# MICROBIOLOGY LIBRARY

J. S. HERON LTD.
*Printers & Bookbinders*
46a WEST PRINCES STREET
GLASGOW 041-332 1883

This thesis is dedicated to my mother and to the memory of my father.

# Studies of the Lysozyme of *Mytilus edulis* L

John G. McHenery

Presented for the degree of Doctor of Philosophy in the Faculty of Science, University of Glasgow.

Department of Microbiology

January, 1980

"Discovery consists of seeing what everybody has seen and thinking what nobody has thought".

Albert von Szent-Gyorgyi

## Acknowledgements

I should like to thank Dr. T.H. Birkbeck for the seemingly limitless patience and guidance he has shown me during my research.

For the financial support which enabled me to carry out my research I thank the Science Research Council.

I should like to record my gratitude to Prof. J.A. Allen of the Marine Station, Millport for dissecting the bivalves examined in the course of my research, and to Prof. A.C. Wardlaw for initiating this collaborative study and for his continued interest. For supplying, at short notice, the large numbers of Mytilus I required, I thank Mr. Finlayson and his staff at the Marine Station and all who made me welcome while working there. For their interest, advice and humour I am indebted to all in the Microbiology Department.

The preparation of this thesis has been eased greatly by Dr. Birkbeck and Prof. Wardlaw, and I am indebted to them for reading the manuscripts and for their advice. I thank Mrs. A. Strachan for deciphering my writing, typing the thesis and her good humour and Mr. I. McKie for his photographic services.

Finally, I should like to thank my mother, my fiancee and relations for the patience and support they afforded me, in so many ways.

# SUMMARY

Mytilus edulis lysozyme responded differently from hen egg-white (HEW) lysozyme to sodium chloride when assayed by an agar-diffusion (lysoplate) technique with Micrococcus luteus as substrate. However at 1% (w/v) NaCl both enzymes gave similar dose response curves and this concentration was used in all lysoplate assays.

The distribution of lysozyme in Mytilus edulis and other bivalves was investigated. In Mytilus the highest concentrations were found in structures associated with digestion, particularly in the style and digestive gland. Similar distributions were found in Modiolus modiolus, Chlamys opercularis and Tellina tenuis. However, in Mya arenaria, highest concentrations were found in gill and digestive gland which may reflect differences in the choice of food particle if lysozyme is related to the proportion of bacteria utilized from the food.

Lysozyme was purified from crystalline styles of Mytilus edulis by chromatography on Amberlite CG-50 and carboxymethyl cellulose. The product was 90% pure, with a specific activity of 16,200 enzyme units per mg of protein, representing a 216-fold purification from crystalline style and 48,000-fold purification compared to homogenates of whole animals (less shells). Collagenase and β-glucuronidase activities, which were found in style homogenates were not detected in purified lysozyme. The enzyme satisfied Salton's criteria for lysozyme, lysing bacterial cells and reducing the turbidity of cell wall suspensions with the liberation of reducing groups and an amino sugar complex. The enzyme cleaved the β1-4 glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine in the bacterial cell wall and thus was a mucopeptide N-acetylmuramylhydrolase (E.C.3.2.1.17).

By sodium dodecyl sulphate polyacrylamide gel electrophoresis, the molecular weight was approximately 18,000 daltons and on isoelectric focusing the isoelectric point was 9.2. On polyacrylamide gel electrophoresis in acid gels Mytilus lysozyme migrated more slowly than HEW lysozyme. Both Mytilus and HEW lysozymes were eluted from Bio-Gel P-60 later than expected from their molecular weights. Also, there was a low recovery of the applied Mytilus lysozyme, possibly due to electrostatic interaction with the gel matrix.

Purified Mytilus lysozyme was heat stable in the presence of gelatin and sodium chloride but was inactivated at room temperature in solution in distilled water. At 100°C in barbital acetate buffer (pH 7.1, ionic strength (I) 0.006) containing 0.5% (w/v) gelatin and 0.2M NaCl, the half life of purified enzyme was 2.5 min.

The apparent affinity constant (Ka app) of Mytilus lysozyme for M. luteus cells, 505 mg $l^{-1}$, was higher than most published values for other lysozymes.

Mytilus lysozyme enzymic activity responded in a similar manner to HEW lysozyme to changes in the pH and I of suspending buffers, being optimally active at pH 7.1, I = 0.011. Under these conditions Mytilus lysozyme was optimally active at 46°C although a lower optimum was found in pH 4.6, I = 0.054 buffer. The activity of Mytilus lysozyme was sensitive to the cation concentration of suspending buffers, being activated at low concentrations and inhibited at higher concentrations, particularly by the divalent cations of $Mg^{2+}$ and $Ca^{2+}$.

In addition to lysing M. luteus cells, Mytilus lysozyme lysed a variety of marine isolates, including several gram-negative bacteria. Although sea water inhibited Mytilus lysozyme activity against M. luteus,

bacteriolytic activity was still detectable. It is suggested that the primary role of lysozyme in Mytilus is in utilization of bacteria as food rather than as a host defence mechanism against infection.

Contents — Page

## Index of Tables

## Index of Figures

Page

## Abbreviations

| | |
|---|---|
| B.S.A. | Bovine serum albumin |
| c | Y-axis intercept |
| CC | Chitin-coated |
| Disc | Discontinuous |
| EDTA | Ethylenediaminetetraacetate |
| HEW | Hen egg-white |
| I | Ionic strength |
| Ka app. | Apparent affinity constant |
| m | Gradient |
| NAG | N-acetylglucosamine |
| NAM | N-acetylmuramic acid |
| n.t. | Not tested |
| PAGE | Polyacrylamide gel electrophoresis |
| PYEA | Peptone yeast extract agar |
| PYEW | Peptone yeast extract water |
| r | Correlation coefficient |
| Rf | Relative mobility |
| S | Substrate concentration |
| SDS | Sodium dodecyl sulphate |
| SW | Sea water |
| v | Velocity |
| Ve | Elution volume |
| Vo | Void volume |
| wt | Weight |

# INTRODUCTION

## Historical Aspects

In 1922 Fleming detected a factor in the secretions and tissues of humans, guinea-pigs, dogs, hen egg-white (HEW) and turnip which had the ability to dissolve certain bacteria, particularly an isolate he named Micrococcus lysodeikticus which is now known as Micrococcus luteus (Buchannan and Gibbons, 1974). He called this factor "lysozyme" as its properties were similar to those of ferments and demonstrated that it could be precipitated from solution by protein precipitants.

Since Fleming's initial report "lysozymes" have been detected and isolated from a variety of additional sources, including bacteriophage (Tsugita et al, 1968), fungi (Hash, 1963), amoebae (Upadhyay et al, 1977), invertebrates (Jolles and Zuilli, 1960), fish (Fletcher and Grant, 1968), amphibians (Nace, Suyama and Iwata, 1965), reptiles (Gayen et al, 1977) and plants (Howard and Glazer, 1967). These enzymes exhibit differences in their physico-chemical characteristics and their action spectra suggesting that the term lysozyme encompasses a variety of enzymes which share certain common activities. Lysozyme-like activities have been detected in the common mussel, Mytilus edulis (Jolles, Jolles-Thaureaux and Fromageot, 1957; Hardy, Fletcher and Gerrie, 1976) and this thesis is concerned with the properties, distribution and function of the lysozyme found in Mytilus edulis and a comparison with those of other lysozymes as reviewed in the following pages.

## Structure and Mode of Action of Lysozymes

Fleming (1922) noted that hen egg-white contained high

concentrations of lysozyme and because of its availability this has become the most widely and thoroughly studied of this group of enzymes.

Abraham and Robinson (1937) first prepared crystalline HEW lysozyme but it was not until 1963 that Jolles et al. (1963) and Canfield (1963), working independently, elucidated the primary structure; later the position of its disulphide bands was determined (Jolles, Jaurequi-Adell and Jolles, 1964; Brown, 1964). The HEW lysozyme molecule consists of a single polypeptide chain of 129 amino acids with a molecular weight of 14,300 daltons and an N-terminal lysine residue (Thompson, 1951, 1952; Jolles et al, 1963; Canfield, 1963).

Although most lysozymes so far isolated are of a similar molecular weight to that of HEW lysozyme, some are larger, notably those of the plants Papaya latex and turnip which have molecular weights of 28,000 daltons (Table 1).

Phillips and his colleagues described the three-dimensional structure of HEW lysozyme based on X-ray crystallographic studies (Blake et al, 1965; Phillips, 1967). The molecule is roughly ellipsoidal (45 x 30 x 30 Å) consisting of a right hand alpha-helical wing (residues 1-40) with a less rigid left hand wing (residues 41-95). The remaining residues partially close the gap between the two wings leaving a deep cleft containing the active site which is partially lined with hydrophobic residues.

Lysozyme became of increasing interest to those studying bacterial cell walls and HEW lysozyme was shown to act against the mucopolysaccharide of the cell wall of M. luteus (Salton, 1952). Later, it was shown that the enzyme degraded chitin, a linear polymer of β(1-4) linked N-acetylglucosamine (NAG) (Fig 1a) suggesting that it possessed

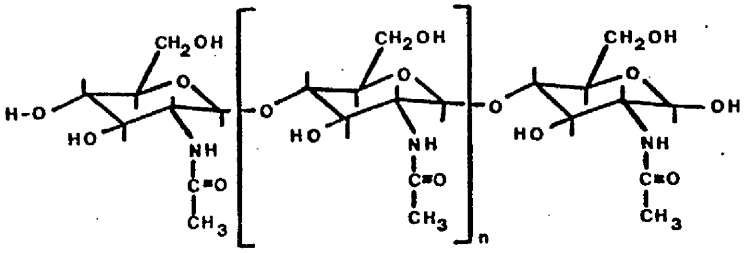
| Source | Molecular Weight | Isoelectric Point | Optimum pH at I | Reference |
|---|---|---|---|---|
| Homeothermic vertebrates | | | | |
| Hen egg white | 14,300 | | | Canfield (1963) |
| | | 11.18 | | Sophianopoulos and Sasse (1965) |
| | | | 8.6 at 0.025 | Saint-Blancard et al (1970) |
| | | | 9.2 at 0.02 | Davies, Neuberger and Wilson (1969) |
| | | | 5 at 0.2 | " " " " " |
| Human milk | 16,000 | 10.5-11 | 6.35 | Parry, Chandon and Shahani (1969) |
| | | | 5.5-7 at 0.1 | Saint-Blancard et al (1970) |
| | | | 8.2 at 0.0375-0.05 | " " " " |
| Human leukemia | 14,000 | | | Canfield et al (1971) |
| | | 11.1 | | Canfield, Collins and Sobell (1976) |
| | | | 8-8.2 at 0.025-0.0375 | Saint-Blancard et al (1970) |
| Goose | 20,000 | | | Arnheim, Inouye and Laudin (1973) |
| | | | 3.8 and 5.2 at 0.125-0.25 | Saint-Blancard et al (1970) |
| Black swan | 14-15,000 | | | Morgan and Arnheim (1974) |
| | 19,500-21,500 | | | " " " " |
| Poikilothermic vertebrates | | | | |
| Rana pipiens | 17,800 | basic | | Ostrovsky et al (1976) |
| | 15,000 | " | | " " " |
| Pleuronectes platessa | 14-15,000 | | 5.4 at 0.1 | Fletcher and White (1973) |
| Trionyx gangeticus | 15,400 | > HEW | 7.5 at 0.014-0.07 | Gayen et al (1977) |
| Plants | | | | |
| Turnip | 28,000 | | 5 | Bernier et al (1971) |
| Papaya latex | 28,000 | | 4.6 | Howard and Glazer (1969) |
| Microorganisms | | | | |
| Bacteriophage $T_4$ | 19,000 | | 7.2 - 7.4 | Tsugita et al (1968) |
| Streptomyces erythraeus | 18,500 | 9.5 | 4 at 0.1 | Morita, Haru and Matsushima (1978) |
| Pyocinogenic Pseudomonas aeruginosa | 24,000 | 9.4 | 6.4 | Ochi, Azegami and Ishi (1978) |
| Chalaropsis | 23,000 | 7.53 | | Hash (1974) |

Fig 1. The structures of chitin and peptidoglycan.

(a) Structure of chitin. The reducing terminus is to the right. One of the smallest lysozyme substrates $(NAG)_3$ consists of 3 pyranose rings.

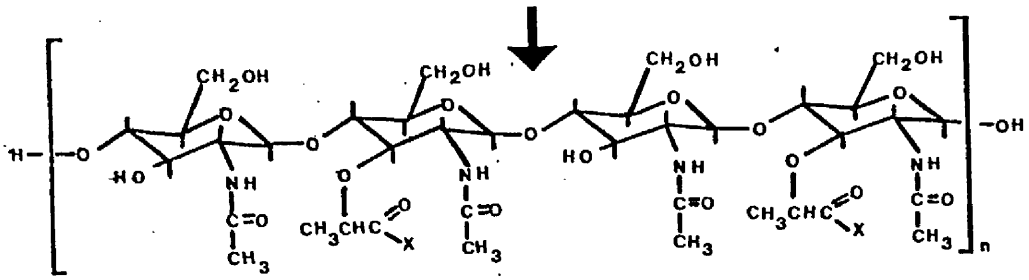
(b) Structure of the bacterial cell wall glycopeptide. In native cell walls the X-groups may be free carboxyl groups or amino groups of peptide through which the polysaccharide chains are cross-linked. The NAG-NAM bond is not cleaved by the enzyme. The smallest cell wall saccharide which is a substrate is the tetra-saccharide. (After Chipman and Sharon, 1969).

a)



LYSOZYME

b)



NAG NAM NAG NAM

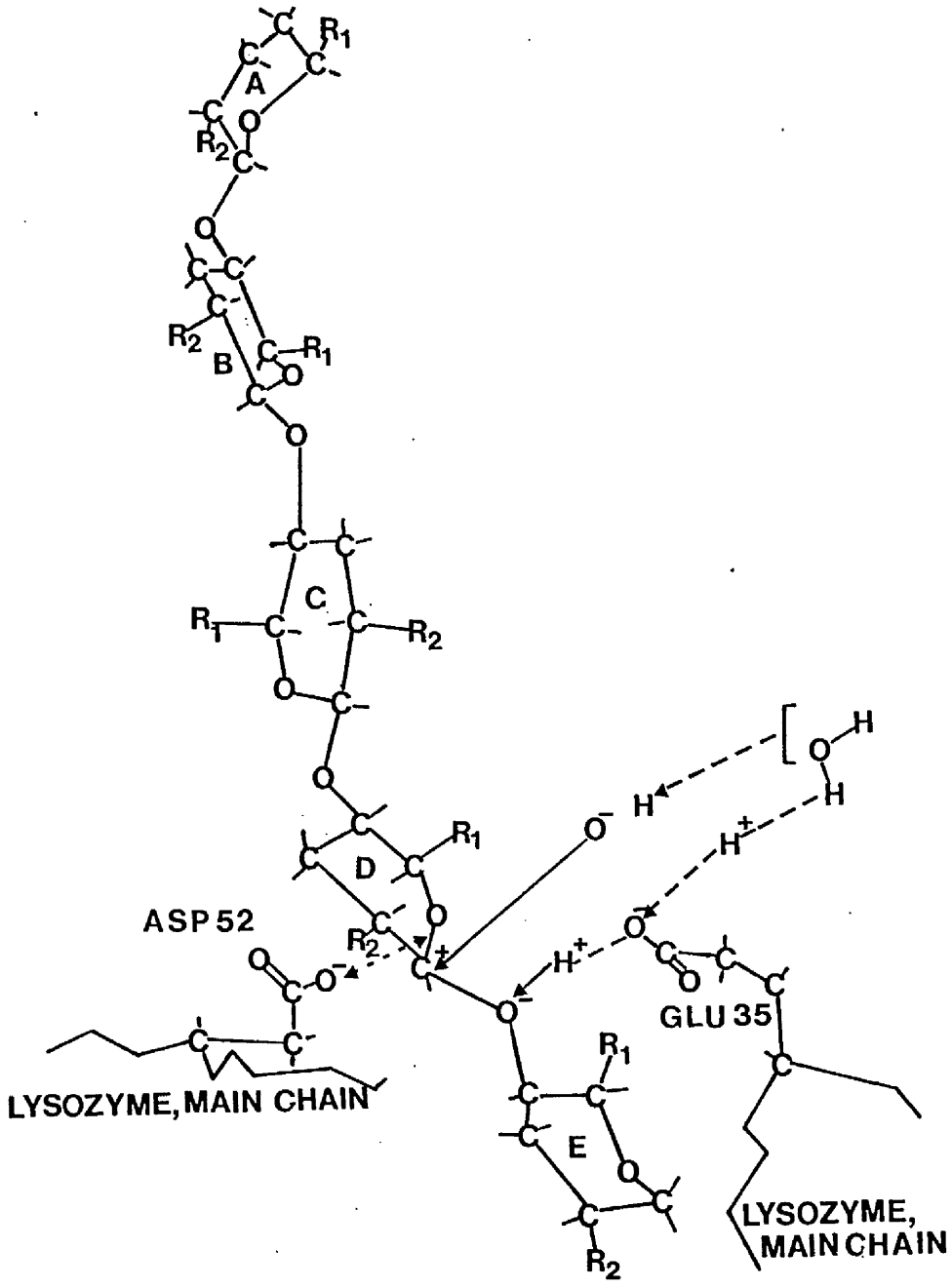
β(1-4) glucosaminidase activity (Berger and Weiser, 1957). It was proposed that the enzyme splits the β(1-4) link between N-acetylmuramic acid (NAM) and NAG (Brumfitt, Wardlaw and Park, 1958) and Salton and Ghuysen (1959) subsequently isolated a tetrasaccharide containing equimolar amounts of NAG and NAM from lysozyme digests of M. luteus cell walls. On further incubation with lysozyme this tetrasaccharide yielded a disaccharide of NAG and NAM. It was deduced that the tetrasaccharide was a dimer of this disaccharide linked by a lysozyme sensitive β(1-4) linkage. The complete structure of the tetrasaccharide was later established as: N-acetylglucosaminyl-β(1-4)-N-acetylmuraminyl-β(1-4)-N-acetylglucosaminyl-β(1-4)-N-acetylmuramic acid (Fig 1b) (Jeanloz, Sharon and Flowers, 1963).

The key to understanding the action of HEW lysozyme was the determination of its tertiary structure by Blake et al (1965). Observations by Phillips (1966), Blake et al (1967) and Vernon (1967) suggested that rupture of the bacterial cell wall proceeded along the following course (as reviewed by Jolles, 1969):

1. The substrate becomes attached to the enzyme and is held in position by hydrogen bonds and other forces. In the process, the ring of the fourth sugar residue becomes distorted and takes up a conformation favouring formation of a carbonium ion.
2. A proton is transferred to the glycosidic oxygen atom from the side chain of the glutamic acid residue 35 (Fig 2).
3. Heterolysis of the $C_1$ carbon-oxygen band gives a carbonium ion which is stabilized by interaction with the negative charge on the side chain of the aspartic acid residue 52 (Fig 2).
4. The disaccharide obtained from the hexasaccharide diffuses away and a water molecule attacks the carbonium ion, thus completing hydrolysis.

Fig 2. Splitting of substrate by lysozyme.

Digestion of substrate is believed to involve the proximity and activity of two side chains, residue 35 (glutamic acid) and residue 52 (aspartic acid). It is proposed that a hydrogen ion ($H^+$) becomes detached from the OH group of residue 35 and attaches itself to the oxygen atom that joins rings D and E, thus breaking the bond between the two rings. This leaves the carbon atom 1 of the D ring with a positive charge, in which form it is known as a carbonium ion. It is stabilized in this condition by the negatively charged side chain of residue 52. The surrounding water supplies an $OH^-$ ion to combine with the carbonium ion and an $H^+$ ion to replace the one lost by residue 35. The two parts of the substrate then fall away, leaving the enzyme free to cleave another polysaccharide chain (Phillips, 1966).


A
B
C
D
E
ASP 52
GLU 35
LYSOZYME, MAIN CHAIN
LYSOZYME, MAIN CHAIN

Three factors are responsible for the catalytic effect: general acid catalysis, activation of the substrate by distortion and electrostatic interaction.

Sharon and Seifter (1964) reported that the action of HEW lysozyme on the cell wall tetrasaccharide led to the formation of products of higher, as well as lower, molecular weights and similar observations were made with chitin-derived saccharides (Kravchenko and Maksimov, 1964). These observations led to the suggestion that the higher molecular weight products arose by "transglycosylation", or glycoside transfer, to acceptor saccharides which in these cases were the substrates themselves. The new bonds are of beta configuration (Pollock, Chipman and Sharon, 1967a) and the reaction is indeed transglycosylation and not simply the reverse of hydrolysis (Chipman, Pollock and Sharon, 1968).

It has been suggested that the reaction of a polysaccharide with lysozyme occurs in several steps (Chipman and Sharon, 1969). The saccharide is cleaved at the glycosidic bond of the residue in subsite D (Fig 2), and the portion of the substrate lying in E and beyond is released from the cleft, leaving an intermediate glycosyl enzyme which may then react with water (hydrolysis) or with an acceptor molecule which comes into the cleft (transfer). Postulation of an intermediate is necessary as the stereochemistry of the reaction demands that the leaving saccharide and the acceptor saccharide moieties occupy the same region and they cannot do so at the same time (Chipman and Sharon, 1969). The acceptor involved in the above reaction is not necessarily the substrate as lysozyme can transfer NAG-NAM residues to a variety of mono- and disaccharide acceptors. Lysozyme can also form and cleave $\beta(1\text{-}2)$ glycosidic bonds (Pollock, Chipman and Sharon, 1967a and b).

A possible mechanism for the hydrolysis of the cell wall tetrasaccharide not involving direct cleavage, but involving a transfer mechanism has been proposed by Sharon (1964) and is outlined in Fig 3.

It should not be assumed that these mechanisms apply to all lysozymes; it is the action on bacterial cell walls which is the common identifying feature of all lysozymes, but it is not necessarily their only activity.

Apart from the role of aspartic (52) and glutamic acid (35) residues in HEW lysozyme little else is known of the function of the various amino acid residues in the lysozymes so far purified. However, it has been shown by fluorescence quenching that oligosaccharides of NAG which inhibit HEW lysozyme do so by interacting with tryptophan residues at the active site (Shinitzky et al, 1966).

Jolles et al (1966) proposed that cystine, while not being necessary for biological activity, determined the heat stability and specific activity of the lysozymes. It was suggested that a high cystine content would lead to a low specific activity of the enzyme since the greater degree of crosslinking would increase the amount of folding, in turn, making it more difficult for the substrate to bind. Although analyses of vertebrate lysozymes were compatible with this suggestion the invertebrate lysozymes so far analysed fail to support it (Table 2).

