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Biogeochemistry of Brachiopod Intracrystalline Molecules [and Discussion]

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Biogeochemistry of brachiopod intracrystalline molecules

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SUMMARY

Brachiopods contain both proteins and lipids within the biocrystals of their shell. One intracrystalline chromoprotein causes red shell coloration, whereas the other molecules may be involved with biomineralization, may strengthen the biocrystal, or may simply have been inadvertently engulfed by calcite during shell growth. Evidence is presented which suggests that at least some of the breakdown products of indigeneous intracrystalline molecules can be recovered from the biocrystals of fossil brachiopod shells. Investigations of the remains of these intracrystalline molecules in fossils have geological application in fields such as environmental reconstruction, isotopic determinations, taxonomy and the interpretation of colour in extinct organisms.

1. INTRODUCTION

This paper deals with molecules that occur within the calcite of the brachiopod shell. These intracrystalline molecules are quite distinct from the intercrystalline organic membranes that surround the calcite biocrystals, and also from the 'metabolizing' evaginations of mantle tissue which penetrate many brachiopod shells (the caeca). This is not to say that the molecules recovered from within brachiopod calcite are uniquely distributed within the shell; indeed it is certain that they are synthesized within the secretory outer epithelium, and it is also possible that they have a function within the soft tissues of the organism.

The geological interest in intracrystalline molecules stems from the possibility that *in vivo* incorporation within resistant biocrystals may provide long-term protection from the various physical, chemical and biological factors that would otherwise bring about the destruction of these molecules. The implication is that the inevitable decay of these molecules over geological timespans may not only proceed at much slower rates within the enclosing biocrystal, but that it may occur essentially *in situ*. Intracrystalline molecules may therefore provide unique access to fossilized remains of the original organic constituents of an organism which have not been subjected to significant post-depositional ingress by the abundant organic debris of various sources and ages which occurs within, and migrates through, enclosing sediments. Brachiopod shells are composed of robust low magnesium calcite that remains stable and in its original configuration for hundreds of millions of years (Williams 1984). Intercrystalline molecules situated between, rather than within, brachiopod biocrystals do, by contrast, decay rapidly and are prone to bacterial infection (Collins 1986).

Intracrystalline molecular fossils will undoubtedly be greatly altered over geological time irrespective of the stability of their enclosing biocrystal. Factors that will affect fossilization potential include the range of molecules present inside biocrystals, their location within the shell, their interactions with the inorganic phase, their chemical stability, the environmental conditions (temperature, pressure, etc.), and the extent to which they can react with other intracrystalline molecules. As some of these factors are likely to have changed over geological time, there is no doubt that the breakdown of intracrystalline molecules will be extremely complicated. In this paper evidence for the *in situ* degradation of intracrystalline proteins and lipids in brachiopods is presented from the investigation of living and closely related fossil shells. The ultimate aim of this research is to determine the biological function of these molecules during biomineralization, and the geological applications of their preserved remains in fossils.

2. METHODS

Surface contaminant molecules and remnants of body tissue were removed by thoroughly cleaning the shells and incubating them for 2 h at 22 °C in an aqueous solution of bleach (5 % by volume). The shells were then powdered in a ceramic pestle and mortar before incubating overnight at 4 °C in an aqueous

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solution of bleach (1% by volume). The bleach was removed by repeated washes with MilliQ water followed by centrifugation (8 *g*.h). The precipitate was washed until no bleach could be detected (typically ten 2-litre washes) and the precipitate was then lyophilized.

The $CaCO_3$ shell was dissolved using EDTA (200 g l^{-1}) at a ratio of 23 ml to 1 g shell. The entire mixture was agitated at 4 °C for 72 h or until the inorganic phase had dissolved. Following centrifugation (20 k*g*.h) the supernatant was concentrated, washed, and the EDTA removed using the Minitan tangential flow system for Millipore. The preparation was further concentrated in a Minicon concentrator (Amicon) to obtain sufficient quantities of intracrystalline proteins for biochemical analysis.

EDTA is very difficult to remove, and various investigators have shown spurious spectrophometer readings, non-reproducible gel electrophoresis patterns and other distortions which have been attributed to the incomplete removal of EDTA (Weiner 1984; Benson *et al*. 1986). During our study the success in removing EDTA from preparations was monitored using an amino acid analyser, as EDTA produces a characteristic pattern of peaks on the reverse phase chromatography in this system. The results confirmed that EDTA cannot be entirely removed by dialysis or simple ultrafiltration. However, the Minitan system, which has a much greater filtration area and tangential flow preventing the blocking of filters, allowed the removal of EDTA down to a level undetectable on the amino acid analyser (i.e. less than 10^{-12} M). Typically the final solution represented a 10000-fold concentration. Samples intended for bulk amino acid analysis were prepared by dissolving the shells in 2 M HCl.

