A Molecular Approach to Palaeontology: Biochemical Method applications of Brachiopod Proteins

by

Ikbal Modhir AL-Rikabi

B.SC. University of Baghdad

Thesis submitted for the degree of M.Sc. (by research) at the University of Glasgow, Department of Geology and Applied Geology, 1991.

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To my parents and my husband Zeid with lots of love

Acknowledgement

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Finally I would like to finish these acknowledgments by remembering and thanking my parent, my sisters and brothers for their moral support.

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Chapter 3 Materials and Methods

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Abstract

This work represents a contribution to the larger project of applying immunological or biochemical techniques to gain access to the molecular structure of geological samples. Intra-crystalline molecules were extracted from various brachiopod shells and analysed using SDS-PAGE, which enables the estimation of their molecular weight. The separated molecules were further characterised by employing electroblotting on PVDF membrane and immunostaining techniques (Alkaline phosphatase-labeled antibody). Polyclonal sera were used in this work for their wide range of specificity. The body tissue homogenate of representatives of the two main brachiopod classes were also analysed and characterised using the above mentioned techniques.

The amino acid content of the intra-crystalline shell extract and the body tissue homogenate samples were analysed using an ABI 420H analyser. The SDS-PAGE of each of *Terebratulina retusa* and *Neocrania anomala* present different separating pattern indicating dissimilarities in their molecular weight. Examined by SDS-PAGE *Terebratulina retusa* intra-crystalline shell extract displays one band at 29-36 k Da in molecular weight, while *Neocrania anomala* intra-crystalline shell extract did not show any band under such examination. However, the amino acid analysis of *Neocrania anomala* sample revealed a significant amount of each amino acid present. Great differences were found in the amino acid contents of the intra-crystalline shell extract of each of *T. retusa* and *N. anomala*.

The above techniques were applied to hundreds of brachiopod samples and a standard or blank sample were always run according to each method.

CHAPTER ONE

INTRODUCTION

1.1- Proteins and Molecular Palaeontology

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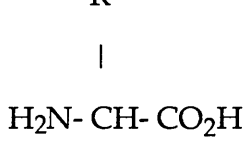
Molecular palaeontology deals with organic material in fossils and related living organisms. The organic material in which we are interested is primarily proteins found in the body and shell of the organism. Proteins are linear polymers formed by the condensation of smaller molecules called the amino acids. Most of these amino acids have the following general structure:



The main difference between individual amino acids lies in their side chain (R) which gives the protein its biological reactivity. Twenty of these side chains are normally found in proteins and they are different in their size, shape, charge, bonding capacity and chemical reactivity (see Fig 1.1, 1.2, and 1.3). These basic units (amino acids) are linked together through peptide bonds, with the exclusion of water, to form the polypeptide chains. Usually 50- 2000 units are linked in this way to form the three-dimensional conformation of each protein molecule. Peptide bonds are formed between

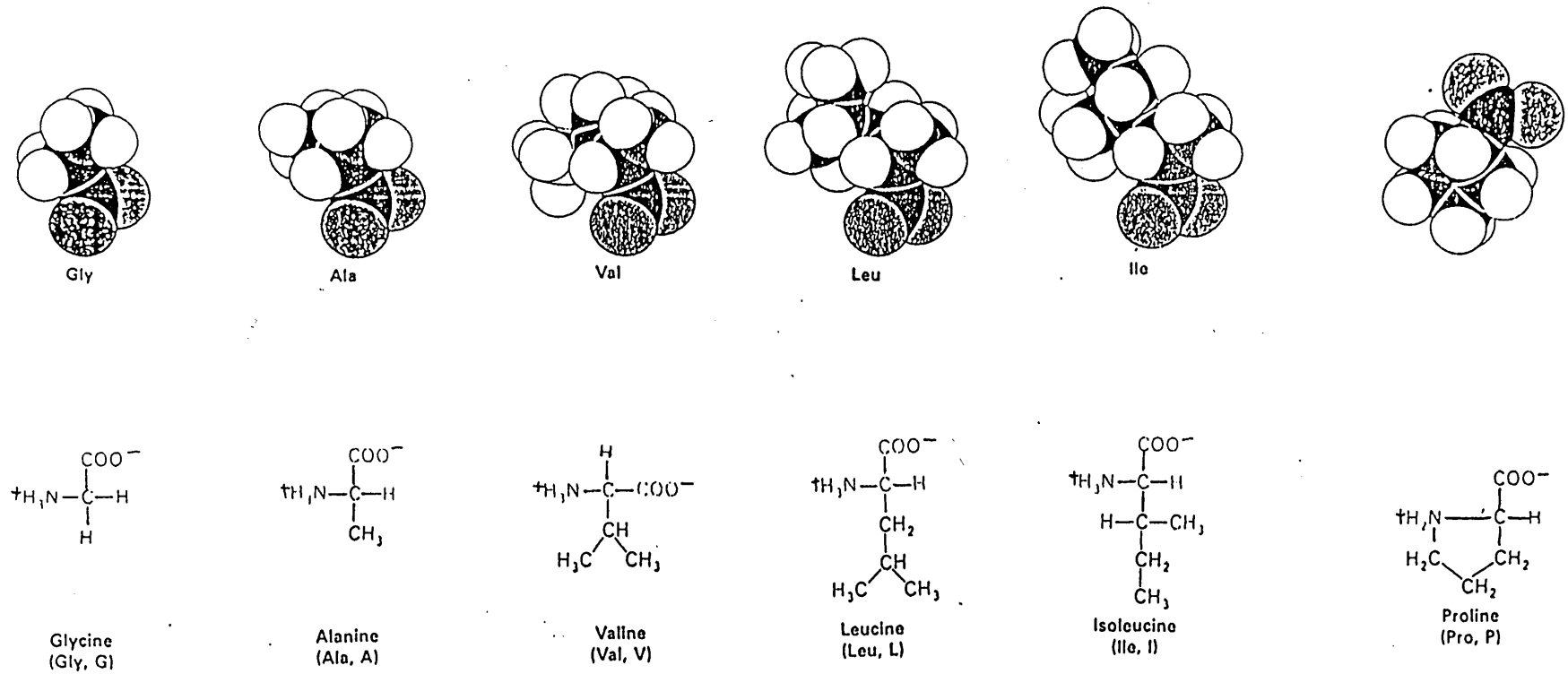


Fig. 1.1- Chemical structures and models of amino acids with aliphatic side chains. Modified from Stryer (1989).

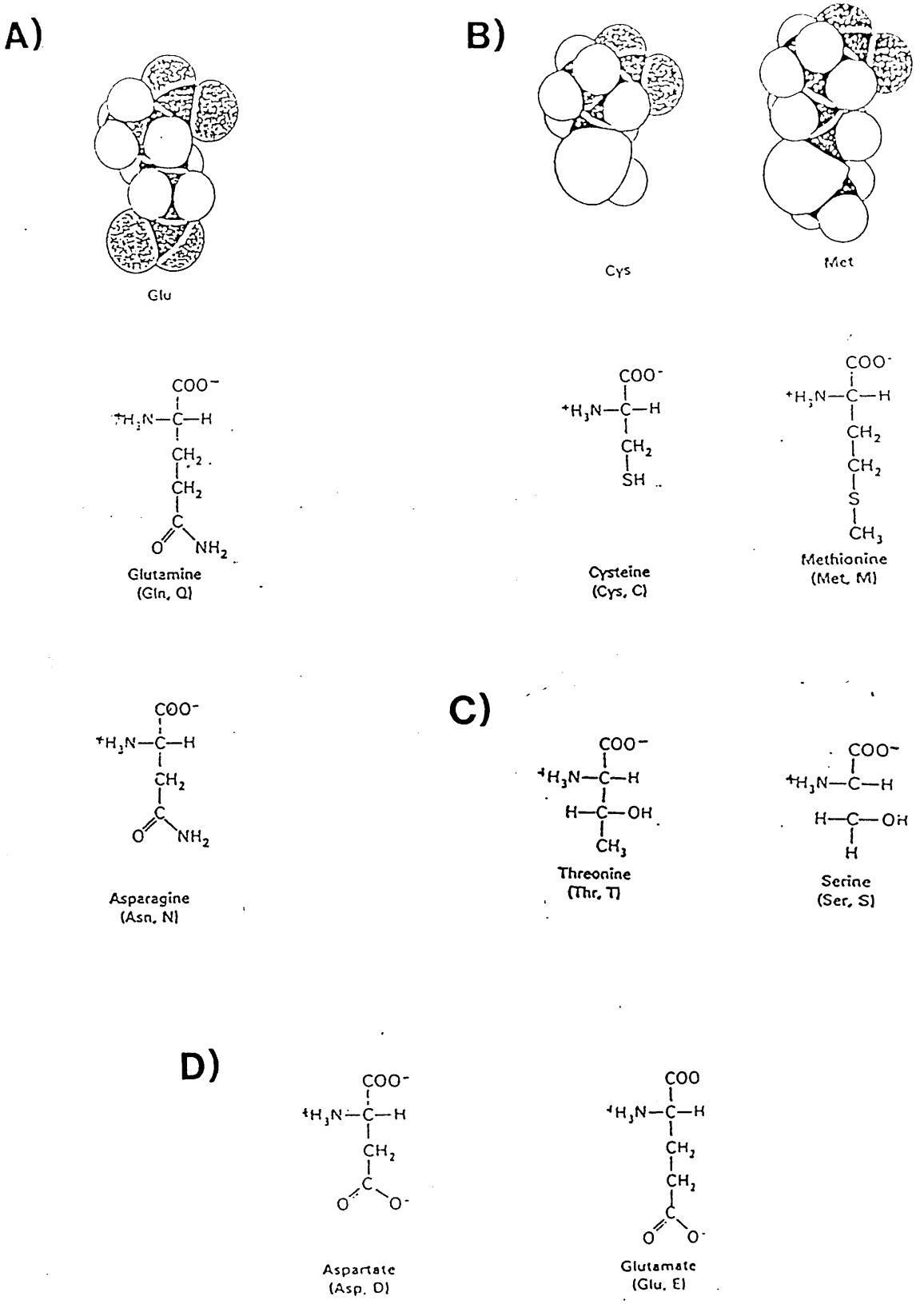
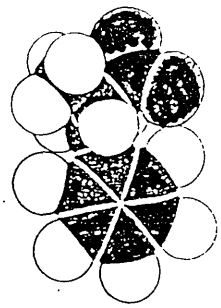
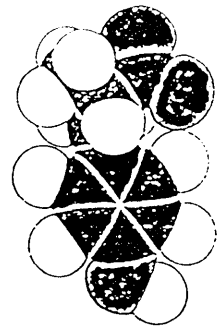


Fig. 1.2- Chemical structures and models of amino acids with, A) amide side chain, B) sulfur side chain, C) hydroxy aliphatic side chain, D) acidic side chain. Modified from Stryer (1989).

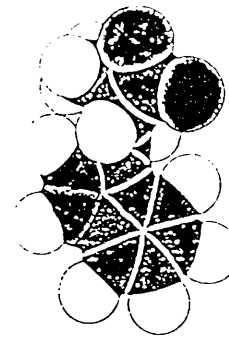
A)



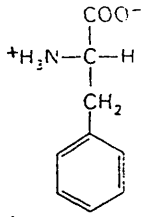
Phe



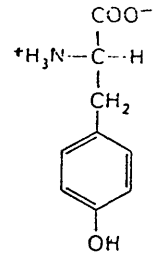
Tyr



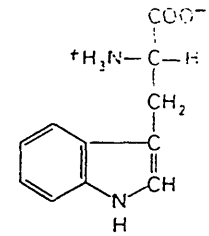
Trp



Phenylalanine
(Phe, F)

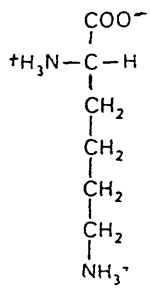


Tyrosine
(Tyr, Y)

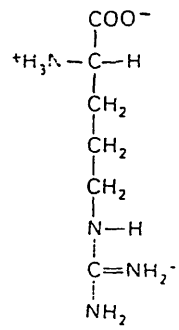


Tryptophan
(Trp, W)

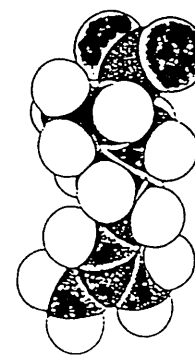
B)



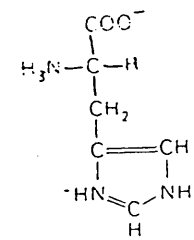
Lysine
(Lys, K)



Arginine
(Arg, R)



Arg



Histidine
(His, H)

Fig. 1.3- Chemical structures and models of amino acids with A) aromatic side chain, B) basic side chain. Modified from Stryer (1989).

the α– carboxyl group of one amino acid and the α- amino group of another amino acid. Each polypeptide chain possesses a direction which begins from the α- amino group and ends with the carboxyl group. Another type of bonds linking polypeptide chains are the disulfide bonds (S-S) formed by the oxidation of cysteine residues.

The specific sequence of amino acid, assembled in polypeptide chains to form protein molecules, is controlled by genes like DNA and RNA. Determination of amino acid sequence in a protein molecule is essential to gain information about the mechanisms controlling protein conformation and their biological activity. It is also important in the pathological field as the alteration of amino acids will affect the protein normal functions and produce abnormality and disease. Molecular paleontology is another field where amino acid sequence are valuable in tracing the evolutionary history of proteins. Proteins in organisms showing common ancestor bear resemblance in their amino acid sequence.

Proteins show four levels of regularly repeating conformation of polypeptide chains. The primary structure of a protein is the amino acid sequence joined by disulfide bonds, the secondary structure refers to the linear sequence of the amino acids. Some of the regular conformation of this structure are α helix, (where the polypeptide is tightly coiled) *B* sheets, which consist of extended sheets of polypeptides and collagen helix (shown in Fig.1.4). Tertiary structure displays the overall topology of the folded polypeptide chain and finally the quaternary structure refers to the aggregation of the polypeptides by specific interactions. Protein structure plays a crucial role in determining the function of the molecule.

Proteins display a significant and wide range of functions in all biological

processes. Their important activities can be summarized in the following points:

1. *storage and transport-* specific proteins are responsible for the transportation of ions and small molecules for example, *haemoglobin* transports oxygen in erythrocytes; *myoglobin* transport oxygen in muscles while iron is carried in the plasma of the blood by *transferrin* and then stored as a complex with *ferritin* (protein) in the liver.

2. *Enzymatic catalysis* - most of the chemical reactions in biological systems require catalization by specific molecules (enzymes), and a large number of the characterized enzymes are proteins. Enzymes have the ability to increase the reaction rate. The ability of certain proteins to make, stabilize or break chemical interactions with substrate molecules, accounts for their properties.

3. *Coordinated motion-* proteins are the main component of muscles. Muscle contraction is accomplished by the sliding motion of two kinds of protein filaments.

4. *The presence of a fibrous protein* (collagen) in skin and bone, enhances their strength.

5. *Immune protection* - antibodies are specific proteins that recognize and combine with foreign substances (bacteria, viruses etc.). Antibodies are the body defence system against diseases.

6. *Proteins are responsible for the generation and transmition of the nerve impulses.* The response of nerve cells to specific stimuli is mediated by receptor proteins.

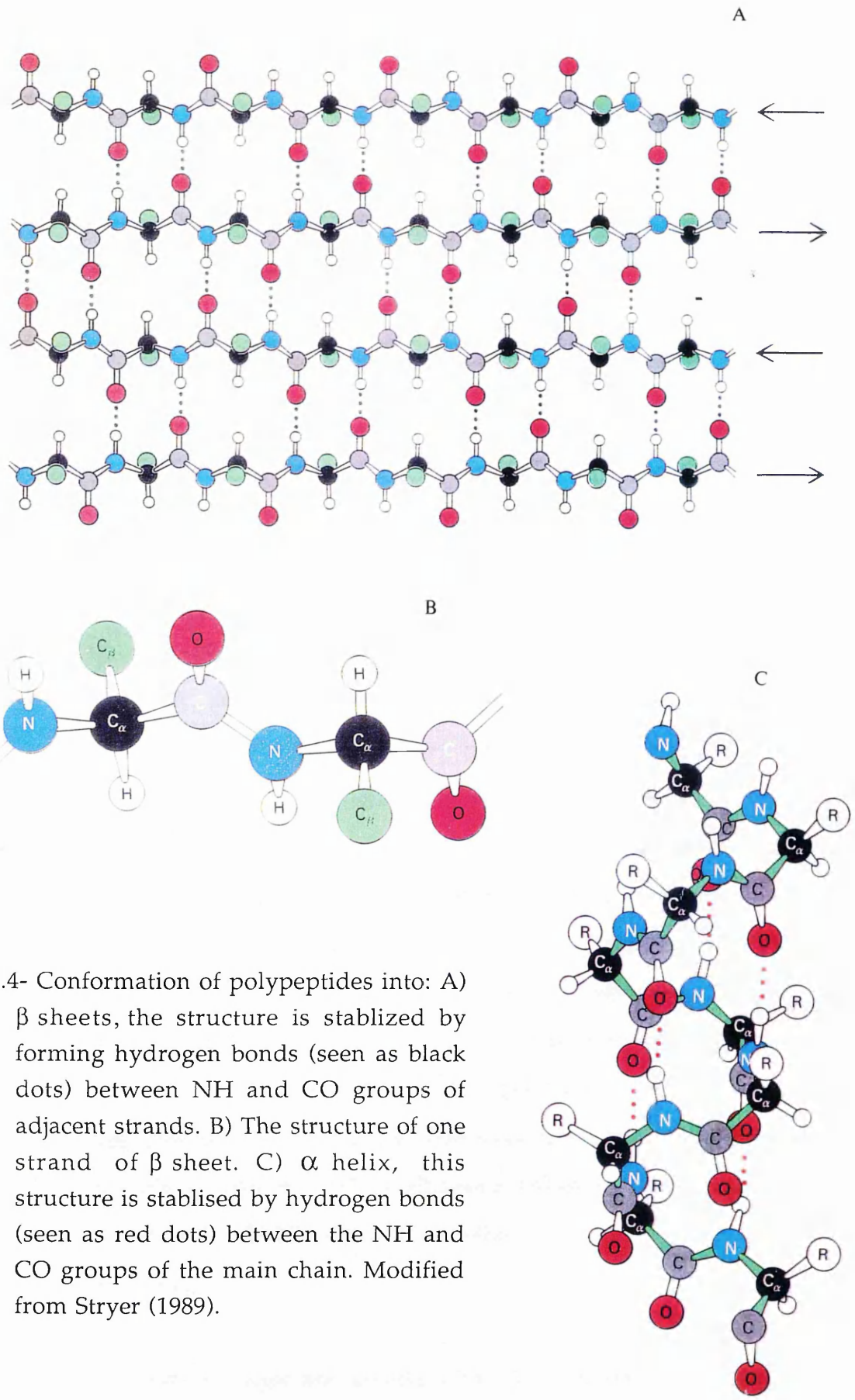


Fig. 1.4- Conformation of polypeptides into: A) β sheets, the structure is stablized by forming hydrogen bonds (seen as black dots) between NH and CO groups of adjacent strands. B) The structure of one strand of β sheet. C) α helix, this structure is stablised by hydrogen bonds (seen as red dots) between the NH and CO groups of the main chain. Modified from Stryer (1989).

7. *Growth and differentiation are controlled by growth factor proteins*, for example many of the hormones that coordinate the functions of different cells in multicellular organisms are proteins such as insulin and thyroid stimulating hormone. In fact proteins serve in the cells as sensors to control the energy and matter flow.

Runnegar (1990) stated that molecular palaeontology has three main components:

1. organic geochemistry.
2. molecular phylogeny.
3. palaeobiochemistry.

Great advances have occurred in these three fields during the last 20-30 years primarily because of the development of new and powerful analytical methods of recovering, purifying and identifying organic compounds. As a result, paleontologists are now able to deal with the minute quantities of molecules found in fossils and rocks and to address the three components in a significant way. The first report of preserved amino acids in fossil shells was by Abelson (1954). Since then many scientists have detected the remains of macromolecules in fossils from different ages (eg. Wyckoff 1972; Weiner *et al.* 1976 , 1979). Amino acids have been detected in fossils ranging back in age from the Holocene to the Cambrian, and indeed Precambrian strata are known to contain amino acids which are thought to be the molecular remnants of ancient Precambrian organisms. Therefore it has been established that organic molecules survive through fossilization (Abelson 1956) and that these molecules appear to hold information vital for many branches of molecular palaeontology.

The sources of these amino acids are uncertain and they are likely to include contamination from various sources over geological time. Therefore working

with organic molecules from well protected sites for example the intracrystalline matrix of shells is of great importance because these molecules are embedded within biominerals which protect them from degradation and contamination with extraneous organic material. Protein molecules can be found also in the inter-crystalline organic matrices of the shell (between the biocrystals) which are more vulnerable to contamination. A significant change in the abundance of these amino acids occurs through geological periods, and in living brachiopods it was found that the secondary layer organic sheaths (ie, the inter-crystalline matrix) are shown to decay in less than a year (Collins 1986).

In the first of Runnegar's categories, organic geochemistry, the use of what may be described as chemical techniques to study the organic fossils is well demonstrated in several applications in geology. For example, extracting molecules from rocks or fossils has proved to be of fundamental importance to the oil industry, in oil diagenesis (Brooks 1981; Mackenzie *et al.*, 1982) and to the study of the early history of life (Schopf, J. W 1978; Runnegar 1986).