## Criteria for Identification of Lysozymes

At the 1972 Lysozyme Conference (Osserman, Canfield and Beychock, 1974) the following recommendation on the nomenclature of lysozyme was drafted and unanimously approved:

"The present position is that while the Commission on Enzyme

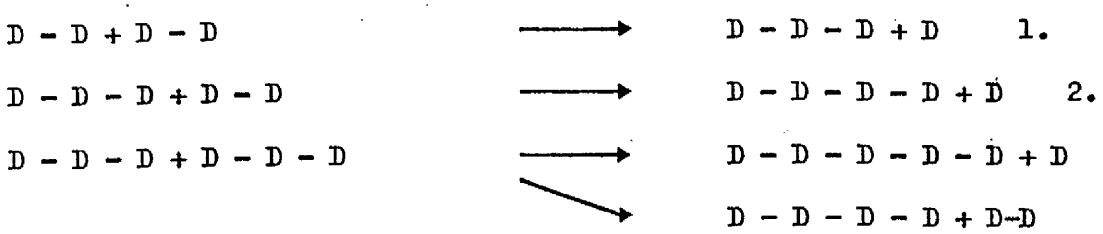



Fig 3. Course of the transglycosylation reaction induced by lysozyme. D is the cell wall disaccharide isolated from M. luteus. In reaction 1 two d-tetraose molecules (D - D) are converted into one hexaose (D - D - D) and one disaccharide (D) molecule. (After Sharon, 1964).

Table 2. Cystine contents and specific activities of lysozymes from a variety of sources.

| Source | Half-Cystine Residues | Specific* Activity | Reference |
|---|---|---|---|
| Hen egg-white | 8 | 1 | Jolles (1969) |
| Duck | 8 | 1 | " " |
| Human | 6 | 3 | " " |
| Goose | 4 | 5 | " " |
| *Ceratitis capitata* | 4 | < 0.02 | Fernandez-Sousa *et al* (1977) |
| *Galleria mellonella* | 8 | 5.9 | Powning and Davidson (1976) |
| *Nephthys hombergi* | 10 | 4 | Perin and Jolles (1972) |
| *Asterias rubens* | 12 | 14 | Jolles and Jolles (1975) |

*The specific activity of HEW lysozyme is taken as 1 and all others are based on this value.

Nomenclature in 1961 decided that E.C.3.2.1.17 should be called muramidase, it reversed this decision in 1964. The recommendation now reads:

E.C.3.2.1.17 Systematic name: mucopeptide N-acetylmuramylhydrolase; recommended trivial name: mucopeptide glucohydrolase, lysozyme; other name not recommended: muramidase.

This recommendation should be followed since otherwise confusion in indexing will result.

The term lysozyme should include only enzymes which are β-1,4-glycan-hydrolases. They may be of animal, plant or microbial origin. In a full description, the species of origin should be given and any allelic or other variation indicated.

The primary identification of a lysozyme is usually made by determining its lytic activity against Micrococcus lysodeikticus (NCTC 2665). Chitinases have no action on this microorganism and are, therefore, excluded."

Previously Salton (1957) had proposed that before an enzyme could be called a lysozyme it must satisfy three criteria. It must:

1. Lyse intact bacterial cells or reduce the turbidity of isolated cell wall suspensions.
2. Liberate reducing sugars.
3. Liberate an amino-sugar complex.

Jolles (1964) subsequently suggested further restrictions and stated that a lysozyme must fulfil six criteria. It must be:

1. A basic protein.
2. Of low molecular weight (near 15,000).
3. Stable at acidic pH (even at 100°C and pH 4.5 for one minute).

4. Unstable at alkaline pH especially at higher temperatures.
5. Active against suspensions of M. luteus.
6. Active on an appropriate substrate to liberate compounds which can be detected by reagents for reducing and amino sugars.

Later however, Jolles (1969) admitted that his criteria were somewhat exacting as not even some avian lysozymes could fulfil them. For example, goose egg-white lysozyme is labile at high temperatures, even at acidic pH (Jolles et al, 1968) and has a molecular weight of 20,000 daltons (Arnheim et al, 1973). In addition, numerous lysozymes have been isolated of molecular weights above 15,000 daltons (Table 1).

A further complication in the acceptance of the formulated criteria may lie in the transglycosylation reaction of lysozymes (q.v.). Rana pipiens lysozyme decreased the amount of free amino sugars on reaction with chitobiose (Ostrovsky et al, 1976) and similar observations have been made for HEW lysozyme (Sharon, 1967; Kravchenko, 1967). Thus, Salton's third criterion may require revision.

## Activities of Lysozymes

Generally, the most sensitive bacteria to lysozyme belong to the gram-positive genera Micrococcus, Sarcina, Staphylococcus and Bacillus whereas untreated, living gram-negative bacteria are more resistant (Salton, 1957). However, the presence of 6-O-acetyl groups on the acetyl-muramic acid of M. luteus confers resistance to lysozyme degradation (Brumfitt, Wardlaw and Park, 1958) as does the absence of N-acetyl groups from muramic acid in B. cereus (Araki et al, 1972) or the presence of free amino groups, which confer a positive charge to the cell wall (Perkins, 1967).

Whereas resistance may be mediated by an altered peptidoglycan other structural polymers such as teichoic acids may confer resistance. This polymer, present in the cell walls of Staph. aureus and many other bacteria, is highly negatively charged and can bind basic proteins, such as HEW lysozyme, thus conferring a degree of resistance (Strominger and Tipper, 1974).

Although the structure conferring, rigid R-layer of the Escherichia coli cell envelope is sensitive to lysozyme (Primosigh et al, 1961), the enzyme must first cross the outer membrane of the intact cell, which is essentially impermeable to hydrophilic molecules larger than 600 daltons (Nikaido, 1976). Then, as it acts on the inner edge of the murein layer (Braun, Rehn and Wolf, 1970), it must penetrate this layer. Gram-negative bacteria can be lysed by lysozyme when treated at low pH (Peterson and Hartsell, 1955) or in conjunction with ethylenediaminetetraacetate (versene, E.D.T.A.) (Repaske, 1956) suggesting that the outer membrane must first be destabilised for the enzyme to function. In the case of Paracoccus denitrificans no such treatment is required for lysis by lysozyme (Wilkinson, 1977). A possible further barrier to lysozyme reaching the murein layer is the interaction of the enzyme with the periphery of the gram-negative cell, as found with Pseudomonas aeruginosa (Cheng, Ingram and Costerton, 1970). The lysozyme forms a complex with the lipopolysaccharide which dissociates in the presence of magnesium ions (Day, Marceau-Day and Ingram, 1978).

Gram-negative bacteria normally resistant to lysozyme may be lysed by the enzyme when exposed to antibody and/or complement (Muschel, Carey and Baron, 1959; Wardlaw, 1962). A further method for the lysis of gram-negative bacteria, by leucocytes, was proposed by Miller (1969).

Free radicals generated by hydrogen peroxide and ascorbic acid may disturb the integrity of the cell wall allowing lysozyme to reach and disrupt the murein layer.

In addition to disrupting wall polymers, lysozyme may degrade polymers in the capsule of B. megaterium (Tomcsik, 1956) and may also cause flocculation of bacteria due to the basic nature of the lysozyme and the negative charge on the cell surface (Salton, 1957). Lysozyme is also active against a number of marine and fresh water blue green algae, causing disintegration (Crespi, Mandeville and Katz, 1962) or sphaeroplast formation (Fulco, Karfunkel and Aaronson, 1967).

It has been pointed out that the role of lysozyme in the resistance to bacterial infection, as proposed by Fleming (1922) has not been clearly established (Hirsch, 1960) however the major bactericidal agent in normal human sera for B. subtilis is lysozyme (Selsted and Martinez, 1978). Intracellular lysozyme may play a role in the destruction of pathogenic bacteria after phagocytosis (Brumfitt and Glynn, 1961) and the main function of lysozyme may be to digest glycopeptide debris from cell walls of bacteria killed in other ways (Chipman and Sharon, 1969). In recent years the involvement of lysozyme in immunostimulating or adjuvant activities has been widely studied (Jolles, 1976) and it has been shown that lysozyme digests of Staph. epidermidis exhibit immunoadjuvant activity (Kotani et al, 1977).

Although lysozymes from different species and sometimes from different parts of the same animal may be chemically different it has been stated that they exhibit qualitatively the same biological activity (Jolles, 1967). However, it has been proposed that the variety of isozymes of lysozyme found in frogs may increase the spectrum of defence

against bacteria (Ostrovsky et al, 1976) and also that certain isozymes may be involved in protecting the animal from the virus implicated in the Lucke renal adenocarcinoma (Nace and Ostrovsky, 1977) suggesting a degree of specialisation of function within the isozymes and possibly that not all of the isozymes are primarily antibacterial (Snyder and Harrison, 1977). Frog lysozymes are not unique in having antiviral activity as similar properties have been attributed to human lysozyme (Arimura, 1973).

## Assay of Lysozyme Activity

The majority of assays for lysozyme rely on the susceptibility to lysis of M. luteus, as reported by Fleming (1922). Alternative assays have been proposed, such as that with the 3,4 dinitrophenyl tetra-N-acetyl-β-chitotetraoside of Ballardie and Capon (1972) as substrate (Turner, Ghneim and Freeman, 1979), however, such assays require complex equipment.

The earliest spectrophotometric assay methods involved prolonged incubation of the enzyme-substrate mixture (Boasson, 1938). The first rapid, accurate lysozyme assay was developed by Smolelis and Hartsell (1949); M. luteus cells were suspended in buffer and the increase in transmission caused by the addition of lysozyme recorded. The other routinely employed assay, the lysoplate system (Osserman and Lawlor, 1966) also utilizes M. luteus as substrate; the cells are suspended in an agarose base into which samples are applied and allowed to diffuse, causing lysis. As with the turbidometric, spectrophotometric, assay, the experimental procedures employed in the lysoplate assay have been adapted to meet the experimental requirements of the investigators cited in this thesis.

The spectrophotometric assay is dependent on the rate at which the absorbance of a suspension of M. luteus decreases being proportional to the concentration of lysozyme added, at low enzyme concentrations. The lysoplate assay is dependent upon the diameter of the zone of clearance being proportional to the log of the enzyme concentration (Peeters and Vantrappen, 1977).

Whereas the spectrophotometric assay is rapid the lysoplate assay can cover a wider concentration range (Peeters and Vantrappen, 1977), is simpler, requires less complex equipment and permits numerous samples to be assayed simultaneously. The results obtained with either system are dependent on ionic strength (I), pH, choice of standard and method of reading the diameter of the zone of clearance or turbidity (Greenwald and Moy, 1976). While the lysoplate assay is less time-consuming, it offers less discrimination and is less accurate at low concentrations than the spectrophotometric assay (Zucker et al, 1970), and using the same samples and standards the lysoplate assay gives values three to four times greater for the unknowns than the spectrophotometric assay, illustrating the danger in drawing comparisons between concentrations gathered with different assays (Greenwald and Moy, 1976).

It has been suggested that the spectrophotometric assay is preferable as constituents of the sample may alter the diffusion rate of lysozyme in the lysoplate system. As the same changes cannot easily be effected in the standards, it was proposed that with lysoplates the unknowns should be standardised against the same type of lysozyme under the same conditions (Ensinck and van Haeringen, 1977).

## Classes of Lysozyme

Comparison of lysozymes of various origins led Jolles et al

(1974) to distinguish the following classes:

1. Lysozymes which are primarily muramidases with only a weak chitinase activity (c type lysozyme): e.g. the "classic" HEW lysozyme, several other avian egg-white lysozymes, all the studied human and some invertebrate lysozymes.
2. Lysozymes which are only muramidases (g type lysozyme): e.g. goose egg-white lysozyme.
3. Lysozymes which are primarily chitinases but also possess a muramidase activity: e.g. plant lysozymes.
4. The phage lysozymes constitute a fourth class.

The lysozymes of different classes differ in amino acid sequence, molecular weight and enzymatic properties. Also the antigenic specificity of enzymes of the c- and g-types is different.

The literature on animal lysozymes is extensive and will be considered under three groupings: vertebrate, invertebrate and bivalve lysozymes.

Vertebrate Lysozymes

The literature on vertebrate lysozymes is considerable and has been comprehensively reviewed elsewhere (Salton, 1957; Jolles, 1969; Chipman and Sharon, 1969; Imoto et al, 1972). The most studied of the lysozymes is that of hen egg-white which has a molecular weight of 14,300 daltons (Canfield, 1963) and an isoelectric point of pH 11.18 (Sophianopoulos and Sasse, 1965). The effect of pH on activity is well documented, the enzyme being active over a wide pH range with the optimum depending on ionic strength. Maximum activity was found at pH 9.2 where I = 0.02 with a further peak at pH 5, I = 0.2 (Davies, Neuberger and Wilson, 1969). The optimum was reported to be pH 8.6

at I = 0.025 by other workers (Saint-Blancard et al, 1970) who studied a variety of vertebrate lysozymes and found that alkaline pH improved the rate of lysis at low ionic strength in all cases except goose lysozyme which had optimum activity at acidic pH (pH 3.8 and 5.25) and at higher ionic strengths. The molecular weights, isoelectric points and pH optima for a variety of lysozymes are listed in Table 1.

That salt enhances lysozyme activity was reported in Fleming's (1922) original paper; HEW lysozyme requires cations for activity but at high concentrations these become inhibitory, divalent cations being more inhibitory than monovalent ones (Smolelis and Hartsell, 1952) and trivalent ones even more inhibitory (Ostroy et al, 1978).

Lysozymes have been detected in a wide variety of homeothermic vertebrates but less is known of the occurrence of lysozyme in poikilothermic vertebrates. In fish, lysozyme was first detected in the surface mucin of the plaice, Pleuronectes platessa by Fletcher and Grant (1968) and it was claimed that following immunisation of rainbow trout the serum lysozyme concentration paralleled the increase in antibody concentration (Vladimirov, 1968) and was implicated in the bacteriolytic activity of the fish. In the plaice, lysozyme was present in most tissues examined, but absent from the gill lamellae, intestine and liver (Fletcher and White, 1973). As the lysozyme was associated with monocytes and neutrophils in plaice blood it was proposed by Fletcher and White (1973) that plasma lysozyme originated in these cells as does the plasma lysozyme in mammals (Hansen et al, 1972).

Secretory lysozyme in mucus may have a different origin from that in serum as distinct lysozyme-containing cells, which did not

appear to be leukocytes were identified in plaice epidermis (Fletcher and White, 1973). The plaice lysozyme has a molecular weight between 14,000 and 15,000 daltons with a pH optimum of 5.4 at I = 0.1 (Table 1). In addition, plaice lysozyme is more heat labile and less basic than HEW lysozyme (Fletcher and White, 1976). High levels of lysozyme have also been detected in the wolf fish (Fange, Lundblad and Lind, 1976) and lumpsucker (Fletcher, White and Baldo, 1977).

The leopard frog, Rana pipiens, possesses eight isozymes of lysozyme, whose molecular weights vary (Table 1) as do their antigenic specificities, salt requirements and pH optima. However, they are all basic proteins and are heat stable at acidic pH (Ostrovsky et al, 1976).

To date only one reptile lysozyme has been purified, that from the egg of the tortoise, Trionyx gangeticus. Two forms of the enzyme were found; both were more basic than HEW lysozyme and they differed antigenically and in their electrophoretic mobilities (Gayen et al, 1977). The molecular weight of the more common variant was 15,400 daltons with optimum enzyme activity at pH 7.5, I = 0.04. Salt was required for activity but at higher concentrations was inhibitory, magnesium salts being more so than sodium or potassium salts (Gayen et al, 1977).

## Invertebrate Lysozymes

The discovery of a lysozyme in invertebrates was first reported by Jolles, Jolles-Thaureaux and Fromageot (1957) who detected lysozymes in an annelid, Nephthys hombergi and in a bivalve, the common mussel Mytilus edulis. The lysozyme of the former has since been well characterised (Table 3) and can be classified with goose lysozyme based on the inhibitory action of NAG polymers, although other characteristics

Table 3. Physico-chemical characteristics of non-bivalve, invertebrate lysozymes

| Source | Molecular Weight | Isoelectric Point | Optimum pH at I | Reference |
|---|---|---|---|---|
| Annelids | | | | |
| *Nephthys hombergi* | 13,000 | basic | 6-6.5 at 0.073 | Perin and Jolles (1972) |
| *Arenicola marina* | | | | Schubert and Messner (1970) |
| *Nereis diversicolor* | | | | " " " " |
| *Tubifex* spp. | | | | " " " " |
| *Enchytraeus* spp. | | | | " " " " |
| *Allobophora caliginosa* | | | | " " " " |
| *Hinido medicinalis* | | | | " " " " |
| *Haemopsis sanguisuga* | | | | " " " " |
| *Herpobdella octoculata* | | | | " " " " |
| Insects | | | | |
| *Galleria mellonella* | 14,700 | basic | 7 at 0.1 | Powning and Davidson (1973) |
| *Bombyx mori* | 16,500 | basic | 6-6.5 at 0.2 | " " " " |
| *Ceratitis capitata* | 23,200 | > 11 | 6.5 at 0.1 | Fernandez-Sousa *et al* (1977) |
| *Spodoptera eridania* | | | 5.5 | Anderson and Cook (1979) |
| *Periplaneta americana* | | | 3.5 | Powning and Irzykiewicz (1967) |
| Gastropods | | | | |
| *Helix pomatia* | 21,000 | I < II | | Takeda *et al* (1966) |
| | 24,000 | II ~ 9 | | " " " |
| *Biomphalaria glabrata* | | | | Rodrick and Cheng (1974a) |
| Echinoderms | | | | |
| *Asterias rubens* | 15,500 | basic | | Jolles and Jolles (1975) |
| | | | 5.75 at 0.0757 | Perin and Jolles (1976) |

such as the variation of initial velocity of lysis as a function of ionic strength (Perin and Jolles, 1973) suggested that it might belong to the HEW type.

Malke (1965) first reported a lysozyme from an insect source and it was later claimed that lysozyme was responsible for the immunity of insects to "non-specific germs" (Mohrig and Messner, 1968a). This suggestion was based on an observation that injection of bacteria, "non-specific substances", or injury caused a "defense" reaction consisting of an increase in the haemolymph lysozyme level in Galleria mellonella which decreased after a successful defence. The importance of lysozyme in insect immunity has been disputed, although the lysozyme level increased after infection no corresponding increase in immunity was detected, and in cases where immunity was stimulated the lysozyme level remained high after the immunity declined (Chadwick, 1970). Confirmation that lysozyme levels could be specifically stimulated in G. mellonella and also in Bombyx mori was reported by Powning and Davidson (1973) who determined some of the characteristics of the two lysozymes (Table 3) and reported that they responded to pH and ionic strength variations in a similar way to HEW lysozyme. The serum lysozyme concentration of Spodoptera eridania was also elevated by the injection of bacteria (Anderson and Cook, 1979).

The role of lysozyme in the defence of insects has not been established, but it may be involved in the formation of sphaeroplasts from gram-negative bacteria injected into the haemolymph of G. mellonella (Hanschke and Hanschke, 1977). A further protective role for lysozyme was proposed by Mohrig and Messner (1968b). As lysozyme levels varied throughout the intestine of the cockroach, Periplaneta americana, it was suggested that lysozyme is secreted in the anterior region of the

intestine and resorbed in the terminal region. Thus, acting in combination with passive protective factors of the intestinal tract, lysozyme may protect the insect from the multiplicity of bacteria absorbed with the food and regulate the formation of a typical bacterial flora.

A lysozyme has been isolated from the eggs of Ceratitis capitata which differs from those isolated from other insects (Table 3) and appears to be of the plant type (Fernandez-Sousa et al, 1977).

Three of the four possible combinations of muramidase and chitinase activity were detected in the digestive juice of the edible snail, Helix pomatia, by Takeda et al (1966). These were:-

a) an enzyme with both muramidase and chitinase activities (muramidase I)

b) an enzyme with muramidase activity but little or no chitinase activity (muramidase II)

c) a chitinase with no muramidase activity.

Muramidase II was larger and more basic than muramidase I (Table 3).

More recent studies on another snail, Biomphalaria glabrata, led to the conclusion that "lysozyme is released from phagocytes into serum as a result of challenge with B. megaterium. Although the exact role of the released lysozyme is uncertain it is hypothesized that it may serve as a humoral defence molecule." (Cheng, Chorney and Yoshino, 1977).

The properties of the lysozyme from the sea star, Asterias rubens, some of which are listed in Table 3, indicate that it may be a representative of another, invertebrate, lysozyme type. This lysozyme could be distinguished from that of N. hombergii by its high sensitivity

to changes in ionic strength and by its N-terminal sequence which differs from those of other lysozymes (Jolles and Jolles, 1975).

Bivalve Lysozymes

The first bivalve reported to possess a lysozyme was the common mussel, Mytilus edulis L (Jolles et al, 1957). Prior to this it had been reported that extracts of the digestive gland of Mytilus californianus possessed a thermolabile factor capable of dissolving bacteria (Zobell and Landon, 1937), and later a heat-stable agent which inhibited the growth of B. subtilis was detected in oyster juice (Li, 1960). Neither factor was characterised but haemolymph of the American oyster, Crassostrea virginica, was shown to inhibit growth of B. subtilis and B. megaterium and reduce the turbidity of suspensions of B. megaterium and M. luteus (McDade and Tripp, 1967a). The factor responsible for this activity was shown to be a lysozyme which satisfied the criteria proposed by Salton (1957). The optimum pH was 6.5 at an ionic strength of 0.15 and sodium chloride was required for activity; however, salt was inhibitory at concentrations above 1%. The enzyme was stable to heating at 80°C (McDade and Tripp, 1967a) and was present in the mantle mucus (McDade and Tripp, 1967b).

The optimum pH determined by McDade and Tripp (1967a) has been disputed by Rodrick and Cheng (1974b) who claimed it lay between pH 5 and 5.5 depending on the buffer used, the requirement for salt was not disputed and the optimum ionic concentration was determined as being 0.05 mM. The lysozyme was extremely sensitive to ionic variations (Rodrick and Cheng, 1974b) similar to HEW lysozyme (Davies, Neuberger and Wilson, 1969) and was active against a wide range of bacteria (M. luteus, B. subtilis, B. megaterium, E. coli, Gaffkya tetragena, Salmonella pullorum and Shigella sonnei)(Rodrick and Cheng, 1974b).

The most striking feature of the lysozyme of C. virginica which distinguishes it from others is its association with acidic proteins of different electrophoretic mobilities. Feng (1974a) proposed that "the lysozyme-like activity found in the oyster haemolymph may represent a case of functional convergence during the course of biochemical evolution."

The lysozyme of the soft shelled clam, Mya arenaria is also heat stable but has a slightly lower pH optimum (pH 4.5 to 5, depending on the buffer used). Monovalent cations are required for activity with an optimum salt concentration of 0.1 mM. The action spectrum on bacteria was similar to the lysozyme of C. virginica (Cheng and Rodrick, 1974).