Samples for electrophoresis in 15% acrylamide gels were heated at 100 °C for 4 min in an equal volume of sample buffer containing final concentrations of 0.15 M Tris HCl, pH 6.8, 0.2 M 2-mercaptoethanol, 1 g l^{-1} SDS, 30% (by volume) glycerol and 0.002 g l^{-1} of the tracking dye, bromophenol blue. Electrophoresis of samples in the small gel system (9 × 7 cm) required a constant voltage of 100 V for 2 h. Following electrophoresis, a constant voltage of 50 V was applied to transfer the proteins onto ProBlott membrane in CAPS buffer (10 mM, pH 11) with methanol (10% by volume). The membrane was then washed briefly with water and then methanol before staining with Coomassie Brilliant Blue-R in destain (1 g l^{-1}) for 1 min. Background staining was reduced by using an aqueous dilution of methanol (50% by volume). High performance liquid chromatography (HPLC) analysis of intracrystalline molecules was carried out using a WATERS 650 HPLC fitted with a multiwavelength detector. Amino acid analysis was done using an ABI 420H amino acid analyser fitted with an automatic hydrolysis head. Amino acid sequencing was done on the stained protein bands immobilized on ProBlott membrane using a pulsed liquid protein sequencer (Applied Biosystems 477A).

For lipid analysis, the shells were cleaned as above, and then etched in cold dilute HCl until 20% of their mass was removed. The etched shells were further cleaned by repeated washes in dichloromethane, and then powdered in a ceramic pestle and mortar. Initially the quantities of lipids extracted from Recent and fossil brachiopod shells using a 93/7 (by volume) mixture of dichloromethane/methanol were insignificant. Similarly, demineralization of the shell powders did not yield significant levels of lipids, even with repeated extractions with dichloromethane. Successful extraction of intracrystalline lipids was, however, achieved by a modified method in which 2 M methanolic HCl was added once the shell had been dissolved in cold 6 M HCl, and the resulting aqueous mixture refluxed for 24 h. The solution was extracted with dichloromethane and the dichloromethane layer evaporated to dryness. The lipid fraction thus obtained was separated into two fractions using a short column of silica gel, eluted with 50/50 petroleum ether/dichloromethane (2 ml) and methanol (2 ml).

The individual fractions thus collected were evaporated to dryness and analysed via combined gas chromatography–mass spectrometry (GC–MS). Gas chromatography was done using a Carlo Erba Mega series 5360 gas chromatograph fitted with a DB-5 coated, 30 m fused silica capillary column (0.25 μm film thickness and 0.32 mm internal diameter (i.d.)). The column was temperature programmed from 50 to 300 °C at 4 °C per minute with an isothermal temperature of 300 °C for 20 min. Cold on column injection of the sample was used and a detector temperature of 310 °C was maintained. GC–MS analyses were done on a VG TS250 mass spectrometer (electron energy 70 eV; filament current 4000 mA; acceleration voltage 4 kV; source temperature 200 °C) interfaced with a Hewlett Packard 4890 GC fitted with a DB-5 coated, 30 m fused silica capillary column (0.25 μm film thickness and 0.32 mm i.d.).

The possibility of contamination was monitored at all stages of preparation by analysing reagents. In the text and figures the standard three-letter amino acid abbreviations have been used.

3. INTRACRYSTALLINE PROTEINS IN RECENT BRACHIOPODS

Polyacrylamide gel electrophoresis of intracrystalline molecules reveals a variable number of Coomassie-stained bands in different brachiopod groups. For example, *Neothyris* has three main bands with estimated molecular weights of 47 kDa, 16 kDa and 6.5 kDa (figure 1). In contrast, *Terebratulina* contains only a single band of molecular mass 30 kDa. These bands are sharp and readily stained by Coomassie Brilliant Blue, suggesting that these are proteins rather than glycoproteins. This interpretation has been supported by amino acid analysis which revealed no sign of galactosamine or glucosamine.

Brachiopod intracrystalline proteins appear to be very different from the sparse data available from other phyla. Weiner (1983) isolated two proteins of similar molecular mass to the two smaller proteins in *Neothyris* from the calcite component of the bivalve *Mytilus*, but the amino acid analyses are completely different (the *Mytilus* proteins have over 50% Asp and Glu,

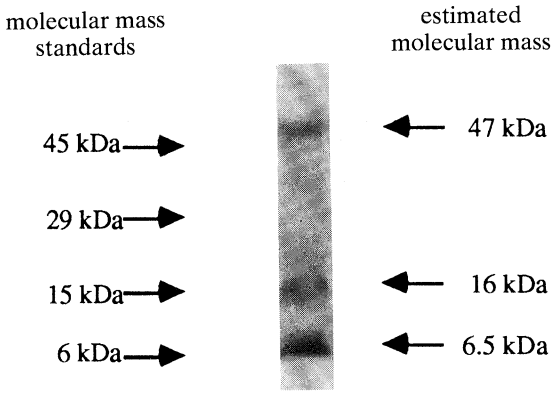

Figure 1. SDS-PAGE of intracrystalline proteins in the Recent brachiopod *Neothyris* from New Zealand.

compared with 20% for the brachiopod). Similarly phosphophryns, the major non-collagenous proteins of dentine, have a distinctive amino acid composition (Veis 1989) unlike that of any brachiopod intracrystalline proteins. In terms of electrophoretic behaviour and amino acid analysis, brachiopod intracrystalline proteins are again different from the glycoproteins extracted from sea urchins, which could not be stained by Coomassie (Benson *et al.* 1986).