Molecular phylogeny attempts to reconstruct the evolutionary relationships of modern day animals by comparing the amino acids and DNA sequences of living organisms. Although complicated by the internal dynamics of biological systems, there is no doubt that this technique has made many important contributions to the understanding of evolution (eg. O' Brien *et. al* . 1986). Attempts have also been made to assess the extent of molecular similarity between taxa using immunological techniques (Collins *et. al.* 1988, 1991 in press; Curry 1991).

Palaeobiochemistry combined the use of biochemical techniques to study the palaeontological material. Several biological and chemical methods have

been used to extract and detect organic molecules in fossil shells and rocks. Immunological techniques have been used in palaeontology to detect macromolecules (proteins) with preserved antigenic determinants in fossil shells and bones (De Jong *et al*, 1974; Westbroek *et al.* 1979, 1983; Muyzer 1988). Lowenstein (1980, 1981) reported the presence of well preserved collagen and albumin in a 1.9 myr. old fossil. Protein fragments have also been reported in chromatography experiments using Jurassic and Silurian fossil brachiopod shells with intact polypeptide chains (Jope, 1967). In the early stages of the present study, protein bands were observed in fossil brachiopod shell (*Neothyris lenticularis*) dated 0.4 myr, using the polyacrylamide gel electrophoresis system.

The present project represents a contribution towards the third of Runnegar's categories, namely Palaeobiochemistry. The aim of this work is to characterize the organic molecules (proteins) in the shell and soft body tissues of living *Terebratulina retusa* and *Neocrania anomala,* which represent two major brachiopod groups, and to investigate the survival of original biopolymeric fragments in related fossils using a combination of electrophoretic and immunological techniques, described in the following sections.

1.2 - Electrophoresis

****

The electrophoresis techniques are based on the principle that charged particles or ions, including molecules, migrate at different rates when placed in an electric field. The rate of migration of a particular molecule is dependent on its size and charge, and therefore mixtures of molecules can be separated into their constituents by electrophoresis.

A sample is usually placed in a jelly-like medium or gel which has a suitable pore size to allow the free migration of the molecules under investigation. When an electric current is applied the various molecules migrate through the gel at different rates, with the larger molecular weight components migrating at a slower rate (Fig. 1.5). The major advantage of gels is their stabilizing effect in minimizing convectional and diffusional movement of the proteins .

The polyacrylamide gel used in this project was first used as a supporting matrix in electrophoresis by Raymound & Weintraub (1959) and in 1964, Ornstein and Davis introduced a method of electrophoresis in gel rods using a discontinuous buffer system. In 1967 Shapiro *et al.*, used polyacrylamide gel electrophoresis (PAGE) in the presence of an ionic detergent, sodium dodecyl sulphate (SDS). Denaturation and binding of SDS to proteins and peptides results in a relatively uniform negative charge.

Laemmli (1970) introduced a new gel system by adding SDS to the Tris-glycine buffer system of Ornstein and Davis to achieve a sharp separation of proteins based largely on their subunit molecular weight. Chambach and Rodbard (1971) reported the advantages of combining a discontinuous buffer system with the SDS system, which includes a high resolution and the ability to apply highly diluted samples. They also emphasizeed the quantity applications of PAGE for the interpretation of the results.

In 1987, Schagger *et al.*, introduced a new gel system based on the Laemmli SDS- PAGE. They subsituted glycine for tricine as the trailing ion in the discontinuous system. Tricine helps to stack and destack bands of protein in the same pH as the separating gel, at low polyacrylamide concentrations. The

two variants of the SDS-polyacrylamide gel (tris-glycine and tris-tricine gels) have been used during this work, and good resolution was obtained with both.

The high resolution and flexibility of PAGE has led to its widespread use as a powerful and versatile technique for separating and detecting molecules. Polyacrylamide gels are synthetic polymers formed by the polymerization and cross-linking of monomeric substances to form a three-dimensional frame work with a suitable pore size. N-N'-methyline bisacrylamide compound is used to crosslink the acrylamide monomer into long chains by reacting with free functional groups in the chain. The final gel structure is shown in Figure 1.6. The gel density depends on the concentration of both monomers. Different pore size is achieved by altering the concentration of the monomer and the cross-linker. The gel is transparent through a wide range of monomer concentrations. In the course of this PAGE study the ionic detergent sodium dodecyl sulphate (SDS) was used. In this system the flow of separated molecules through the gel depends only on the radius (size) of the individual molecules, because this highly charged detergent wipes out the charges on the polypeptide chain so that the net charge per unit mass becomes constant.

The separation conditions can be tailored to particular molecules by changing the pH of the gel, the nature of gel medium, the voltage applied, or the duration of electrophoresis. The relative positions of molecules of interest (in this case proteins), are determined by incubating the gel with a suitable stain which reveals their location as bands. The positions of bands in the gel relative to molecular weight standards provides an indication of their molecular weight.

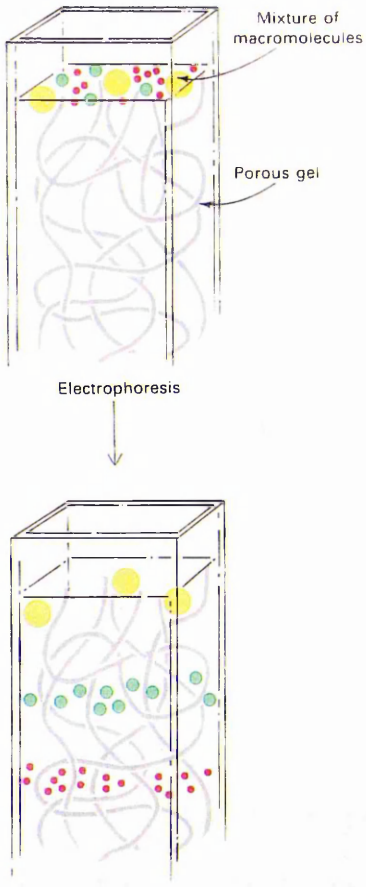


Fig 1.5- The migration of molecules in porous polyacrylamide gel during electrophoresis. From stryer (1989).

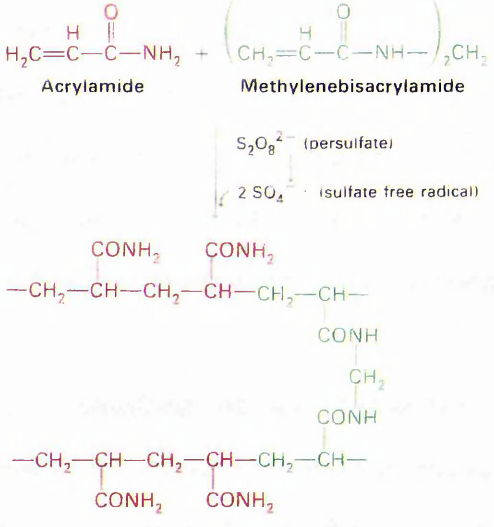


Fig. 1.6- The polymerization of polyacrlyamide gel. After Stryer (1989).

1.3 - Molecular Weight Measurement

****

Shapiro, Vinuela and Maizel (1967), were the first to indicate that during SDS-gel electrophoresis, the mobility of a polypeptide chain is dependant on its molecular weight. Soon after, Weber and Osborn confirmed this observation in a study of forty proteins with known molecular weights.

SDS-PAGE system can be used to determine a protein molecular weight. Most proteins bind the anionic detergent SDS at a constant rate (1.4 g SDS per 1 g of protein). The treatment of protein samples with Laemmli sample buffer (contains 10% w/v SDS, 0.2M DTE) prior to electrophoresis, disrupts the tertiary structure of the protein samples by unfolding and breaking the hydrophobic and the disulfide bonds which join the polypeptides together. A rod-shape (negatively charged) complex of SDS-protein is formed and the length of this complex is proportional to the molecular weight of the protein molecule. The unfolding of the tertiary structure leads to a decrease in solubility and biological activity of the proteins.

After the staining and the destaining of the gel, the relative mobility (Rf) of each band separated in the gel is determined by dividing the protein band migration distance by the migration distance of the tracking dye, which marks the front of the gel (ie. the fastest migrating compounds present). All measurements were taken from the top of the separating gel.

Molecular weight estimation of an unknown protein is obtained by establishing a calibration curve for marker proteins of known molecular weight. The calibration curve is drawn by plotting the relative mobilities of the separated molecules against the logarithmic values of their molecular weight. A separate calibration curve was plotted for each kind of gel.

1.4 - Immunoblotting

****

Immunoblotting may be considered as a three step process: firstly powerful separation of protein mixtures by SDS-PAGE as described earlier in this chapter (see section 1.2). Secondly the transfer of the separated molecules onto an inert membrane, and finally the detection of these molecules using antibodies.

In 1975 the term immunoblotting was first used to describe the transferring of separated DNA molecules from gels onto a membrane by E.M.Southern. The technique was soon applied to RNA by Alwine *et al.* In 1979 the blotting of proteins by means of capillary and electric transfer was introduced by Renart *et al.* and Towbin *et al.* respectively.

The transferring, or blotting procedure of biological molecules (in this case proteins) from the analytical support onto a membrane usually requires a driving force to guide and facilitate the movement of the molecules. Such forces could be electrical, capillary, centrifugal, vacuum pressure or diffusional forces. The use of an electric field during blotting proved to be the fastest and most reliable technique in accomplishing the transfer. This technique is called electroblotting. The main criterion of blotting is to have close contact between the resolving gel and the membrane so the protein molecules will be able to transfer (see Fig. 1.7). The binding forces of the membrane can be covalent or non- covalent.

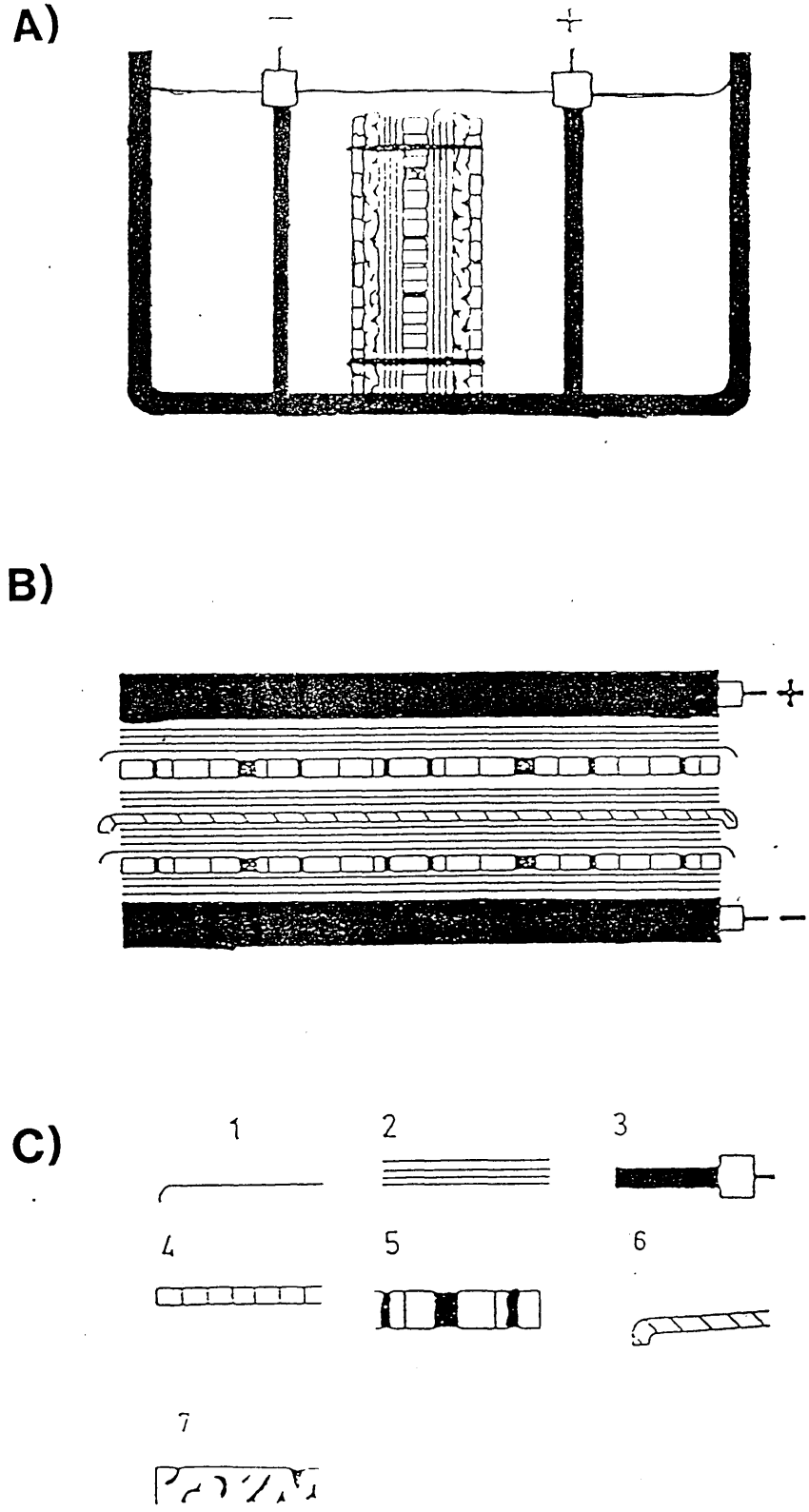
The efficiency of the elution depends on the composition of the separation medium (gel), the size of proteins and the length of the transfer time. The concentration of the two monomers (acrylamide and bis-acrylamide) determine the gel porosity and affect the flow of the molecules through the

resolving gel. It is possible to monitor the efficiency of the transfer by staining the gel with Coomassie Brilliant Blue (CBB) or silver stain after transfer.

Two kind of electroblotting have been used in the course of this work (Fig 1.6), the wet-blotting (the gel and membrane sandwich immersed in buffer) using the Bio-Rad mini trans blot cell, and the semi-dry blotting (without buffer). The semi-dry blotting using the Bio-Rad semi dry electrophoretic transfer cell, did not give completely satisfactory results, i.e. some of the separated molecules at the high molecular weight range were still in the gel after the transfer time was finished.

Electroblotting was followed by immunological detection, which involves the addition of enzyme-linked labelled antibody to recognize the molecules (antigens) already immobilized on the membrane. Polyclonal anti sera are usually used with denatured proteins. The rate and direction of the electroblotting depends on the pH of the transfer buffer.

A major factor in determining the success of an immunological detection is the nature of the antigenic determinant (also called epitopes; regions of an antigene which reacts with an antibody). Immunoblotting procedure includes prior analysis of protein samples on SDS - PAGE which denaturated these proteins. Protein denaturation means unfolding of the protein structure which affects the secondary and tertiary protein structures while the primary structure keeps intact as indicated by the sequencing of denatured proteins separated by PAGE. In such conditions polyclonal anti- sera are widely used because they contain a complex mixture of antibodies with wide spectrum of specifity.



g. 1.7- Electroblotting techniques, A) wet electroblotting, B) Semi-dry electroblotting, C) Symbol explanation; 1. blotting membrane, 2. filter papers, 3. electrode, 4. plexiglass grid, 5. gel, 6. dialysis film, 7. sponge. Modified from Bjerrum (1989).

The high sensitivity of immunoblotting is firstly due to the concentration of the proteins on the transfer membrane, and secondly to the fact that after denaturation of the protein samples with SDS, different epitopes will be exposed and ready to interact with several antibodies at the same molecule. This kind of reaction will increase the strength of the signal.

This technique is attracting increasing interest because of its accessibility where the antigens are concentrated on the membrane surface which facilitates direct detection of the antigene. The binding ability of the blotting membrane immobilizes the protein so diffusion is avoided. Immunoblotting has been used in this study because it is useful to determine the presence and quantity of an antigen (protein molecules) and the efficiency of the extraction of the molecules.

1.5 - Amino Acid Analysis

****

Stanford Moore and William Stein (1940) revolutionized the biological sciences by developing an-ion exchange chromatographic method for quantitative amino acid analysis, and in 1958 they presented detailed information for constructing an automated analyzer using ionic chromatography separation and post column derivitization with ninhydrin. The development of the reverse-phase chromatography in 1970 and all the recent modification in equipment and derivitization chemistry minimized the analysis time and increased the analysis sensitivity to the picomole level, although the basic protocol was still the method of 1958. The amino acid analysis procedure contains three main steps:

1. Hydrolysis - where the proteins are split into their constituent amino acids

by the cleavage of the peptide bonds.

2. Derivitization- the treatment of free amino acids with certain chemicals to produce a stable compound which absorbs (ninhydrin, PTC), this step can be applied either before or after chromatography.

3. Chromatography - amino acids are separated chromatographically for identification and quantification.

Samples are loaded on glass frits in liquid form, hydrolyzed at 160 °C for one hour and then they are derivatized in turn. Each sample derivative is then injected automatically onto a reversed phase column and buffers are applied to elute the amino acid derivatives. A detector housed after the column is linked to a data collection computer which integrates the peak height and estimates the quantities of each amino acid on the basis of comparison with a calibration curve of standards.

1.6 - Brachiopods

****

1.6.1- General Features

Brachiopods are widespread throughout the seas of the world and are found from the intertidal situation down to depths of about 6000 m (Fig. 1.8). Most live epifaunally, attached to solid objects by a fleshy stalk known as the pedicle (Fig. 1.9 C). Brachiopods are grouped together as a single phylum, superficially resembling bivalve molluscs, but differing fundamentally in the arrangement of their shell valves and the organization of their soft tissues. Two shell valves enclose the body dorsally and ventrally as in the bivalve. The brachiopod ventral valve is typically larger and often bears an attachment stalk or pedicle (Fig. 1.9 B).

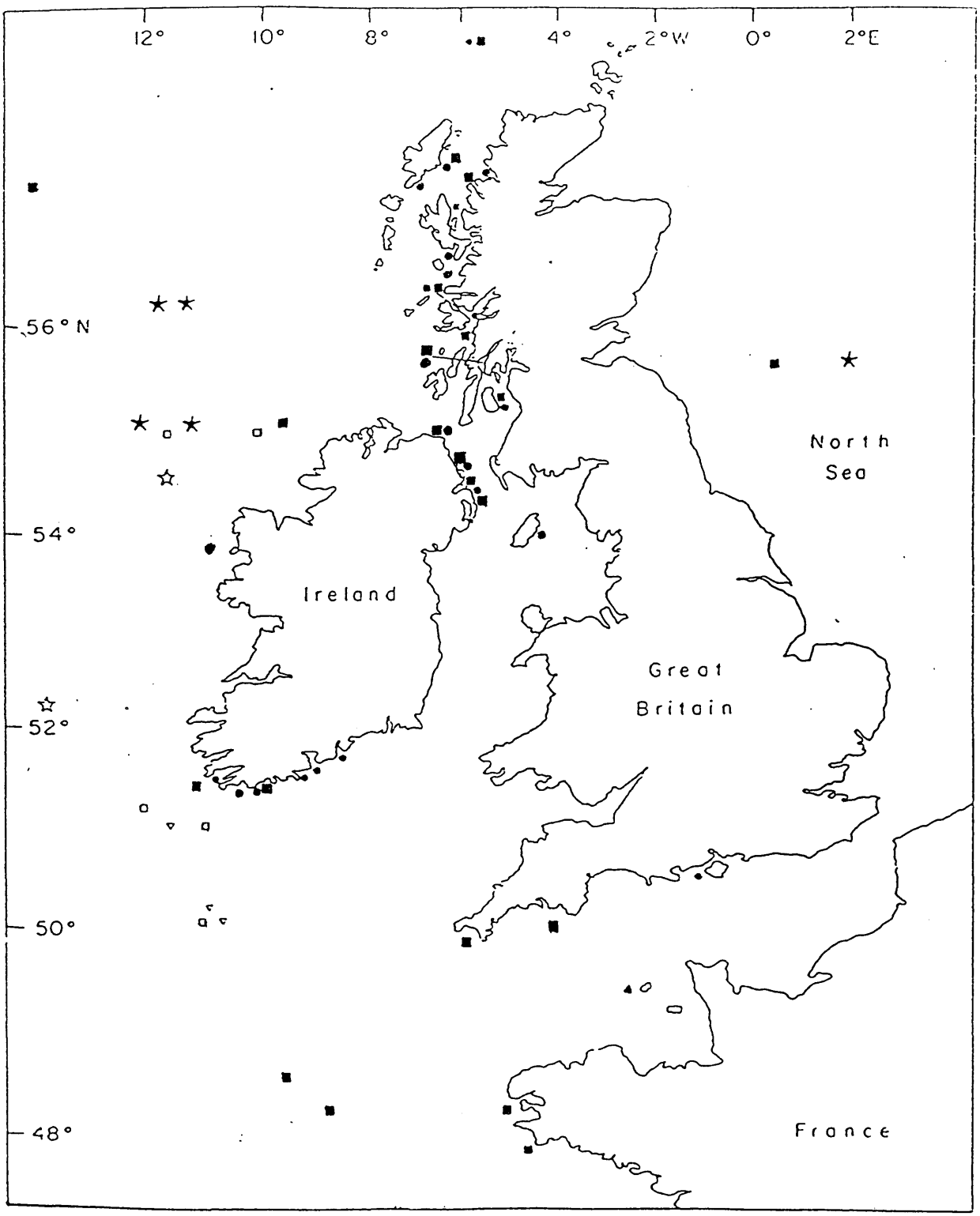


Fig. 1.8- Brachiopod collection cites in the United Kingdom. Legend explanation: □ -Terebratulina septentrionalis; ● -Neocrania anomala; ■ -Terebratulina retusa; ☆-Pelagodiscus atlanticus; ★-Cryptopora gnomon; ▽ – Platidia anomioides. Modified from Brunton et al., (1979).