Lysozymes have also been detected in the fresh water clam, Anodonta anatina (Messner and Mohrig, 1969) and the quahug clam, Mercenaria mercenaria (Cheng and Rodrick, 1975). In addition it has been suggested that there may be lysozymes present in the digestive systems of a number of bivalves (Kristensen, 1972a).

## Mytilus edulis (L)

The common mussel, Mytilus edulis is widely distributed along the coasts of Europe and also inhabits the North Atlantic shores of America (Yonge, 1971); it is a benthic (littoral or sub-littoral) semi-sessile species, and a particulate suspension feeder, relying for its food on a seasonally variable supply of particles within a limited size range. It may experience wide fluctuations in salinity and temperature and in its intertidal habitat it is exposed to air for varying periods of time with resulting problems of potential desiccation, thermal shock and lack of oxygen (Bayne, Thompson and Widdows, 1976). It lives on a variety of substrata, such as rock, stones, shingle, dead shells and

even compacted mud or sand, attached by its byssus threads (Seed, 1976).

Feeding and Digestion

The gills of bivalves serve both as respiratory surfaces and to trap food particles suspended in the water circulating through them (Fig 4) (Owen, 1974). Food particles are bound into mucus strings on the gill lamellae and are carried to the labial palps via ciliated grooves on the lamellar margins. These sort the incoming material and convey it either to the mouth or to the rejection tract which removes unsuitable or excess particles as pseudofaeces (Bayne et al, 1976). The lips, which are formed by a continuation of the outer and inner palps, are thought to function to prevent water being taken into the mouth (Gilmour, 1974).

Food enters the stomach (Fig 5) from the oesophagus in the form of a mucus string which is carried by the cilia of the buttress to the gastric shield. The crystalline style, a hyaline rod of mucoprotein, protrudes from the style sac, where it is secreted by cells of the typhlosoles (Giusti, 1970; Owen, 1974), into the stomach proper and rests against the gastric shield and around this the string is wound, as the style slowly rotates against the gastric shield, appearing to draw in more material from the oesophagus.

The mechanical action of the style against the gastric shield breaks up the food material into fragments which fall onto the typhlosole tongue and the duct tracts (Fig 5). This material can either be rejected as pseudofaeces, carried by the shield and buttress tract for further maceration at the style tip, or digested (Reid, 1965).

The cilia of the duct tracts and typhlosole tongue set up currents in the stomach sac keeping small light particles in suspension

Fig 4. View of the mantle cavity of the common mussel Mytilus edulis. The drawing is from the left side showing the food currents and is enlarged. The arrows on and at the edge of the gill-lamellae (G) indicate the paths of the main food-streams.

C.P. The dotted arrows and line at the ventral edge of the mantle indicate the ciliated path which carries the material rejected by the palps and that collected from the mantle to the point indicated by the arrow above B in the figure. Here the rejected material is pushed into the exhalent current.

A. Arrows indicating the paths of the heavier particles settling out of the main food-stream.

B. A sort of curtain hanging from the dorsal part of the inhalent aperture.

C. The line of attachment of the mantle to the body-wall.

D. Arrows in the supra-branchial chamber indicating the direction of the exhalent current.

E. Uplifted left border of the inhalent aperture to show the curtain, B.

F.H. Points between which the main food-current is drawn into the mantle cavity.

G. Left outer gill-lamella.

P.P. Left palps between which the edges of the left gill-lamellae may be seen to end.

$P^1P^1$ Right palps.

M. Locus of the mouth. (From Orton, 1910)

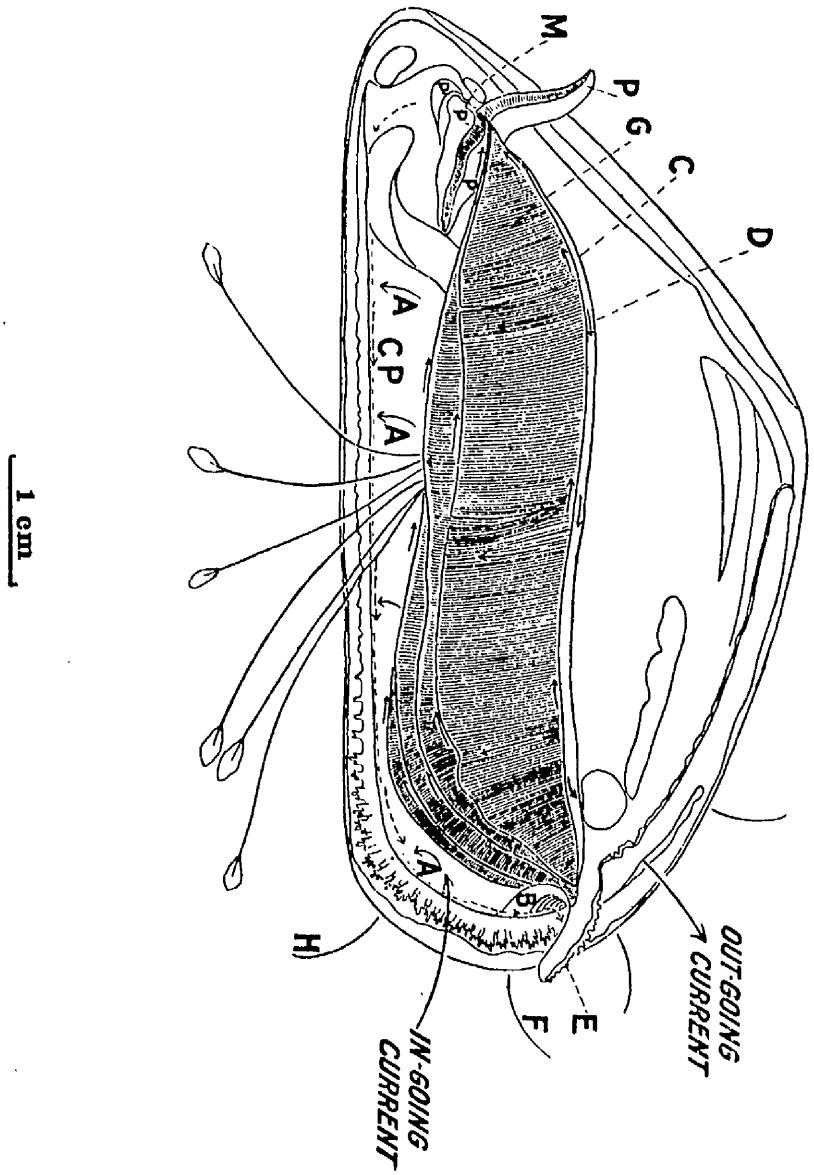
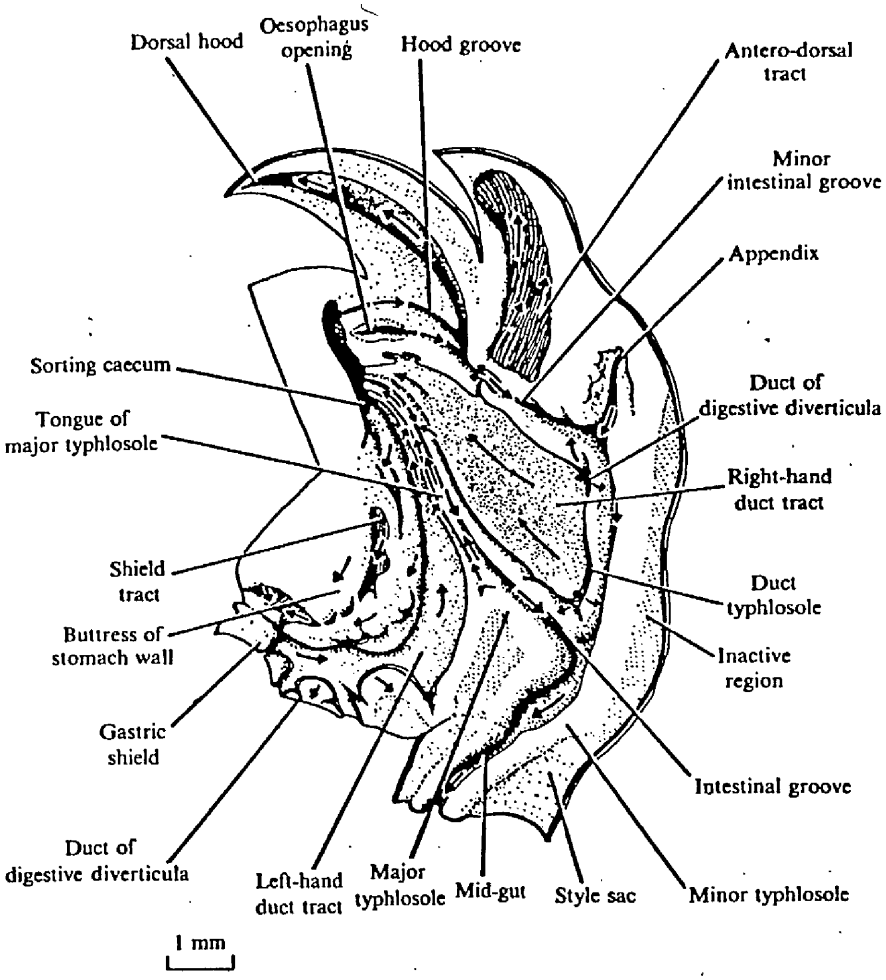

M
P
G
C
D
A
CP
A
A
B
H
E
F
OUT-GOING CURRENT
IN-GOING CURRENT
1 cm

Fig. 5. Interior of the stomach of Mytilus edulis

(From Reid, 1965)


Dorsal hood
Oesophagus opening
Hood groove
Antero-dorsal tract
Minor intestinal groove
Appendix
Sorting caecum
Tongue of major typhlosole
Duct of digestive diverticula
Right-hand duct tract
Shield tract
Buttress of stomach wall
Duct typhlosole
Inactive region
Gastric shield
Intestinal groove
Duct of digestive diverticula
Left-hand duct tract
Major typhlosole
Mid-gut
Style sac
Minor typhlosole
1 mm

and directing them towards the digestive gland duct openings. These ducts possess inhalent currents which channel this material into the digestive diverticula (Owen, 1955).

The digestive diverticula consist of blindly ending tubules which are linked with the stomach by a system of branched partially ciliated ducts. By light microscopy two cell types are discernible in tubule epithelium, one acidophilic, columnar and vacuolated, the other pyramidical and basophilic. The acidophilic cells are responsible for intracellular digestion of food and are called digestive cells. Particulate material from the lumen is taken up by pinocytosis (Owen, 1972), the pinocytic vacuoles so formed appear to fuse (Thompson, Ratcliffe and Bayne, 1974) giving rise to heterophagosomes (Owen, 1972). The origin and identification of the primary lysozomes which contain the hydrolytic enzymes is uncertain.

Among the features of the basophil cell are an extensive granular cytoplasmic reticulum, numerous free ribosomes and an active Golgi body, all of which indicate that it is equipped for extensive protein synthesis. The function of the basophil cells is not known, but they are regarded as either immature digestive cells or as enzyme secreting cells (Sumner, 1966; Owen, 1970).

In addition to its role in the digestion of food the digestive gland of Mytilus edulis serves as a site for storage of metabolic reserves (Thompson et al, 1974). There is general agreement that the digestive diverticula functions primarily as an organ of intracellular digestion and absorption but it may have a secretory function, producing extracellular enzymes (Owen, 1974).

Mytilus feeds largely on phytoplankton and recent detritus

(Bayne et al, 1976) and has a large array of carbohydrases in its digestive system. Extracts of the style degrade starch (Allen, 1921) and many other substrates including maltose, sucrose, melezitose, cellobiose, amylose, glycogen, laminaran, cellulose and chitin (Kristensen, 1972a). Digestive gland extracts exhibited greater activity against these substrates but was also active against trehalose, raffinose, lactose, dextran, methylcellulose, xylan, pectin, alginic acid, sodium alginate, agar and furcellaran (Kristensen, 1972a). It was suggested that lysozyme might be involved in the observed hydrolysis of cellobiose. Other enzymic activities detected in Mytilus include acid phosphatase, aryl sulphatase, β-glucuronidase, N-acetyl-β-glucosaminidase and esterase in the lysosomes of digestive cells (Sumner, 1969) and proteases (Rosen, 1949).

## The Distribution and Function of Lysozyme in Bivalves

When lysozyme was first detected in Mytilus edulis (Jolles et al, 1957) and later in C. virginica haemolymph (McDade and Tripp, 1967a) the enzyme source was unknown. Subsequently specimens of the latter were dissected and the enzyme was found to be localised in mantle and secreted into mantle mucus; the conclusion was drawn that the lysozyme in haemolymph represented "spill over" of material secreted by mantle cells into the mucus covering their external surface (McDade and Tripp, 1967b).

Subsequent examination of haemolymph of C. virginica (Rodrick and Cheng, 1974b) and Mya arenaria (Cheng and Rodrick, 1974) led to the proposal that lysozyme was synthesized by organelles in intact haemolymph cells and stored in the "primary and secondary phagosomes" in which phagocytosed bacteria were degraded, and from which it could be released.

Most invertebrates react against foreign material by phagocytosis, encapsulation or synthesis of humoral components (Cooper, 1976). The intracellular degradation of foreign materials, such as bacteria, by phagocytic cells plays an important role in the internal defence mechanisms of molluscs (Tripp, 1960, 1961), although oyster cells are somewhat reluctant to engulf certain bacteria (Bang, 1961). Lysozyme was released during phagocytosis of B. megaterium by haemolymph cells of Mercenaria mercenaria and thus serum lysozyme has its origin in cells. The function of lysozyme in the serum of molluscs remains uncertain. Released enzyme may neutralize or kill susceptible microorganisms which have invaded the haemolymph but have not been phagocytosed (Cheng et al, 1975).

It has been suggested that "degranulation" of molluscan granulocytes (the explosive discharge of the contents of the cytoplasmic granules from the cell without damage to the cytoplasmic membrane) (Foley, 1974), is a morphological reflection of the release of enzymes into the medium. Degranulation of the granulocytes of both Mercenaria mercenaria and C. virginica in vitro, in the absence of bacteria, has been reported (Feng, 1974b; Foley, 1974), although the process is increased when the cells are exposed to bacteria. Thus, the release of enzymes from oyster cells is a normal process which is enhanced during phagocytosis (Cheng et al, 1975) similar to that of mammalian leukocytes (Wright and Malawista, 1973) and snail phagocytes (Cheng et al, 1977).

Feng and Canzonier (1970) presented evidence that haemolymph lysozyme levels change with haemocyte concentration and activity during the immune response by C. virginica to Bucephalus and Minchinia infections, thus supporting the role of lysozyme in bivalve immunity.

The finding of lysozyme in haemolymph cells, mantle cells and mucus (the surface in greatest contact with the environment) of C. virginica is not unique in marine animals. Lysozyme-secreting cells have been detected in plaice haemolymph and epidermis and lysozyme has been detected in epidermal and gill mucus (Fletcher and Grant, 1968; Fletcher and White, 1973), suggesting that in these cases lysozyme distribution may be an adaptation to the environment, playing a role in both internal and external defence mechanisms (Tripp, 1974a).

Direct evidence for the in vivo function of lysozyme in invertebrates is lacking but if it is indeed a defensive factor then it should not be considered in isolation but as one of a group of possibly defensive factors. Mytilus edulis haemolymph also contains lysins and agglutinins which are active against a wide range of vertebrate erythrocytes and bacterial cell walls (Hardy et al, 1976). Such agglutinins may decrease the mobility of invading organisms and have an opsonic effect similar to other invertebrate agglutinins (Tripp, 1974b). However, Hardy et al (1976) considered that this putative defensive role may be a fortuitous by-product of the primary function of the haemolymph proteins.

While the function of lysozyme in Mytilus is, as yet, unknown, mantle injury and invasion by microorganisms results in an inflammatory response with three characteristic stages (Mikhailova and Prazduckar, 1961, 1962):

1. an initial migration of haemocytes to the damaged area followed by encapsulation of foreign material,
2. intensive phagocytosis with both intra- and extracellular digestion of foreign particles,

3. the migration of haemocytes containing engulfed material to the blood and thence to the digestive gland, kidney and other epithelial linings where they are discharged to the exterior.

Haemocytes play an important role in the digestion and transport of material in the alimentary canal of mussels (Wagge, 1955). They ingest particles of nutrient value which are too large to enter the cells of the digestive diverticula (Yonge, 1926a) and contain lipases (Yonge, 1926b; George, 1952; Zachs and Welsh, 1953), carbohydrases (Takatsuki, 1934) and proteases (Yonge, 1926b; Takatsuki, 1934); acid phosphatase and β-glucuronidase have also been identified in the macrophages (Moore and Lowe, 1975). Although the function of these cells remains obscure, present evidence indicates a role in the transport of nutrient reserves around the body (Bayne et al, 1976b). It has been found that B. megaterium phagocytosed by C. virginica haemolymph cells was degraded and the resultant carbohydrate converted into glycogen which was subsequently discharged into the serum and absorbed by the body tissues (Cheng and Rudo, 1976). Thus, in the oyster the degradation of bacteria in haemolymph cells containing lysozyme (Rodrick and Cheng, 1974b) can be of nutritional value to the animal.

## Bacteria as Food for Mussels

That bacteria might be of nutritional value to mussels was first proposed by Allen (1921). Later, it was shown that other invertebrates could thrive on a diet of bacteria and it was suggested that if animals could live on such a diet, then if bacteria occurred in their food in nature they would be utilized in the proportion in which they occurred (MacGinitie, 1932). Indeed, it had earlier been proposed that one of the most important functions of aquatic bacteria

was the conversion of dissolved organic matter into particulate matter which animals could utilize (Krizencky and Podhradsky, 1927).

The marine environment, particularly sediments, is rich in bacteria (Zobell and Feltham, 1934) and the comparative paucity of bacteria in sea water has been attributed to their being fed upon by marine animals (Waksman and Carey, 1935). The Californian mussel, Mytilus californianus can reduce the concentration of bacteria in sea water $10^3$ to $10^4$ fold within six hours, the bacteria being digested, and not rejected in the pseudofaeces. Mussels gained weight when maintained on a bacterial diet over long periods (Zobell and Landon, 1937). Similar findings were reported by Kincaid with oysters (Zobell and Landon, 1937). An extract from the digestive system of the mussel lysed eighteen species of marine bacteria, but this activity was destroyed by heating at $75^{\circ}C$ for ten minutes. When mussels were maintained on a diet of bacteria, 5 to 10% of the solid matter in the bacteria was assimilated by the mussels which were maintained on a bacterial diet for up to two years (Zobell and Feltham, 1938). A thermolabile bacteriotoxic activity has also been detected in the crystalline style of Mya arenaria (Johansson, 1945).

The importance of bacteria in the nutrition of other bivalves has been investigated; in Macoma balthica, which feeds on a mixture of organic debris and silt. The bacterial coating on these particles is digested, the detritus and silt being rejected in the faeces (Newell, 1965). It has been suggested that filter feeders which would normally obtain insufficient bacterial food to meet their energy requirements (Zobell and Feltham, 1938) may tap a vastly enhanced food supply in regions where deposits are fine (and thus the ratio of bacteria to

weight of deposit is increased) and turbulence occurs which resuspends the deposits (Newell, 1965).

Macoma can remove 95 to 99% of bacteria, all protozoa and 50 to 75% of diatoms from detritus during its passage through the animal (Fenchel, 1972). The high levels of α-amylase found in bivalve digestive systems may reflect the possible involvement of this enzyme in removing microorganisms from sand grains and detrital particles (Kristensen, 1972b).

Although the carbohydrases in the digestive organs of bivalves were found to split reserve polysaccharides and some oligosaccharides at a significant rate, generally they displayed little or no activity against the majority of structural polymers which make up the bulk of organic detritus (Kristensen, 1972a). This was taken to support the theory that the digestion of cell walls of higher plant debris in detritus was insignificant when compared to the digestion of bacteria, fungi and microalgae by bivalves (Fenchel, 1972).

The findings of Reid and Reid (1969) and Fenchel (1972) that bacteria were not immediately killed at the tip of the style, and the high bacterial numbers recorded in the stomach compared with those in the intestine of Macoma (Fenchel, 1972) led Kristensen (1972b) to propose that bacteria were digested in the digestive gland and not in the stomach.

Components of the crystalline style are secreted by cells on the typhlosoles of the style sac (Giusti, 1970; Owen, 1974) and are formed into styles consisting largely of water, carbohydrate (principally hexosamines (Owen, 1974)) and proteins including enzymes, particularly carbohydrases (Owen, 1966; Kristensen, 1972a,b); in Mya and Cardium spp

some of the protein is bound to the carbohydrate. In addition to its enzymic activities the style:

1. has a high buffering capacity (Berkeley, 1959),
2. inhibits bacterial multiplication,
3. emulsifies oil, reduces viscosity and surface tension (surfactants).

Some of these properties might serve to separate food particles from detritus and bind to other substances, thus easing absorption and transport of food particles (Kristensen, 1972b).

Lysozyme activity has been detected in Mytilus edulis (Jolles et al, 1957; Hardy et al, 1976) and although this enzyme has not previously been detected in Mytilus digestive gland and style extracts, it has been suggested that the hydrolysis of cellobiose by these extracts is due to lysozyme (Kristensen, 1972a). A member of the Mytilidae, Mytilus californianus, can digest bacteria (Zobell and Landon, 1937) and Yonge (1937) has suggested that the enzymes possessed by a species reflects the composition of its diet. Therefore, one might expect bacteriolytic enzymes to be present in the digestive systems of members of the Mytilidae which digest bacteria.

It was intended that the investigations undertaken and recorded in this thesis would disclose the distribution and, by inference, the function of lysozyme in Mytilus edulis and provide sufficient enzyme to allow for its characterisation.

## Objects of Research

Lysozyme-like activities have previously been detected in a variety of bivalves, but as yet the properties and functions of these lysozymes are poorly understood. Also there are conflicting reports on the distribution of lysozyme in these animals.

The primary object of this investigation was to purify the lysozyme of Mytilus edulis, to determine its physico-chemical properties and enzymic characteristics for comparison with other lysozymes. As large numbers of fractions must be assayed for lysozyme in the purification stages, the development of a suitable lysoplate assay with an easily available standard was needed.

A secondary objective of this research was to study the distribution of lysozyme in Mytilus edulis and other bivalves to determine, by inference, its role in the life of these animals.

Finally, having determined the optimum conditions for action of Mytilus lysozyme, the study concluded with an examination of the influence of ions present in the marine environment on the bacteriolytic activity of the lysozyme and the activity of the enzyme on a range of bacteria isolated from mussels.

# MATERIALS AND METHODS

## 1. Growth and preparation of bacterial substrates

### 1.1 Micrococcus luteus

a) Growth

An ampoule of freeze dried M. luteus, strain NCTC 2665, was obtained from the Microbiology Department, Glasgow University. The lyophilised cells were reconstituted with five drops of Peptone Yeast Extract Water (P.Y.E.W.) (Appendix 1) for 2 h at 23°C and inoculated onto two plates of Peptone Yeast Extract Agar (P.Y.E.A.) (Appendix 1) which were incubated at 30°C for 24 h.