The sequence of a 50 kDa sea urchin intracrystalline glycoprotein has been determined by nucleotide sequencing of the coding gene (Benson *et al.* 1987), but the development of a protocol which allowed purification of brachiopod intracrystalline proteins to homogeneity has permitted direct amino acid sequencing (Curry *et al.* 1991). As yet no similarity has been detected between the amino acid sequence of brachiopod intracrystalline proteins and published amino acid or nucleotide sequences (Curry *et al.* 1991).

The function of intracrystalline proteins is poorly understood. It has recently been suggested that they strengthen the shell (Berman *et al.* 1990), although it is also possible that some have been inadvertently incorporated into the shell during growth. As discussed below, the investigation of brachiopod shell proteins has revealed that one is responsible for shell colour. The indications are that there is considerable variation in the nature of intracrystalline proteins both within different groups of brachiopods and between different phyla.

4. INTRACRYSTALLINE AMINO ACIDS IN FOSSIL BRACHIOPODS

There are several lines of evidence that suggest that the remains of indigenous intracrystalline molecules can be recovered from fossil brachiopods. Firstly, amino acid analyses of progressively older fossil representatives of a single genus reveal a pattern of decay that is predictable from the chemical characteristics of individual amino acids. Within the shell of *Waltonia*, for example, relatively unstable amino acids (such as Asp, Glu, Pro and Ser) show a steady decline in relative abundance with increasing age (figure 2*a*,*c*). Alanine, one of the breakdown products of serine (Hoering 1980), shows a corresponding increase (figure 2*b*). This suggests that the parent proteins have progressively decayed *in situ* and have not been significantly contaminated by the abundant amino acids that occur in the surrounding sediments (Walton & Curry 1991).

Immunological techniques have also shown that original antigenic determinants, including peptides, have survived in these brachiopods, and such techniques offer an important method of extracting original molecular information from Pleistocene shells (see Lowenstein & Scheuenstuhl, this symposium). The immunological approach has proved less useful with older fossils, possibly due to interference from other intracrystalline compounds, and possibly EDTA, as well as the decay of determinants on the target molecules. However, amino acid analysis has suggested that intracrystalline molecules remain uncontaminated for much greater periods of geological time. For example, 27-million-year-old (Ma) specimens of *Terebratulina* have a similar amino acid composition to that of the single protein found in Recent shells of this genus. The major differences, the increase in Gly and Ala (both products of the decay of Ser), and decrease of Asp, Ser, Thr and Pro, are again predictable consequences of the known chemical properties of these amino acids (figure 3). The absolute abundance of soluble amino acids in 27 Ma shells of *Terebratulina* has dropped to about 50% of that in Recent shells. Some amino acids decay completely, but the most significant reduction is probably caused by incorporation of other amino acids into insoluble molecular agglutinations (melanoidins of Hoering 1980) which increase in abundance in fossils and are excluded from amino acid analysis of the soluble fraction.

The trends apparent from the investigation of *Waltonia* shells (figure 2) indicate that an amino acid such as Asp has decayed from 4% of total amino acids in Recent shells to about 2% in 2 Ma shells and to 1% in 2.5 Ma shells, and by extrapolation would be expected to disappear entirely in shells older than about 5 or 6 Ma. However, only a small decrease in Asp (and other amino acids) is apparent when comparing Recent and 27 Ma *Terebratulina* (in which Asp declines from 6% of the total to 1% – figure 3). This inconsistency may in part be explained by the different number and composition of the intracrystalline proteins present in these two genera, but may also reflect variations in the extent to which these proteins are intimately associated with the calcite matrix. Adsorption of amino acids such as Asp to calcium carbonate may increase their survival potential and retard their incorporation into the insoluble high molecular mass fraction. As more data become available it may be possible to reconcile these two results by investigating the stability of amino acids in a single taxon over much longer periods of time.

A third line of evidence for the *in situ* decay comes from principal component analyses (PCA) of amino acid analyses of intracrystalline proteins. In Recent shells, differences in homologous amino acid sequences, and the variation in the number and possibly also the proportions of proteins present, have produced differences in amino acid composition which can be revealed