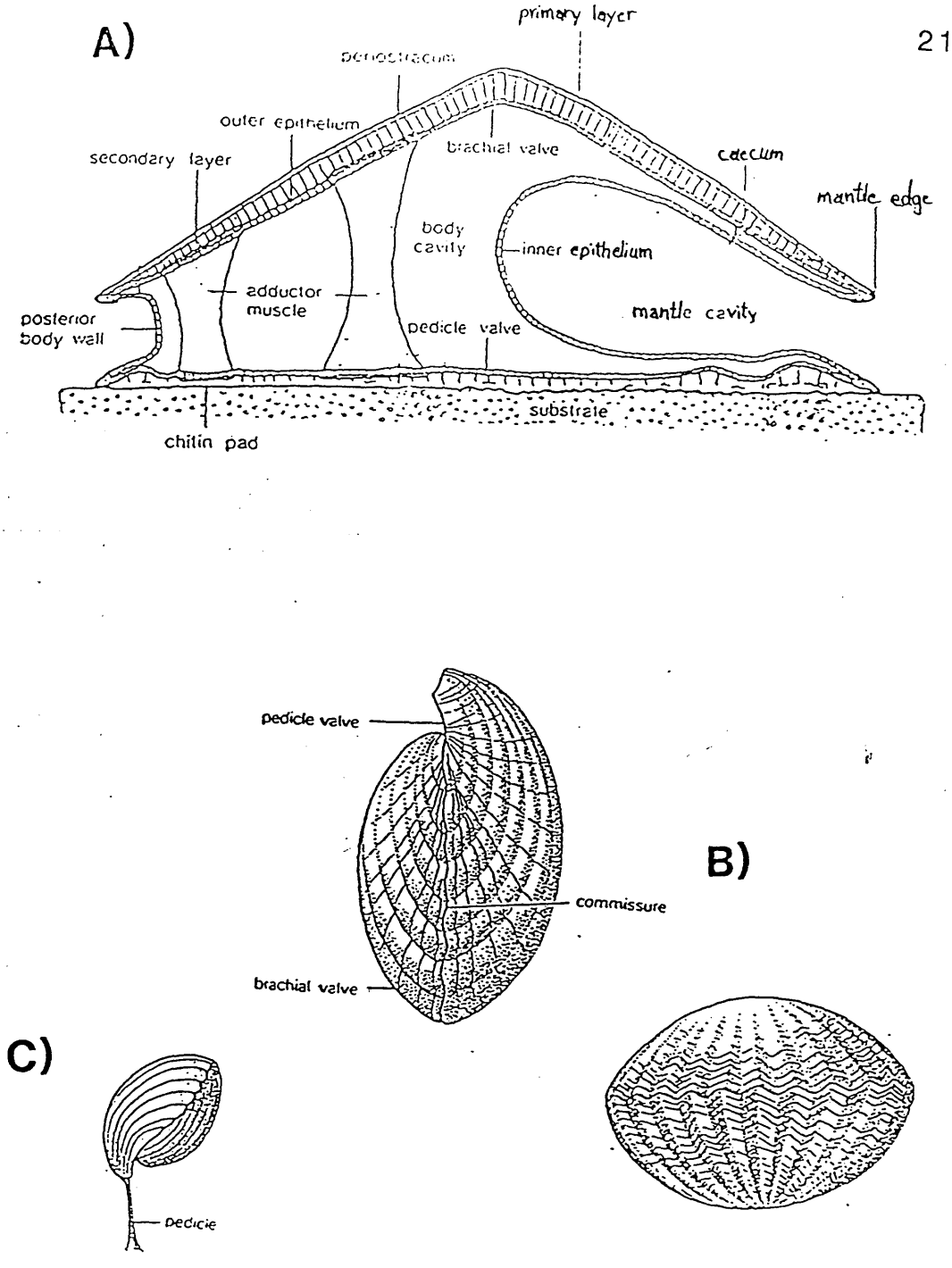


Fig. 1.9- General features of brachiopods. A) *Neocrania anomala*, cemented to the substrate, showing the brachial and pedical valve structures. B) Order Terebratulida (*Magellania flavescens*) showing the lateral and anterior views, C) Life position. Modified from Williams & Wright (1970) and Clarkson (1986).

Brachiopods are of great palaeontological and biostratigraphical importance because they have an exceptionally long recorded history and their remains are often abundant in fossil-bearing rocks. Fossil brachiopods are known from almost every geological age from the earliest Cambrian strata. However, relatively few representatives have survived to the present day. Some fundamental morphological features are common in all brachiopods, such as the two-valved shell, the lophophore, their mode of attachment (or relation) to the substrate, which is either by pedicle and spines or cemented or free lying .

1.6.2- Internal Anatomy of Articulate brachiopods

The two shell valves enclose all the vital organs suspended in a fluid- filled cavity (Fig. 1.10). The inner side of the valves is lined with a cellular epithelium tissue (the mantle) which separates the mantle cavity at the front from the posterior body cavity by the anterior body wall. Most of the mantle cavity is occupied by a coiled food gathering and respiratory organ (lophophore), which takes up much of the space, and the remaining organs tend to be simple. The lophophore is attached to the brachial valve interior at the rear of the mantle cavity and it consists of elongated calcareous brachia, supported by a brachial canal (brachial axis). These brachium is not always preserved in fossils. From the brachial canal emerge lines of slender parallel filaments lined with cilia. A continuous water current is drawn in and out of the mantle cavity by the movement of these cilia, while the sticky filaments filter or trap food particles and algae out of the sea water. Food passes to a small gut through the mouth (located in the anterior body wall) then to the stomach and blind intestine. Brachiopods have simple digestive, excretory and reproductive systems, in addition to the central parts of circulatory and

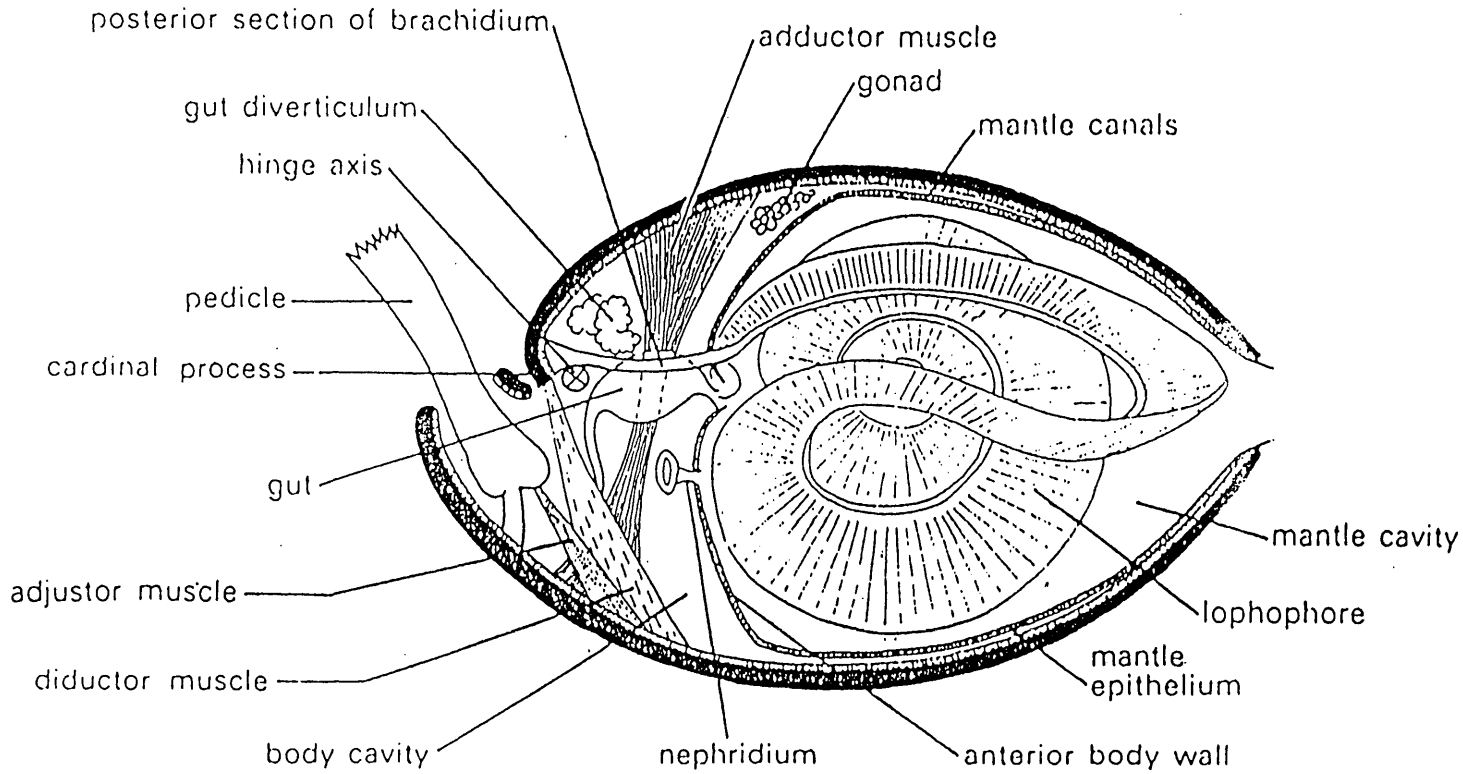


Fig. 1.10- Digrammatic structure of brachiopod internal anatomy. After Clarkson (1986).

nervous systems. There is no fusion of the mantle margins and the characteristic mobile foot of the molluscs is absent. The sexes are separate and fertilization is external and gives rise to a trochophore- like larva which can have a long planktonic life.

Within the body cavity there are two sets of paired muscles, namely the diductor muscles to open the shell, and the adductor muscles to close it. In addition there are adjustor muscles which allow the shell to swing into or away from the current. The scars of these muscles can be seen inside fossil brachiopod shell. Finally the body cavity includes the pedicle, located at the posterior part of the shell and in most brachiopods the pedicle is responsible for attaching the shell to a substrate.

1.6.3- Traditional Brachiopod Classification

All early classification systems were based on external and internal morphological characters of the shell. In 1869, Huxley introduced the terms Articulate and Inarticulate to describe the two major divisions of the brachiopod phylum (Muir-Wood 1955). This classification system was primarily based on the presence or absence of interlocking hinges between the two valves. Since then, the classification system for brachiopods has became more refined, for example; Schuchert (1896, 1897, and 1913), Walcott (1912), Thomson (1927), Allan (1940) and Cooper (1944) .

Williams *et al.*, (1965) adopted a systematic procedure in their classification system which reflects, to some degree, the phylogentic complexities of brachiopod evolution. Accordingly the phylum was categorized into two distinct classes:

(A) Articulate - brachiopods with valves hinged by interlocking teeth and sockets. These structures lie just inside the posterior edges of the valves. In turn this class is divided into six orders: *Orthida, Strophomenida, Pentamerida, Spiriferida, Rhynchonellida and Terebratulida.*

Order Terebratulida was segregated depending on the geometry and ontogeny of the loop (a supporting structure for the lophophore), into two superfamilies (Williams *et.al.*, 1965; Williams and Hurst 1977): Terebratulacea-with a short loop and Terebratellacea-with a long loop (Fig. 1.11).

(B) The other main class is the Inarticulates - brachiopods which lack the articulation structures, but possess a complex series of muscles to hold the valves together. This class was represented by four orders; *Obolellida, Paterinida, Acrotretida* and *Lingulida.*

Lingula, a member of the order Lingulida, one of the two main orders of the class Inarticulata has the distinction of being the oldest surviving genus with fossil representatives stretching back over 400 million years old. The order is unique because they are the only brachiopods that live in burrows.

The two classes of articulate and inarticulate brachiopods are also distinguished by aspects of their digestive system, the alimentary canal of the articulates ends blindly, while that of the inarticulate terminates in an anus (William *et al.* Treatise -H).

The two classes again differ in shell chemistry and structure. All articulate brachiopods have calcareous shells consisting primarily of calcium carbonate with a small percentage of organic material (0.5 % protein), while most

Inarticulata have chitino-phosphatic shell with a high percentage of organic material (25% organic). These major distinctions of shell composition provide a broad basis for classification, although a number of forms are difficult to fit into either group (Jope, in Williams *et al* 1965). There is one exception for this generalization; *Neocrania* [superfamily Craniacea] is a member of the Inarticulata but has the carbonate shell similar in composition to that of the Articulata.

Jope (1967, 1980) demonstrated that brachiopod shell proteins hold phylogenetic information in their amino acid constitution, reporting a major taxonomic distinction between the two classes, (i.e. the presence of hydroxyproline in phosphatic shells and its absence in carbonate shells including the inarticulate *Neocrania).*

Brachiopoda classification involves some problems at the genera level due to homeomorphy; the repetitive evolution of one or more characteristic features in unrelated stocks through time (Fig. 1.12). Immunological techniques can be used to clarify such confusion. This phenomenon is well recognized in brachiopods, for example, the tubular spines, a characteristic feature which was partially responsible for the abundance of the late Palaeozoic Strophomenides. When they evolved again in the Jurassic Rhynchonellides they characterized only pairs of short lived genera. Similarly, the repetitive development of brachidia, firstly in the Ordovician Atrypides, secondly in the Silurian Spiriferides led in both cases to their abundance while the development of the brachidium in Triassic strophomenides resulted in the survival of a few short lived genera.

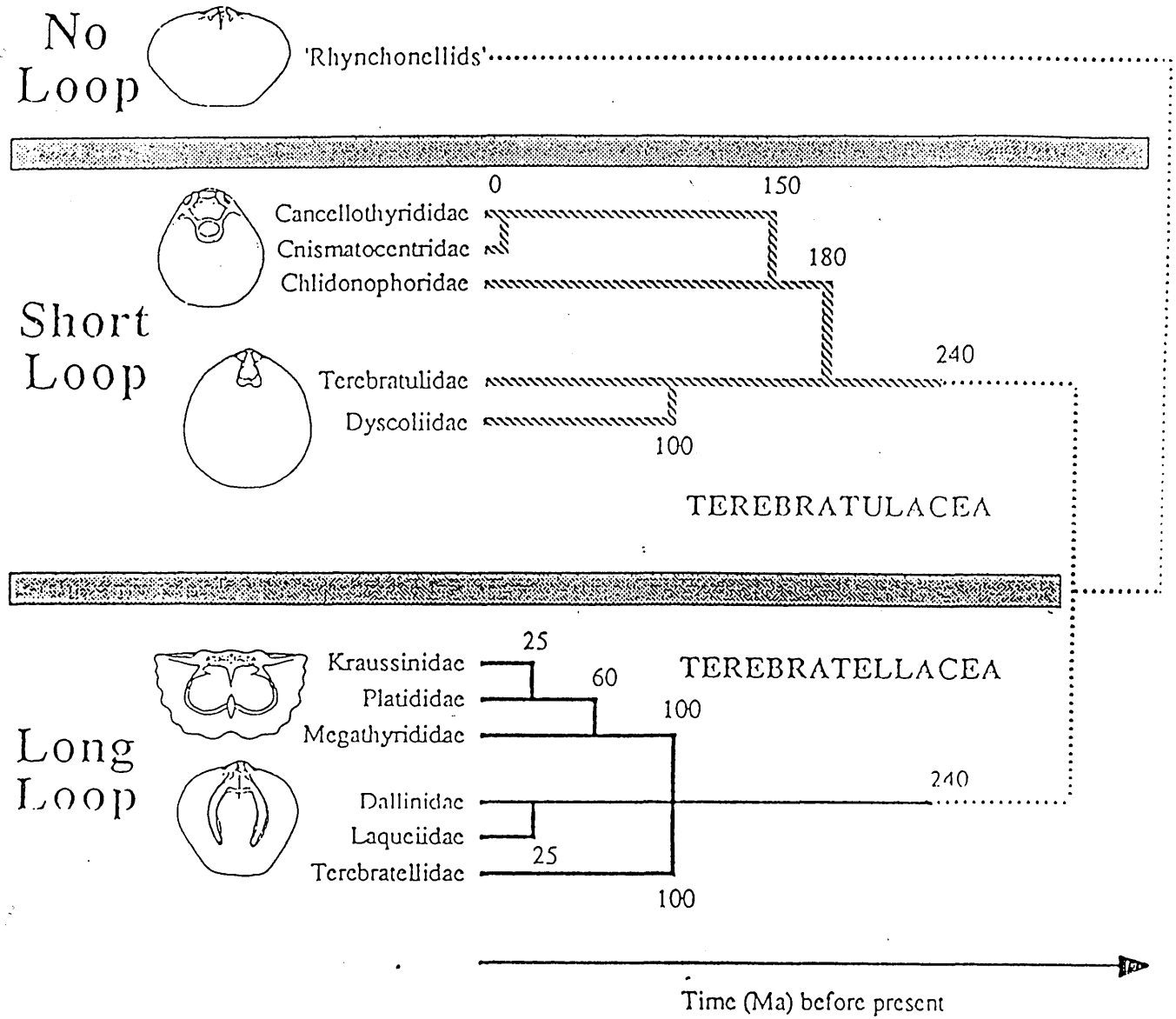


Fig. 1.11- Classification of order Terebratulida. From Williamset.al., (1965).

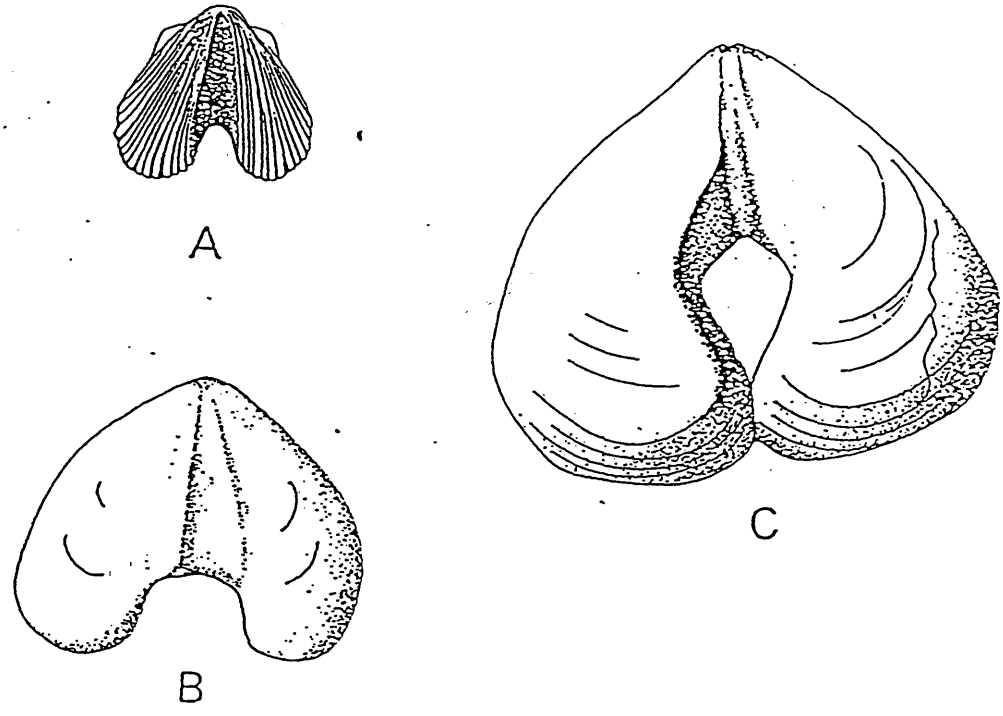


Fig. 1.12- Homeomorphy in brachiopods, A) Devonian orthid *Diccoelosia*. B) Jurassic terebratulid, *Pygope* (juvenile). C) *Pygope* (adult). The juvenile *Pygope* shows similar external morphology to *Dicoelosia* but in the adult form the aliform expansion is joined. After Babin, C. (1980).

Homeomorphy occurs in two ways, *ISOCHRONOUS* (at the same time) and *HETEROCHRONOUS* (at different times), they may occur either within the same taxon or in different taxa (Clarkson, 1983). Heterochronous homeomorphy is more common in brachiopods.

1.6.3 - Immunological Approach to Classification

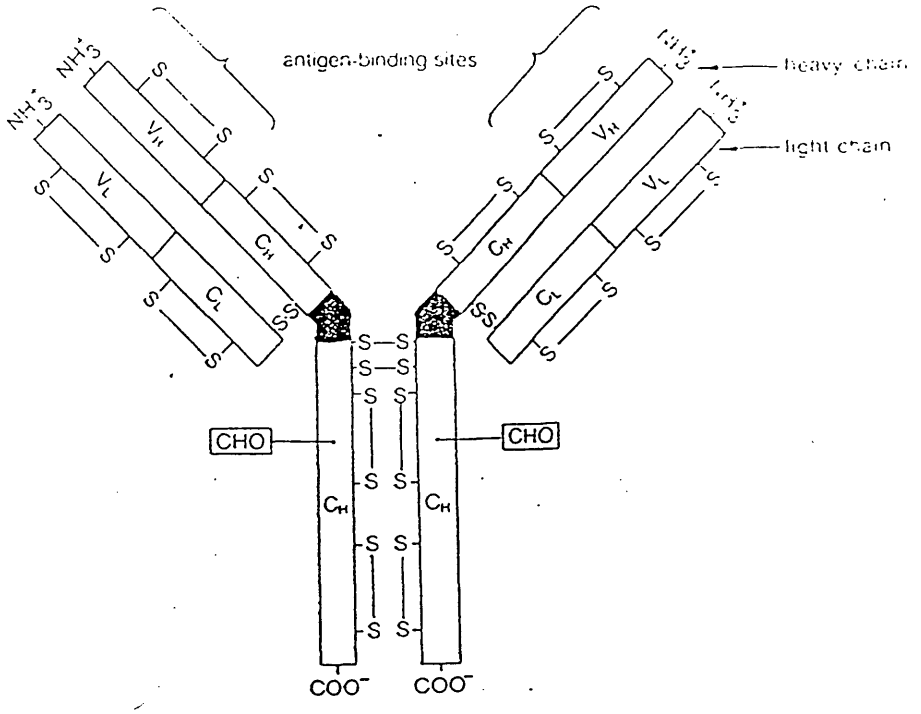
More recently immunological techniques have been applied to investigate brachiopod taxonomy, immunology and proteins within the shell can be considered as providing 'indirect' access to the primary structure of peptides (Curry, 1988) .