An isolated colony was inoculated into 100 ml of P.Y.E.W. which was incubated overnight at 30°C at 150 RPM in an orbital incubator. After checking culture purity by microscopy the culture was divided between six 2 l flasks each containing 630 ml of P.Y.E.W. and incubated as above.

b) Harvesting

The broth cultures were centrifuged at 2000 g for 30 min at 4°C and the cells washed twice with one litre aliquots of 0.06M sodium phosphate buffer (Appendix 2) and thrice with 500 ml aliquots of distilled water. The cell pellet was resuspended to a volume of 150 ml in distilled water, shell frozen and lyophilised, yielding six grams of cells.

c) Preparation of cell walls

Bacteria were grown and harvested as above and disrupted by agitation in a Braun Homogeniser (Braun, West Germany) with Ballotini beads at 2,000 oscillations $min^{-1}$ for 3 min under $CO_2$ cooling. The

walls were then prepared by the method of Sharon and Jeanloz (1964).

The supernates were transferred into a 2 l beaker, kept on ice and the beads washed by decantation with five aliquots of 200 ml of ice cold distilled water. The supernates and wash fluids were combined, centrifuged at 1,400 g at 4°C for 15 min and the precipitate discarded. The supernate was centrifuged at 12,200 g at 4°C for 15 min and the top, white layer of the precipitate suspended in 500 ml distilled water, transferred into clean centrifuge bottles and centrifuged again for 15 min at 12,200 g. This procedure was repeated twice followed by centrifugation at 1,400 g for 15 min to remove impurities and the supernates finally centrifuged at 12,200 g for 15 min. The packed cell walls were suspended in 250 ml distilled water, heated for 20 min at 100°C, treated with 12.5 mg trypsin at 37°C for 2 h, washed thrice more with 500 ml aliquots of distilled water and finally lyophilised.

## 1.2 Micrococcus roseus

An ampoule of freeze dried M. roseus was obtained from the Microbiology Department, Glasgow University, reconstituted with five drops of Marine Broth (Appendix 1) and used to inoculate two plates of Marine Agar (Appendix 1) which were incubated at 22°C for four days. Culture purity was checked and the bacteria were grown and maintained on Marine Agar slopes.

## 1.3 Marine isolates

Swabs were taken from various fluids and surfaces of Mytilus edulis and plated on to Marine Agar. After incubation at 22°C for 2 days followed by 4°C for 2 days to allow for pigment development, the plates were examined; isolated colonies were subcultured on marine agar plates and maintained and grown on Marine Agar slopes.

Each isolate was inoculated on to triplicate plates of Marine Agar which were incubated at 4°C, 22°C or 37°C and plates of Nutrient and MacConkey Agar (Appendix 1) which were incubated at 22°C for 5 days during which the growth was monitored to determine the optimum culture conditions and possible origins of each isolate.

## 2. Assay techniques

### 2.1 Lysozyme

#### a) Spectrophotometric assay

The method was modified from those of Shugar (1952) and Litwack (1955). Freeze dried M. luteus (30 mg) was suspended in 10 ml 0.06M sodium phosphate buffer (pH 6.4) (Appendix 2) containing 1% (w/v) sodium chloride by exposure to ultrasound (Ultrasonic Cleaner, Millipore, London) for 15 sec and added to 90 ml 0.06M sodium phosphate buffer (pH 6.4); 2.9 ml aliquots were dispensed into 1 cm pathlength cuvettes giving an $E_{600}$ of approximately 0.8 against a distilled water blank in a Pye Unicam SP500 spectrophotometer at 23°C. Test solution (0.1 ml) was added to the cuvette with mixing and the $E_{600}$ recorded 15 sec after the addition and at one minute intervals for 5 min on an SP20 series chart recorder (Pye Unicam, Cambridge). A duplicate with distilled water substituted for the test solution was run in parallel to check for spontaneous reduction in the absorbance of the cell suspension.

One unit of enzyme was defined as that amount which produced a reduction of 0.001 per minute in the $E^{1\,cm}_{600}$ of the bacterial suspension.

#### b) Comparison of lysoplates with the spectrophotometric assay

Lysoplates, modified from those described by Osserman and Lawlor (1966) were prepared as follows. One gram of agarose (B.D.H. Ltd.,

Poole, England) was dispensed into each of four bottles and 0.1 g, 0.5 g and 1 g sodium chloride added to three of them. Ninety millilitres of 0.06M sodium phosphate buffer (pH 6.4) (Appendix 2) was added to each bottle and the pH re-adjusted to 6.4 with 40% (w/v) sodium hydroxide where necessary. The bottles were then autoclaved (121°C/15 min), cooled to 60°C and 60 mg freeze dried M. luteus, suspended in 10 ml buffer by treatment with ultrasound for 15 sec, added to each bottle with mixing. Aliquots of 5 ml were dispensed into 5 cm diameter petri dishes; when the agarose had set seven 3.4 mm diameter wells were cut in each with a No. 1 cork borer (Gallenkamp Ltd., London) and the plugs removed by suction.

Doubling dilutions of hen egg-white (HEW) lysozyme (Sigma, London) over the range 40 to 0.3125 μg $ml^{-1}$ were prepared in the above buffer as were doubling dilutions of partially purified Mytilus lysozyme (Amberlite eluate) (200 units $ml^{-1}$). Aliquots of each dilution (20 μl) together with buffer controls were applied in duplicate to each lysoplate system, incubated at 20°C for 20 h and the diameters of the resultant zones of clearance recorded.

Each concentration was assayed spectrophotometrically (see above) and the diameters of the zones of clearance plotted against log activity (units $ml^{-1}$ in the spectrophotometric assay). Based on the results of this experiment the lysoplates in all further assays contained 1% (w/v) sodium chloride.

c) Effect of gelatin on the lysoplate assay

Doubling dilutions of HEW lysozyme (10 to 0.078 μg $ml^{-1}$) prepared in 0.1M acetic acid or in 0.1M acetic acid containing 0.04% (w/v) gelatin were assayed in duplicate on lysoplates.

## 2.2 Protease (collagenase)

The method employed was modified from that of Smyth and Arbuthnott (1974). Azocoll (Wellcome Reagents Ltd., Beckenham), 5 mg, was weighed into a test tube, 0.95 ml diluent A (Appendix 2) added and the tube was incubated at 37°C for 5 min. Test solution (0.05 ml) was added and the mixture incubated for 3 h at 37°C with agitation at 10 min intervals. The mixture was centrifuged at 2100 g for 2 min and the $E^{1\ cm}_{510}$ of the supernate measured against a reagent blank.

One unit of enzyme gave an increase of $E_{510}$ of 0.01 $h^{-1}$ and enzyme units per ml $= \frac{\Delta E_{510} \times 100}{3 \times 0.05}$. Where no activity was detected the incubation period was increased to 25 h and the volume of the test solution added increased to 0.2 ml and the volume of diluent A reduced to 0.8 ml.

## 2.3 β-Glucuronidase

The method was adapted from Allison and Sandelin (1963). The reaction mixture, prepared in test tubes, was as follows:

0.25 ml 0.1M sodium acetate buffer (pH 5) (Appendix 2)

0.05 ml 0.01M phenolphthalein-β-glucuronide (Sigma, London)

0.05 ml test solution.

The mixture was incubated at 37°C for 30 min and the reaction stopped and colour developed by addition of 1 ml 0.4M glycine buffer (pH 10.7) (Appendix 2). The mixtures were centrifuged at 2000 g for 10 min and the $E^{1\ cm}_{545}$ of the supernate measured against a reagent blank.

One unit of enzyme activity gave an increase of $E_{545}$ of 0.01 $min^{-1}$. The units of enzyme per ml of sample $= \frac{E_{545} \times 1.35 \times 100}{0.05 \times 30}$. Where no activity was detected the incubation period was increased to 1 h.

## 2.4 Protein estimation

Protein was estimated by the method of Lowry et al (1951).

Reagents: (A) 2% (w/v) $Na_2CO_3$ in 0.1N NaOH

($B_1$) 1% (w/v) $CuSO_4.5H_2O$

($B_2$) 2% (w/v) Sodium-potassium tartrate

(C) Folin-Ciocalteau Reagent (B.D.H., Poole) diluted 1:1 with distilled water to 1N.

(D) 50 ml A + 0.5 ml $B_1$ + 0.5 ml $B_2$.

Standards: Bovine serum albumin (B.S.A.) (Sigma, London) at 0, 25, 50, 75, 100, 125 and 150 $\mu g\ ml^{-1}$.

Procedure: 0.6 ml of sample, or standard, was mixed with 3 ml of reagent D and maintained at room temperature. After 10 min 0.3 ml reagent C was added, immediately mixed and maintained at room temperature for 30 min. The $E^{1\ cm}_{750}$ of the fluids were recorded and compared to the standards.

## 3. Distribution of lysozyme-like activity in bivalves

The bivalves; Mytilus edulis, Modiolus modiolus, Chlamys opercularis, Mya arenaria, Tellina tenuis and Nucula nucleus were collected from the Firth of Clyde and maintained in running sea water at the University Marine Biological Station, Millport. Tissues, organs and structures, obtained during dissection by Professor J.A. Allen, were collected in tared 50 mm x 13 mm ampoules and cooled to $-20^{\circ}C$ for transport and storage. Samples were thawed when required, the ampoules reweighed and the samples homogenised in 1 ml 0.1M acetic acid in glass/teflon tissue homogenizers (Jencons Ltd., Hemel Hempstead). The lysozyme content of homogenates was assayed on lysoplates and the zones

of clearance compared to those of HEW lysozyme standards. Results were expressed as μg of HEW lysozyme-like activity per gram wet weight.

## 4. Purification of Mytilus edulis lysozyme

### 4.1 Ion exchange on CM- and DEAE-celluloses

Twenty-four gram (wet weight) aliquots of Whatman CM-32 or DEAE-32 were equilibrated with 0.01M potassium phosphate buffers, pH 6.5 and pH 7 respectively (Appendix 2).

Two aliquots (15 ml) of the 0.1M acetic acid extracted material from the lyophilisate of the post-amberlite treated whole animal homogenate, were dialysed at 4°C overnight against 0.01M potassium phosphate buffers, pH 6.5 and 7, and 13 ml aliquots of the dialysates were mixed with the respective equilibrated exchangers at 4°C for 1 h and centrifuged at 1200 g for 15 min. The exchangers were then sequentially washed with 20 ml aliquots of 0.01, 0.05, 0.1, 0.2, 0.4 and 0.6M buffers at pH 6.5 and 7 respectively (Appendix 2). The lysozyme content of each wash was determined by the lysoplate assay and the protein content as in 2.4.

### 4.2 Purification procedure

Crystalline styles, dissected from 350 live Mytilus edulis at the Marine Station and frozen at -20°C, were homogenised in 0.1M acetic acid in a glass/teflon homogenizer and the pH adjusted to 6.5 with 40% (w/v) sodium hydroxide giving a volume of 28 ml of which 2.5 ml was retained at -20°C for later analysis and the remainder mixed with 10 ml Amberlite CG50 resin (B.D.H., Poole), previously equilibrated with 0.1M potassium phosphate buffer (pH 6.5) (Appendix 2) after the method of

Hirs et al (1953), for 1 h. The mixture was poured into a 10 ml column and washed with 11 column volumes of 0.1M buffer (pH 6.5) followed by a linear gradient of 0.1M to 1M potassium phosphate buffer (pH 6.5) (Appendix 2) formed from 115 ml and 100 ml of the respective buffers, and finally with 1M buffer. The absorbance at 280 nm of each fraction was recorded with a Pye Unicam SP500 spectrophotometer, SP40 sample changer and SP20 recorder and the lysozyme assayed on lysoplates.

The peak activity fractions were pooled (50 ml), 2.5 ml was removed and frozen and the remainder dialysed against two aliquots of 2 l 0.01M sodium acetate buffer (pH 5) (Appendix 2) overnight. Of the 95 ml recovered 2.5 ml was removed and frozen and the remainder applied to a column of CM-32 (4 ml) equilibrated with 0.01M sodium acetate buffer (pH 5). The column was washed with four volumes of buffer before a linear gradient formed from 30 ml buffer and 28 ml buffer containing 1M sodium chloride was applied, followed by buffer containing 1M sodium chloride. Each fraction was assayed as above ($E_{280}$ and lysozyme) and the peak activity fractions pooled (9.5 ml). One ml was removed and frozen and, if required, the remainder was dialysed overnight against two aliquots of 2 l distilled water. The recovered volume (15.5 ml) was dispensed in 2 ml aliquots and stored at $-20^{\circ}C$. The above procedures were carried out at $4^{\circ}C$ in a cold room.

The lysozyme activity and protein content of each frozen sample was determined spectrophotometrically and by the method of Lowry et al (1951) respectively. The presence of collagenase and β-glucuronidase activities were determined by the methods in 2.2 and 2.3.

## 4.3 Acid gel electrophoresis

Samples were dialysed against distilled water overnight at

4°C and to 0.5 ml of each was added two drops of glycerol and one of Pyronin·Y solution (0.005% w/v). A control of HEW lysozyme (100 μg $ml^{-1}$) was included in each examination. The disc electrophoresis system of Reisfeld, Lewis and Williams (1962) was employed and consisted of a 15% small pore running gel (pH 4.3) with a 2.5% large pore upper gel (pH 6.8) in a β-alanine buffer (pH 4.5) (Appendix 3). Aliquots (200 μl) of each sample and control were applied to duplicate sets of gels and run at 0.5 mA per 55 mm x 5 mm diameter gel for 45 min followed by 5 mA per gel for 2 h at 4°C. Protein bands were stained with Coomassie blue (Appendix 3). The lytic activity was located by cutting the duplicate unstained gels into 1 mm sections on a gel slicer (Mickle Laboratory Engineering Co., Gomshall). The slices were placed in order on an agarose base prepared as for lysoplates but dispensed in 15 ml volumes into 9 cm diameter petri dishes. The plates were examined after 20 h at 20°C and the zones of clearing in the plates associated with specific slices was correlated with stained protein bands in the duplicate gels by reference to their relative mobilities.

Stained gels were scanned in a U.V. gel scanner (Joyce Loebl and Co. Ltd., Gateshead) and the resultant traces used to determine the purity of the samples.

## 4.4 Chitin-coated (CC-) cellulose as an adsorbent for *Mytilus* lysozyme

CC-cellulose prepared by the method of Imoto and Yagashita (1973) was poured into a 0.7 ml column in a 2 ml syringe.

Lysozyme eluted from Amberlite CG50 was dialysed against 0.2M potassium phosphate buffer (pH 6.8) (Appendix 2) containing 0.5M sodium chloride for 2 h and applied to the CC-cellulose column which was then washed with 24 ml of the buffer containing salt followed with 24 ml 0.1M acetic acid. Eluted material was collected in 3 ml fractions.

4.5 Effect of gelatin on CC-cellulose as an adsorbent for Mytilus lysozyme

Gelatin was added to the buffer and acetic acid employed as above (4.4) at a concentration of 0.04% (w/v). A fresh CC-cellulose column, 0.7 ml, was poured and washed with 15 ml buffer containing salt and gelatin. Lysozyme solution prepared as above (4.4) was applied to the column which was then washed with 30 ml buffer containing salt and gelatin followed with 95 ml 0.1M acetic acid containing gelatin and finally with 30 ml 0.1M acetic acid.

## 5. Characterisation as a lysozyme

The following mixtures were prepared in 100 mm x 13 mm test tubes with a 2 mg $ml^{-1}$ M. luteus cell wall suspension in barbital acetate buffer (pH 7.1, I = 0.012) (Appendix 2).

A. 2 ml cell wall suspension + 0.2 ml distilled water.

B. 2 ml cell wall suspension + 0.2 ml HEW lysozyme solution (50 µg $ml^{-1}$).

C. 2 ml cell wall suspension + 0.2 ml Mytilus enzyme solution (900 units $ml^{-1}$).

D. 2 ml buffer + 0.2 ml Mytilus enzyme solution (900 units $ml^{-1}$).

E. 2 ml buffer + 0.2 ml HEW lysozyme solution (50 µg $ml^{-1}$).

F. 2 ml buffer + 0.2 ml distilled water.

The mixtures were incubated at 37°C for 2 h with frequent agitation before centrifugation at 2000 g for 45 min to remove undigested debris. The supernates assayed as below for liberated reducing groups and N-acetylamino sugars.

## 5.1 Liberation of reducing groups

The method was that of Thompson and Shockman (1968) adjusted to a one-fifth scale.

Reagents: Potassium ferricyanide, 0.05% (w/v)

Carbonate-cyanide, 5.3 g $Na_2CO_3$ + 0.65 g KCN in 1 l distilled water.

Colour reagent, made up freshly from the following stock solutions which were prepared in 0.05N $H_2SO_4$:

a) Ferric ammonium sulphate 15 g. $l^{-1}$

b) Sodium dodecyl sulphate (S.D.S.) 3 g. $l^{-1}$

c) Carbowax 20M 10 g. $l^{-1}$

d) 0.05N $H_2SO_4$

Equal volumes of a, b, c and d were mixed.

Procedure: 0.4 ml of each reaction supernate was mixed with 0.4 ml potassium ferricyanide reagent and 0.4 ml carbonate cyanide reagent, heated in boiling water for 15 min and cooled in tap water for 5 min; 0.1N $H_2SO_4$ (1 ml) was added with mixing and the solutions were centrifuged at 2000 g for 10 min. The supernatant fluids (1 ml) were then mixed with 0.4 ml colour reagent and after 15 min the $E_{700}$ was measured in semi-micro cuvettes against the reagent blank, F. Glucose standards of 2.5, 5, 7.5, 10, 15 and 20 µg in 0.4 ml of buffer were also used and the liberated reducing power was expressed in terms of the equivalent amount of glucose.

## 5.2 Liberation of N-acetylamino sugars

The method was based on that of Ghuysen, Tipper and Strominger (1966).

Morgan-Elson Reagent: p-Dimethylaminobenzaldehyde, 16 g, was dissolved in glacial acetic acid to a volume of 95 ml and 5 ml concentrated hydrochloric acid added. This stock reagent was diluted 1:8 with glacial acetic acid to give the colour reagent.

Procedure: 20 µl of 10% (w/v) $K_2B_4O_7$ solution was added to 180 µl of supernate and heated for 30 min in a boiling water bath. After cooling, 0.9 ml fresh colour reagent was added, the mixtures incubated for 20 min at 37°C and $E^{1\ cm}_{585}$ measured against the reagent blank. Standards containing 5-25 x $10^{-8}$ moles of N-acetylglucosamine per ml of buffer were used as standards and the quantity of N-acetylamino sugar liberated was expressed in terms of N-acetylglucosamine.

## 5.3 Characterisation of the glycosidic linkage split

*M. luteus* cell walls were suspended in barbital acetate buffer (pH 7.1, I = 0.012) (Appendix 2) at 4 mg $ml^{-1}$ and the following mixtures prepared:

A: 2 ml cell wall suspension + 0.4 ml *Mytilus* lysozyme solution (900 units $ml^{-1}$).

B: 2 ml cell wall suspension + 0.4 ml distilled water.

C: 2 ml buffer + 0.4 ml *Mytilus* lysozyme solution (900 units $ml^{-1}$).

The mixtures were incubated at 37°C for 3 h and centrifuged

at 2000 g for 45 min. The supernatant fluids were removed and divided into two equal volumes.

Standards of N-acetylglucosamine (10 mg $ml^{-1}$) and N-acetylmuramic acid were prepared and duplicate aliquots of each (50 µl and 60 µl respectively) were dispensed into vials.

The samples and standards were freeze dried and treated as detailed by Work (1969) except that ten times the volumes were used. Samples and standards were resuspended in 0.3 ml fresh unbuffered 0.1M $NaBH_4$ and maintained at room temperature for 3 h; controls were similar samples containing 0.3 ml 0.1M $NaBH_4$ destroyed by acidification with acetic acid to pH 5 followed by neutralization. Products and controls were acidified with 0.15 ml concentrated hydrochloric acid and hydrolyzed in sealed tubes for 3 h at 95°C.

Both samples and standards were evaporated to dryness, resuspended in 0.5 ml distilled water, 10 µl aliquots applied to Polygram G (20 cm x 20 cm) plates (Camlab, Cambridge) and components separated by chromatography in two dimensions in freshly prepared butanol-acetic acid-water (3:1:1, v/v) and then pyridine-water (4:1, v/v).

Hexosamines were revealed as red spots by spraying the plates with ninhydrin (0.05% in n-butanol) and heating at 105°C - 110°C for 10 min.

## 6. Physico-chemical characteristics

### 6.1 Isoelectric focusing

Focusing was performed in a Zone Convection apparatus (Talbot and Caie, 1975) with a solution of 1% pH 7-9 and 1% pH 9-11 ampholines (L.K.B., Sweden) at 4°C. The ampholytes were prefocused in the trough

for 18 h at 0.3 watts and 1 ml Mytilus lysozyme solution (682 units $ml^{-1}$, 32 µg protein), dialysed against 1% glycine, was added to the middle of the trough with removal of the corresponding volume of ampholyte solution. After 76 h at 0.3 watt the gradient was divided into 36 fractions, each of 1 ml, and transferred into 100 mm x 13 mm test tubes which were held on ice while the pH of each was measured.

The three peak activity fractions were pooled and re-focused in a 1% solution of the above ampholines and treated as above.

## 6.2 Molecular weight determination

### a) Gel filtration on Bio-Gel P60

An 85 cm x 2 cm column of Bio-Gel P-60 (Bio-Rad Laboratories Ltd., California) was prepared in 0.06M sodium phosphate buffer, pH 6.4 (Appendix 2) containing 0.1M sodium chloride as directed by the manufacturers. Mytilus lysozyme solution (2 ml containing 110 µg of HEW lysozyme equivalent) was applied to the column, eluted with buffer and 3 ml fractions collected. The lysozyme was detected with lysoplates. A series of standard proteins, ovalbumin, myoglobin, cytochrome c and HEW lysozyme (Sigma Chemical Co. Ltd., London) and Blue Dextran 2000 (Pharmacia, Uppsala) were applied to the column in turn (2 mg in 2 ml of buffer) and eluted as above.

### b) Polyacrylamide gel electrophoresis

A freeze-dried sample of Mytilus lysozyme (900 enzyme units and 69 µg protein) was reconstituted in 0.5 ml alanine/acetic acid buffer (Appendix 3) and analysed by acid gel electrophoresis (section 4.3).

A duplicate sample was resuspended in 0.2 ml Tris/glycine

buffer (Appendix 3) and denatured by addition of 0.2 ml solubilizing buffer (Appendix 3) and heating at 100°C for 5 min. HEW lysozyme (100 μg $ml^{-1}$) and trypsin (Sigma, London) solutions (200 μg $ml^{-1}$) were similarly treated and a solubilized standard solution (1.2 ml) containing human serum albumin, ovalbumin, myoglobin, cytochrome c and insulin (Sigma, London) at a concentration of 100 μg $ml^{-1}$ of each was added to the solubilized trypsin.