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

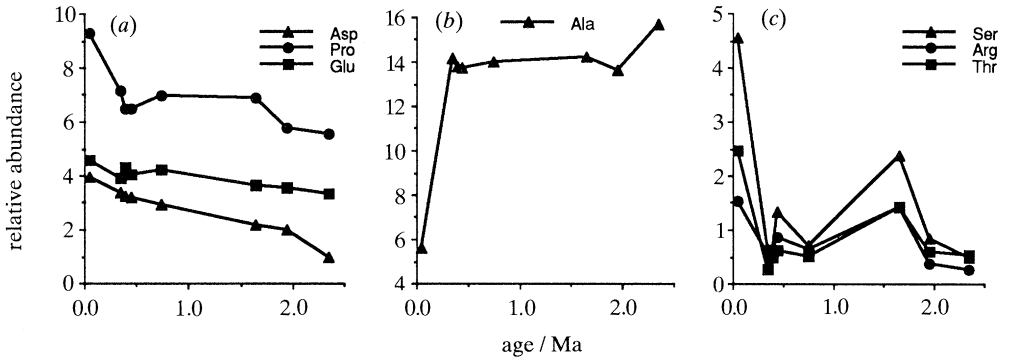

Figure 2. Relative abundance of intracrystalline amino acids in Recent and fossil shells of *Waltonia* from New Zealand.

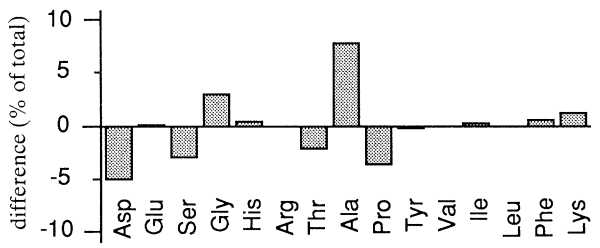

Figure 3. Comparison of the intracrystalline amino acid compositions of Recent *Terebratulina* and 27 Ma fossil shells of the same genus from New Zealand. The data for each analysis have been expressed in mole percentages, and the vertical axis indicates the difference detected in relative abundance for each amino acid between the fossil and Recent shells.

by PCA (figure 4). For example, four New Zealand brachiopod genera are readily distinguished on the basis of amino acid composition of their shells (figure 4*a*). The raw data from amino acid analyses are difficult to compare, and PCA not only has the advantage of presenting a clear graphical summary of the dispersion of the data but the eigenvector values provide a clear indication of which amino acids are important in distinguishing between different taxa (Walton & Curry 1991). The same four genera were collected as fossils from within a single horizon of the Shakespeare Group in New Zealand (approximately 600000 years before present (BP)), and were again distinguished by PCA analysis of their intracrystalline amino acid composition (figure 4*b*). The shells of the four genera have not homogenized in the fossil record, as would be the case if they had absorbed amino acids from the surrounding sediments. Amino acids are abundant in sediments, but in all cases investigated their compositions are readily distinguished from brachiopod shell amino acids when subject to PCA (Walton & Curry 1991). The differences detected by PCA most probably reflect back to the original compositional variability detected in their living representatives. Although shell amino acid composition may be affected by environmental conditions during the life of the organism, in this case these effects have been identical because the specimens lived together and certainly experienced identical diagenetic histories.

5. LIPIDS IN RECENT BRACHIOPODS

GC and GC–MS analysis of intracrystalline molecules from Recent brachiopod shell extracts reveals the presence of an homologous series of saturated acyclic

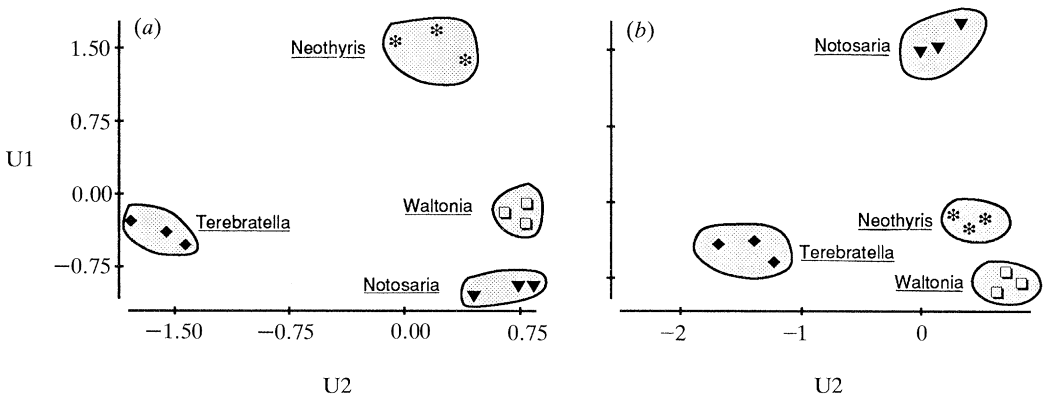

Figure 4. Plot of first (U1) and second (U2) principal component axes of amino acids in the shells showing the differentiation (*a*) between 4 Recent brachiopod genera and (*b*) between fossil representatives of the same taxa, collected from the Shakespeare Group, near Wanganui, N. Island, New Zealand. In (*a*) Gly has a high positive scoring on the first principal component axis, and Ser, Ala, Ile, Leu and Lys have high negative scores, whereas for the second principal component axis, Asp and Glu have high positive scores and His, Arg, Thr and Pro have high negative scores. In (*b*) Thr has a high positive scoring on the first principal component axis, and Gly, Arg, Valine and Leucine have high negative scores, whereas for the second principal component axis, Asp and Glu have high positive scores and Pro, Ile and Ala have high negative scores.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