Antibodies, or immunoglobulins (Ig) are highly sensitive protein molecules, produced by the immune system against foreign macromolecules (antigens). The lymphocytes, a group of cells found only in the vertebrates, produce the immunoglobulin molecules to be carried on the cell surface. These molecules are called antibodies only when they are released into the body fluids. Each immunoglobulin molecule is made up of two identical heavy peptide chains and two identical light peptide chains joined together by disulphide bonds and every chain is folded into a globular shape (which have *B* - pleated sheet protein structure) by the internal disulphide links which form loops in the peptide chain. The sequence of immunoglobulin molecules reveals that the heavy and light peptide chains possess variable and constant regions (Fig. 1.13). The N-terminal end of the molecule (where the variable regions are) shows a remarkable diversity localized in three hypervariable segments on each of the two chains. The grouping of these immunoglobulins into five classes [Immunoglobulin G (IgG), Immunoglobulin A (IgA), IgD, IgE, IgM] was based upon the structure of their heavy chains and they show highly variable

physical and chemical properties. These specialized molecules can detect and bind specifically to an antigenic - determinant (epitopes consist of 1-6 amino acids in proteins). Antibodies possess very specific three dimensional requirements for binding to an antigenic determinant (Cooper, 1977). Antigenic determinants are protected inside the intraskeletal matrix of the organisms, and they stand a good chance of being preserved over geological time due to their limited size (Muyzer 1988). Antibodies recognize 80% of the amino acid sequences that make up the macromolecules (protein). Any evolutionary or diagenetic change in the antigenic determinants, will alter the antibody response to the macromolecules (Lowenstein 1988). The non-covalent reaction between the antigens and the antibodies, is the basic principle in most immunological techniques and it produces a precipitate that can be detected because of its insolubility. The assay of such specific reactions is carried out with a labelled antibody. Antibodies may be linked to a fluorescent or radioactive marker or an enzyme leading to a dye development.

One of the well established detection techniques is the use of a radio-labelled antigen to compete with an unlabelled antigen (the sample) for a limited number of antibody binding sites. Addition of I^{125} is usually the method for labelling soluble proteins because the isotope is cheap and easily detected in addition to the possibility of obtaining high specific activity preparations (Johnston & Thorpe, 1982). By using a known quantity of the sample with a fixed quantity of each of the labelled antigen and the antibody, an estimate of the protein sample is determined by the degree to which it successfully competes with the radioactive protein for binding to the antibody. This method is the principle of the radioimmunoassay techniques (RIA). After years of applying RIA techniques to phylogenetic problems, Lowenstein (1981,

A)



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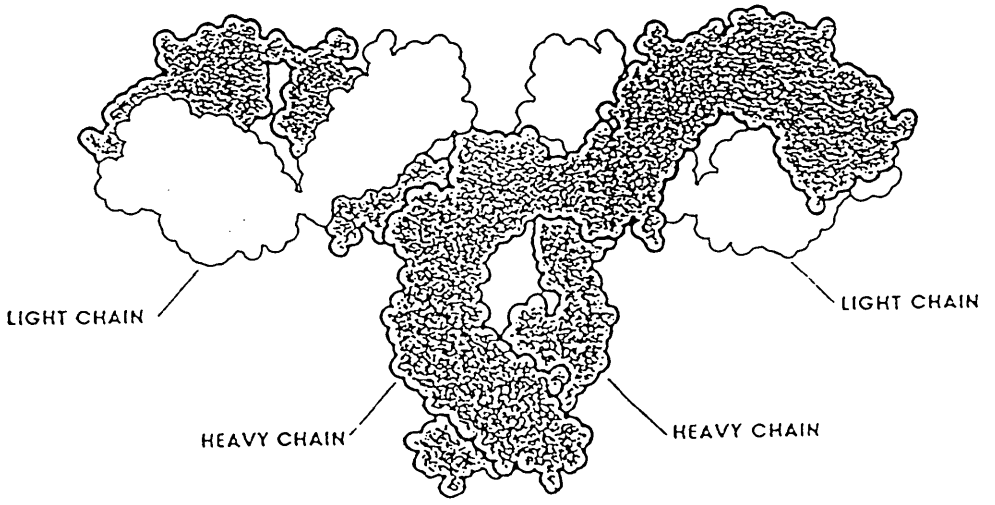

Fig. 1.13- The structural features of the immunoglobulin molecule, (A) digramatic structure ; H-heavy chain. L- light chain. V- variable region. C- constant region. S-S- disulphide bond. CHO- carbohydrate group attached to the heavy chain. (B) Model showing the heavy and light chain. Modified from Hood et al., (1984) and Silverton *et al.*, (1977).

1988) reported that this technique has the advantage of measuring cross-reactions between very distantly related groups such as the mammals and the amphibians.

Another detection method is the enzyme-linked immunosorbent assay (ELISA) where antibodies or antigens are coupled to an easily assayed enzyme. Horseradish peroxidase or alkaline phosphatase are commonly used as conjugate.

The principle of the ELISA procedures is to build up a three layered sandwich on the inner surface of each well in the microtiter plate (Fig. 1.14), each layer consisting of antigen, first antibody and finally the second antibody (attached to an enzyme). The resulting fluorescence is measured by an automated plate reader as a function of the antigene-antibody combining ability. ELISA may be used for assaying antigens either by a competitive or double antibody method or by assaying a specific antibody by an indirect method.

The immunological principles are commonly used because they have succeeded in a wide variety of applications, for example; phylogeny and diagenesis studies (Lowenstein 1980, 1981; O' Brian *et. al,* 1985) and the detection of macromolecules in fossils (De Jong *et. al.* 1974 ; Westbroek *et. al.* 1979, 1983 and Muyzer 1984, 1988). Collins *et. al,* (1988), (Fig. 1.15) used serotaxonomy to introduce an immunological-based classification of living Terebratulida. By extracting proteins from the secondary layer fibres, Collins proved two important points :

1. Indigenous protein material is found within the shell structure, protected by a mineral skeleton and not on the surface.

2. Serological data contradict some aspects of the morphologically based classification.

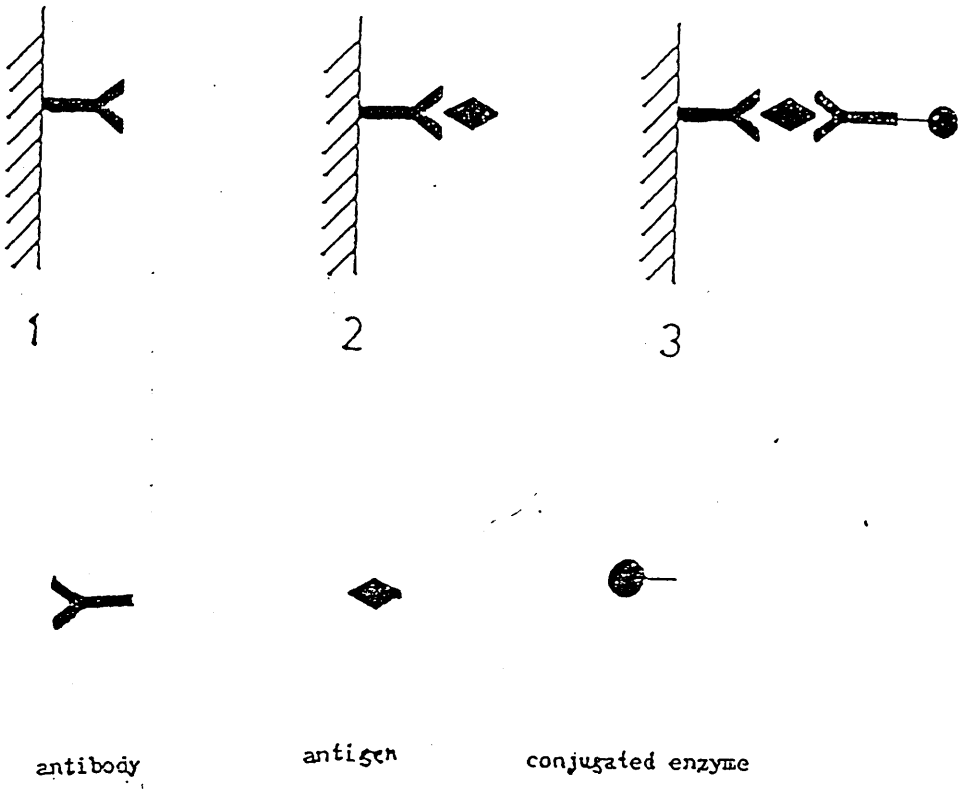

Fig. 1.14- ELISA techniques, digram showing the three layers on the microtiter plate. From Gaastra, W. In: Walker (1984).

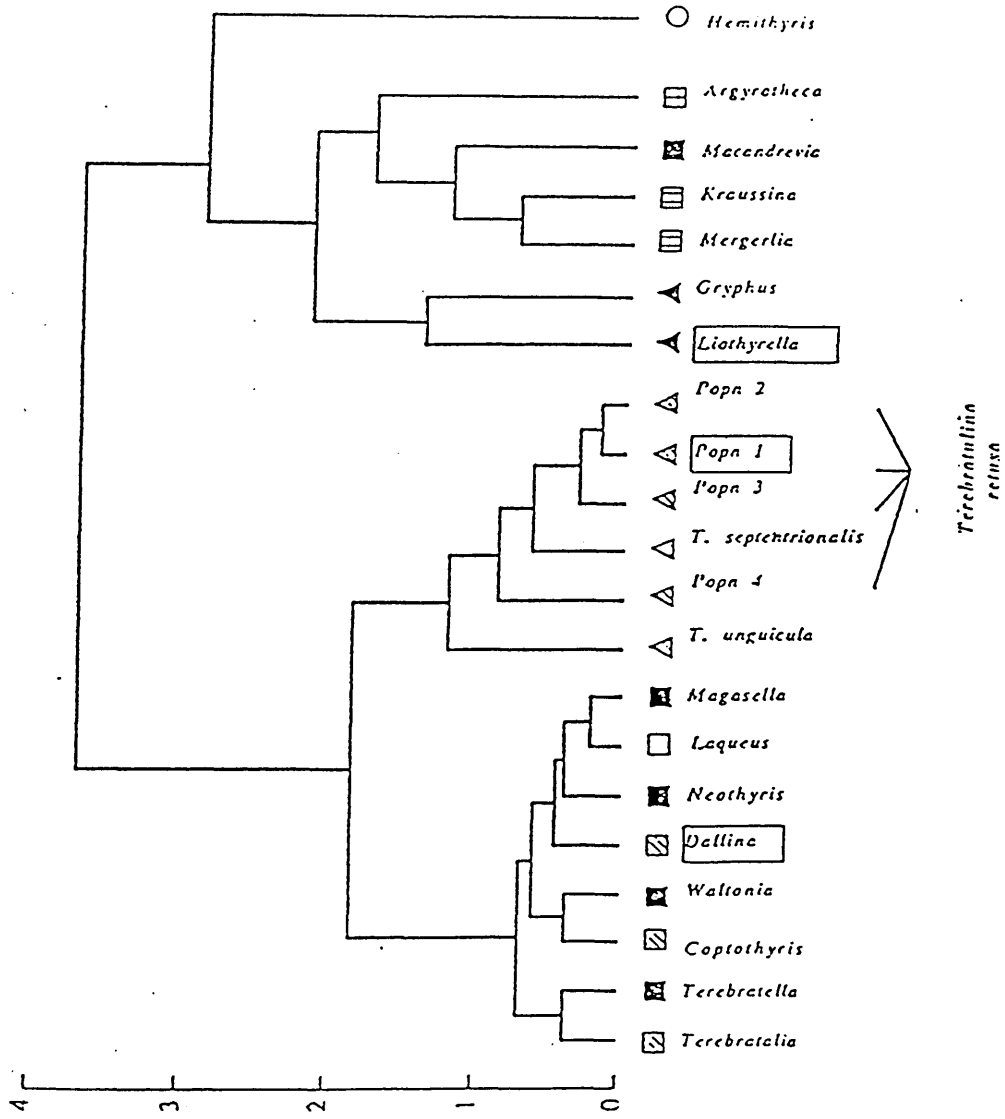

Fig. 1.15- Cluster analysis of some brachiopod genera based on serotaxonomy. Similar symbols indicate related groups. From Collins *et al.*, (1988).

1.7 - The Shell

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The shell is the most conspicuous part of a brachiopod, and consists of two dissimilar valves which enclose almost all the rest of the organism. In calcareous shelled articulate brachiopods up to 50% of the total tissue mass of an individual is within the shell (Curry & Ansell, 1986). The main function of the shell is to provide a protective armour for the organism. The shell has bilateral symmetry, in which the plane of symmetry runs through the two valves and not between them as in most bivalve molluscs. Very few shell shapes have been adopted by brachiopods, presumably for functional reasons. Shell growth is a chain of interlinked processes, which are biochemically regulated and the shell grows by accreting new material from the mantle at the valve edges. The ultimate form of any developing brachiopod shell is a product of the relative growth rates of different parts of the valve edge (Rudwick, 1959). The adult shell is effectively dead tissue which can have only repairs or additions to the inner layer, but during growth it takes an integral part in the basic metabolic processes of the animal.

The shell layers [periostracium, primary and secondary layers] are all secreted by the outer mantle epithelium in a pattern that changes as the shell grows (Jope, 1977). The outer protective coating, the periostracum, is composed of polysaccharide, protein, and some lipid. But this protein is highly tanned and usually poorly preserved. There is also a small amount of protein material in the primary calcareous layer, appearing as a fine threads or trails of microvilli. The main protein material is found within the secondary layer of the shell. This protein bears phylogenetic information (Collins *et al.*, 1988).

The protein in calcareous articulates appear as a cushioning matrix between the calcite fibres of the secondary layer (Terebratulida), or as a series of

alternating layers of hard dark coloured and softer light coloured proteins with different amino acid compositions (Rhynchonellida). The light soft layer is the main shell source of inter-crystalline proteins (Jope 1967 , 1971). New material is added continuously to the lower end of the fibre in the secondary layer by the surface of each cell.

The calcareous inarticulate *Neocrania* has a complex shell structure (Fig. 1.16), where there is a well developed three-layered brachial valve and a pedicle valve with different morphology and layer succession which results from its adhesion to a substrates (Williams & Wright, 1970) (See Fig. 1.9A). The ultrastructure of the three-layered *brachial valve* begins with the periostracium, which include mucopolysaccharide films segregated partially, by proteinaceous fibrils and sheets, into lenticular masses up to 9μm wide. The characteristic fabric of the following layer (primary layer) is composed of acicular crystallites, usually about 0.015μm thick. These crystallites usually starts off as isolated nuclei or seeds, the first formed seeds being separated by continuous protein sheets. The secondary layer is made up of laminae (tubular crystallites) which are separated from one another by monolayers of proteins. The spacing and thickness of the protein sheets vary with the growth of the valve.

The first layer of the *pedicle valve* to be secreted is a mucopolysaccharide layer which binds the valve to the substrates of cemented brachiopods. This layer forms an external film to the outer bounding membrane of the periostracium and is found to be squeezed against the substrate causing the flattening of the fibrillar extensions of the outer bounding membrane. The second layer to appear consists of small calcite rhombs which grow into platelets accumulated parallel to the periostracal surface to form a 2-3 μm thick layer.

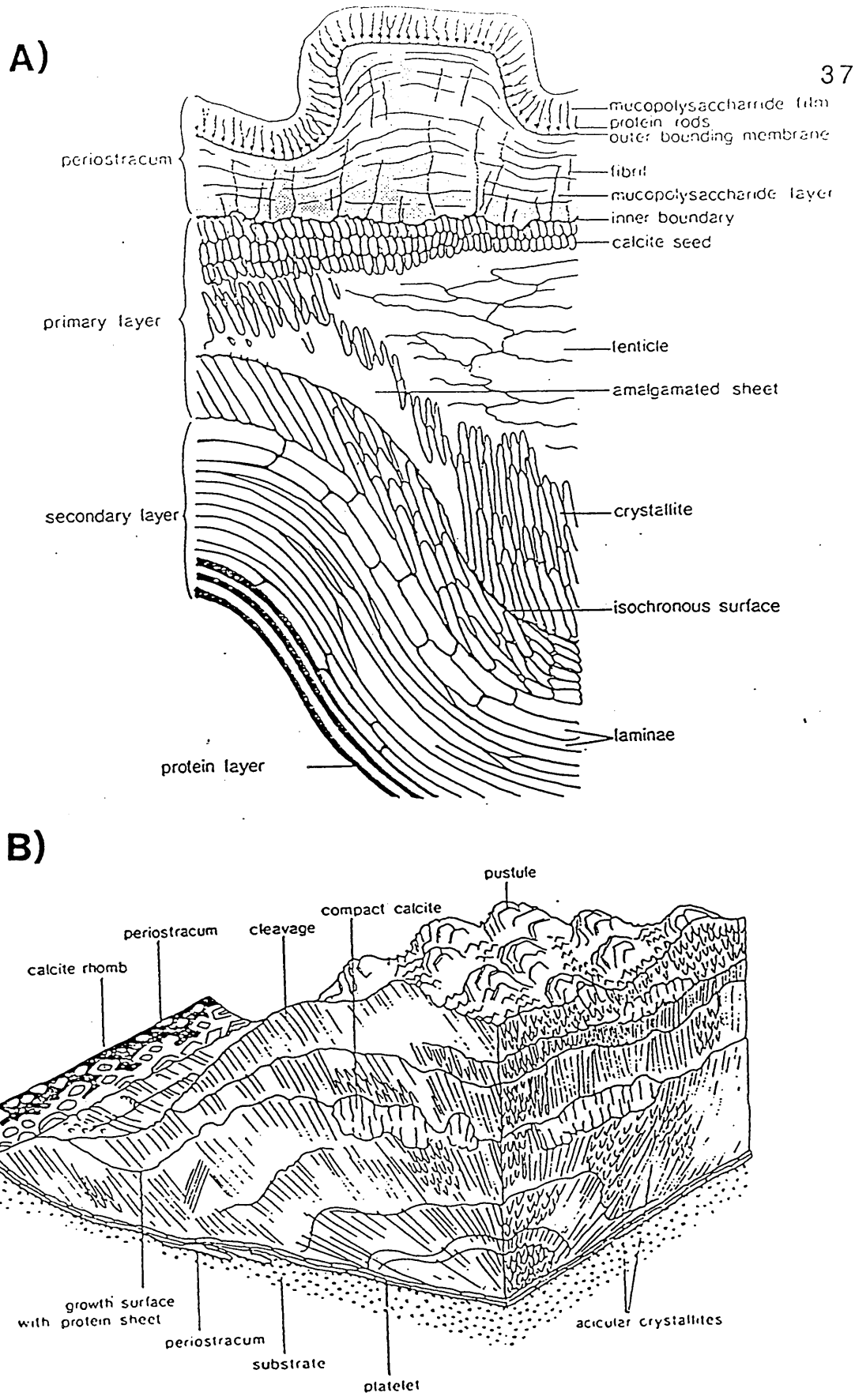

Fig. 1.16- Digrammatic sections of *Neocrania anomala* shell microstructure, A) brachial valve, B) pedicle valve. Modified from Williams and Wright (1970).

These flat lying platelets become the surface on which a series of needle-like crystallites set are secreted. These acicular crystallites may be enlarged radially or concentrically developing strong radial cleavages which are usually clothed in protein sheets. Throughout this layer small patches of compact calcite containing small quantities of proteins are found. These calcite lenses represent localized centres of calcite secretion.

The phosphatic shell of the Inarticulata has no three-layer structure, here the protein material is found as protein or chitino-protein layers 2.5 μm thick with varying mineralisation, and these layers are arranged in a series of laminae subparallel to the surface of the valve (Jope, 1971). The proteinaceous material within the shell is protected by a biomineral skeleton which hardens the shell structure.

CHAPTER TWO

Historical Background of Brachiopoda

2.1- Introduction

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Brachiopods classification have been a matter of controversy due to the long duration of their existance (Cambrian-Recent) which was disrupted by several extinction events. A review of these events along with their main causes will be presented later in this chapter (see section 2.4). Another important factor contributing to this debate is homeomorphy, a well known phenomenon in brachiopods. Therefore, before dealing with the electrophoretic patterns found in brachiopod shells, it is necessary to examine the geological history of brachiopods.

2.2- Brachiopod Phylogeny

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Brachiopods appear in the geological record at the base of the Cambrian, where they show a diversity indicative of a substantial Precambrian ancestry. A conflict of views regarding the phylogeny of brachiopods has surfaced recently between the proponents of the traditional monophyletic origin and the more recent polyphyletic view.