Three sets of duplicate SDS-containing polyacrylamide gels (11% w/v), 55 mm x 5 mm diameter were prepared after the methods of Laemmli (1970) and Ames (1974) (Appendix 3). Aliquots of either 100 μl or 50 μl of each of the solubilized protein solutions were applied to the duplicate gels and electrophoresed at 1 mA per gel for 20 min and then at 3 mA per gel until the marker dye reached the anodal end of the gel. Gels were removed and stained with Coomassie blue (Appendix 3); the distance migrated by each protein was measured and related to the marker dye front.

6.3 Determination of heat stability

Aliquots of 230 μl of Mytilus lysozyme in distilled water (682 units $ml^{-1}$) were dispensed into 50 mm x 13 mm diameter glass ampoules and placed in water baths at 20°C, 40°C, 60°C, 80°C and 100°C for periods of 5, 10, 20 and 30 min after which the ampoules were removed and maintained on ice until the enzymic activity was determined spectrophotometrically; the activities were related to a control ampoule which had been maintained on ice during the experiment.

In later experiments the above lysozyme solution was diluted 1:1 with barbital-acetate buffer (pH 7.1, I = 0.012) (Appendix 2) containing 1% (w/v) gelatin and in some cases 0.4M NaCl. The enzyme solutions

were treated as above at 25°C, 50°C, 75°C and 100°C and activity related to a cold control.

### 6.4 Examination of active and inactive Mytilus lysozyme preparations

Samples of Mytilus lysozyme (0.5 ml) found to be inactive after 2 h at 25°C were electrophoresed, in duplicate in parallel with active samples, also in duplicate, on polyacrylamide gels prepared after Reisfeld et al (1962) and examined as described in section 4.3.

### 6.5 Effect of dialysis on Mytilus lysozyme

Aliquots (1 ml) of the acetic acid extract of style adjusted to pH 6.5 and containing 5 µg of HEW lysozyme equivalent, were placed in 40 mm lengths of 6 mm diameter Visking dialysis tubing previously boiled in distilled water. The dialysis tubes were tied to seal them and were then placed in test tubes containing 5 ml distilled water at 4°C for periods of 2.5, 4, 7.5 and 24 h, after which they were removed from the tubing and frozen as was the dialysate. Aliquots of 0.25 ml were removed from a control sample maintained under the same conditions, but not dialysed to check for any spontaneous loss of activity. The frozen samples were thawed and assayed on lysoplates with HEW lysozyme standards.

## 7. Enzymic activity

### 7.1 Determination of optimum pH and ionic strength (I) for activity

Barbital-acetate buffers of constant ionic strength (I = 0.177) were prepared over the pH range 3 to 9 after the method of Michaelis (1931) and diluted to give the ionic strength range 0.006 - 0.177 (Appendix 2).

Two millilitre aliquots of a 3 mg $ml^{-1}$ suspension of freeze

dried M. luteus cells were mixed with the buffers (20 ml) and 2.9 ml volumes of the mixtures dispensed into two cuvettes. To one was added 0.1 ml distilled water and to the other 0.1 ml Mytilus lysozyme solution (172 units $ml^{-1}$) or HEW lysozyme (10 µg $ml^{-1}$). Cuvettes were inverted to mix the contents and 15 sec after addition of the enzyme the $E^{1\,cm}_{600}$ of the suspensions were recorded at one min intervals for 10 min at 25°C on a Pye Unicam SP500 spectrophotometer with SP20 chart recorder.

For both Mytilus and HEW lysozymes, enzyme activity was related to that obtained under optimum conditions.

## 7.2 Determination of optimum temperature

A 10 ml suspension of 3 mg freeze dried M. luteus cells in distilled water was added to 100 ml barbital-acetate buffer, pH 7.1 (Appendix 2) giving an ionic strength of 0.011 or to 100 ml of pH 4.6 buffer (Appendix 2) giving an ionic strength of 0.054. Volumes of 2.9 ml were dispensed into cuvettes as detailed above (7.1) and 0.1 ml Mytilus lysozyme solution (172 units $ml^{-1}$), HEW lysozyme (10 µg $ml^{-1}$) or distilled water was added. Cell suspensions were maintained in a water-jacketed cuvette holder enabling the reaction temperature to be maintained over the range 10°C to 60°C. The change in absorbance of the suspensions was measured in a Pye Unicam SP800 spectrophotometer as described above. The activity of the enzymes at each temperature was related to the maximum activity found, which was taken as representing 100% activity.

## 7.3 Apparent affinity constant (Ka app) for M. luteus cells

Freeze dried M. luteus cells were suspended in barbital acetate

buffer (pH 7.1, I = 0.011) (Appendix 2) at a concentration of 0.5 mg $ml^{-1}$ and diluted with buffer to 100 to 450 mg $l^{-1}$. Duplicate 2.9 ml volumes at each concentration were dispensed into cuvettes and the $E^{1\,cm}_{600}$ of each was recorded on a SP500 spectrophotometer with SP20 chart recorder at 25°C. Mytilus lysozyme (622 units $ml^{-1}$, 0.1 ml), or buffer was added and the $\Delta E^{1\,cm}_{600}$ recorded as above over 3.5 min. The $E^{1\,cm}_{600}$ at the beginning and end of each timed period were checked against a standard curve of cell concentration ([S]) against $E^{1\,cm}_{600}$ and the initial velocity of lysis of substrate per minute (V) calculated.

The apparent affinity constant (Ka app) was determined graphically according to the method of Lineweaver and Burk as adapted by Locquet, Saint-Blancard and Jolles (1968).

## 7.4 Apparent affinity constant for M. luteus cell walls

This was determined according to the method above (7.3) substituting M. luteus cell walls for cells over the concentration range 56 to 1000 mg $l^{-1}$ and recording $\Delta E^{1\,cm}_{540}$. The Mytilus lysozyme solution contained 900 units $ml^{-1}$ and a standard curve of cell wall concentration (mg $l^{-1}$) ([S]) against $E^{1\,cm}_{540}$ was prepared.

## 7.5 Effect of cations

Salts were added to separate batches of 0.01M Tris maleate buffer, pH 6.4 (Appendix 2) to the following concentrations: $MgCl_2$, 0.05M; $CaCl_2$, 0.05M; NaCl, 1M. In each case the pH was re-adjusted to 6.4 with 40% (w/v) sodium hydroxide. Freeze dried M. luteus cells were added to the buffer and to the buffers containing salts to a concentration of 0.3 mg $ml^{-1}$. The stock cell suspensions containing the above salt concentrations were then diluted with the cell suspension to

which no salts had been added, thus providing cell suspensions over a range of concentrations of each salt.

Duplicate volumes of 2.9 ml of each of the buffered cell suspensions were dispensed into cuvettes to which were added either 0.1 ml distilled water or 0.1 ml of an acetic acid extract of styles adjusted to pH 6.5. After mixing the $E^{1\,cm}_{600}$ of the sample and control suspensions were recorded as above (7.1).

To determine whether the lysozyme was forming sphaeroplasts from the cells, which were being stabilized by the salts present, the following procedure was employed. Freeze-dried M. luteus cells were suspended at a concentration of 0.3 mg $ml^{-1}$ in 0.01M Tris maleate buffer (pH 6.4) (Appendix 2) and in buffers containing 0.55M NaCl or 0.05M $MgCl_2$. The activity of the above style lysozyme preparation against the three cell suspensions was determined as above (7.1) and, after 10 min, 0.5 ml 0.5M sodium hydroxide was added with mixing and the $E_{600}$ recorded for a further 10 min. This procedure was repeated substituting SDS (10% w/v) for the sodium hydroxide.

## 7.6 Effect of cations on enzymic activity against cell walls

Tris-maleate buffers containing a range of concentrations of NaCl (0 to 0.6M) or $MgCl_2$ (0 to 0.05M), were prepared as above (7.5) with freeze dried M. luteus cell walls substituted for cells at a concentration of 0.7 mg $ml^{-1}$. Duplicate volumes of 2.9 ml of each suspension were dispensed into cuvettes and 0.1 ml of style extract or distilled water added and the $E^{1\,cm}_{540}$ of the sample and control suspensions recorded for 10 min as above (7.1).

The sample containing 0.6M NaCl was incubated at 22°C overnight, and after agitation the $E_{540}$ of the sample and control cuvettes were again determined.

7.7 Effect of sea water on enzymic activity

Freeze dried M. luteus cells were suspended in distilled water, sea water and 0.06M sodium phosphate buffer (pH 6.4) (Appendix 2) containing 0.1% (w/v) NaCl at a concentration of 0.3 mg $ml^{-1}$. Mixtures of the distilled water and sea water suspensions were prepared at the ratios 9:1, 4:1, 3:2, 2:3, 1:4.

The $\Delta E_{600}$ of the suspensions caused by the addition of distilled water or style extract (see 7.5) was measured as above (7.1). The pH of each suspension, excluding the buffered suspension, was in the range pH 6.9 to 7.2.

## 8. Sensitivity of marine isolates to lysozyme

Marine bacterial isolates, M. roseus cells grown on Marine Agar and freeze dried M. luteus cells were suspended in 0.06M sodium phosphate buffer (pH 6.4) (Appendix 2) containing 0.1% (w/v) sodium chloride. The $\Delta E^{1\,cm}_{600}$ of each suspension after the addition of Mytilus lysozyme solution (900 units $ml^{-1}$) HEW lysozyme (100 µg $ml^{-1}$) or distilled water was recorded as above (7.1). The activity of the enzymes against M. luteus was taken as 100%.

# RESULTS

## 1. Assay of lysozyme activity

Lysozyme is normally assayed spectrophotometrically by measuring the reduction in turbidity of a suspension of M. luteus whole cells or cell walls or, alternatively, in a 'lysoplate' assay by measuring the zone of clearance produced in an agarose layer containing M. luteus whole cells or cell walls. While the spectrophotometric assay is more accurate and more sensitive than the lysoplate assay the former is more time consuming. Thus for procedures such as column chromatography, which give rise to large numbers of fractions, the lysoplate assay is preferable.

Ideally, in enzyme assays the same lysozyme should be used in the standards as is in the unknowns. The ready availability of large quantities of purified HEW lysozyme of known stability made this a suitable standard provided that a similar dose response curve could be obtained with Mytilus lysozyme. Peeters and Vantrappen (1977) and Ensink and van Haeringen (1977) reported that sodium chloride concentration influences the size of the zone of clearance formed in lysoplates. Therefore the influence of sodium chloride on the lysoplate assay was investigated.

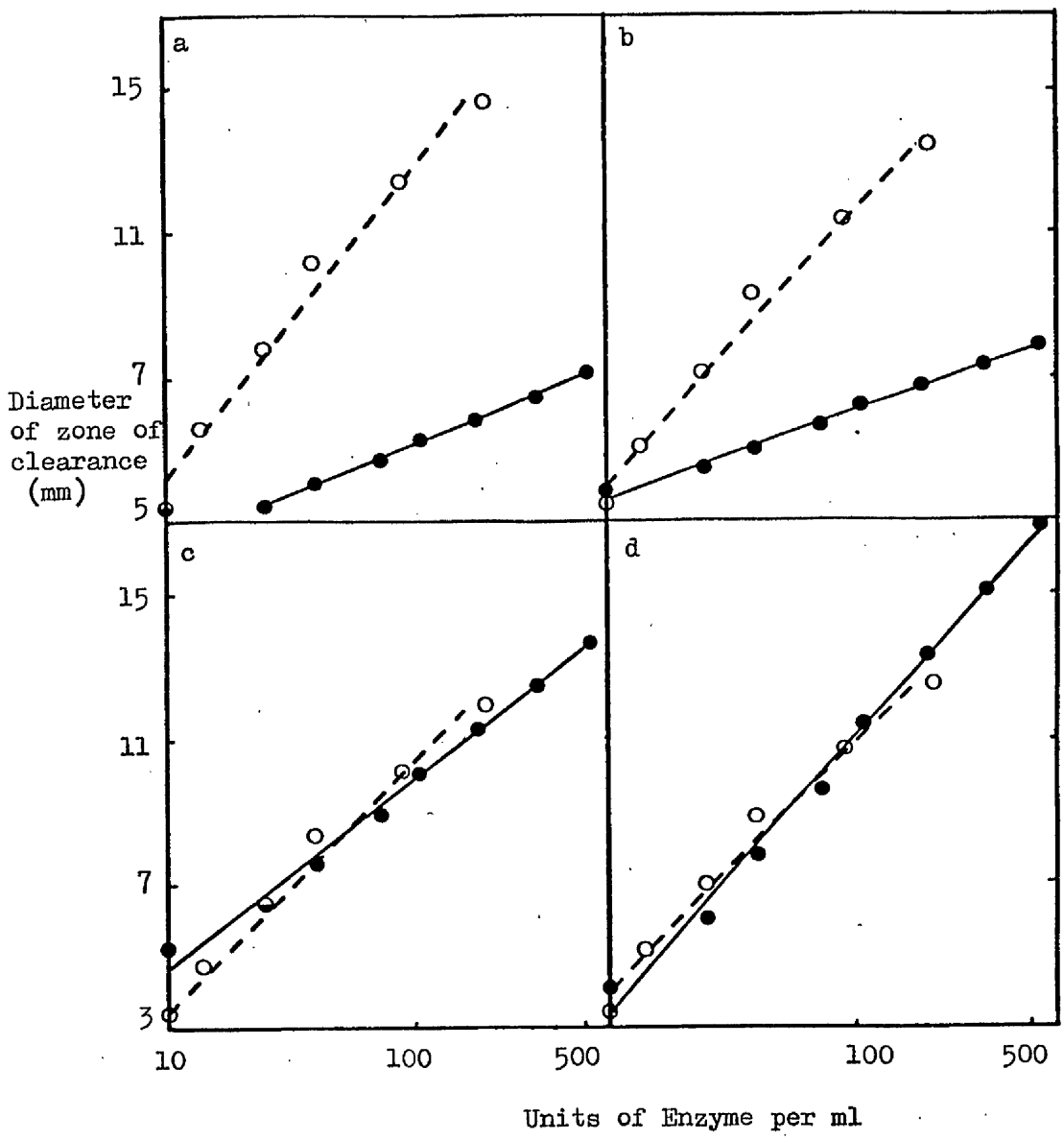
### 1.1 Comparability of assays

The enzymic activity of several dilutions of stock solutions of HEW and Mytilus lysozyme was determined spectrophotometrically and compared to the zones of lysis produced in lysoplates. When log (enzyme activity) was plotted against zone diameter a straight line response was obtained both with HEW and Mytilus lysozyme (Fig 6a). When the concentration of sodium chloride in the lysoplates was increased

Fig 6. The effect of NaCl on the zones of clearance produced by lysozymes in agarose assay plates.

Where the NaCl concentrations are: a) 0%, b) 0.1%, c) 0.5% and d) 1% w/v.

*Mytilus* lysozyme, O- - -O; HEW lysozyme, ●——● .


a
b
c
d
15
11
7
5
3
Diameter of zone of clearance (mm)
10
100
500
100
500
Units of Enzyme per ml

to 0.1%, 0.5% and 1% w/v the slopes of the response line for Mytilus lysozyme decreased and those of HEW lysozyme increased until, in 1% NaCl, the lines were almost parallel (Figs 6a-6d). In all cases the lines were fitted by regression analysis (Appendix 4) and the parameters of the lines are listed in Table 4. At all sodium chloride concentrations a high correlation coefficient ($r > 0.990$) was obtained with Mytilus lysozyme and HEW lysozyme but when the combined results were analysed at each salt concentration a high correlation was obtained only with 1% NaCl (Table 4).

Thus, by incorporating 1% w/v NaCl in lysoplates almost parallel dose response curves were obtained with the two lysozymes enabling HEW lysozyme to be used as a standard in lysoplate assays for Mytilus lysozyme. This NaCl concentration was adopted for all subsequent lysoplate assays.

Replotting the diameters of the zones of clearance obtained with four of the Mytilus lysozyme dilutions (200, 90, 40 and 25 units $ml^{-1}$) and four of the HEW lysozyme dilutions (180, 105, 40 and 25 units $ml^{-1}$) against the concentration of sodium chloride added to the lysoplate agarose base showed differences between the responses of the two lysozymes. Whereas the diameters of the zones of clearance obtained with HEW lysozyme increased as the sodium chloride concentration was raised, levelling off above 0.085M (Fig 7a), the diameters of the zones obtained with Mytilus lysozyme decreased to a minimum at 0.085M NaCl as the concentration was raised (Fig 7b).

## 1.2 Effect of gelatin on the lysoplate assay

At certain stages in the purification of the lysozyme unexplained losses in activity were experienced and it was thought that

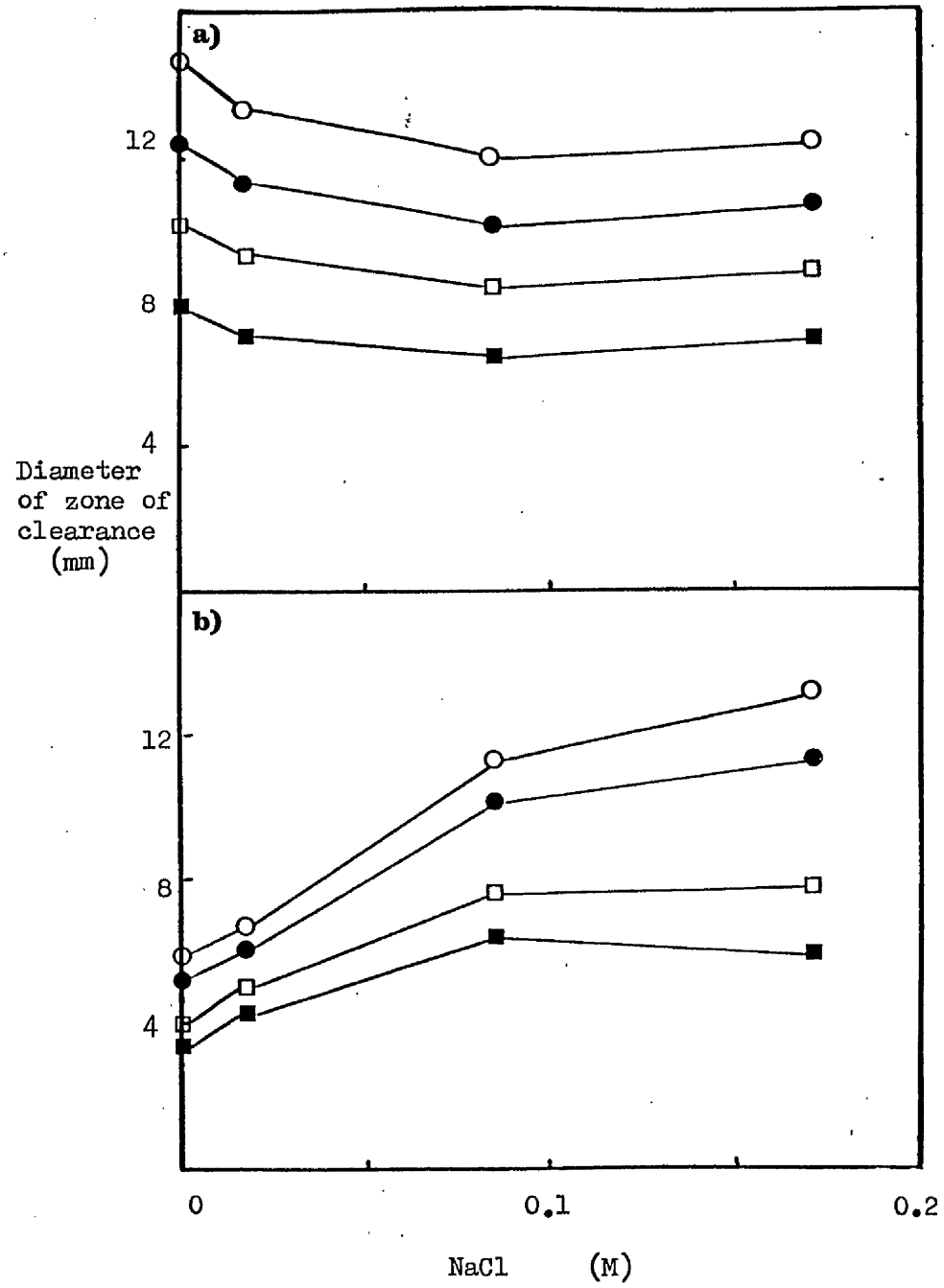
Table 4. Effect of sodium chloride concentration on the lysoplate assay.

The parameters of the lines fitted by linear regression for Mytilus and HEW lysozymes (Figure 6) and those which would be found for a composite of both are shown below where r = correlation coefficient, m = gradient and c = y-axis intercept.

| | | Linear regression analysis parameters obtained at the following NaCl concentrations (% w/v) | | | |
|---|---|---|---|---|---|
| Lysozyme | Parameter | 0 | 0.1 | 0.5 | 1 |
| Mytilus | r | 0.990 | 0.993 | 0.991 | 0.991 |
| | m | 8.470 | 7.654 | 6.804 | 6.891 |
| | c | 4.189 | 3.891 | 3.593 | 4.026 |
| Hen egg-white | r | 0.999 | 0.996 | 0.996 | 0.996 |
| | m | 2.736 | 2.395 | 5.125 | 7.655 |
| | c | 2.339 | 3.603 | 4.750 | 3.420 |
| Both (composite) | r | 0.263 | 0.585 | 0.988 | 0.994 |
| | m | 1.869 | 3.015 | 5.618 | 7.362 |
| | c | 5.565 | 4.524 | 4.322 | 3.722 |

Fig 7. The effect of increasing salt concentration on the diffusion of a) Mytilus and b) HEW lysozyme in lysoplates. Concentrations of enzymes (units $ml^{-1}$)

| | | | | |
|---|---|---|---|---|
| ○ | a) | 200 | b) | 180 |
| ● | a) | 90 | b) | 105 |
| □ | a) | 40 | b) | 40 |
| ■ | a) | 25 | b) | 25 |


a)
b)
12
8
4
12
8
4
Diameter of zone of clearance (mm)
0
0.1
0.2
NaCl (M)

this might be due to the highly basic lysozyme binding non-specifically to glassware. The inclusion of 0.04% (w/v) gelatin in all buffers was considered as a means of overcoming this but as other proteins are known to affect the diameter of the zones of clearance obtained on lysoplates (Peeters and Vantrappen, 1977), the effect of gelatin on the lysoplate assay was examined.

Over the range of concentrations of HEW lysozyme examined (0.078 to 10 μg $ml^{-1}$) gelatin increased the size and clarity of the zones, particularly at lower concentrations, and decreased the steepness of the gradient (Fig 8). However, as gelatin offered no advantage in the purification of Mytilus lysozyme with CC-cellulose (vide infra) it was not incorporated in the buffers.