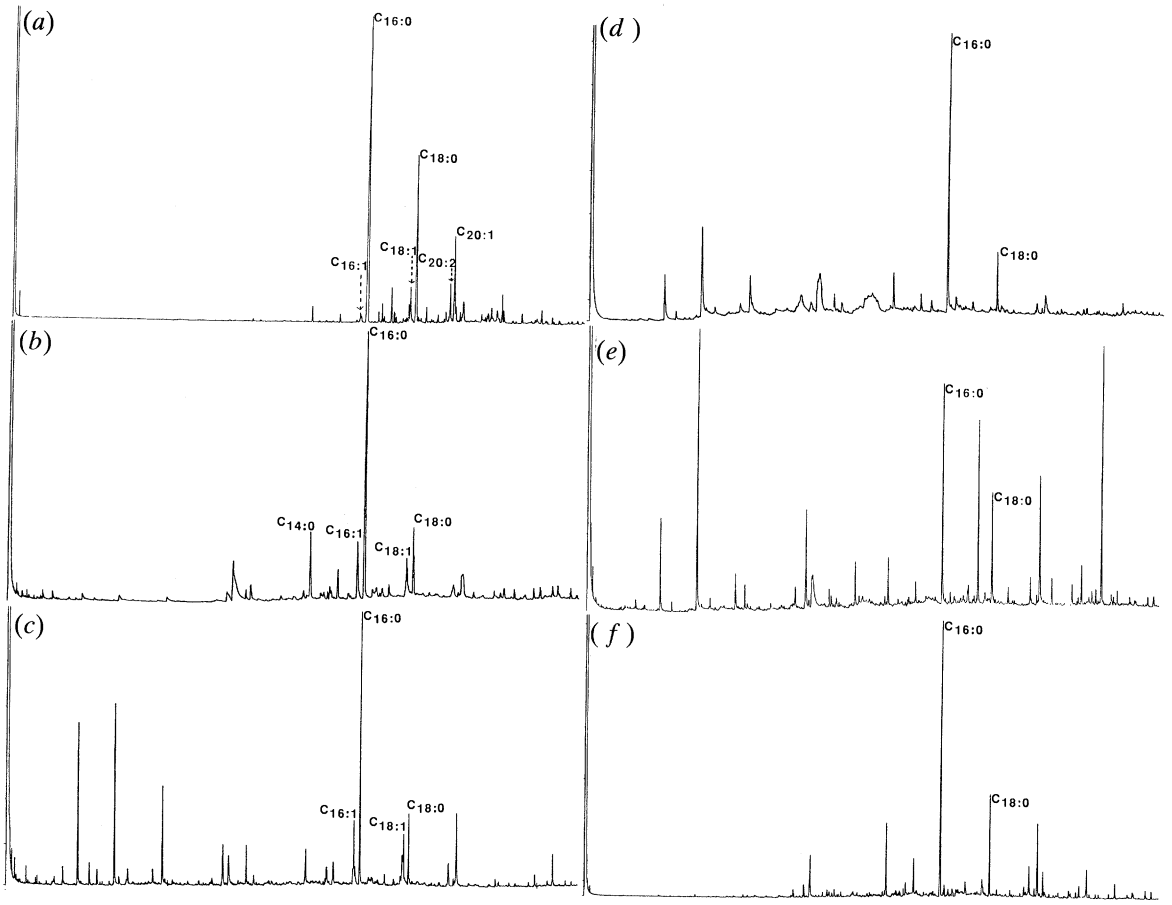

Figure 5. Gas chromatograms of petroleum ether : dichloromethane (50 : 50 by volume). Carbon numbers of the *n* fatty acids are indicated. (*a*), Recent *Neothyris*; (*b*), Shakespeare Group *Neothyris*; (*c*), Tewkesbury Fm *Neothyris*; (*d*) Castlepoint Fm *Neothyris*; (*e*), Gee Greensand Fm *Pachymagas*, (*f*), McDonald Limestone Fm *Liothyrella*. In (*e*) the major peak on right of chromatogram (retention time = 51 min) has been assigned to contamination.

carboxylic acids (derivatized as the methyl ester) as well as a number of unsaturated components. The dominant lipids detected in *Neothyris* in order of decreasing abundance are: *n*-$C_{16:0}$, *n*-$C_{18:0}$, *n*-$C_{20:1}$, *n*-$C_{17:0}$, *n*-$C_{18:1}$, *n*-$C_{20:2}$, fatty acids (figure 5*a*).