Williams & Rowell (1965) ; Williams & Hurst (1977) and Rowell (1982) all support the view which indicates that brachiopods are a distinct phylum and were developed from a single phoronid-like ancestor at the beginning of

Cambrian times, with close relations with other lophophorates (the phoronoid worms and bryozoans; Hyman, 1959). This view is built on the assumption that resemblances observed between the principale brachiopod lineages were clear indications of their monophyletic ancestry. These strong similarities include the lophophore, the trochophore-like larva, the location of the nerve ganglia in the mesosome and the possession of mantle canals. Rowell (1982) employed a cladistic analysis of living and fossil brachiopods to support his view of monophyletic origin and Eldredge & Cracraft (1980) accepted the phoronids as the "out group" regarding the relations between the major taxa of Cambrian and Recent brachiopods.

The proponents (Caven & Valentine, 1973) of polyphyletic origin argued that it is not possible to ignore the large and constant differences between the articulate and inarticulate stocks, and have suggested that the various early brachiopod lineages arose separately and independently from different groups of infaunal phoronid-like worms which developed an exoskeleton as an adaptation for epifaunal existence. This view leads to the belief that brachiopods are not a taxon by themselves but a grade in organization, and therefore should be grouped together with phoronids and bryzoans as Lophophorates (Emig, 1977a). Wright (1979) concluded that brachiopods were developed from a series of infaunal lophophorates that had already undergone diversification in the late Precambrian prior to emerging as epifaunal brachiopods with mineralized valves.

Neither argument could stand when examined, because none of the similarities or differences between brachiopod stocks can be used alone as basis to support one view rather than the other (Rowell 1982), which leaves the door open to look for new and convincing evidence to settle this argument one way or another.

2.3- Evolutionary History of Brachiopoda

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The brachiopods have an abundant fossil record which shows the effects of major extinction and radiation events. The earliest radiation event was in the Cambrian and led to great structural diversity, while the second radiation event took place during the Ordovician and resulted in the domination of calcareous skeletal material. There is no record of brachiopods in Precambrian sediments, yet the sudden radiation of this phylum in Cambrian times attracts various explanations. One of which indicates that members of the phylum developed an ability to secrete a mineralized skeleton and thats increased the chances of preservation into the fossil record. Another reason is the adaptation of new methods for feeding, in addition to the moderate climate following the severe glaciation in the late Precambrian. Rudwick (1970), suggested that radiation was influenced by ecological interaction with simultaneous development in other phyla, for example the emergence of carnivorous organisms early in the Ordovician times may have affected the brachiopod faunas as in the development of the quasi-infaunal and the burrowing habits.

During their history, brachiopods were distinct from any other phylum and expressed variable generic diversity (as seen in Fig. 2.1). In general, throughout the Cambrian Period, Inarticulata were more common and varied than the Articulates but neither class was very abundant until the subsequent Ordovician Period (Rudwick, 1970). The expansion of the phylum during the Ordovician was thought to be due partly to the ability of different groups to utilise carbonate instead of the much scarcer calcium phosphate (Rudwick 1970). The Silurian and the following Devonian were times of consolidation for the brachiopod fauna. There were few changes in the fauna and most of the genera reached their peak of diversity. Towards the end of the

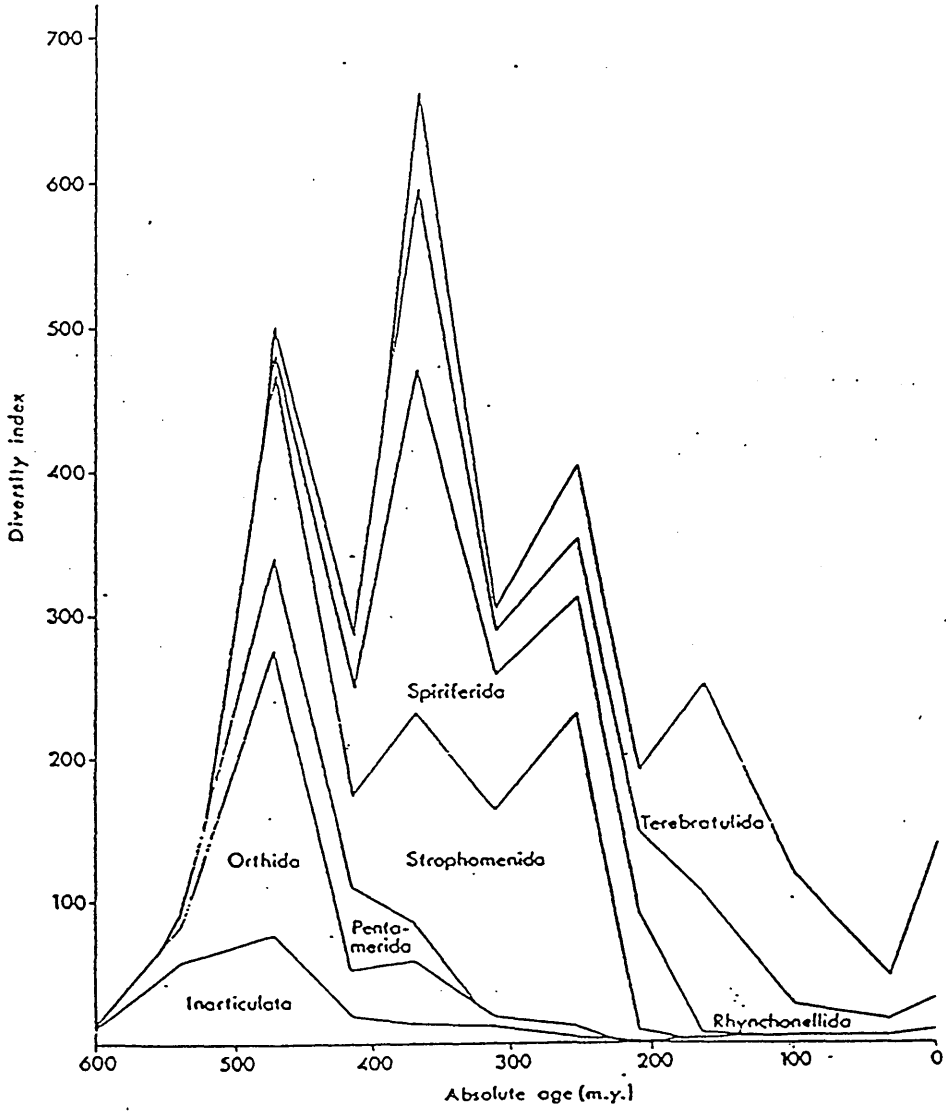

Fig. 2.1- Variations in generic diversity of Articulata and Inarticulata through Phanerozoic time. From Williams and Hurst (1977).

Devonian, the steady expansion and radiation of the phylum were brought to an end by a sudden wide spread extinction (Copper 1966). This event did not affect all groups equally. Indeed some groups survived and flourished later and some of these survivors were particularly characteristic of the reef environment.

The Carboniferous period was a time of abundance for brachiopods where the fauna experienced some significant development in feeding and habitat modes. Sedimentation in the late Carboniferous(especially in northern hemisphere) was mainly coal-swamp deposition, therefore brachiopods of this age were not so well known since they prefer to live in strata representing occasional marine incursions.

During the late Permian - early Triassic the phylum suffered its greatest and gradual decline in its history. Whole orders disappeared completely leaving only a few rare survivors. This mass extinction did not only affect the brachiopods but many other phyla of marine invertebrates. Triassic faunas are poorly known, largely because they were limited to certain parts of the world and confined to deep water environments and to sediments which were later involved in large tectonic movement. These conditions made the fauna vulnerable, so they became extinct at the end of the Triassic.

From the Jurassic until the present times, the characteristics of the fauna have changed a little and only a few representatives of the brachiopod fauna survived to the late Mesozoic. The end of Cretaceous Period marked a time of crisis in several phyla, although brachiopods were relatively unaffected by the Cretaceous-Tertiary extinct in terms of abundance and diversity (except at the generic level). In fact, the terminal Cretaceous extinction was a selective event, because it did not affect most of the small land vertebrates and the

fresh water communities. However, it must be emphasized that adaptation to new environments and modes of life helped the brachiopods to survive the extinction.

2.4- Extinctions and the Brachiopod Fossil Record

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There is a controversial debate over the brachiopod fossil record and whether it proceeds gradually or in jumps. The number of these extinction or replacement events is highly debatable, a group of palaeontologists suggest that brachiopod faunas have suffered several mass extinction events (Alvarez 1980, Alvarez 1984 and Surlyk 1971), others accept the less catastrophic view but recognizing two main extinction events in the brachiopod record, the late Devonian and the Permian-early Triassic extinctions, and arguing that most of these other 'events' were man made artefacts (see Ager 1988) produced by the way the samples and the information obtained from them have been handled. Alvarez *et. al,* (1984) suggested the Cretaceous-Tertiary extinction event in brachiopod record had an extra-terrestrial cause.

One of the main causes of confusion here is the over classified phylum. The brachiopod phylum has been subjected to several classification schemes which has led to great confusion regarding the numbers of subdivisions. Since the Treatise was published, a great effort has been made to adapted the phylum to every new classification scheme by adding or deducting few groups which led to artificial events. Palaeontologist from all over the world are trying to address this problem and find an acceptable solution.

The reasons for these extinction events are uncertain but in general the causes of such extinction events which have been proposed may be categorized into three groups; Firstly, *the earth bound causes (terrestrial)*

which include the falls in sea level, climatic changes and increased volcanicity. Sea level regression will alter the environmental stress by affecting the habitat of these marine invertebrates. Reducing the habitat area will certainly led to crowded habitat, increased competition and lower diversity. The correlation between extinction events among the Phanerozoic marine invertebrates and the changing in sea level was first made by Newell (1967) and accordingly he distinguished six extinction events, end-Cambrian, end-Ordovician, late-Devonian, end-Permian, end-Triassic and end-Cretaceous. Changing in the sea level may be caused by tectonic (continental movement) or melting and freezing of the polar ice.

Stanley (1984, 1987) put forward his temperature control hypothesis and argued that changes of temperature in marine regions are the key control of extinction events, for example, the end Permian extinction that took place during an (elevated) moderate temperature climate. In fact this event could be induced by temperature rising (Hallam 1990). Volcanism is another terrestrial cause, which accounted for some drastic events among marine invertebrate but not brachiopods. Volcanism cause the injection of large quantities of gases into the earth's surface which in turn will produce, intense acid rain, reduce the alkalinity and pH of the ocean, change the ozone layer and cooling of the global atmosphere. The Cretaceous-Tertiary extinction is a good example of such joint effects. Loper and McCartney (1986) mentioned the correlation between increased volcanism and changes in the geomagnetic field at the end-Cretaceous time. However, non of the three terrestrial causes can stand alone as a major cause in extinctions through the geological times. Fischer (1984) linked the effect of change in the sea level, climate and volcanicity to be accounted for mass extinction during the Phanerozoic.

The second suggested cause for mass extinction is the *extra-terrestrial.*

Variable solar heat output, collision with comets and asteroids, immense solar flares are some of the extraterresterial causes for mass extinction. Extraterrestrial impact will generate global darkness (due to the cloud of fine dust) accompanied by cold climate, followed by the greenhouse effect and global warming which results in nitric acid rain. Intense acid rain will damage the land foliage and in the long term lower the pH of the ocean enough to dissolve calcite and stress calcareous animals, all this will lead to disintegration of the marine and terrestrial food chain. Jablonski (1990) explained that extraterrestiral impact theory requires more research to exploit its potential explanations for these events in the fossil record. However, Alvarez (1986), Alvarez (1987) and Hallam (1987) presented some physical evidence to support this theory, which include the discovery of anomalous concentrations of iridium world wide at the Cretaceous-Tertiary boundary, which may indicate an extra-terrestrial cause. Mclaren (1970) in his interpretation of the sudden Devonian event, suggested that mass extinction could be induced by the impact of extraterrestrial body.

Thirdly, *periodicity* was suggested as a possible cause for mass extinction. Periodicity means that through the geological times extinction events took place at regularly spaced intervals (cyclicity). This theory, based upon statistical analysis of fossil record, was proposed first by Fischer & Arthur (1977) and Raup & Sepkoski (1984), the latter two reported a 26 million year periodicity in extinction of marine families. Volcanism and extra-terrestrial causes have been proposed for the periodicity in extinction.

In conclusion, most of the brachiopod extinction events in their earlier record can be interpreted as an ecological replacement of some group with more adaptive group, except the Permian-Triassic event which affect all the marine populations including the well adapted forms such as the Strophomenida.

CHAPTER THREE

MATERIALS AND METHODS

3.1- Materials

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3.1.1- Sample Collection

Specimens of *Terebratulina retusa* and *Neocrania anomala* were obtained by dredging in the Firth of Lorne between the island of Kerrera and the west side of the isle of Mull (west coast of Scotland) near Oban. After initial cleaning the animals were frozen alive. Other samples of living *Terebratulina retusa* has been obtained from Loch Fyne, Scotland(See appendix 3 for sampling location). Fossil brachiopod samples from New Zealand (see Table 3.1) were provided by Dr. M.J. Collins (University of Bristol).

3.1.2 - Production of Antisera

Antisera were raised against different brachiopod taxa to test the reactivity of the antigens prepared from representatives of major brachiopod classes, ie. *T. retusa* and *Neocrania anomala*. These polyclonal antisera were raised in New Zealand white rabbits (see Harlow and Lane 1988 for antibody production protocol). Table 3.1 shows a list of thirteen antisera used in immunostaining procedure which will be explained later in the method section (see section 3.6.3).

Table 3.1-antibodies preparation used in immunostaining

Antisera	Location	Titration
Terebratulina retusa.	Firth of Lorne, Scotland	1:40 000
Liothyrella uva	Antarctic Peninsula, Antarctica	1:40 000
Liothyrella neozelandica	Fyordland, South Island, New Zealand.	1:500
Dallina septigera.	Rockall Trough, off west Scotland	1:40 000
Notosaria nigricans.-	Karitane, near Dunedin, South Island, New Zealand.	1:500
Waltonia inconspicua.- 0.6 Ma.	New Zealand-Kupe formation	1:500
Terebratulina crossi.	Ostuchi, Japan	1: 80
T. septentrionalis.	Bay of Fundy, West Atlantic (Canada)	1: 800
T. unguicula.	USA- East coast of Pacific (Oregon)	1: 5 000
Laqueus rubellus	Sagami Bay, Japan	1: 10 000
Pictothyris picta	Sagami Bay, Japan	1: 5 000
Neothyris lenticularis .	New Zealand	1: 150
Pachymagus sp.- 22 Ma, Miocene.	Gee greensand, Oamarn, South Island, New Zealand.	1:500

3.2 - Methods

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3.2.1. - Sample Preparation

3.2.1.1 - Whole Shell Fibre Preparation :

The shells were first scrubbed and rinsed with water to remove all the surface epifauna, then immersed in a dilute solution of sodium hypochlorite (5% for recent shells, 10% fossil shells) for 24 hours to weaken the organic shell matrix. They were then scrubbed again thoroughly and rinsed with Milli Q ™ water, sonicated a few times, and left to dry at room temperature.

Shells were disaggregated using a mortar and pestle or, for hard shells a crushing machine. The shell powder was then washed in NaOCl (1% for recent material, 10% for fossil material) overnight with continuous motion, which separates the fibres from the primary shell layer because of their differential suspension in water. Fibres were then collected, rinsed in Milli Q ™ water and sonicated several times until all bleach was removed. The fibres were then lyophilised, weighed and stored.

3.2.1.2 - Extracting Organic Material From Fibres :

Fibres were decalcified by stirring with 20% (w/v) EDTA at 4°C for at least 48 hours, in the ratio of 40 g/1L of 20% EDTA (pH 8). Samples were then centrifuged for 20 minutes at 4^{o}C (25000 rpm), and the EDTA-Ca complex was removed using the Mini-Tan ultrafiltration system which filters the sample across several filters which allows EDTA through but retains the organic molecules of molecular weight greater than 10kDa. The sample is then subjected to further concentration in a Mini-Con cell down to 10-20X. This

concentrate is then stored at -20^{o}C in eppendurf tubes.

3.2.1.3 - Whole Body Tissue Homogenates :

To release the proteins and enzymes from the body tissues, each animal was individually homogenized in 200 µl of 0.5M tris buffer pH 6.8. The homogenate was stored in the freezer at -20°C in eppendurf tubes. Prior to the sample application, the homogenate was centrifuged for 2-3 minutes and the supernatant was applied to the gel.

3.3 - Electrophoresis

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3.3.1- Tris-glycine-SDS PAGE

SDS-PAGE was carried out in a BIO-RAD mini protein II dual slab cell using the discontinuous buffer system of Laemmli (1970). The mini cell uses small gel of size 7cm. X 8cm X 0.75mm thickness.

The separating gel (12 -15) % acrylamide was polymerized by adding 50 µl of 10 % APS and 7.5 µl of Temed per 15 ml. of solution. The separation gel mixture was poured between the glass plate sandwich, covered with isopropyl alcohol or methanol to level the gel surface, and then left for 20-30 minutes to polymerise. After setting the separating (resolving) gel, the alcohol was removed and the space for the stacking gel rinsed with distiled water and dried. The stacking gel mixture was then poured over the polymerized separating gel. The stacking gel (4 % acrylamide) polymerized due to the addition of 50 µl of 10 % APS and 10 µl of Temed per 10 ml.of solution. The ammonium persulfate (APS) yields a persulfate free radical which activates the temed. The temed in

turn acts as an electron carrier to activate the acrylamide monomer. A comb of 10 wells was inserted into the gel mixture in order to create the lanes where the samples were to be loaded, and the gel was left to polymerize for 20 minutes. These polymerized gels can be kept for a week in a fridge well wrapped in cling film and aluminium foil.

The protein samples were made up in a solvent containing : 2% SDS; 12% glycerol ; 25 M tris-HCL pH: 6.8; 0.2M DTT; and pyronin Y as a dye marker. During this procedure the protein mixture is denatured and disulphide bonds are cleaved by heat in the presence of a reducing agent such as Dithiothreitol (100 °C for 5 minutes in a water bath). In such conditions proteins bind the anionic detergent SDS yielding molecules with approximately uniform charge per unit of protein. This uniformity with strong negative charge on the molecules results in similar electrophoretic mobility, although the rate of migration depends on the size of the unfolding molecule. Samples were loaded in duplicate and 10-20 µl of the samples were loaded in each lane. A mixture of marker proteins were run in parallel with the samples. Marker proteins and their correspondent molecular weights are listed in Tables 3.3, 3.4, 3.5.

The electrode buffer used with the tris-glycine gel was 1% (w/v)SDS, 0.025 M tris-HCl buffer, 0.9M glycine. The two gel sandwiches are clamped together to form the upper chamber, while the mini cell tank represents the lower chamber and is filled with electrophoresis buffer to cover only the lower end of the glass plates. The upper chamber is filled with transfer buffer above the rim of the shortest glass plate. The electrophoresis of the samples needed a constant voltage setting of 200 V for 40-45 min. approximately. The separation of the macromolecules (proteins) by the gel electrophoresis is accomplished by the sieving action of the porous polyacrylamide gel and is based on the differences in the sizes of the proteins (ie. molecular weight). Details of the composition of

the gels are presented in Table 3.2.

Table 3. 2 SDS-polyacrylamide gel composition used in mini protein Bio-Rad system.

A- Separating gel solution (tris-glycine) for two 0.75 mm thick gels

% acrylamide	12 %	15 %
Distiled water (ml)	3.35	4.3
Acryl-bisacrylamide (ml)	4.0	7.5
1.5 M Tris-HCl (ml)	2.5	3.0
10% SDS (ml)	0.1	0.15
10% (w/v) APS (µl)	50	50
Temed (µl)	5.0	7.5

B - Stacking gel solution (4% acrylamide)

DDW	6.1 ml.
Tris-HCl pH: 6.8	2.5 ml.
10% (w/v) SDS	0.1 ml.
Acryl-bisacrylamide	1.3 ml.
10% APS	50 µl
Temed	10 µl

3.3.2 - Tricine-SDS PAGE

The same Bio-Rad apparatus was used to run this kind of gel, where the glycine in the Laemmli system was substitute with tricine as the trailing ion, following the work of Schagger *et al*, 1987. This system allows improved resolution of small proteins at lower acrylamide concentration. The procedure is identical to that described above (see section 3.2.1). However the separating gel (10% T 3%C) polymerization was initiated by using 75µl of 10% (w/v) APS and 7.5µl of Temed per 10 ml solution. The stacking gel (4%T 3%C) polymerized by the addition of 40µl of 10% APS and 7.5µl Temed.

In addition, different buffers were used for Tricine-SDS gels as the upper cathode chamber was filled with buffer pH 8.25 (0.1 M tris, 0.1 M tricine, 0.1% W/V SDS), while the lower anode chamber was filled with buffer pH 8.9 (0.2 M tris).

The running condition for the Tricine-SDS gel electrophoresis is a constant 100V using the 200/2.0 power pack (Bio-Rad). It is also possible to produce a better molecular separation regarding band sharpness when the electrophoresis is run at 50-70V for a longer period. The compositions of the tricine-SDS polyacrylamide gel are explained in the following Table 3.3.

Table 3. 3 - Tricine -SDS polyacrylamide gel system used in mini-protein Bio-Rad system.

acrylamide solution	48 g. acrylamide
	1.5 g. bisacrylamide
gel buffer	3 M Tris
	0.3% (w/v) SDS
separating gel (10 % acrylamide)	2 ml. acrylamide solution.
	3.3 ml gel buffer
	1 ml. 98% glycerol.
	2.75 ml DDw
	75 µl 10% APS
	7.5 µl Temed.
stacking gel	0.35 ml acrylamide solution
	1.03 ml gel buffer
	DDW
	40 µl 10% APS
	7.5 µl Temed.