## 2. Distribution of lysozyme-like activity in marine bivalves

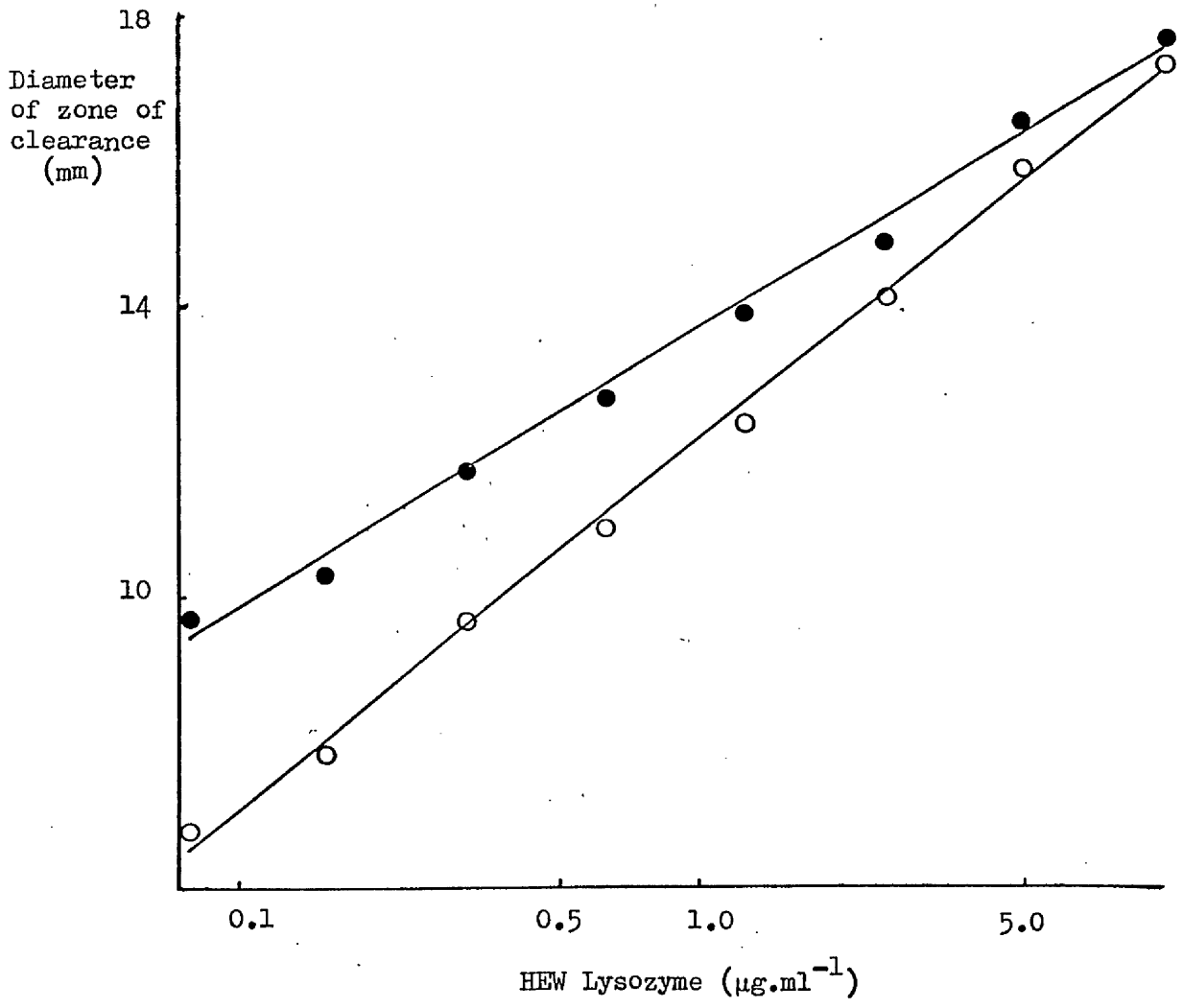
Studies on the distribution of lysozymes in bivalves are comparatively few and have been concentrated within narrow boundaries, thus reaching different conclusions. A detailed examination of the distribution of the enzyme in a range of bivalves was undertaken to determine whether there was any common pattern of distribution which might be related to the function of the enzyme in the natural habitat of the animals.

### 2.1 Initial survey

Initially one specimen of Mytilus edulis, Chlamys opercularis, Mya arenaria and Tellina tenuis was dissected and examined. In Mytilus, Chlamys and Tellina the highest concentrations of lysozyme-like enzyme, as indicated by the lysoplate assay, were found in the style but in

Fig 8. Effect of gelatin on HEW lysozyme in the lysoplate assay.

Stock enzyme solutions were diluted in 0.1M acetic acid (○) and in acid containing 0.04% (w/v) gelatin (●).


18
14
10
Diameter of zone of clearance (mm)
0.1
0.5
1.0
5.0
HEW Lysozyme (μg.ml$^{-1}$)

Mya the highest concentration was found in the gill, followed by the palps and digestive gland (Table 5). In Tellina relatively high concentrations were found in gill, digestive gland and style sac tissue, while the only other samples to exhibit any activity in Chlamys were the digestive gland and body fluid (Table 5). In Mytilus the style was followed by the digestive gland, byssus gland and foot while other samples displayed, at most, trace amounts of lysozyme, which could have been due to contamination from adjacent tissue (Table 5).

Thus, for Mytilus, Chlamys and Tellina highest concentrations of lysozyme-like activity occurred in tissues and structures associated with digestive processes.

## 2.2 Mytilus edulis

Studies on the distribution of lysozyme were principally concerned with Mytilus edulis. Two batches of Mytilus edulis (13 and 5 animals respectively) were dissected and the lysozyme content of those tissues and structures previously found to contain lysozyme was measured. The lysozyme content of tissues and structures in each of the 19 animals (i.e. including the animal from the previous experiment) is plotted in Figure 9 together with the geometric mean concentrations. The highest concentration of lysozyme found in Mytilus was 245 µg (HEW lysozyme equivalent) per gram wet weight in style. In all but one of the 19 animals examined the concentration in style exceeded that in all other samples. The distribution followed the same pattern found in the initial study, with the style being followed by the digestive gland, style sac, byssus gland and foot with regard to enzyme concentration (Tables 5 and 6; Fig 9). The concentrations in the style sac and byssus gland may be higher than detected as the size and

Table 5. Lysozyme-like activity ($\mu g.g^{-1}$ wet weight) in four marine bivalves.

| Sample | Mytilus edulis | Chlamys operecularis | Mya arenaria | Tellina tenuis |
|---|---|---|---|---|
| Style | 151.0 | 30 | 3.6 | 62.5 |
| Style sac | n.t. | n.t. | 0 | 25.9 |
| Digestive gland | 40.0 | 3.8 | 9.0 | 24.1 |
| Gill | 3.7 | 0 | 14.7 | 27.4 |
| Palps | 0 | 0 | 10.1 | 0 |
| Foot | 16.0 | 0 | 0 | 0 |
| Mantle fluid | 1.4 | 0 | 1.0 | n.t. |
| Mantle edge | 4.4 | 0 | 2.3 | 0 |
| Ovary | 3.7 | 0 | n.t. | 0 |
| Testis/sperm | 6.1 | 0 | 3.0 | 2.5 |
| Heart blood | 0.9 | n.t. | 0 | n.t. |
| Body fluid | n.t. | 0.7 | 1.7 | n.t. |
| Kidney | 0 | 0 | 0 | n.t. |
| Pericardial gland | 0 | n.t. | n.t. | n.t. |
| Adductor muscle | 0 | 0 | 0 | n.t. |
| Byssus gland | 29.0 | - | - | - |

Fig. 9. Lysozyme content of structures and tissues of marine bivalves. Data from 19 *Mytilus edulis*, five *Modiolus modiolus*, 11 *Chlamys opercularis* and six *Mya arenaria* are shown. Each point represents the lysozyme content of an individual structure or tissue from a single animal. Lysozyme concentration is shown on a logarithmic scale and the geometric mean for each structure is represented by a horizontal bar.

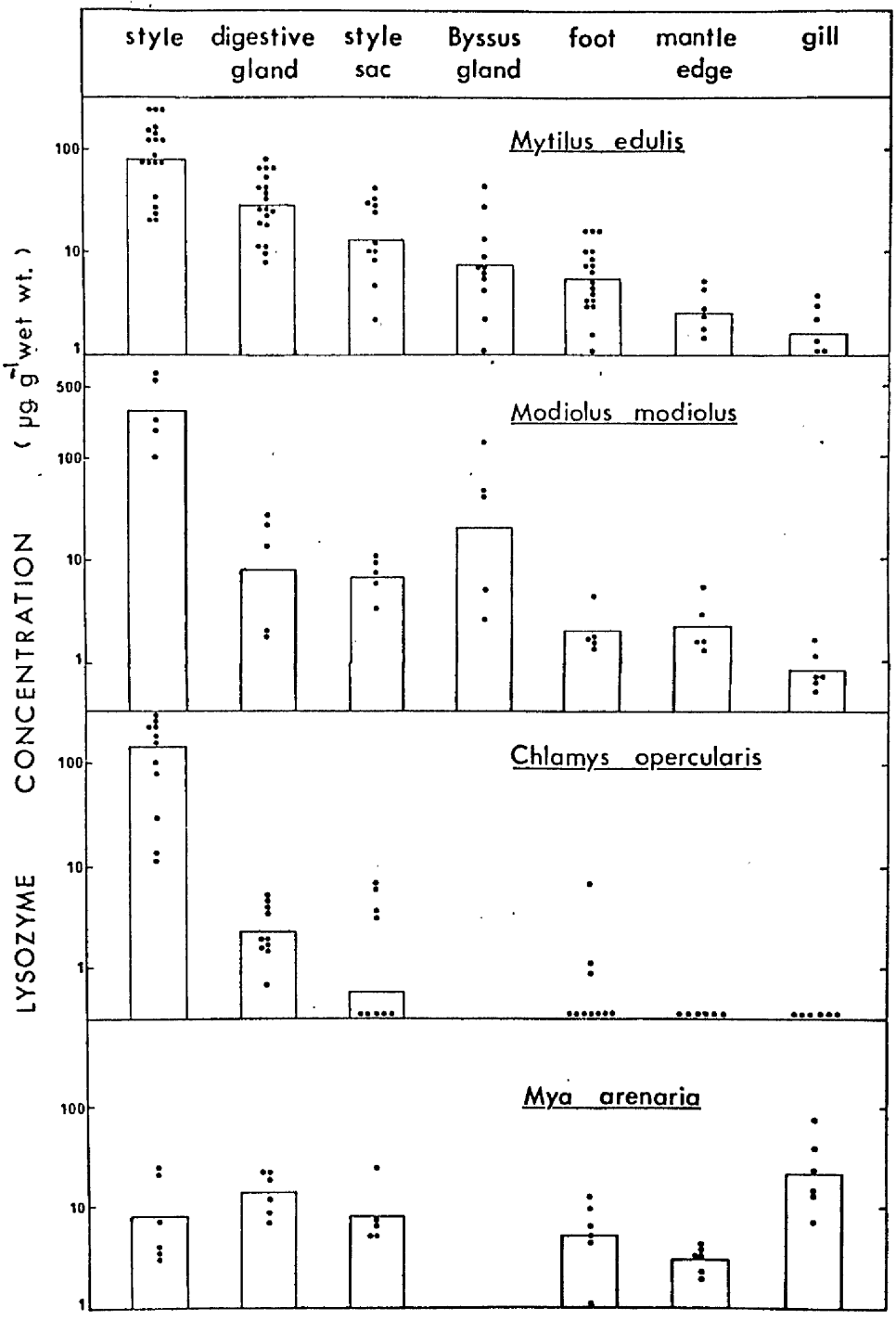

style
digestive gland
style sac
Byssus gland
foot
mantle edge
gill
Mytilus edulis
Modiolus modiolus
Chlamys opercularis
Mya arenaria
LYSOZYME CONCENTRATION ( µg g⁻¹ wet wt. )
100
10
1
500
100
10
1
100
10
1
100
10
1

Table 6. Variation in lysozyme-like activity ($\mu g.g^{-1}$ wet wt) in Mytilus edulis and Chlamys opercularis

| | Mytilus edulis | | | Chlamys opercularis | | |
|---|---|---|---|---|---|---|
| | June '77 | Dec '77 | Feb '78 | June '77 | Dec '77 | Feb '78 |
| Style | 151 | 104 (13) | 35.7 (5) | 30 | 114 (5) | 249 (5) |
| Style sac | n.t. | 16.7 (7) | 8.9 (5) | n.t. | 0 (4) | 2.2 (5) |
| Digestive gland | 40.0 | 35.2 (13) | 13.0 (5) | 3.8 | 3.6 (5) | 1.4 (5) |
| Byssus gland | 29.0 | 6.9 (5) | 5.4 (5) | - | - | - |
| Foot | 16 | 5.0 (12) | 5.7 (5) | 0 | 0.3 (4) | 0.2 (5) |
| Mantle edge | 4.4 | n.t. | 2.0 (5) | 0 | n.t. | 0.1 (5) |

In June 1977 a single specimen was examined; for other times the geometric mean value is shown with the number of samples in brackets.

positioning of these secretory organs makes them difficult to dissect free of contaminating tissue: mantle and gill tissue contained only low concentrations of enzyme (Tables 5 and 6; Fig 9).

The maximum value recorded for Mytilus style, 245 μg $g^{-1}$ wet wt, represented a six-fold increase over that found in whole animal homogenates, 35.9 μg $g^{-1}$ wet wt (Table 7).

2.3 Other bivalves

Further specimens were dissected to confirm the results of the initial survey. In Figure 9 the lysozyme content of individual structures and tissues of a total of five Modiolus modiolus, eleven Chlamys opercularis and six Mya arenaria are also shown. The highest concentration of lysozyme in any of the bivalves, 664 μg $g^{-1}$ wet weight was found in Modiolus style. As in the other member of the family Mytilidae, Mytilus edulis the highest concentrations in Modiolus were in styles. However, unlike Mytilus the next highest level was in the byssus gland followed by digestive gland and style sac, with the foot, gill and mantle edge containing lower concentrations than equivalent samples from Mytilus (Fig 9).

In Chlamys, the highest concentrations were detected in style followed by digestive gland and style sac, with no detectable enzyme in gill, mantle edge and seven of the ten samples of foot examined (Fig 9; Table 6).

In Mya the distribution pattern differed from that in other bivalves. Highest concentrations were found in gill tissue followed by digestive gland, style, style sac, foot and mantle edge (Fig 9). The lysozyme concentrations in Mya gill were higher than those found in any other gill specimens (Fig 9).

Table 7. Lysozyme-like activity in whole body homogenates of marine bivalves.

| | Lysozyme content ($\mu g.g^{-1}$ wet wt) |
|---|---|
| *Mytilus edulis* (pool of 300) | 35.9 |
| *Tellina tenuis* (4 individuals) | 76.8, 47.0, 88.4, 20.9 |
| *Nucula nucleus* (3 individuals) | 1.0, 22.9, 0.9 |

In addition to analysis of individual tissues and structures the lysozyme contents of individual body homogenates of the small bivalves Tellina tenuis and Nucula nucleus were compared to those of Mytilus edulis. Tellina specimens had the highest lysozyme concentrations with a mean value of 58.3 μg $g^{-1}$ wet weight compared with 35.9 μg $g^{-1}$ wet weight for Mytilus with the value in Nucula varying from 0.9 to 22.9 μg $g^{-1}$ wet weight (Table 7).

2.4 Seasonal variation

In the above studies Mytilus and Chlamys were dissected on three separate occasions and the geometric mean results on each occasion were compared to determine if there was seasonal variation in the lysozyme content and distribution in bivalves (Table 6). In Mytilus the concentrations in style and other samples decreased from their maximum value (a single sample) in June '77 during the sampling period. In Chlamys the concentrations in style increased eight-fold over the sampling period while the concentration in digestive gland dropped to the same extent as the concentration in Mytilus digestive gland, three-fold (Table 6). The logarithms of the lysozyme concentrations approximated to a normal distribution and were examined by the t-test (Appendix 4). The data were also examined by the Mann-Whitney U-test (Appendix 4) as the use of the t-test for samples with no detectable lysozyme is questionable. Generally, the conclusions drawn from the t- and U-tests were in agreement; both tests indicated significant differences in the lysozyme concentrations between the two sampling periods in style and digestive gland of Mytilus and Chlamys (Table 8). However, while the t-test indicated that the lysozyme concentrations in Chlamys style sac at the two sampling periods differed significantly the U-test did not (Table 8).

Table 8. Significance of the differences between the lysozyme-like activities found in December '77 and February '78 in Mytilus edulis and Chlamys opercularis samples

| Sample | t-Test[a] | | | Mann-Whitney U-Test[b] | | | | |
|---|---|---|---|---|---|---|---|---|
| | t Statistic | Degrees of freedom | Probability[c] % | $U_1$ | $U_2$ | Number of observations | | Probability[c] % |
| Mytilus edulis | | | | | | | | |
| Style | 2.88 | 16 | < 2 | 9 | 56 | 13 | 5 | < 5 |
| Style sac | 1.05 | 9 | > 25 | 31 | 63 | 7 | 4 | > 5 |
| Digestive gland | 3.72 | 16 | < 1 | 60 | 5 | 13 | 5 | < 5 |
| Byssus gland | 0.8 | 8 | > 25 | 35 | 10 | 5 | 5 | > 5 |
| *Foot | 2.09 | 15 | > 5 | 23 | 37 | 12 | 5 | > 5 |

[a] The found values of t and U were compared with those of the tabulated values with the relevant degrees of freedom or numbers of observations.

[c] The probability is the tabulated value for the found value of the test statistic and relates to the probability that the differences in means could be due to random sampling fluctuation.

## 3. Purification of lysozyme from Mytilus edulis

Mytilus edulis possesses enzymes which degrade a wide range of carbohydrates and other organic compounds (Kristensen, 1972a,b) and purification of Mytilus lysozyme was necessary to study the specificity and characteristics of the enzyme.

The highly basic nature of lysozymes facilitates the use of acidic ion-exchange resins for purification and the method adopted for the purification of Mytilus lysozyme exploited this property.

### 3.1 Purification from whole-animal homogenates

Before the above distribution studies had revealed the potential of crystalline style as a starting material, attempts were made to purify the lysozyme from whole-animal homogenates.

Two hundred fresh, living Mytilus were obtained from the Marine Station, Millport, and maintained in aerated seawater at 4°C overnight. The 1.4 Kg of meat and associated fluid obtained on dissection was homogenised in 0.1M acetic acid in a Silverson Homogeniser (Silverson Machines, London) and the pH adjusted to 6.5 with sodium hydroxide (40% w/v). The homogenate was maintained at 4°C to allow flocculation and precipitation to occur (which did not affect the activity) and was then centrifuged at 2000 g for 1 h at 4°C. The supernate contained 50.3 mg of HEW lysozyme-equivalent with a specific activity of 0.34 units $mg^{-1}$ protein.

The supernate was mixed with Amberlite CG-50 and the activity eluted with 0.4M potassium phosphate buffer (pH 6.5). Ninety-eight percent of the applied activity was recovered and the specific activity elevated to 15.8 units $mg^{-1}$ (Table 9).

Table 9. Purification of lysozyme from homogenates of Mytilus edulis (less shells)

| Purification Stage | Activity | Overall Recovery* | Purification* |
|---|---|---|---|
| | enzyme units per mg protein | % | |
| Acetic acid extract of Mytilus | 0.34 | 100 | 1 |
| Amberlite CG-50 | 15.8 | 98 | 46 |
| Dialysis, freeze drying, re-extraction with acetic acid, pH to 6.8 | 7.9 | 34 | 23 |
| CC-cellulose and dialysis | 400 | 3.1 | 1,180 |
| CM-cellulose, dialysis (distilled water), freeze drying | 3,930 | 2.9 | 11,600 |

*Compared to the acetic acid extract of Mytilus

Following dialysis against distilled water and lyophilisation a large proportion of material became insoluble in water and was thought to contain a large amount of lipid. The lyophilised material was extracted with 0.1M acetic acid, the resultant extract containing 34% of the initial activity with a specific activity of 7.9 units $mg^{-1}$ (Table 9). The pH of the extracted material was adjusted to 6.8 with sodium hydroxide (40% w/v) and the solution applied to a column of chitin-coated cellulose (CC-cellulose), washed with 0.2M potassium phosphate buffer (pH 6.8) containing 0.5M sodium chloride and eluted with 0.1M acetic acid.

Of the enzyme applied to the column 19% was recovered, of which 9% was in peak activity fractions, representing an overall recovery of 3% with an increase in specific activity to 400 units $mg^{-1}$ (Table 9). The peak activity fractions were dialysed against 0.01M acetate buffer (pH 5), applied to a column of CM-cellulose and eluted with a gradient of 0 to 1M sodium chloride added to the buffer. Ninety-five percent of the applied activity was recovered from the column, dialysed against distilled water and freeze dried without further loss of activity. The freeze dried product had a specific activity of 3930 units $mg^{-1}$, representing an overall increase in purity of 11,600-fold (Table 9).

In view of the low recovery from CC-cellulose this stage was omitted from the purification process.

## 3.2 Purification of Mytilus lysozyme on CM and DEAE-cellulose

While the above purification was in progress alternative steps were examined. Material from the same batch as that applied to CC-cellulose (3.1) was applied to CM and DEAE-cellulose after dialysis

against 0.01M potassium phosphate buffer, pH 6.5 and 7 respectively.

CM-cellulose. All of the enzyme was bound to CM-cellulose and remained bound until 0.2M or greater molarity buffers was applied. The material eluted with 0.4M buffer had the greatest lysozyme:protein ratio but 38% of the applied enzyme was not recovered under the conditions used (Table 10).

DEAE-cellulose. Under half of the lysozyme applied was bound and all of the enzyme was recovered by washing the DEAE-cellulose with low molarity buffers. The highest purity was found in the material which had not bound to the ion exchanger (Table 10).

## 3.3 Purification of Mytilus lysozyme from crystalline style

As previously noted, the distribution studies revealed that the style contained up to 245 μg HEW lysozyme equivalent per g wet weight compared to 35.9 μg $g^{-1}$ wet weight found in whole animal homogenate. Of greater significance was the specific activity of 75 units $mg^{-1}$ which was 220 times that of whole animal homogenate. The high specific activity and the apparent absence of lipid from the styles, which created difficulties in the purification from whole animal homogenates, indicated that crystalline style should provide an excellent starting material for the purification of the lysozyme.

Batches of approximately 350 styles had an average wet weight of 5 g and contained between 0.68 and 2.12 mg of HEW lysozyme equivalent. No correlation between total protein and lysozyme content was apparent in the style homogenates (Table 11).