6. LIPIDS IN FOSSIL BRACHIOPODS

A range of *Neothyris* fossil extracts as analysed using the method described above. *Neothyris* shells from the Shakespeare Group (600 000 years BP) contained a range of saturated and unsaturated carboxylic acids, namely *n*-$C_{16:0}$, *n*-$C_{18:0}$, *n*-$C_{14:0}$, *n*-$C_{16:1}$, *n*-$C_{18:1}$ in order of decreasing abundance (figure 5*b*). With increasing age, low molecular mass lipids begin to increase in relative abundance to the above acids, but as yet their assignments are equivocal. *Neothyris* from the Tewkesbury Formation (Fm) (1.6 Ma) contained *n*-$C_{16:0}$, *n*-$C_{18:0}$, *n*-$C_{16:1}$, *n*-$C_{18:1}$ fatty acids (figure 5*c*), whereas the same genus from the older Castlepoint Fm (1.85 Ma) contained *n*-$C_{16:0}$ and *n*-$C_{18:0}$, although there is a notable absence of unsaturated fatty acids (figure 5*d*).

Analyses of older fossil brachiopods of a different genus (*Pachymagas*) from the Gee Greensand Fm (South Island, New Zealand; 23 Ma) revealed *n*-$C_{16:0}$ and *n*-$C_{18:0}$ fatty acids (figure 5*e*), and these same compounds were also recovered from the shells of *Liothyrella* from the McDonald Limestone Fm (30 Ma). These fossil extracts did not yield unsaturated fatty acids (figure 5*f*).

The presence of lipids within brachiopod shells is well known (Jope 1965; Stoyanova 1984) but until now there has been no attempt to distinguish between intercrystalline and intracrystalline lipids. The distinction is important as the intercrystalline lipids are more prone to post-depositional ingress. In this study intercrystalline lipids have been detected in extractions of the shell powders, and these are quite distinct from the intracrystalline lipids released from the subsequent demineralization of the residues. These results have therefore shown for the first time the presence of lipids within brachiopod biocrystals.

All Recent and fossil brachiopod shells examined contained normal saturated carboxylic acids, with $C_{16:0}$ (palmitic) and $C_{18:0}$ (stearic) fatty acids the dominant compounds present. With increasing age there is a decrease in the levels of lipids extracted from the shells, and a decrease in the levels of unsaturated fatty acids. In the samples studied, the unsaturated carboxylic acids disappear in shells older than approximately 2 Ma. Low molecular mass compounds increased in relative abundance with increasing age, with the exception of *Liothyrella* from the McDonald Limestone Fm (figure 5*b–f*); these compounds are not present in the shells of Recent brachiopods.

The function of these intracrystalline lipids is poorly understood, although these compounds may be responsible for establishing some of the essential conditions for calcification, as has been suggested for the lipid fraction of ostrich egg shells (Kriesten *et al.* 1979). The distribution and abundance of intracrystalline lipids may well be amenable to the kind of multivariate analysis applied to amino acids, and could augment the molecular discrimination between Recent and fossil taxa.

7. APPLICATIONS OF INTRACRYSTALLINE MOLECULES

The demonstration that brachiopod intracrystalline molecules do not appear to be contaminated by extraneous amino acids or lipids over time suggests several geological applications; their taxonomic uses have been discussed above and in immunological papers (Lowenstein & Schenenstahl, this symposium) and will not be expanded here. The short amino acid sequences recoverable from fossils have little geological application, but extensive research has already highlighted important applications for amino acids in absolute dating, stratigraphic correlation (including correlation from land to sea (Bowen *et al.* 1989)), environmental reconstruction, and nitrogen and carbon stable isotope determinations to investigate the diets of ancient organisms. It has been suggested that racemization studies and stable isotope ratios from individual amino acid enantiomers will also allow the indigenity of the amino acid to be demonstrated (Bada, this symposium; Engel & Macko 1986), especially from biominerals (such as the apatite in bone) which has a more porous structure than brachiopod calcite.

Previous isotopic determination from shells must inevitably have included carbon and nitrogen from molecules as well as from calcite, but as the absolute quantities of molecules involved is probably of the order of 1% of shell mass (amino acids alone account for about 0.04% of shell mass in *Terebratulina*, not including the substantial quantities of lipids, carbohydrates and other molecules present) the difference is likely to be comparatively insignificant and within the range of experimental error. However, if there was any preferential release of carbon and nitrogen from intracrystalline molecules during the preparation of shells for isotopic investigation then the significance of these molecules would increase.

There are indications that the decay rates of proteins in brachiopod shells provide useful environmental information. For example, several relatively unstable amino acids appear to be much better preserved in the *Waltonia* specimens from the Tewkesbury Fm than in most other Plio-Pleistocene horizons investigated (i.e. the peak at approximately 1.6 Ma in figure 2*c*). The Tewkesbury Fm represents a shallow marine environment, as compared with the fully marine faunas of the other formations investigated (Fleming 1953). The improved preservation of serine and other usually fragile amino acids in the Tewkesbury Fm may reflect superior preservation conditions or a relation between environmental conditions and the types of amino acids incorporated. These possibilities are being investigated given the widespread distribution of Recent brachiopods and their fossil ancestors in New Zealand ecosystems and Plio-Pleistocene successions. Other applications may appear once the function of intracrystalline molecules is revealed; for example, if any were toxic they would discourage predation.