3.4 - Measurement of Molecular Weight by Electrophoresis on Tris-glycine-SDS- PAGE and Tricine -SDS- PAGE

The molecular weight of a protein is determined by comparing its relative mobility (Rf) with the relative mobility of the standard marker protein, where both are run in the same gel.

Rf = distance of protein migration/distance of tracking dye migration.

Two different sets of standard molecular weight proteins were used during the course of this study to accommodate both the high and low molecular weight ranges. Sigma, Bio-Rad and BRL (Bethesda Research Laboratories) molecular weight protein standard kits were applied (in different lanes) to the gel alongside the samples. Each kit is a mixture of known molecular weight proteins, and the components of each set are listed in Tables 3.4, 3.5, and 3.6. A calibration curve is plotted using the relative mobilities of the standard molecular marker run on the same gel against the log values of their molecular weights. Two calibration curves were obtained for the two kinds of gels (Fig 3.1) with a correlation coefficient of 0.99.

Table 3. 4 -Bio-Rad molecular weight standards.

Proteins	Molecular weight
Rabbit muscle phosphorylase b	97,400
Bovine serum albumin (BSA)	66,200
Hen egg white ovalbumin	45,000
Bovine carbonic anhydrase	31,000
Soybean trypsin inhibitor	21,500
Hen egg white lysozyme	14,400

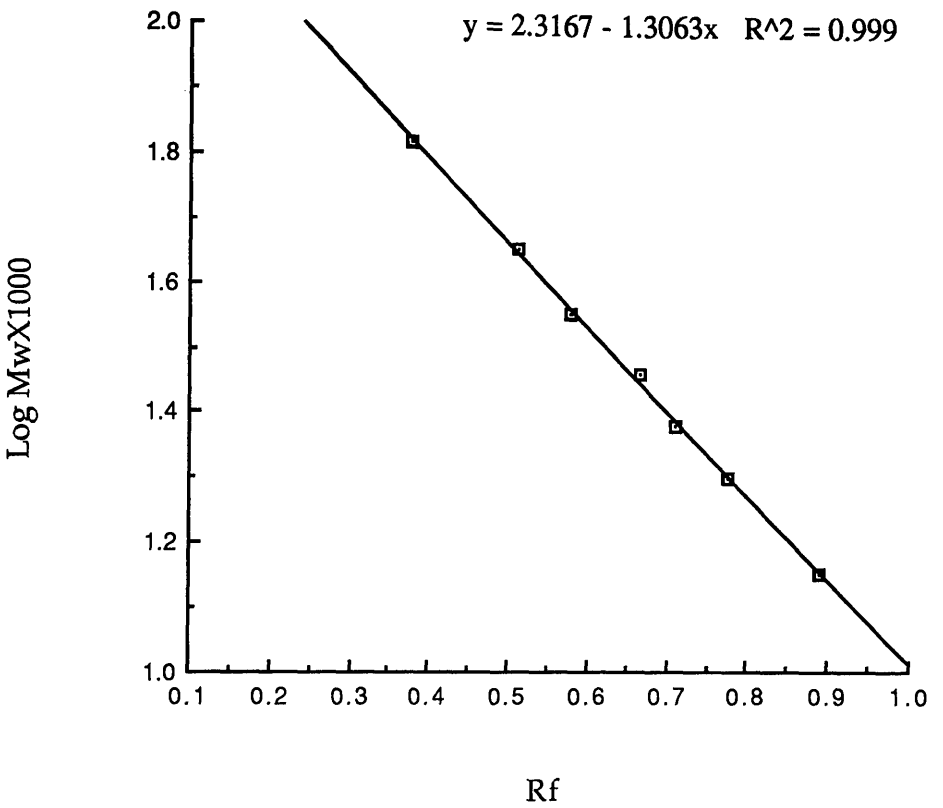

Figure 3.1- Calibration curve of the electrophoretic mobility of SDS protein standards as a function of molecular weight (log 10) of the proteins.

Rf = distance of protein migration/distance of tracking dye migration.

Table 3. 5 Sigma standard molecular weight proteins.

Proteins	Molecular weight
Albumin, bovine	66,000
Ovalalbumin	45,000
Glyceraldehyde-3-phosphate dehydrogenase carbonic anhydrase	36,000
Carbonic anhydrase	29,000
Trypsinogen	24,000
Trypsine inhibitor	20,100
Lactoalbumin	14,200

Table 3. 6 BRL protein molecular weights standards.

Proteins	Molecular weight
Ovalbumin	43,000- 43,525
Carbonic anhydrase	29,000-29,550
B- Lactoglobulin	18,400-18,700
Lysozyme	14,300-15,500
Bovine trypsin inhibitor	6,200-5,900
Insulin (A and B)	2,300-2,925

3.5 - Electroblotting Transfer

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3.5.1- Wet Electroblotting Transfer

A replica of separated proteins on SDS-PAGE was transfered to different blotting membranes (PVDF, ProBlott) using the Bio-Rad mini trans blot cell. The driving force in this kind of cell is the voltage applied over the short distance (4cm) between the electrodes, which allows the generation of high driving forces to produce an efficient transfer. The mini trans blot cell includes a cooling unit to absorb the heat energy generated during the transfer application.

Following SDS-PAGE the gel is equilibrated in transfer buffer (25 mM Tris, 20% v/v Methanol, 192 mM glycine, pH 8.3) for 15 minutes in order to remove the electrophoresis buffer salts and detergents. Meanwhile the membrane and 10 filter papers (3 Mm Whatman) were cut to the size of the gel. The orientation of the membrane was marked at the upper right hand corner. The membrane was then wetted (for Problott membrane use 100% methanol), and kept in transfer buffer. Filter papers, and supporting sponges were immersed in transfer buffer for 5 minutes. The transfer sandwich was assembled according to the following order starting from the clear panel of the transfer cassette (the anode); supporting sponges, filter papers (5), membrane, polyacrylamide gel, filter papers, supporting sponges. Air bubbles between layers were removed by rolling a glass rod or pipette over each layer, in order to get a close contact between the different layers which ensures complete transfer. The components of the transfer sandwich require wetting with a few millilitres of transfer buffer

during their stacking. The sandwich was then assembled in the transblot cassette.

This cassette was placed in the tank with approximately 300ml of transfer buffer to cover the upper rim of the shortest glass plate in the sandwich. A complete transfer was accomplished using 40V for 2-3 hours, but with Tris-glycine gels 100V for one hour was used. After the transfer was completed, the sandwich was disassembled and the membrane was rinsed with water before staining.

3.5.2- Semi-Dry Blotting

The trans-blot semi dry electrophoretic transfer cell from Bio-Rad was used to obtain a copy of the protein pattern in the SDS-PAGE. Nitrocellulose membrane, PVDF and Problot membranes (both from Applied Biosystems) were also used with the Tris-Glycine transfer buffer (section 3.1.2.4). The membrane and filter papers were cut to gel size, soaked in transfer buffer for few minutes and the gel was equlibrated as mentioned before (see section 3.5.1). In this kind of blotting, a sandwich of filter papers and polyacrylamide gel were assembled between the bottom plate (anode) and the upper plate (cathode). A close contact between the gel and the membrane is achieved by excluding any air bubbles and saturating the layers with drops of transfer buffer. The transfer was achieved using 15V (5.5 mA/ cm2) for 30-45 minutes.

3.6 - Detection of Protein Molecules

****

The separated protein bands on the gel were visualized by applying one or two of the following methods:

3.6.1 - Coomassie Brilliant Blue Stain (CBB)

Coomassie Brilliant Blue R 250 (CBB) was used in this work to reveal the total composition of proteins on the gel and to determine the position of molecular weight standards.

After electrophoresis, the polyacrylamide gel was incubated in 0.05% (W/V) CBB in aqueous methanol-acetic acid solution (Weber & Osborn 1972) for one hour with gentle shaking. The dye forms a noncovalent complex with the proteins, through an electrostatic bond with NH_3^+ groups and by Van Der Waals forces. The dye-protein complexes will be insoluble in the gel and the excess dye is removed by diffusion against several changes of destaining solution (750 ml. methanol, 250 ml. acetic acid, 1.5 L. distiled water) to give a clear background. The gel was then dried on a filter paper using the Bio-Rad gel dryer. The moisture in the gel was absorbed by applying heat (80°C) for two hours under vacuum pressure.

3.6.2 - Silver Stain

The silver stain method of Morrissey (1981) demonstrates a higher level of sensitivity than CBB. The responsible reactions include the reduction of silver from the ionic to the metallic form which precipitate in gels. In order to estimate the efficiency of the transfer, gels were stained with silver stain after transfer.

3.6.3- Immuno-detection

Immunodetection is used after electroblotting to reveal proteins by staining with antibodies. Blotting membranes bind the transferred proteins nonspecifically through a non-covalent bond.

The immunoassay procedure can be outlined in the following steps:

1. The non-specific binding sites on the membrane were blocked with 2% ovalbumin / TBS to prevent the adsorbtion of the immunological reagent. The blot was incubated for 45 minutes at room temperature or overnight at 4°C with gentle agitation. Blots were then rinsed three times for 10 minutes with TBS/Tween with gentle agitation.

2. The unlabelled primary antibody, diluted basically to 1/500 with 0.1% OVA/TBS, was applied to bind the antigen on the membrane. To achieve this, the blot was incubated with the primary antibody for 2-4 hours at room temperature using orbital shaker to provide gentle agitation. Brachiopod antibodies were diluted according to their titration rate when possible (Table 3.1). The blot was washed again for 10 minutes with TBS/Tween solution .

3. The primary antibodies directed against brachiopod molecules have been attached to any of these antigens in the membrane. To reveal the positions of these antibodies the blots were incubated with a second antibody which binds to the primary antibody. In this case goat anti-rabbit antibodies are used, as the brachiopod antibodies were produced in rabbits. The blots were incubated with Goat anti-rabbit sera (GAR), mixed with alkaline-phosphatase, diluted 1/1000 with 0.1 OVA/TBS/Tween for 2 hours at room temperature or overnight at 4°C. After repeated washing of the blot with TBS/Tween, the AP-substrate is

added to initiate the colour reaction, which will take 5-15 minutes for the final colour development. The blot was then washed with distiled water and left to dry at room temperature.

3.7-Amino Acid Analysis of Brachiopod Body Tissue and Shell Proteins

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An automated Applied Biosystem 420 H amino acid analyser is currently used for the analysis of brachiopod body tissue and shell protein extract. This system consists of three machine components designed to carry out the main analysis steps under Argon which is used as an inert medium and to pressurise the sample lines. The first unit is the **420 H** which hydrolyses the samples using 6N HCL at 165^{o}C for one hour and then derivitizes the amino acids using the coupling reagent 5% Phenylisothiocyanate (PITC) with n-heptane in the presence of Diisopropylethylamine (DIEA) vapour. The PTC-amino acids are then transferred to a reverse phase hplc **130 A** using 29 mM sodium acetate (pH 5.0) transfer buffer made up from; 3M sodium acetate, pH 3.8 and 3M sodium acetate, pH 5.5 with 0.01% tripotassium EDTA, all in Milli Q^{TM} water (Millipore). Peaks corresponding to amino acids are detected by UV absorbence. The solvents for the mobile phases are, solvent A (50 mM sodium acetate buffer, pH 5.4) and solvent B (70% acetonitrile, 32 mM sodium acetate, pH 6.1, made up in Milli Q^{TM} water). The data are collected and analysed by a **920 A** computer. Programming of each unit allows synchronisation between hplc, data analysis, derivatization / hydrolysis and the automated analysis of up to 72 samples.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1- Introduction

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In this chapter the results obtained using the two kinds of polyacrylamide gel electrophoresis (PAGE) are presented and analyzed. Initially, this study concentrated on the proteins within skeletal fibres in both fossil and living brachiopod shells. Samples of fossil brachiopods are less readily available, but abundant living specimens were available. Initial extraction of shell proteins was achieved using the Amicon ultrafiltration system. This system separated intracrystalline proteins from the EDTA solution used to demineralize the shell (see section 3.1.2) and the resulting samples were stored at -20°C. However, after a period in the freezer these samples turned viscous and did not reveal any bands when examined by SDS-PAGE, suggesting that these samples still contained some EDTA. Subsequently a new filtering and cleaning system, the Minitan™ cell from Millipore was used and this gave excellent results (see section 3.1.2).

4.2- Results

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4.2.1- Molecular Weight Measurement

4.2.1.1- Molecular Weight of Living Brachiopod Body Tissue

SDS-PAGE reveals a distinctive pattern of proteins for each brachiopod class (see Fig. 1.4). The body tissue homogenates of living *Terebratulina retusa* and *Neocrania anomala* display several protein bands (indicating heterogeneous samples) ranging between 6kDa - 200kDa. Some of these bands, especially the low molecular weight bands, became degraded after prolonged storage (i.e. 1-1.5 year). The molecular weight of the separated bands were calculated from the standard calibration curve (see section 3.3). *Terebratulina retusa* body homogenate contains 7-17 bands with varying staining intensity in the gel, while *Neocrania anomala* revealed 8-14 of such bands. Calculated molecular weights of the separated body tissue bands are shown in Tables 4.1 and 4.2.

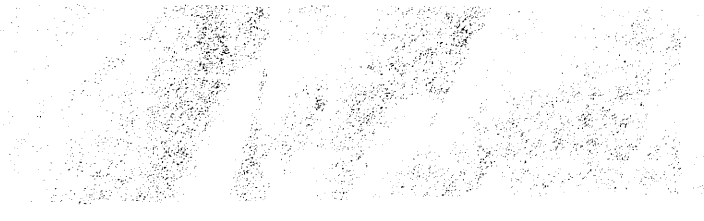
4.2.1.2.- Molecular Weight of Living and Fossil Brachiopod Shells

Recent brachiopod shell samples were analyzed by SDS-PAGE and the molecular weights of their protein bands calculated using a standard calibration curve. The extract of *Terbratulina retusa* intra-crystalline shell fibres reveals only one protein band with estimated molecular weights ranging between 29-36 kDa, but the *Neocrania anomala* shell extract did not show any band when examined with PAGE. Recent *Liothyrella uva* shell displays three bands ranging between 65kDa-99kDa, but *Terebratalia transversa* shell fibre extract gave two bands only with molecular weight of 57 kDa and 54kDa

respectively.

The samples of recent *Neothyris lenticularis* shows three bands in 54 - 69 kDa molecular weight range, and the fossil *Neothyris* samples also contain three bands ranging between 48-62kDa. Recent *Waltonia inconspicua* shell proteins reveal four bands with molecular weight range of 8-46 kDa, and the fossil *Waltonia* shows two protein bands with 47 kDa and 36 kDa in molecular weight.

A few shell protein preparations were also extracted from recent coral shell and fossil *Pachymagus sp.* shell, and analysed with SDS-PAGE to determine the molecular weights of any proteins detected. The fossil *Pachymagus* secondary shell extract contains three protein bands between 9-69 kDa, and the coral samples contain four very dense protein bands spanning from 43 kDa to 58 kDa. Calculated molecular weights of protein bands extracted from the secondary shell fibres of different brachiopod shells are shown in Table 4.3.



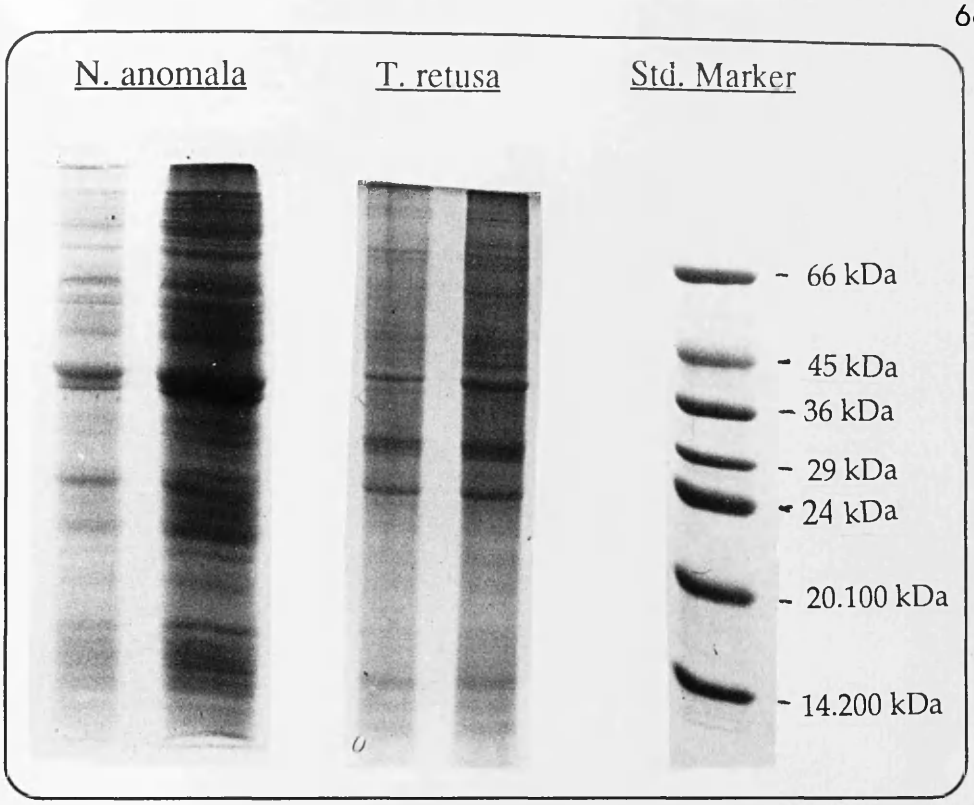


Fig. 4.1- Protein bands from the body tissue of the brachiopds *T. retusa* and *N. anomala* separated by SDS PAGE.

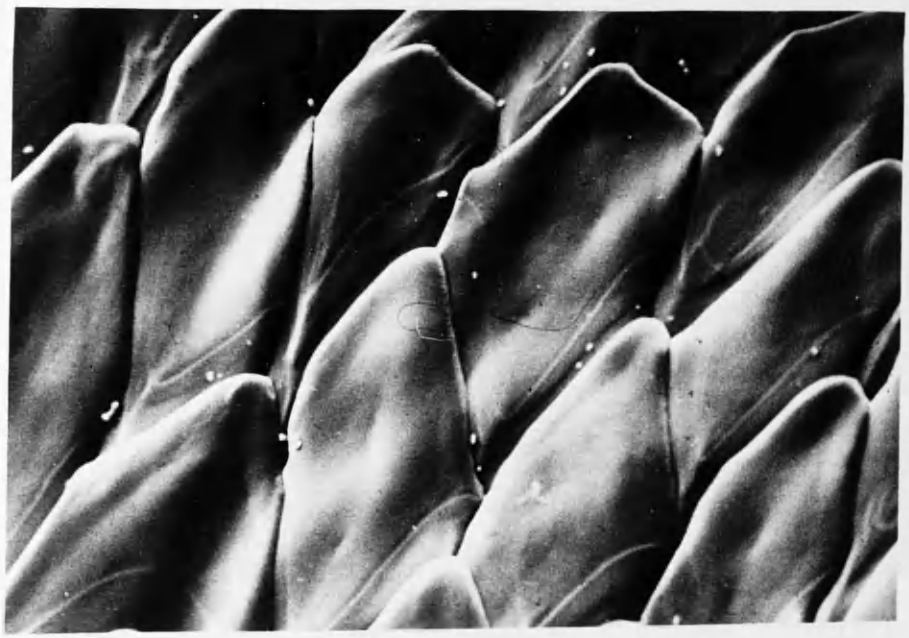


Fig. 4.2- An inside section of the secondary layer from brachiopod shell (*Notosaria nigricans*) under SEM. The section shows calcite fibers from which intracrystallin proteins are extracted.

Table 4.1 Calculated molecular weight in kDa of *Terebratulina retusa* body tissue protein bands.

Sample Number					
S1 *	S2 *	S3 *	S4 "	S5 "	S6 "
97.683	89.782	73.617	123.521	162.514	155.970
82.313	86.553	70.326	95.076	147.601	155.720
74.853	81.759	51.179	73.181	134.056	92.500
57.714	77.387	44.616	47.256	86.671	43.860
46.665	73.250	35.580	27.997	78.481	32.540
38.686	65.493	32.470	18.139	71.279	26.010
33.548	51.511	18.799	13.962	25.712	20.800
29.165	46.150		8.693	21.210	15.430
	38.348			17.495	13.290
	35.566				
	33.034				
	22.001				
	18.964				
	15.758				
	15.242				
	14.118				
	13.610				
0.992	0.981	0.995	0.995	0.999	0.988

(*) samples were run in Tris- glycine gels.

(") samples were run in Tris- Tricine gels.

Table 4. 2 Calculated molecular weights in kDa of *Neocrania anomala* body tissue.

Sample Number						
N1	N2	N3	N4	N5	N6	N7
195.110	182.600	131.780	215.194	74.520	94.053	190.651
167.560	147.300	119.240	177.644	50.561	83.600	160.478
155.040	86.600	102.900	141.421	46.426	70.150	112.952
105.500	67.700	88.740	121.371	29.700	59.018	99.260
84.080	41.020	72.820	100.193		55.715	91.068
61.820	33.100	65.890	79.763		41.555	73.181
42.190	14.080	54.070	71.168		34.961	39.778
22.880	10.300	46.640	41.730		23.239	19.771
21.170		42.300	33.221		16.406	
Correlation Coefficient						
0.988	0.985	0.982	0.986	0.976	0.977	0.995

Table 4.3 Molecular weight measurements (in kDa) of brachiopod intracrystalline and coral shell protein.