### Fractionation of extracts of crystalline style on Amberlite CG50

The crystalline style extract prepared in October 1978 (54 ml

Table 10. Recovery of *Mytilus* lysozyme from CM- and DEAE-cellulose. *Mytilus* lysozyme solution was mixed with the ion exchangers and eluted with pH 6.5 and 7 phosphate buffers respectively.

| Treatment | CM-Cellulose | | | | DEAE-Cellulose | | | |
|---|---|---|---|---|---|---|---|---|
| | Lysozyme* µg HEW lysozyme equivalent | Protein* mg | Lysozyme: Protein $\mu g.mg^{-1}$ | Yield % | Lysozyme* µg HEW lysozyme equivalent | Protein* mg | Lysozyme: Protein $\mu g.mg^{-1}$ | Yield % |
| Enzyme Solution applied to exchanger | 44.2 | 76.7 | 0.58 | 100 | 34.8 | 58.8 | 0.59 | 100 |
| Supernate | 0 | 9.3 | 0 | 0 | 18.8 | 10.8 | 1.74 | 54 |
| Eluting buffer 0.01M | 0 | 9.8 | 0 | 0 | 9.5 | 9.0 | 1.06 | 27.3 |
| 0.05M | 0 | 6.3 | 0 | 0 | 9.9 | 6.1 | 1.62 | 28.5 |
| 0.1M | 0 | 10.0 | 0 | 0 | 4.4 | 7.4 | 0.61 | 12.6 |
| 0.2M | 6.8 | 10.1 | 0.67 | 15.4 | 2.9 | 7.2 | 0.4 | 8.3 |
| 0.4M | 13.4 | 6.1 | 2.2 | 30.3 | 0 | 5.0 | 0 | 0 |
| 0.6M | 11.7 | 6.1 | 1.9 | 26.5 | 0 | 4.6 | 0 | 0 |

*Total values are given in each case.

Table 11. Lysozyme and protein content of batches of approximately 350 styles of Mytilus edulis.

| Sampling Period | A. HEW lysozyme equivalent (µg) | B. Total Protein (mg) | Ratio A:B (µg/mg) |
|---|---|---|---|
| October '78 | 2120 | 667 | 3.17 |
| December '78 | 675 | 812 | 0.83 |
| February '79 | 897 | 351 | 2.56 |
| Mean values | 1230 | 610 | 2.02 |

containing 621 mg protein and 2 mg of HEW lysozyme equivalent) was mixed for 1 h with 4 g Amberlite CG50, poured into a column and eluted with 0.1M phosphate buffer, pH 6.5 until almost all 280 nm absorbing material had been washed through the column. Lysozyme was bound to the column and was eluted by a 0.1 to 1M phosphate buffer gradient, pH 6.5 (Fig 10). The fractions containing Peak activity represented a 60% recovery of activity with a 44-fold increase in purity to a specific activity of 3340 units $mg^{-1}$ (Table 12).

Dialysis against 0.01M acetate buffer (pH 5)

Fractions containing peak activity obtained from Amberlite CG50 were pooled and dialysed against acetate buffer. Dialysis further increased the purification to 99-fold with specific activity of 7,500 units $mg^{-1}$ (Table 12).

Fractionation on CM-cellulose (CM-32)

The above fraction was applied to a 4 ml column of CM-cellulose (CM-32) equilibrated in 0.01M sodium acetate buffer pH 5 and, after washing with 50 ml sodium acetate buffer, a 0 to 1M NaCl gradient in sodium acetate buffer was applied. The peak of lysozyme activity was associated with the shoulder following the main $E_{280}$ absorbing peak (Fig 11). The fractions containing peak activity represented an average recovery of 31.5% of the initial activity, the specific activity of 11,800 units $mg^{-1}$ representing 151-fold purification (Table 12).

Dialysis against distilled water

For some experiments salt free lysozyme was required and the above fraction was dialysed against distilled water. This led to a considerable loss of protein and activity in the October 1978 sample

Fig 10. Elution of Mytilus lysozyme from Amberlite CG-50.

A 0.1M acetic acid extract of 350 Mytilus crystalline styles, adjusted to pH 6.5 was mixed with 10 ml Amberlite CG-50 resin for 1 h, poured into a column and eluted with pH 6.5 potassium phosphate buffer: A, 0.1M; B, a 0.1M to 1M linear gradient; C, 1M buffer. ●——●, $E_{280}$; ■——■, lysozyme ($\mu g.ml^{-1}$) compared with HEW lysozyme as a standard.

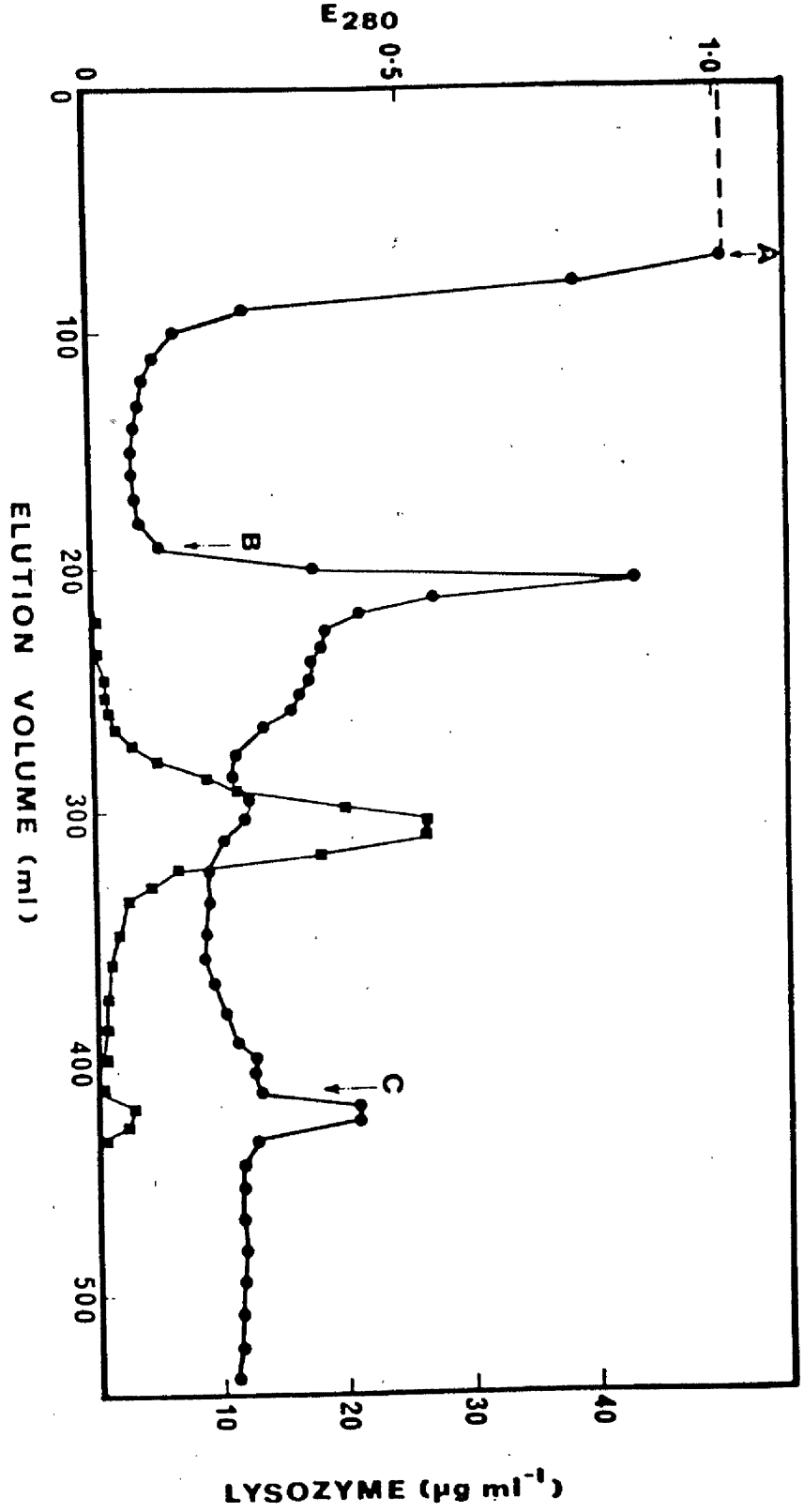

E280
0
0·5
1·0
0
100
200
300
400
500
A
B
C
ELUTION VOLUME (ml)
10
20
30
40
LYSOZYME (μg ml⁻¹)

Table 12. Purification of lysozyme from crystalline styles of Mytilus edulis

| Purification stage | October '78 (2120 µg lysozyme)+ | | | December '78 (675 µg lysozyme)+ | | | February '79 (897 µg lysozyme)+ | | | Average values | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Specific Activity units $mg^{-1}$ | Overall* Recovery | Purification* | Specific Activity units $mg^{-1}$ | Overall* Recovery | Purification* | Specific Activity units $mg^{-1}$ | Overall* Recovery | Purification* | Specific Activity units $mg^{-1}$ | Overall* Recovery | Purification* |
| Acetic acid extract of Mytilus styles | 76 | 100 | 1 | 49 | 100 | 1 | 100 | 100 | 1 | 75 | 100 | 1 |
| Amberlite CG-50 | 3340 | 59.8 | 43.9 | 3820 | 97.5 | 77.9 | 3800 | 84.2 | 38 | 3650 | 80.5 | 48.7 |
| Dialysis (0.01M $CH_3COONa$, pH 5) | 7530 | 66.9 | 99.1 | 7200 | 80.8 | 147 | 3380 | 75.8 | 33.8 | 6040 | 74.5 | 80.5 |
| CM-cellulose | 11500 | 31.5 | 151 | 16500 | 24.3 | 336 | 13000 | 41.6 | 130 | 13700 | 32.5 | 182 |
| Dialysis (distilled water) | 11100 | 12.9 | 146 | 21300 | 35.3 | 435 | - | - | - | 16200 | 24.1 | 216 |
| HEW lysozyme | 16200 | | | 12200 | | | 18000 | | | 15500 | | |

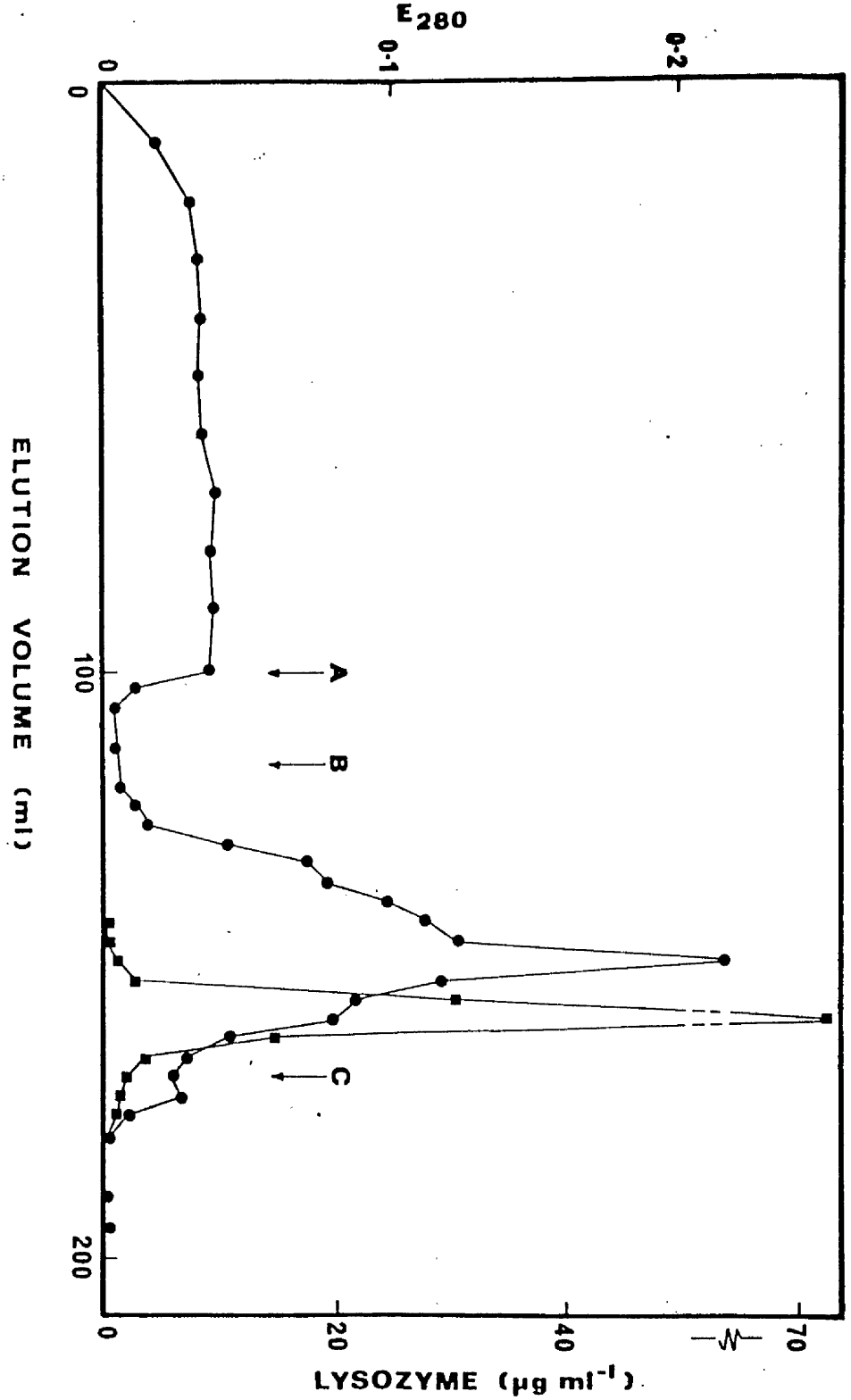
+ Lysozyme content of style extract expressed as HEW lysozyme equivalent.

* Compared to the acetic acid extract of Mytilus styles.

Fig 11. Elution of Mytilus lysozyme from CM-cellulose.

Peak fractions from Amberlite CG50 (Fig 10) were pooled, dialysed against 0.01M sodium acetate pH 5 buffer, applied to 4 ml column of CM-cellulose (Whatman CM32) and eluted with A, 0.01M sodium acetate, pH 5; B, a 0.0 to 1M NaCl gradient in 0.01M sodium acetate, pH 5; C, 1M NaCl in 0.01M sodium acetate, pH 5.

●——●, $E_{280}$; ■——■, lysozyme ($\mu g.ml^{-1}$) using HEW lysozyme as a standard.


E280
0
0·1
0·2
0
100
200
ELUTION VOLUME (ml)
A
B
C
0
20
40
70
LYSOZYME (μg ml⁻¹)

but dialysed material had a similar specific activity to the fraction recovered from CM-cellulose (Table 12). Further batches of lysozyme were purified in December 1978 and February 1979 and the results were similar to those of the first batch (Table 12). Unless salt free lysozyme was specifically required the fraction obtained from CM-cellulose was not dialysed as this rendered the enzyme less stable to storage at -20°C or -70°C. The specific activity of the final product was comparable with that found for commercially available HEW lysozyme, 15,500 units $mg^{-1}$ (Table 12).

Presence of other style enzymes in lysozyme fractions

The crystalline style contains a variety of enzymes (Reid, 1968; Kristensen, 1972a,b) and the activity of two enzymes, protease and β-glucuronidase, was measured in the fractions obtained during lysozyme purification. Both enzymes were detected in the acetic acid extract prepared in February 1979 and in the lysozyme fraction obtained from Amberlite CG50 but the enzymes were not detected in any of the active fractions obtained after dialysis or eluted from CM-cellulose (Table 13). This represented an alteration in the lysozyme:protease ratio of > 1,900 and in the lysozyme:β-glucuronidase ratio of > 84 during the purification procedure.

## 3.4 Monitoring of purity by disc-gel electrophoresis

Electrophoresis of duplicate samples from each stage of the purification process was carried out in acid polyacrylamide gels, to monitor the complexity of the fractions obtained in purification. Gels were either stained with Coomassie Brilliant Blue dye to reveal protein bands or sectioned, to determine the location of the lysozyme.

Table 13. Residual β-glucuronidase and collagenase activities in purified *Mytilus* lysozyme

| Purification Stage | Activity enzyme units per mg protein | Overall[1] Recovery % | Purification[1] | Ratio of lysozyme/ protease activity | Ratio of lysozyme/ β-glucuronidase |
|---|---|---|---|---|---|
| Acetic acid extract of *Mytilus* style | 100 | 100 | 1 | 23 | 238 |
| Amberlite CG50 | 3,800 | 84.2 | 38 | 37 | 5510 |
| Dialysis (0.01M $CH_3COONa$, pH 5) | 3,380 | 75.8 | 33.8 | > 12,000[2] | > 5,300[3] |
| CM-cellulose | 13,000 | 41.7 | 130 | > 45,000[2] | > 20,000[3] |

1. Compared to the acetic acid extract of *Mytilus* styles.
2. Figures obtained by increasing sample volume four-fold and incubating sample-substrate mixture for 25 h.
3. Figures obtained by incubating sample-substrate mixture for 1 h.

Following electrophoresis of the acetic acid extract of style 13 stained bands were visible in the separating gel; the lysozyme activity was associated with the third fastest moving band (A, Fig 12). The lysozyme fraction eluted from Amberlite was less complex (B, Fig 12) and the fraction eluted from CM-cellulose yielded one major staining band with a second fainter band (C, Fig 12). The lysozyme activity was associated with the major staining band. In a purified HEW lysozyme control electrophoresed at the same time activity was associated with the major staining band which migrated fractionally more rapidly than the active band in the Mytilus preparation (D, Fig 12). A second minor band without associated lysozyme activity was found in the HEW lysozyme gel. Scanning of the gels with a U.V. gel scanner (Joyce Loebl, Gateshead) revealed that the major staining band with which activity was associated in the purified Mytilus lysozyme gel represented 90% of the total staining material, compared with 98% in the HEW lysozyme.

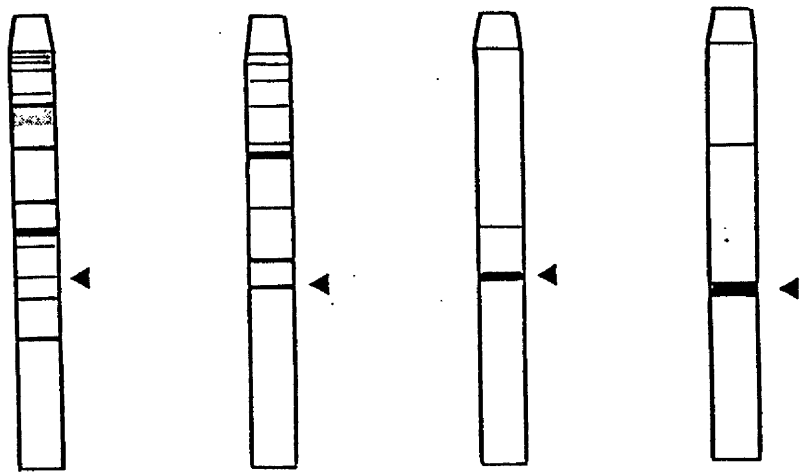
## 3.5. CC-cellulose as an adsorbent for Mytilus lysozyme

Whilst the recovery of lysozyme from CC-cellulose was low with whole animal homogenates as starting material it represented a 51-fold purification of the material applied. Therefore experiments were conducted to determine whether CC-cellulose could be used in the purification of style lysozyme without the same loss of activity.

Lysozyme eluted from Amberlite CG-50 was dialysed against 0.2M potassium phosphate buffer (pH 6.6) containing 0.5M NaCl and 64.9 µg enzyme (HEW lysozyme equivalent) in 141 ml was applied to a CC-cellulose column. All lysozyme was absorbed but on elution with 0.1M acetic acid only 4.7 µg of enzyme (HEW lysozyme equivalent) was recovered, which represented 7.3% of the applied lysozyme (Fig 13a).

Fig 12. Purity of Mytilus lysozyme as indicated by disc electrophoresis. Disc electrophoresis in 15% polyacrylamide gels (pH 4.3), of dialysed samples of style lysozyme during the purification stages:

A - acetic acid extract of style, B - post amberlite, C - post CM-cellulose and D - HEW lysozyme. The direction of protein migration is from anode (top) toward cathode (bottom). The location of activity is denoted thus: ◀



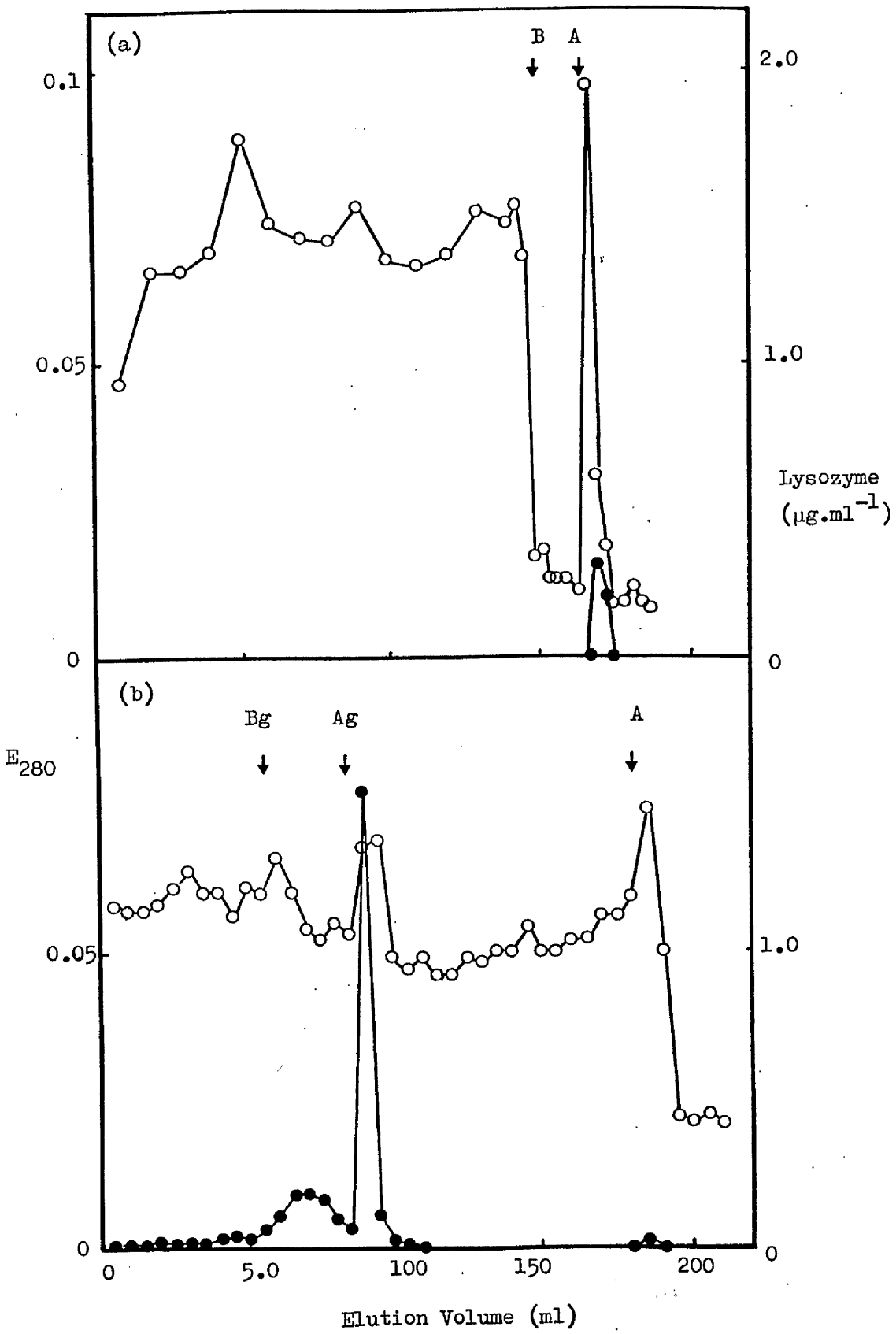
A B C D

Fig 13. Elution of Mytilus lysozyme from CC-cellulose.