8. SHELL COLOUR

During the biochemical investigation of Recent brachiopod intracrystalline molecules, it became clear that red shells retained their colour throughout the preparation protocol which stripped off all intercrystalline molecules. The solution produced by dissolving the calcium carbonate of the shell was also red, suggesting that shell coloration is caused by a soluble intracrystalline molecule. Reverse-phase HPLC of these samples allowed the isolation of the coloured fractions, which were then re-analysed by SDS-PAGE (polyacrylamide gel electrophoresis). In all six red-shelled brachiopod genera analysed, the molecule responsible for shell colour is an intracrystalline protein (ICP1) with an apparent molecular mass of 6.5 kDa. All other intracrystalline molecules are colourless, and non-coloured shells do not have ICP1.

Detailed analyses of ICP1 have confirmed that this chromoprotein has a very similar N-terminal amino acid sequence in the three red brachiopod genera *Waltonia*, *Neothyris* and *Terebratella*. The proteins are clearly homologous (there is no need to insert gaps to align them) but searching of recent versions of the protein and DNA sequence databases (EMBL, NBRF, GenBank & SwissProt using the GCG package) has so far failed to reveal any significant match with the primary structure of any known protein.

UV spectrophotometry and standard colour tests (Dunning 1963) have suggested that the prosthetic compound may be a carotenoid, which as a similar role in crustacean chromoproteins (Zagalsky 1976). ICP1, like other chromoproteins, is soluble in water, but the prosthetic chromophore becomes insoluble when separated, which is characteristic of a lipid–protein association. This emphasizes the importance of preparation technique because the chromophore could not be isolated for analysis in organic solvents until it had been stripped from the protein segment of the molecule.

The importance of showing that the red colour of brachiopods is caused by an intracrystalline protein, results from the fact that fossil brachiopods with preserved colour patterns have been documented from many geological horizons. During the investigation of New Zealand Pleistocene brachiopods a number of specimens with red coloured shell corresponding to that of Recent con-generic species have been recovered from sediments up to 400 000 years old. Records of colour pattern do extend much further back in time, indeed as far back as the Palaeozoic. Surprisingly perhaps, some of the best recorded examples of patterned brachiopod shells occur in Devonian and Carboniferous rocks, with ages ranging from 350 to over 400 Ma (Blodgett *et al.* 1988).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

At these ages the colour has usually faded or altered, although there are some reports of original colours being preserved that fade rapidly on exposure to light and oxygen. Most fossil 'coloured' brachiopods simply show colour patterns that are reddish brown or black, suggesting that the original prosthetic molecule has altered over geological time. The majority of fossil brachiopods do not show preserved colour patterns, although shells which were originally uniformly coloured, rather than patterned, are easily overlooked. If some molecules do indeed survive within the shell for long periods of geological time, then it may be possible to recover the alteration or breakdown products of the chromoprotein from shells that have lost their colour as a result of the light or oxygen sensitivity of the prosthetic group. This may open up the possibility of reconstructing the colour of fossil shells from an intracrystalline biomarker, an important new ability given that colour may be a crucial factor in evolutionary history (radiations into shallow water and onto land) because it provides protection from predators and radiation. Blumer (1965) has shown that this is possible by determining the breakdown pathway of a chromophore found in the skeleton of fossil crinoids more than 160 million years old.

9. DISCUSSION

Apart from lipids and proteins, it is thought that other types of molecules (e.g. carbohydrates) are present inside biocrystals. The full exploitation of this phenomenon will only be realized when these compounds have been localized, characterized and their function determined. It is possible to recover amino acids, lipids and probably other types of molecules from early Mesozoic or even Palaeozoic brachiopods (i.e. up to 550 Ma), but undoubtedly it becomes progressively more difficult to utilize these molecules in older specimens. Potential problems which must be addressed include the possibility of regeneration (e.g. compounds decay and then are produced *de novo* from simple building blocks within the biocrystal), coelution and hence misidentification of decay products due to identical chromatographic behaviour, and the generation of entirely new molecular species. The precise location of molecules within the shell, and their relation with the calcite matrix, are also crucial factors that have considerable bearing on the extent to which intracrystalline molecules survive in the fossil record, are free to interact with other components, and can be utilized in palaeobiological studies.

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Discussion

J. L. Bada (*Scripps Institution of Oceanography, University of California at San Diego, U.S.A.*). The pigments Dr Curry finds in the shell layers are likely photosynthetic in origin. Is it thus possible that the protein components that are present are derived from the surrounding seawater and are not directly synthesized by the brachiopods?

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

G. B. Curry. We certainly consider it likely that the pigment portion of the chromoprotein is derived from the brachiopod diet, possibly from some planktonic organism present in the surrounding seawater. We have not considered the possibility that the entire carotenoprotein had been directly assimilated, rather than synthesized by the brachiopod. We may get some information on this from a current survey of the pigments present in other parts of the brachiopod (for example the gonads) and of the pigmentation present in pelagic microorganisms that occur in the vicinity of brachiopod populations.