Waltonia inconspicua	46.302	19.659	11.276	8.374
Waltonia inconspicua (fossil)	47.256	36.474	/	/
Liothyrella uva	99.549	76.400	65.880	
Terebratalia transversa	57.132	54.95	/	/
Coral	58.138	48.075	45.889	43.802
Neothyris lenticularis	69.28	65.88	54.36	
N. lenticularis (fossil)	62.552	52.364	48.630	
Pachymagas Sp. (fossil)	69.280	61.308	9.980	

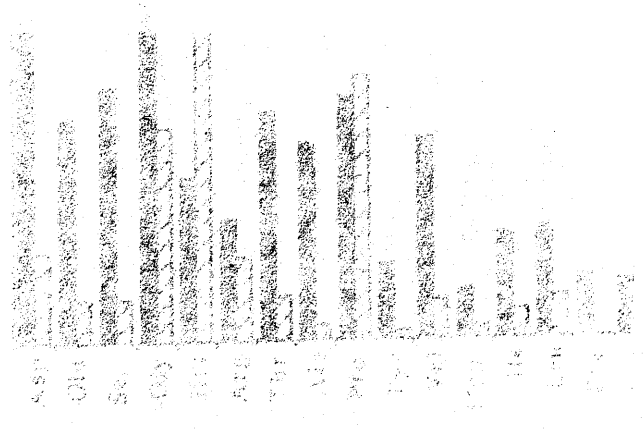
4.2.2- Amino Acid Analysis of Living Brachiopod (body and shell)

The results presented in this section represent a preliminary investigation of amino acids in brachiopods. This analysis was performed using an ABI 420 H analyser, following the procedure of Dupont, D. R. *et al.* (1989). Table 4.4 shows the quantities (in picomol = 10^{-12} mole) of amino acids found in *Terebratulina retusa* intracrystalline proteins. The table reveals variable amounts of each amino acid. This variability is due in part to the initial quantity of shell used in preparing the sample. Acidic amino acids make up approximately a quarter of the whole amino acids recovered in each sample. (see Table 4.5). Great differences were observed in the amount of Glycine identified in several samples. Some of these samples contain 22-23% glycine while others contain only 12%. Methionine was not detected in some samples. Arginine is present in moderate percentage (4.1- 5.7%) in all *T. retusa* intracrystalline samples.

Table 4.6 shows the amount and percentages of amino acids identified in *Neocrania anomala* shell proteins. In comparison with the results from *Terebratulina retusa,* it appears that they both share similar percentages of Arginine and Threonine. These tables also reveal the differences between the two species. *Terebratulina retusa* intracrystalline proteins contain higher percentages of Glycine, Alanine, Tyrosine, Valine, Isoleucine, Leucine. On the other hand *N. anomala* contains higher Histidine and Proline. Lysine and Phenylalanine were not detected in *N. anomala* in contrast with *Terebratulina retusa* samples.

Body tissue samples for SDS-PAGE were homogenized with Tris buffer (0.5 M). Unfortunately, Tris buffer interferes with the amino acid analyses because it contains an amine group. The amino acid analysis of *T. retusa* and *N. anomala* body tissue undertaken in this work are presented in Tables 4.7 and 4.8. The

average amount of the individual amino acid detected in the analysis along with its percentages are also given in the above mentioned tables. The body tissue of *T. retusa* contains a high percentage of acidic amino acids, 43% of the total amount recovered (Table 4.7). Similar percentages of each of Threonine, Alanine and Phenylalanine were detected in *T. retusa* and *N. anomala* body tissue, while higher percentages of Serine, Glycine, Histidine, Tyrosine, Valine, Methionine and Isoleucine were noticed in body tissue of *N. anomala*. Finally, the body tissue of *T. retusa* was found to contain higher percentages of each of Arginine, Proline, Leucine than *N. anomala* body tissue.



amino acids

amino acid analysis of intracystidine phyll
retusa and *Nemania anomala*.

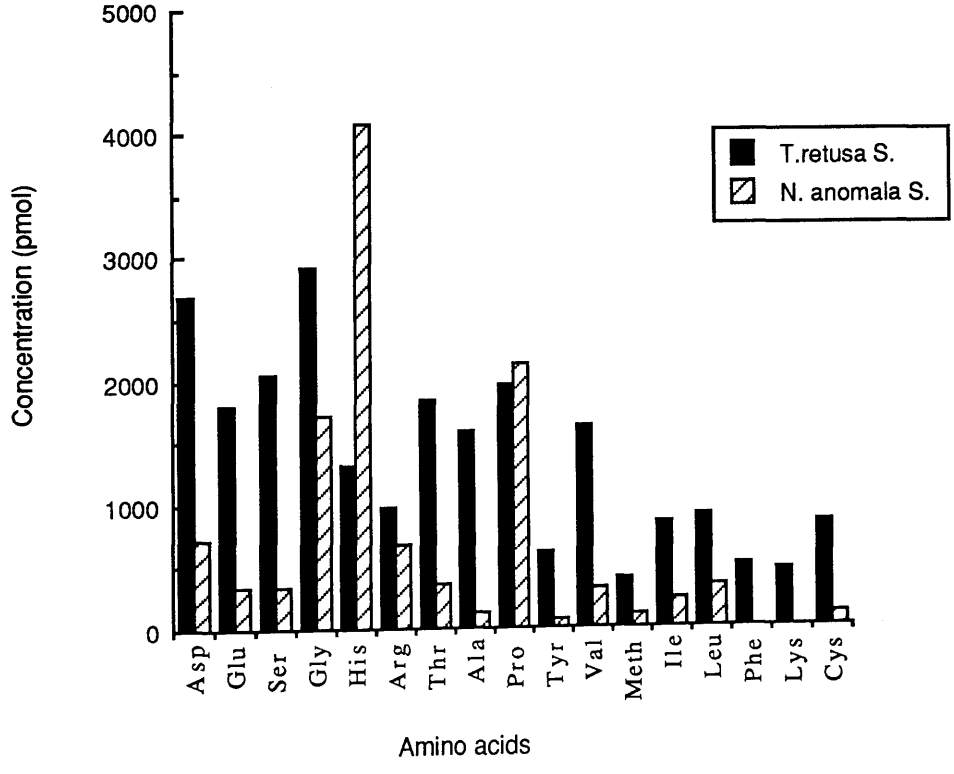


Fig.4.3 Amino acid analysis of intracrystalline shell extract from *Terebratulina retusa* and *Neocrania anomala.*

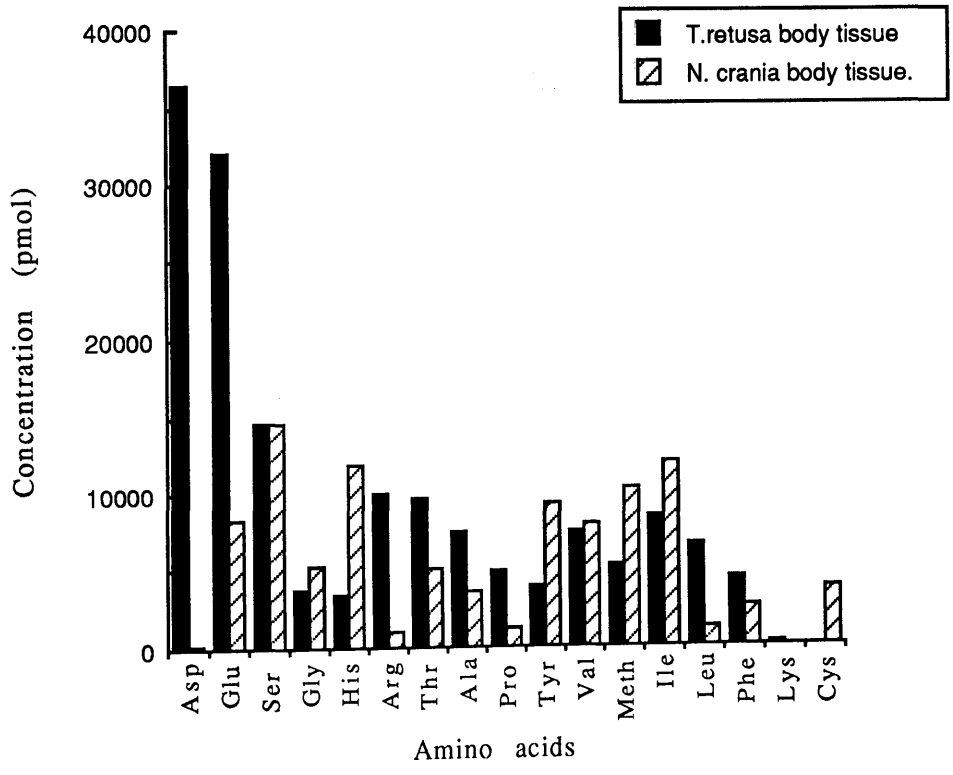


Fig. 4.4 Amino acid analysis of body tissues from *Terebratulina retusa* and *Neocrania anomala.*

Table 4.4 Amino acid analysis of *T. retusa* shell proteins (averages of absolute abundances).(*)Data represent the mean values from two analyses.

Amino acids	Aver *. S1 (PMol)	Aver*. S 2 (PMol)	Aver*. S 3 (PMol)
Asp	60.35	141.62	7875.05
Glu	40.8	112.93	5279.31
Ser	51.46	136.85	5993.08
Gly	180.137	276.306	8338.63
His	28.5	27.19	3855.82
Arg	45.27	52.62	2822.02
Thr	28.32	57.86	5474.26
Ala	29.05	57.82	4652.63
Pro	39.11	31.34	5829.15
Tyr	50.1	76.67	1741.3
Val	25.34	52.94	4835.71
Meth	Un	Un	1237.79
Ile	17.01	47.4	2499.93
Leu	91.9	50.89	2620.79
Phe	55.02	15.06	1436.30
Lys	58.93	33.02	1330.27
Cys	Un	Un	2528.0
Total	801.32	1170.79	68349.56

(Un) Undetectable values.

Table 4.5 Amino acid analysis of *T. retusa* shell protein (%). Data represent the mean values from three analysis.

Amino acids	Average S1 %	Average S2 %	Average S3 %	MEAN
Asp	7.53	12.1	11.52	10.383
Glu	5.09	9.65	7.72	7.486
Ser	6.42	11.7	8.77	8. 963
Gly	22.48	23.6	12.2	19. 426
His	3.56	2.3	5.64	3. 833
Arg	5.65	4.5	4.13	3.426
Thr	3.53	4.9	8.01	5.48
Ala	3.63	4.94	6.81	4. 946
Pro	4.88	2.68	8.53	5. 363
Tyr	6.25	6.55	2.55	5.11
Val	3.16	4.52	7.07	4.9166
Meth	Un	Un-	1.81	Un
Ile	2.12	4.05	3.66	3. 278
Leu	11.47	4.35	3.83	6.55
Phe	6.87	1.29	2.1	3.42
Lys	7.35	2. 8	1.95	4.03
Cys	Un	Un	3.7	1.233
Total	801.32	1170.79	68349.43	

Table 4.6 - Amino acid of *N. anomala* shell protein (averages and % of absolute abundance).

Amino acids	Average (PMol)	Percentage %
Asp	728.315	6.23
Glu	346.8	2.97
Ser	340.8	2.91
Gly	1720.09	14.71
His	4084.42	34.93
Arg	686.56	5.87
Thr	369.65	3.16
Ala	120.1	1.03
Pro	2136.85	18.27
Tyr	71.68	0.61
Val	310.38	2.65
Meth	110.095	0.94
Ile	228.51	1.95
Lue	331.39	2.83
Cys	107.85	0.92
Total	11693.49	

Table 4.7 Amino acid analysis of *T. retusa* body tissue (without Tris buffer).

Body tissue	22601007	16301008	13702005	Average	Percentage %
Amino acids	Pmol- Tris	Pmol- Tris	Pmol- Tris	Pmol	Pmol %
Asp.	136.62	33726.73	8051.78	36427.277	22.98
Glu.	5399.51	24108.04	7369.43	31964.027	20.17
Ser.	7235.58	30419.22	6011.37	14555.39	9.2
Gly.	2994.81	6995.6	1314.05	3768.15	2.38
His.	2132.86	7240.7	907.50	3427.02	2.2
Arg	6853.9	20417.3	2674.08	9981.76	6.3
Thr.	4646.86	20043.82	4191.73	9627.47	6.1
Ala.	4327.34	13408.66	4575.87	7437.29	4.7
Pro.	2891.66	8971.01	3004.14	4955.60	3.13
Tyr.	3588.27	6960.48	1367.18	3971.98	6.31
Val.	4901.8	13443.38	3968.53	7437.90	4.7
Meth.	4293.08	9698.58	1689.20	5226.95	3.3
Ile.	6437.47	15968.33	2954.44	8443.31	5.33
Leu.	5791.75	11171.95	2924.12	6629.27	4.2
Phe.	4553.01	7112.28	1596.15	4420.48	2.79
Lys.	-	-	641.58	213.86	5.3
Total	66184.52	229686.08	63469.08	158487.73	

Table 4.8 Amino acid analysis of *N. anomala* body tissue (without Tris buffer).

Amino acids	22601008 (Pmol) - Tris	16301012 (Pmol) -Tris	Average Pmol	Percentage % (from average) s)
Asp	47.86	107.07	131.0	0.2
Glu	5092.91	11450.64	8271.775	10.3
Ser	6861.88	15426.58	14575.17	18.2
Gly	2050.77	6558.45	5329.995	6.6
His	5769.59	12315.27	11927.225	14.9
Arg	1912.49	/	956.245	1.2
Thr	2345.59	5632.50	5161.84	6.4
Ala	1539.96	3959.96	3519.94	4.4
Pro	573.12	1369.36	1257.8	1.6
Tyr	2943.47	12909.96	9398.45	11.7
Val	3822.25	8324.65	7984.575	9.9
Meth	5978.81	8655.31	10306.465	12.8
Ile	4191.44	15727.37	12055.125	15.0
Lue	2410.65	/	1205.33	1.5
Cys	7479.66	/	3739.83	4.7
Phe	5131.12	/	2565.56	3.2
Total	58151.57	102437.12	80294.345	

4.2.3- Immunostaining Results

4.2.3.1- Shell Protein Immunostaining

Immunoblotting depends on the specific recognition and reaction between the antibody and the antigen. Polyclonal anti-sera have been used because of their wide reaction spectrum (see section 1.4). The application of immunoblotting in this study (using polyclonal anti-sera raised against the crude shell preparation from several brachiopod species) is intended to detect the presence of protein molecules in brachiopod intracrystalline secondary shell. The intensity of the reaction reflects the degree of relatedness between the taxa. Table 4.9 (a, b) shows the results obtained by detecting the antigen-antibody reaction. This table shows a distinctive reaction pattern between *Terebratulina retusa* intracrystalline shell protein and several antibody preparations generated against a range of brachiopod species.

Antibodies raised against species which have traditionally been classified as closely related to *Terebratulina retusa* show strong reaction with four bands in *T. retusa* extracts. For example, anti-*Terebratulina septentrionalis*, anti-*Terebratulina unguicula* and anti-*Terebratulina crossei* (all from superfamily Cancellothyridacea) all reacted with four *T. retusa* intracrystalline molecules in the molecular weight range of 31-97 kDa. A positive reaction was also revealed for other antibodies when detected against *Terebratulina retusa* intracrystalline extracts. These antibodies are anti-*Laqueus*, anti-*Pictothyris*, anti-*Dallina* and anti-*Neothyris lenticularis*. The first two antibodies reacted with two proteins occupying similar position in molecular weight of 97kDa and 66kDa. However, the reaction of the other two antibodies also revealed two bands although in different molecular weight positions(see Table 4.9).

Antibodies raised against *Liothyrella uva* and *Liothyrella neozealandica* reacted with *Terebratulina retusa* intracrystalline shell molecules similarly by indicating four bands. Anti- *Waltonia* reacted positively with three protein bands of molecular weight 97kDa, 45kDa and 36 kDa, while anti-*Waltonia* raised against fossil *Waltonia* did not show any reaction with *Terebratulina retusa* intracrystalline shell protein. Also, no reaction was obtained by applying fossil anti-*Pachymagus* against *Terebratulina retusa* intracrystalline protein.

Table 4.9 (a, b) also reveals the different reaction patterns obtained from the treatment of *Neocrana anomala* shell protein with several antibodies. Two bands were picked out of the *N. anomala* shell extract by reacting with six antibody preparations. Three of these six (anti-*Gryphus*, anti- *Neothyris* and anti- *Notosaria*) reacted with two bands in the range of 45k Da to over 97k Da. Anti-*Laqueus* and anti- *Waltonia* show similar reactions but with bands of different molecular weight (66k Da and 36k Da).

Anti-*Terebratulina retusa* preparation reacted with *N. anomala* shell protein also revealing two bands, one in the high molecular weight range (over 97k Da) the other in the low molecular weight range (29 - 31). Other antibodies reveal different patterns of reaction for example, anti-*Liothyrella*, anti-*Terebratulina crossei*, anti- *Terebratulina unguicula* and anti- *Dallina* all reacted with one band in the *N. anomala* shell protein, although with different molecular weight positions. Antibodies prepared against *Waltonia inconspicua* (fossil 0.4-0.5 Myr) reacted with *N. anomala* shell extract with bands at 66k Da. Anti-*Neothyris* (fossil-0.4-0.5-Myr) also shows one faint band in the molecular weight of 97 kDa while anti- *Pachymagus* (fossil-22 Myr old) did not react at all with *N. anomala* shell protein.

Antiserum	Antigens (shell protein)	over 97 k Da	97 k Da	66-97 k Da	66 k Da	43-45 k Da	31-36 k Da	29-31 k Da	20-21 kDa	14-18 k Da
Anti-Dallina K 5007	T. retusa		+			+				
	N. anomala						+			
Anti-T.retusa K 4962	T. retusa					+	+	+	+	
	N. anomala	+						+		
Anti-Waltonia K 5040	T. retusa		+			+	+			
	N. anomala				+		+			
Anti-Notosaria K 5038	T. retusa									
	N. anomala	+				+				
Anti-Neothyris lenticularis 427	T. retusa									
	N. anomala	+			+	+				
Anti-Neothyris Fossil 428	T. retusa									
	N. anomala		+?							
Anti-Waltonia Fossil 490	T. retusa									
	N. anomala				+					

Table 4.9 (A) Immunostaining results of intracrystalline shell proteins of *T. retusa* and *N. anomala*

Antiserum	Antigens	over 97 k Da	97 k Da	66-97 k Da	66 k Da	43-45 k Da	31-36 k Da	29-31 k Da	20-21 kDa	14-18 k Da
Anti-Gryphus vitrus 802	T. retusa				+		+			
	N. anomala	+				+				
Anti-Liothyrella 803	T. retusa		+		+	+	+			
	N. anomala	+								
Anti-T. crossei 171	T. retusa		+		+	+	+			
	N. anomala				+					
Anti-septentrionalis 173	T. retusa		+		+	+	+			
	N. anomala									
Anti-T.unguicula 174	T. retusa		+		+	+	+			
	N. anomala							+		
Anti-Laqueus 1191	T. retusa		+		+					
	N. anomala				+		+			
Anti-Pictothyris 1192	T. retusa		+		+					
	N. anomala	+			+					
Anti-Liothyrella uva K 5010	T. retusa	+	+		+	+				
	N. anomala									

Table 4.9 (B) Immunostaning Results Of Intracrystalline Shell Proteins of *T. retusa* and *N. anomala*

4.2.3.2- Body Homogenate Immunostaining

Table 4.10 (a, b) presents immunoblotting results of the body tissue homogenates from *Terebratulina retusa* and *N. anomala*. Body tissues were treated with similar antibodies to those used to detect the shell proteins (see Table 3. 1).

Terebratulina retusa body homogenate expreses different reaction pattern from the intracrystalline extract. The primary molecule recognized by staining with enzyme-labeled antibodies has a molecular weight of 66kDa, and was picked out by eight of the thirteen antibodies used. These antibodies include anti-*Liothyrella neozealandica*, anti-*Liothyrella uva*, anti-*Laqueus*, anti-*T. septentrionalis*, anti-*Terebratulina retusa*, anti-*Waltonia*, anti-*Dallina* and anti-*Notosaria*. A strong signal was detected with anti-*Terebratulina retusa* when it reacted with six protein bands ranging between 31kDa to over 97kDa. Another strong reaction in *Terebratulina retusa* body homogenate expressed in four bands was revealed with anti-*Waltonia*, anti-*Dallina*, anti-*Liothyrella neozealandica*, anti-*Liothyrella uva* and anti-*Terebratulina septentrionalis*. Two of these antibodies reacted with protein bands at 20-30 kDa and anti-*Dallina* picked up a 5-6k Da band in *Terebratulina retusa* body homogenate.

Antibodies against *Neocrania anomala* body homogenate were able to recognize more bands with molecular weight in the range of 45k Da to over 97k Da. Anti-*Gryphus* reacted with five bands between 45 and 97k Da. Five antibodies (anti-*Liothyrella neozealandica*, anti-*T. septentrionalis*, anti-*T. retusa*, anti-*Notosaria* and anti-*Neothyris*) reacted with four bands in the body homogenate although at different molecular weight positions (see Table 4.10). Three bands in *Neocrania anomala* were revealed by anti-*Terebratulina crossei*, anti-*Laqueus* and anti-*Liothyrella uva*. Another two bands were revealed with anti-*Waltonia*, anti-

Dallina and anti-*Terebratulina unguicula* when stained or blotted against the body homogenate.

Antiserum	Antigens body tissue	over 97kDa	97 k Da	66-97 kDa	66 k Da	45-66 kDa	43-45 kDa	31-45 kDa	29-31kDa	20-30kDa	14-18kDa	5-6 kDa
Anti-Dallina K 5007	T. retusa				+		+	+				+
	N. anomala				+		+					
Anti-T. retusa K 4962	T. retusa	+	+	+	+		+	+				
	N. anomala	+	+		+	+						
Anti-Waltonia ·K 5040	T. retusa				+		+	+		+		
	N. anomala				+	+						
Anti-Notosaria K 5038	T. retusa			+	+							
	N. anomala	+	+				+	+				
Anti-Neothyris lenticularis 427	T. retusa	/	/	/	/							
	N. anomala	+	+	+						+		
Anti-Gryphus vitrus 802	T. retusa		+			+	+					
	N. anomala		+	+	+	+	+					
Anti-Liothyrella NZ. 803	T. retusa											
	N. anomala											

Table 4.10 (A) Immunostaining reactions of body tissues of *T. retusa* and *N. anomala*

Antiserum	Antigens (body tissue	over 97kDa	97 k Da	66-97kDa	66 kDa	45-66kDa	43-45 kDa	31-45 kDa	29-31kDa	20-30 kDa	14-18 kDa	5-6 kDa
Anti-septentrionalis 173	T. retusa		+		+	+		+				
	N. anomala	+		+	+		+					
Anti-unguicula 174	T. retusa						+					
	N. anomala					+	+					
Anti-T. retusa 171	T. retusa			+		+				+		
	N. anomala		+		+	+						
Anti-Laqueus 1191	T. retusa				+							
	N. anomala		+		+		+					
Anti-Pictothyris 1192	T. retusa	+				+						
	N. anomala	+										
Anti-Liothyrella uva K 5010	T. retusa	+		+	+	+						
	N. anomala	+		+		+						

Table 4.10 (B) Immunostaning results of body tissues of *T. retusa* and *N. anomala*

4.3 -Discussion

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A variety of biochemical techniques were used to detect and characterize the protein molecules found in brachiopod intra-crystalline shell extracts. The techniques used include SDS-PAGE, electroblotting, enzyme immunostaining and amino acid analysis, to investigate the range of macromolecules present within the calcite of brachiopod shells. The soft body tissues of brachiopods have also been examined by these techniques to allow comparison between shell and body tissue molecules.

4.3.1 Molecular weight Measurment of body tissue and shell protein

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SDS-PAGE provide precise comparative information in the range of protein present in the soft tissue of the two species investigated (ie. *Terebratulina retusa* and *Neocrania anomala*). Tissue samples (homogenates primarily compounded of mantle, muscles and gonads) from both animals display numerous bands stained with CBB in the molecular weight range of 6-200 kDa(see Table 4.1). Jope (1969a) using disc electrophoresis reported a homogeneous fraction of 100.000 in molecular weight extracted from the mantle only. From the results presented in tables 4.1 and 4.2, it is clear that molecular weight measurment (of body homogenate) alone will not show clear distinction between *Terebratulina retusa* and *Neocrania anomala* despite the difference displayed by their band separation patterns in the gel. Another important point is the origine of the detected proteins in these body homogenates. It is possible that some of these proteins are from the surrounding environment(during the feeding process).

The intra-crystalline shell samples showed one band only in *T. retusa* at the molecular weight range of 29-36 kDa, and did not show any bands in *N. anomala* intracrystalline samples. The lack of bands may be due to staining characteristics, CBB reveals protein but not glycoprotein nor carbohydrate. However, by extracting macromolecules using the whole shell, Jope (1980, 1986) identified two bands in the Recent samples of *T. retusa* and *N. anomala*. The estimated molecular weight for *Terbratulina retusa* bands were between 40-60 k Da. The differences between the two analyses can be explained as in brachiopod shells proteniaceous materials are present between the crystals which form the biomineral skeleton (Jope 1971) as well as within the crystals (Collins *et al.*, 1988). The intercrystalline proteins are present in higher amounts than the intracrystalline proteins in the shell (Fig.4.1). So by using the whole shell, Jope has extracted the intercrystalline molecules without any subsequent concentration steps. It is likely, therefore that the presence of intracrystalline molecules were not detected by Jope because of their low concentration in her preparation.

4.3.2 Amino Acid Analysis of Body Tissue and Shell Proteins

Amino acid analysis of *Terebratulina retusa* and *Neocrania anomala* intracrystalline shell extract reveal considerable variations in their amino acid content (see Fig. 4.2). In general, the data show higher yield of amino acids in *T. retusa* (intracrystalline shell extract and tissue homogenate) than in *N. anomala* samples. Lysine (one of the basic amino acids) was absent from *N. anomala* shell and body samples. In comparison with the data obtained from the amino acid analysis of Recent whole shells of *T. retusa* (Jope 1967, 1969a, 1977), ten out of the seventeen analysed amino acids are similar to these described by Jope. During the course of the present study, however higher Tyrosine and Proline were detected in *T. retusa* intracrystalline shell samples,

while Jope detected high Arginine, Valine and Isoleucine in the whole shell extract of *T. retusa* . In *Neocrania anomala* intracrystalline samples, Histidine and Proline were present in very high concentrations and in addition to Lysine, Phenylalanine was also undetectable. Once again the major cause of these differences is likely to be the difference in the samples used, with Jope including the intercrystalline macromolecules which were excluded by preparation techniques used in this study.

The body tissue data indicate that *Terebratulina retusa* contains higher concentrations of acidic amino acids compared to *N. anomala* body tissues (Fig. 4.3). High percentages of Glycine, Histidine, Tyrosine, Valine, Methionine, Isoleusine and Cysteine were observed in *N. anomala* body homogenates.

Several factors may contribute to the detected variations in amino acid yield. For example, the initial rough quantity of shells used in each sample could introduce considerable variability. However, the data was expressed as percentages (%) to attempt to overcome such variability, and such data still revealed considerable variations (Table 4.5). One of the main factors which affect the amino acid yield is the differences in growth stage, as the quantity of proteins incorporated in the shell and the degree of calcification, may vary through the life time of the animal. The distribution of certain proteins may also vary with environment (Degens et al 1967). Finally, differences in the analytical patterns of some amino acids may cause some variations. Some amino acids are sensitive to acid hydrolysis conditions with variable loss percentages. For example, the reported loss percentage for Serine is (15-20%), Threionine (10-15%), Tyrosine (15-20%), Methionine (variable) and 50% or greater for Cysteine.

4.3.3 Immunostaninig

Benson *et al.* 1987 used polyclonal antisera to localize and follow the accumulation of 50 kDa glycoprotein in sea urchin embryo spicules. In this work polyclonal antisera were used as probes to characterize the macromolecule fractions separated by SDS-PAGE in the body tissue and intra-crystalline shell extract of *T. retusa* and *N. anomala*. Immunostaining techniques rely on the specific ability of antibodies to recognize and bind specifically to any antigenic molecules. Most macromolecules such as proteins, nucleic acids and carbohydrates are known to be antigenic. The polyclonal antisera were raised against unpurified preparations (the crude mixture of the shell), and it is expected that they may reveal other macromolecules presence in addition to the proteins. Tissue samples from both animals were expected to react in a similar pattern towards different antibodies, since these samples are a crude mixture of all proteins, but different patterns of reaction were obtained for each animal. Antisera of closely related species to *T. retusa* (traditionally) reacted clearly to *T. retusa* intracrystalline shell proteins and similar reactions were obtained with *N. anomala* (Table 4.9 A&B). These results reflect the traditional classification of brachiopod phylum in placing each of *Terebratulina retusa* and *Neocrania anomala* in different classes.

As a result, immunological techniques used during this study detected more compounds or molecules in the intracrystalline shell extract than dose the SDS-PAGE system and this is due to the extreme sensitivity of the immunostaining technique to detect even non-protein macromolecules. Frequently, molecules are able to change their conformation (denaturation) and small molecules can group together and viseversa, which will increase the chance of exposing more antigenic determinant (epitope) sites able to bind

more antibodies which in turn enhance the strength of the reaction.

Recently, It has been established that lipids and other types of macromolecules (probably carbohydrates) are present in brachiopod shells in addition to proteins (Curry *et al*, 1991). The staining of the separated bands in the SDS-PAGE with CBB (which is known to be a specialized stain for proteins) supported by the amino acid analysis, suggest that these macromolecules are predominantly of proteinous material. However, the immunostaining results do not support such a suggestion because of the wide specifity of polyclonal antisera which posses the ability to react against any antigenic molecules as mentioned before. In SDS-PAGE the presence of heavily glycolated molecules (ie. proteins with carbohydrates attached) will be indicated by smearing in the gel after staining. During the course of this work a good resolution was obtained with SDS-PAGE system, which indicates that such molecules are unlikely to be present.

In other phyla, macromolecules extracted from the hard parts of the organisms show some differences relative to brachiopod intracrystalline extract. From the teeth of the sea urchin Weiner (1985) extracted protein molecules rich in glycine (25 %). While Benson *et al*, (1986) isolated four glycoprotein residues from the spicules of the embryo with molecular mass of 47, 50, 57, and 64 k Da. In mollusks, glycoprotein molecules rich in aspartic acid (50%) were extract from the shell (Simkiss 1965, Crenshaw 1972a, Weiner 1979). A glyccoprotein fragment of 15.000 molecular mass has been isolated from the bivalve *Mytilus californianus* (Addadi *et al*, 1987). The molecular weight and amino acid analyses of the two brachiopod genera analysed in this study, are unlike any previously published for shell-related macromolecules

4.4 Conclusions

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The principal conclusions drawn from this work are summarized as follows:

1. This work is a preliminary study demonstrating the possibility of extracting informative molecular material from brachiopods. Each species (the body homogenate samples) provides a distinctive gel pattern which appeared promising for taxonomy, but the uncertainty concerning the origin of the analysed molecules limits the application. This work is also valuable in defining the limits for the application of SDS-PAGE analysis of brachiopod shell proteins in palaeontology.

2- Brachiopod shells contain other molecules such as carbohydrates and lipids in addition to proteins, although purified protein preparations did not show clear indication. At present protein molecules are more informative because of the ability to trace their genetic codes which is valuable for taxonomy.

3- Immunostaining is more sensitive as a detection method than CBB. Although the polyclonal antisera (used in immunostaining) has a disadvantage of detecting all antigenic molecules while CBB is a specific due for proteins only.

4- Extending the applications of the biochemical techniques used in this work to oldest fossils is not possible at the present time. These techniques are currently limited to less than a million year.

Suggestion for further work

This work was built on the improvement in extraction and purification technology and as the possibility of further improvements in general technology may increase sensitivity then analysis of older material may become possible. In serotaxonomy, the generation of monoclonal antibodies against homogeneous protein preparations, although time consuming, should be worthwhile because such antibodies react with specific epitope e.g. a small region of peptide sequence.

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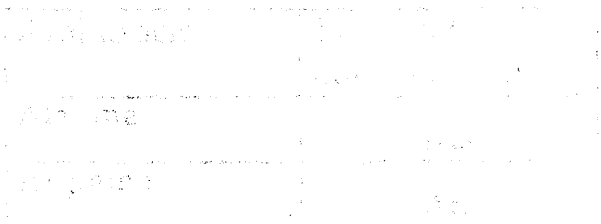


APPENDIX 1. GENERAL ABBREVIATIONS

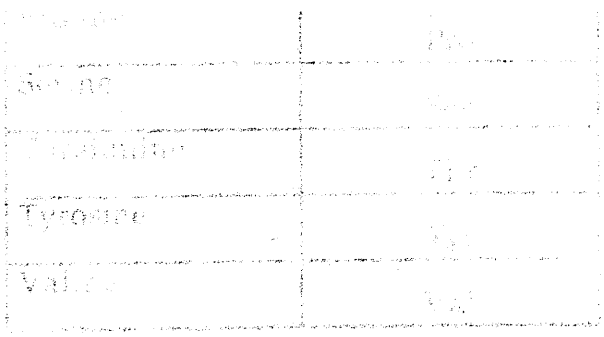


Table 1.1 Abbreviations for amino acids

Amino acid	Three letter abbreviation
Alanine	Ala
Arginine	Arg
Aspartic acid	Asp
Cystine	Cys
Glutamic acid	Glu
Glycine	Gly
Histidine	His
Isolucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenilalanine	Phe
Proline	Pro
Serine	Ser
Threionine	Thr
Tyrosine	Tyr
Valine	Val

Table 1.2 -List of other abbreviations

BCIP	5-bromo-4-chloro-3-indodyl phosphate
NBT	Nitro blue tetrazolium
PAGE	Polyacrylamide gel electrophoresis
PITC	Phenylisothiocyanate
PTC	Phenylthiocarbamyl
PVDF	Polyvinylidene difluoride
SDS	Sodium dodycel sulfate
SEM	Scanning electron microscope

APPENDIX 2. CHEMICALS AND BUFFERS

1- Electrophoresis Chemicals:

Reagents	Suppliers
polyacrylamide C_3H_5NO.	Sigma
N, N'- methyline- bisacrylamide $CH_2(NHCOCH=CH_2)_2$	Sigma
Tris (hydroxymethylamino methane)	Sigma
Temed (N,N,N',N'Tetramethyl ethylne diamine)	Sigma
Pyronin Y.	Sigma
Ammonium persulphate	May & Baker Ltd. Dagenham, England.
Dithiothreitol - $C_4H_{10}O_2S_2$	Sigma
Sodium dodecyl sulphate $C_{12}H_{25}OSO_3.Na$	BDH Chemicals Ltd. Poole, England
Tricin(N-tris[hydroxymethyl]-methylglycine) $C_6H_{13}NO_5$.	Sigma

2 - Blotting Chemicals

Reagents	Supplier
Tween 20 (polyoxyethylene sorbitan monolorate)	Sigma
Albumin - ovalbumin	Sigma
Anti-rabbit IgG (whole molecule) alkaline phosphates conjugate	Sigma
5-bromo-4-choloro-3-indolyl phosphate	Sigma
Nitro blue tetrazolium, Grade III	Sigma

3 - Immuno-detection Buffers

1. TBS (Tris buffered saline) pH 7:

Dissolve 10 mM Tris and 0.9% NaCl in 900 ml of distiled water, adjust the pH to 7.5 then make up to 1 litre.

2. TBS/Tween - 0.5 ml. Tween 20/ L TBS.

3. 0.1% BSA/TBS/ Tween - 0.1 g.BSA/ 100 ml. TBS/Tween.

4. 2% BSA/TBS - 2 g. BSA/ 100ml. TBS.

5. AP (Alkaline phosphates) buffer:

   100 mM Tris-HCl (pH 9.5).

   !00 mM NaCl

   5 mM $MgCl_2$

6. GAR/Alkaline phosphates conjugated second antibody. The antibody is diluted 1:1000 in 0.1% OVA/ TBS/ Tween.

7. Alkaline phosphatase-Substrates, BCIP and NBT.

   BCIP and NBT each dissolved in 70% dimethylformamide. To make the substrates, mix 5 ml. AP buffer (see 5 this section) with 33µl NBT-formamide then add 16.5µl BCIP-formamide. This substrate must be used within one hour and be protected from light.

4 - Other Chemicals and Buffers

1. Coomassie Brilliant Blue R250 Sigma

2. Silver stain:

   Dithiothyretol (DTE)

   Silver nitrate - $AgNO_3$ Sigma

   Developer - $Na_2CO_3.10H_2O$

   formaldehyde

3. EDTA - (Ethylene diamino tetra acetic acid, disodium salt)-Boehringer Mannheim Gmbh.

20% EDTA- 200 g EDTA

22 g NaOH , adjust the pH to 8.0.

4. Tris - glycine transfer buffer : pH 8.3

25 mM Tris

20% v/v Methanol

192 mM glycine

5. Laemmli sample buffer (LSB)-

1.0 ml 0.5M Tris-Hcl pH:6.8

1.0 ml 98% glycerol

1.0 ml 10% (w/v) SDS

0.12g Dithiothreitol (DTE)

pyronin Y.

5 - Amino Acid Analysis Reagents and Buffers

Reagents & Cat. NO.	Supplier or Company
Argon -	British oxygen company
6N HCL - 400939	ABI,
Test peptide - 400909	ABI
PITC - 400208	ABI
Diisopropylethylamine - 400136	ABI
3M sodium acetate, pH 3.8- 400319	ABI
3M sodium acetate, pH 5.5 - 400471	ABI
0.01% Tripotassium EDTA - 400941	ABI
Ammonium hydroxide -	Aldrich
70% Acetonitrile - 400313	ABI

Hplc mobile phases solvents:

Solvent **A**- 50 mM sodium acetate buffer, pH 5.4 (3M sodium acetate, pH 5.5 in Milli Q^{TM} water, adjust the pH to 5.4 with ammonium hydroxide).

Solvent **B**- 70% acetonitrile, 32 mM sodium acetate pH 6.1, all in Milli Q^{TM} water.

32 mM sodium acetate , pH 6.1 is prepared from 3M sodium acetate, pH 5.5 and 3M sodium acetate, pH 3.8.

Hplc column conditions:

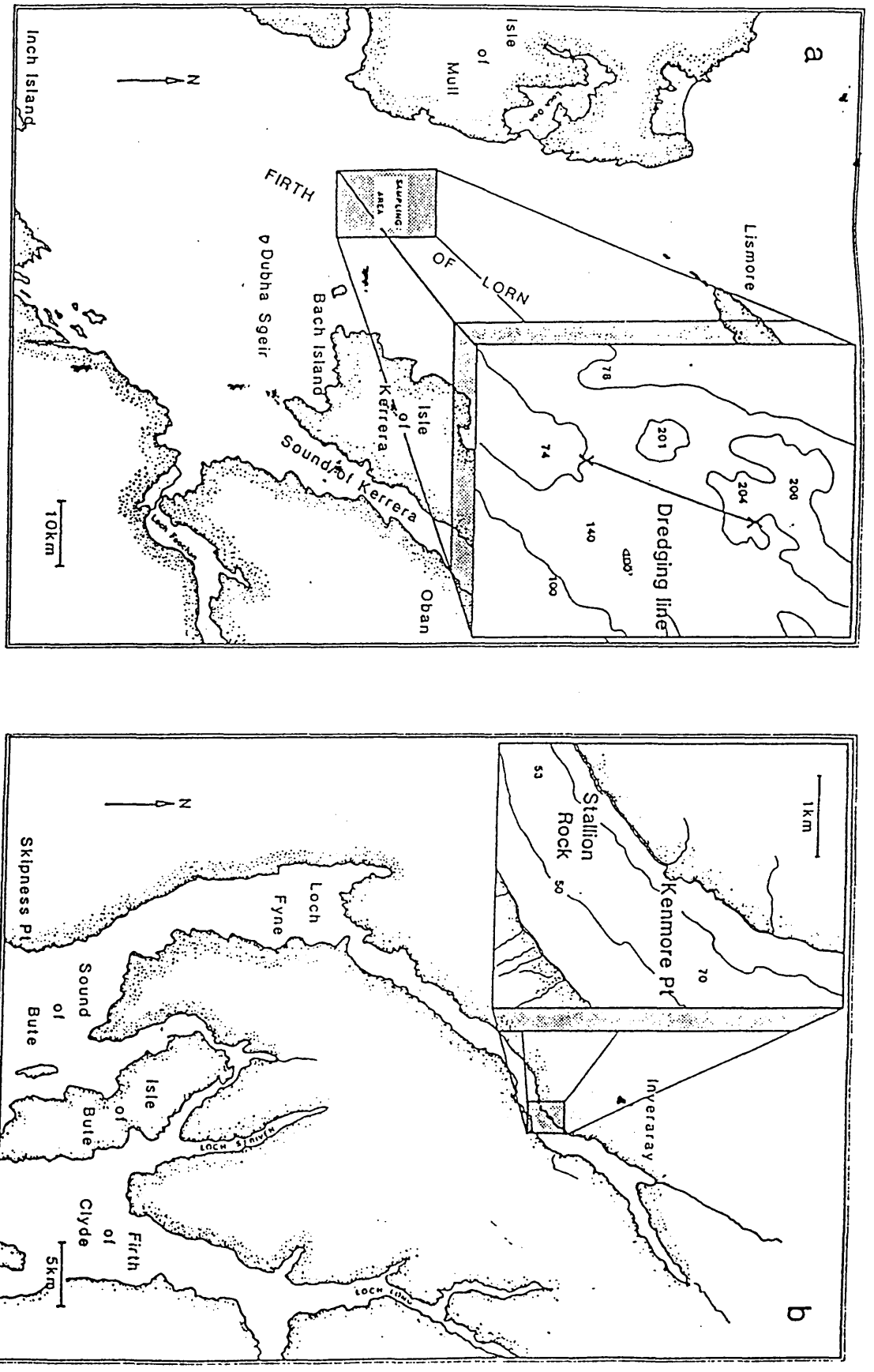
reverse phase column, PTC-C18, 2.1 X 220 mm (0711- 0204)

temperature -37^{o}C, flow rate - 300µl/ min.

Gradient:

Time	% B
0.0	5
4.0	15
10.0	32
20.0	63
25.0	100
31.0	100
32.0	5

APPENDIX 3. SAMPLING LOCATIONS



Locations of brachiopod sample collection from firth of Lorne (a) and Loch fyne (b). Numbers inside sampling area shows depth in meters. After James, M. A 1991.