M. H. Engel (*School of Geology and Geophysics, University of Oklahoma, Norman, U.S.A.*). I was curious about the potential preservation of unstable amino acids (e.g. serine) in some of the higher molecular mass fractions of proteins from fossil brachiopods. Wherever the presence of serine is commonly attributed to a modern overprint (contamination), several investigators (e.g. Weiner, Lowenstein) have reported the presence of relatively high concentrations of serine in proteins isolated from fossil ammonoids that are Cretaceous in age. Perhaps Dr Curry could comment on the relative abundance of serine in his various molecular mass fractions going back in time. Also, I would be interested in any thoughts he might have concerning the racemization of amino acids in the various molecular mass fractions of the brachiopods!

G. B. Curry. Serine and various other 'unstable' amino acids are indeed recovered by hydrolysing the insoluble high molecular mass fraction extracted from brachiopod shells. We have not measured the racemization state of amino acids in fossil brachiopods, but intend to do so in the near future.

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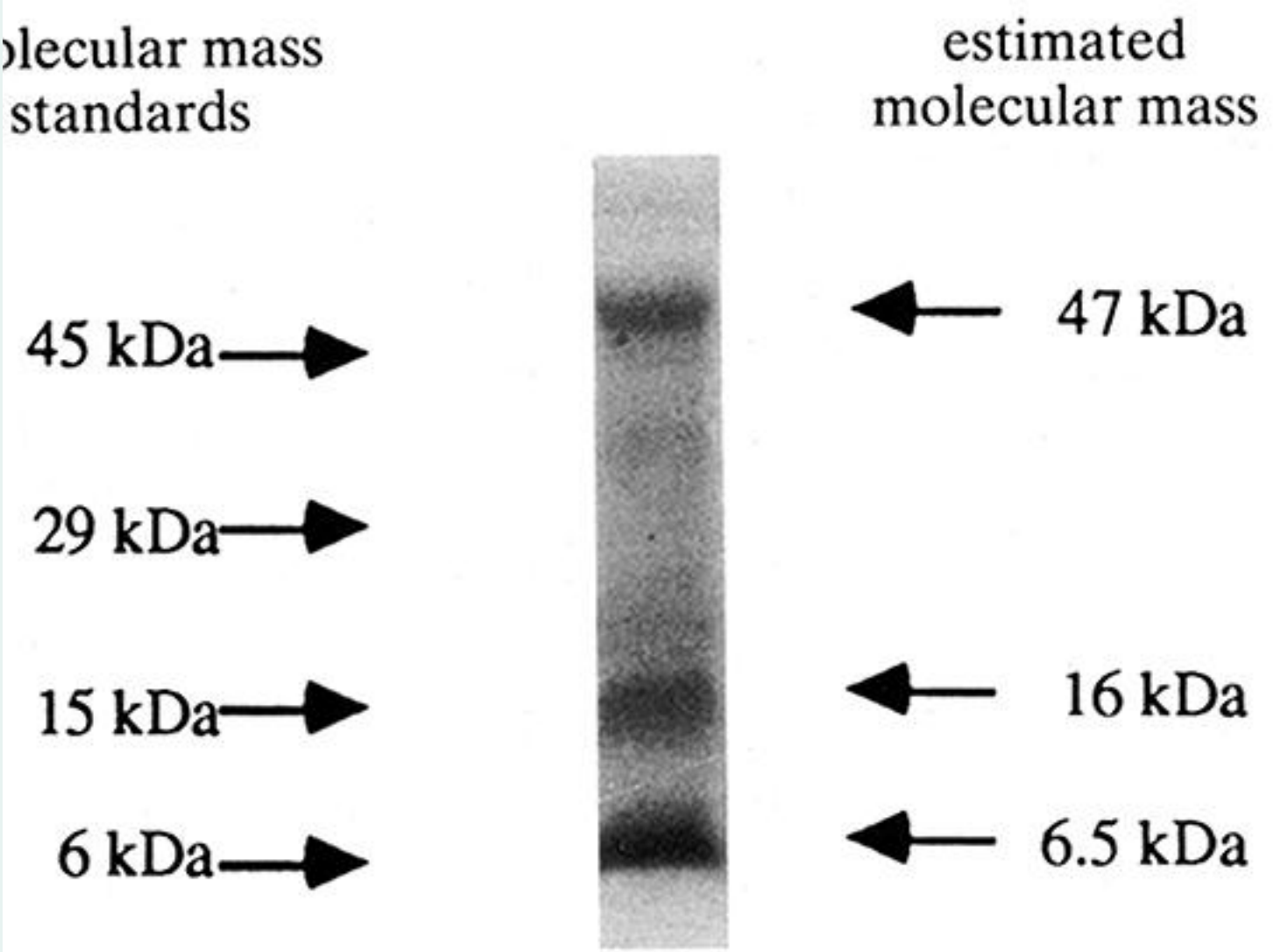

gure 1. SDS-PAGE of intracrystalline proteins in the Recent brachiopod *Neothyris* from New Zealand.