(a) Sixty-five μg enzyme (HEW lysozyme equivalent) in 141 ml was applied to a 0.7 ml column and eluted with 0.2M potassium phosphate buffer containing 0.5M NaCl (B) and 0.1M acetic acid (A).

(b) Twenty-one μg enzyme (HEW lysozyme equivalent) in 59 ml was applied to a 0.7 ml column and eluted with 0.2M potassium phosphate buffer containing 0.5M NaCl and 0.04% (w/v) gelatin (Bg) and 0.1M acetic acid containing 0.04% (w/v) gelatin (Ag) and finally with 0.1M acetic acid (A).

○——○, $E_{280}$; ●——●, lysozyme ($\mu g.ml^{-1}$) using HEW lysozyme as a standard.


(a)
B
A
0.1
0.05
0
2.0
1.0
0
Lysozyme
(μg.ml⁻¹)
(b)
Bg
Ag
A
E280
0.05
0
1.0
0
0
5.0
100
150
200
Elution Volume (ml)

The effect of gelatin on the recovery of *Mytilus* lysozyme from CC-cellulose

It was considered that the highly basic nature of the lysozyme might cause it to bind non-specifically to the resin and/or glass column. In an attempt to overcome this 0.04% w/v gelatin was incorporated into all solutions.

The column was washed with 0.2M phosphate buffer, pH 6.6 containing sodium chloride (0.5M) and 0.04% gelatin (Bg) and 59 ml of Amberlite CG-50 prepared lysozyme solution, containing 21.2 μg lysozyme (HEW lysozyme equivalent) was applied to the column. Gelatin effectively masked any $E_{280}$ changes in the elution profile (Fig 13b) but traces of lysozyme were detected in the non-adsorbed fraction especially when the column was washed with buffer containing gelatin (Bg) (Fig 13b). When the 0.1M acetic acid containing gelatin (Ag) was applied lysozyme was eluted in a single fraction (4.7 ml containing 7.3 μg of HEW lysozyme equivalent) representing 34.4% of the applied activity.

Overall, 62.7% of the applied activity could be accounted for: 6.1% washed through the column, 18.4% was eluted by the buffer containing gelatin, 3.3% followed the peak and 0.5% was contained in the peak eluted by the acid, without gelatin (Fig 13b).

Allowing for the presence of the gelatin the specific activity of the starting material was 2200 units $mg^{-1}$ and that of the eluted peak, 5550 units $mg^{-1}$, a 2.5-fold increase with 34.4% recovery. As this offered no advantage compared to CM-cellulose the use of CC-cellulose was discontinued.

## 4. Characterisation as a lysozyme

The characteristic activity of lysozyme in hydrolysing the glycosidic linkages in bacterial cell walls gave rise to the criteria which were suggested as requirements for recognition of a lysozyme (Salton, 1957). A lysozyme must:

(1) lyse intact bacterial cells or reduce the turbidity of isolated cell walls,

(2) liberate reducing groups,

(3) liberate an amino-sugar complex.

That the Mytilus enzyme could satisfy the first of the criteria was shown by the use of the spectrophotometric and lysoplate assays to determine the activity/concentration of the enzyme. The satisfaction of the second and third criteria require specific chemical assays.

### 4.1 Liberation of reducing groups from cell walls

The presence of reducing groups in the supernates of bacterial cell wall suspensions degraded by HEW and Mytilus lysozyme was determined by a modification of the method of Thompson and Shockman (1968). Both Mytilus and HEW lysozyme liberated reducing groups from M. luteus cell walls; 102 and 138 μg of glucose equivalent respectively were released from 4 mg cell wall suspended in 2 ml buffer (Table 14). Thus Mytilus, and HEW, lysozyme satisfied the second criterion, showing that both were glycosidases.

### 4.2 Liberation of N-acetylamino sugars from cell walls

The concentrations of N-acetylamino sugars in the reaction mixture supernates were determined by a method based on the Morgan-Elson

Table 14. The liberation of reducing groups from M. luteus cell walls by HEW and Mytilus lysozymes.

| Reaction mixture | $E^{1cm}_{700}$ | Glucose Equivalent (μg) | | |
|---|---|---|---|---|
| | | In 0.4 ml sample | In 2 ml supernate | Total (adjusted for control) |
| A. Cell wall control | 0.025 | 0.35 | 1.75 | |
| *B. Cell wall suspension + HEW lysozyme | 0.82 | 14 | 140 | 138 |
| *C. Cell wall suspension + Mytilus lysozyme | 0.643 | 11 | 110 | 102 |
| D. Mytilus lysozyme control | 0.078 | 1.3 | 6.5 | |
| E. HEW lysozyme control | 0.003 | 0 | 0 | |

* Assayed at 1/2 dilution.

reaction (Ghuysen et al, 1966). Both Mytilus and HEW lysozymes liberated N-acetylamino sugars from M. luteus cell walls releasing 2.5 and 9 mμM of N-acetylglucosamine equivalent respectively from 4 mg of cell wall in 2 ml of buffer (Table 15). Thus both Mytilus and HEW lysozymes satisfy the third criterion of Salton (1957).

## 4.3 Characterisation of the glycosidic linkage split

For an enzyme to be called a lysozyme it should be a muco-peptide N-acetylmuramylhydrolase (E.C.3.2.1.17). The nature of the liberated reducing groups from cell walls was determined by a subtractive method involving reduction with borohydride, acidic hydrolysis and two-dimensional chromatography.

When Mytilus lysozyme digests of cell walls were treated as above and the chromatograms sprayed with ninhydrin, two red spots corresponding in position to those formed by N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) were revealed in the control, unreduced sample (Fig 14a), whereas in the sample reduced by borohydride before hydrolysis the intensity and size of the NAM spot was markedly decreased compared to the corresponding spot in the unreduced control. The size and intensity of the NAG spot remained similar to that of the control (Fig 14b).

It was concluded that the Mytilus lysozyme cleaved the β 1→4 linkage between NAM and NAG and is therefore a true lysozyme.

## 5. Physico-chemical characteristics

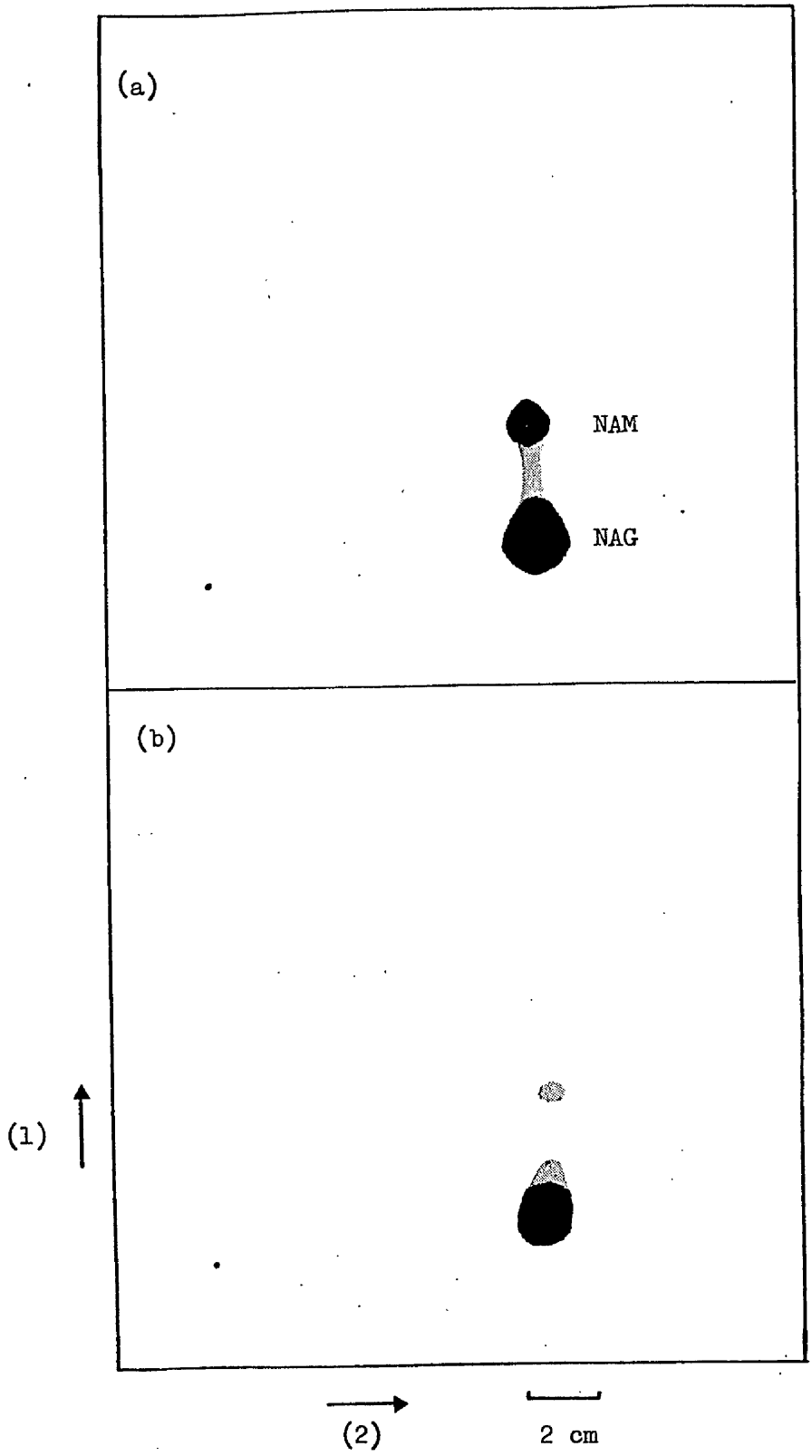
Whereas not all lysozymes have the characteristics attributed to these enzymes by Jolles (1964), all share the same enzymic activity. Jolles noted that most lysozymes were basic proteins of low molecular

Table 15. The liberation of N-acetylamino sugars from *M. luteus* cell walls by HEW and *Mytilus* lysozymes

| Reaction Mixture | $E^{1cm}_{585}$ | N-acetylglucosamine equivalent (x $10^{-8}$M) | | |
|---|---|---|---|---|
| | | In 0.2 ml sample | In 2 ml supernate | Total (adjusted for controls) |
| A. Cell wall control | 0.021 | 0.2 | 2 | |
| B. Cell wall suspension + HEW lysozyme | 0.117 | 1.3 | 13 | 9 |
| C. Cell wall suspension + *Mytilus* lysozyme | 0.055 | 0.6 | 6 | 2.5 |
| D. *Mytilus* lysozyme control | 0.016 | 0.15 | 1.5 | |
| E. HEW lysozyme control | 0.020 | 0.2 | 2 | |

Fig 14. Two dimensional chromatograms of acid hydrolysed *Mytilus* lysozyme cell wall digests.

Both the control (a) and the borohydride reduced sample (b) were run in (1) butanol:acetic acid:water (3:1:1, by volume) followed by (2) pyridine:water (4:1, by volume).


(a)
NAM
NAG
(b)
(1)
(2)
2 cm

weights (around 15,000) which were stable at acid pH (even at higher temperatures) and were labile at alkaline pH.

Several physicochemical parameters of the Mytilus lysozyme were therefore investigated.

## 5.1 Isoelectric focusing

The zone convection apparatus of Talbot and Caie (1975) was used such that the enzyme could be readily recovered for assay after focusing. Lysozyme activity was not sharply focused, being found in 21 of the 36 fractions over the pH range 7.65 to 10. The peak fraction was focused at pH 9.2. However, there was no corresponding peak in absorbance at 280 nm (Fig 15a).

When the three peak enzyme fractions (pH 9.1 to 9.3) were refocused in a similar gradient the lysozyme was again found over a wide pH range (from pH 8.35 to 10.1) with peak activity at pH 9.2 (Fig 15b).

From these results it was concluded that Mytilus lysozyme is a basic protein with an isoelectric point (pI) of 9.2.

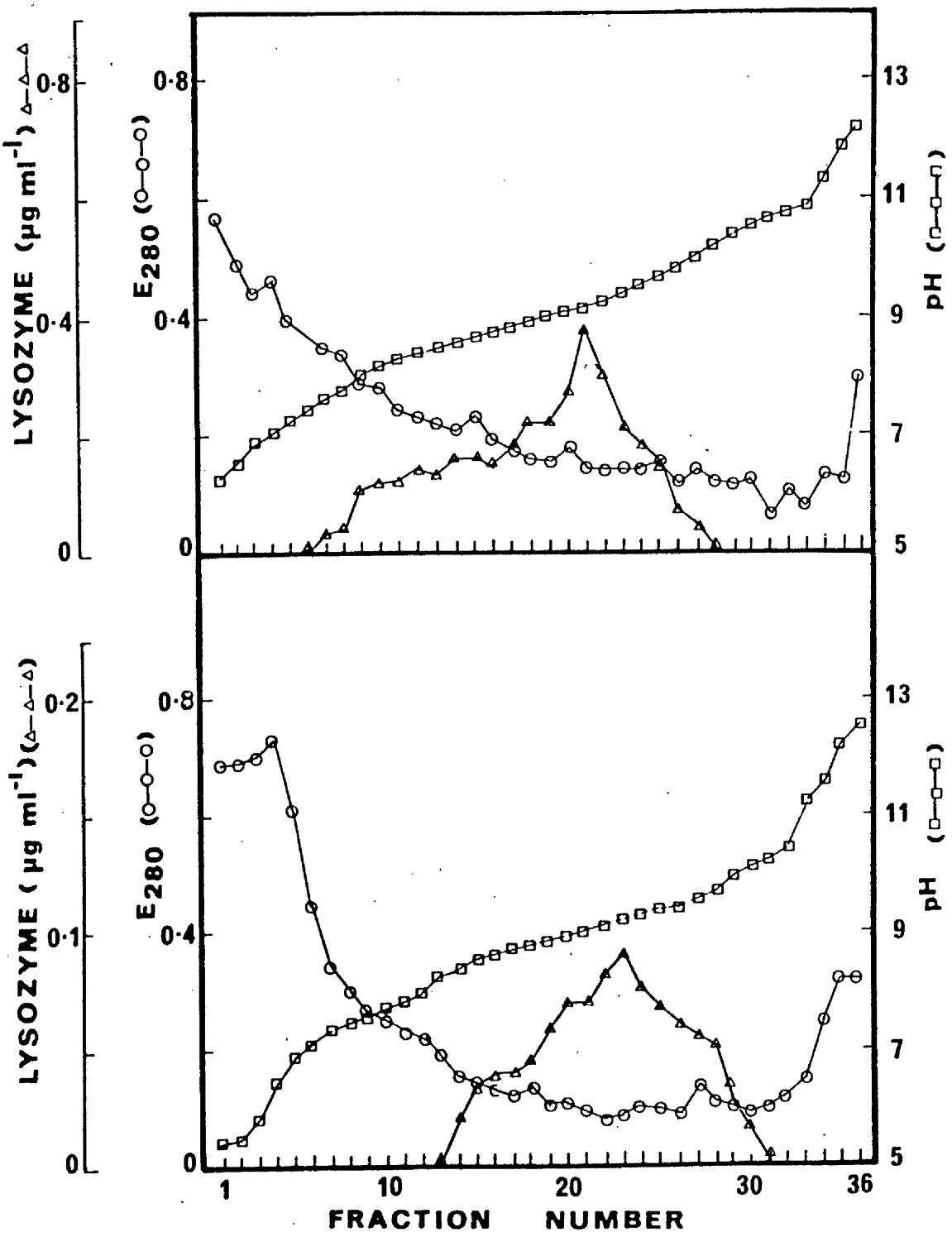
## 5.2 Molecular weight determination

The use of gel filtration for determination of molecular weights is particularly suited to enzymes such as lysozyme, for which simple sensitive assays are available, as it allows the determination to be undertaken with quantities of protein which would otherwise not normally be detected. The finding that HEW lysozyme eluted later than would be expected from dextran columns (Sephadex) (Whitaker, 1963; Fernandez-Sousa and Rodriguez, 1977) and from agarose based gels (Bio-Gel A) (Fernandez-Sousa and Rodriguez, 1977) led to the choice of a polyacrylamide based gel (Bio-Gel P60) in this study.

Fig 15. Isoelectric focusing of Mytilus lysozyme.

a) Mytilus lysozyme, purified by chromatography on Amberlite CG-50 and CM-cellulose was dialysed overnight against 1% glycine, and 1 ml containing 82 µg of protein subjected to isoelectric focusing in a zone convection apparatus.

b) Isoelectric focusing of pre-focused Mytilus lysozyme.


LYSOZYME (μg ml⁻¹) △–△
E280 (○–○)
pH (□–□)
0·8
0·4
0
13
11
9
7
5
LYSOZYME (μg ml⁻¹)(△–△)
0·2
0·1
1
10
20
30
36
FRACTION NUMBER

a) Gel filtration on Bio-Gel P60

A column of Bio-Gel P60 was calibrated with five standard macromolecules. While four of the five standards used (Blue Dextran 2000, ovalbumin, myoglobin and cytochrome c) were eluted in the volumes expected from their molecular weights, the fifth (HEW lysozyme) was eluted at a volume more than 50% greater than that expected. Cytochrome c of molecular weight 11,700 daltons was eluted before HEW lysozyme (14,300 daltons molecular weight) (Fig 16).

Mytilus lysozyme was eluted at a volume between those for cytochrome c and HEW lysozyme (Fig 16) with the peak giving an elution volume to void volume ratio of 2.31 corresponding to a molecular weight of 6,920 daltons (Fig 17). However only 8.1% of the 10 µg enzyme (HEW lysozyme equivalent) applied was recovered and because of this and the retardation of HEW lysozyme such molecular weight estimates were discounted.

b) Disc polyacrylamide gel electrophoresis

Whereas with the above system the purity of the applied enzyme preparation was not critically important, molecular weight determinations by disc gel electrophoresis ideally require a pure homogeneous sample. Disc electrophoresis of Mytilus and HEW lysozyme preparations in 15% polyacrylamide gels (pH 4.3) showed that enzymic activity was associated with the major staining band in each case (C and D, Fig 12).

Duplicate samples of the lysozymes, along with protein standards, were treated with S.D.S. and mercaptoethanol, electrophoresed into S.D.S. containing 11% polyacrylamide gels (pH 8.8) and stained.

Fig 16. Elution of Mytilus lysozyme from a Bio-Gel P-60 column (85 x 2 cm).

Applied standards were eluted at: (1) Blue Dextran 2000, (2) ovalbumin (M.Wt. 45,000 daltons), (3) myoglobin (17,000 daltons), (4) cytochrome c (11,700 daltons) and (5) HEW lysozyme (14,300 daltons).

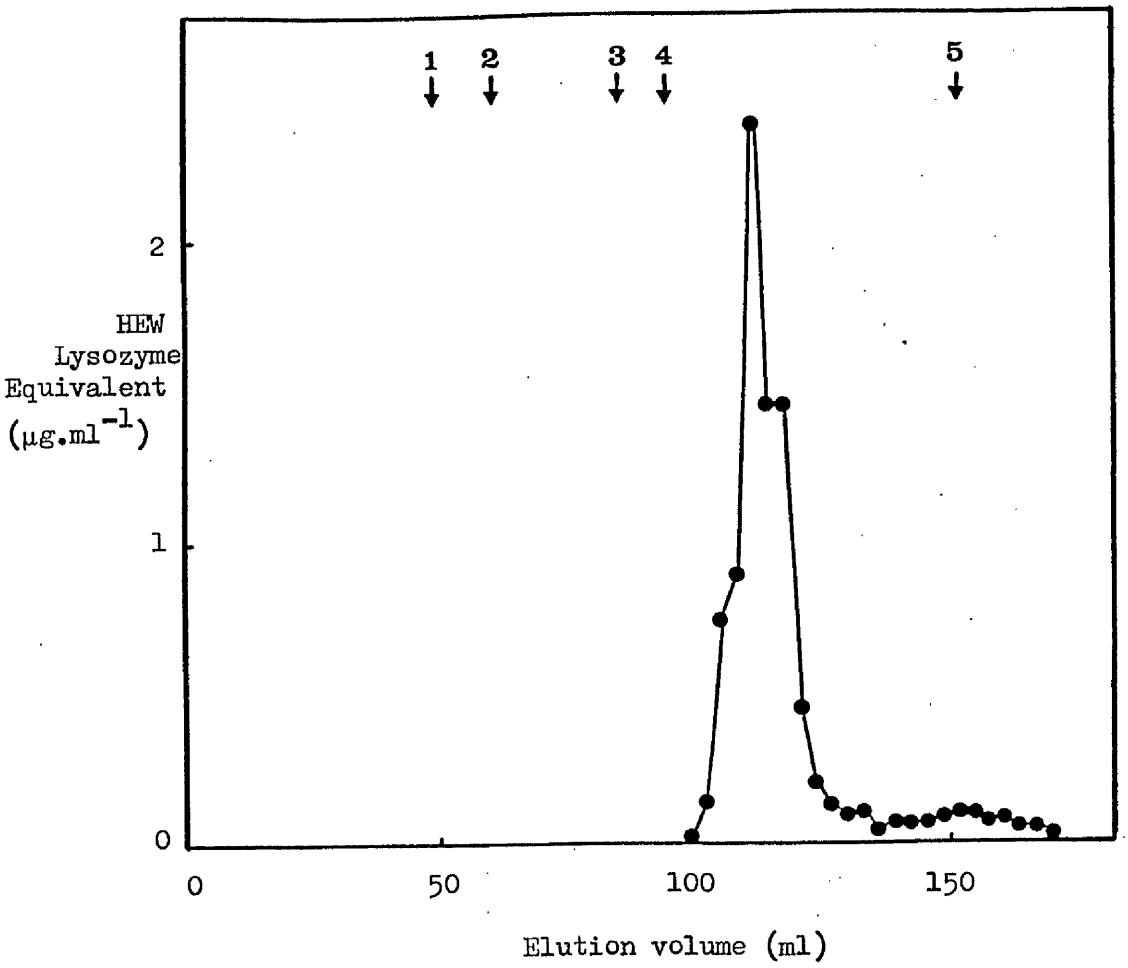
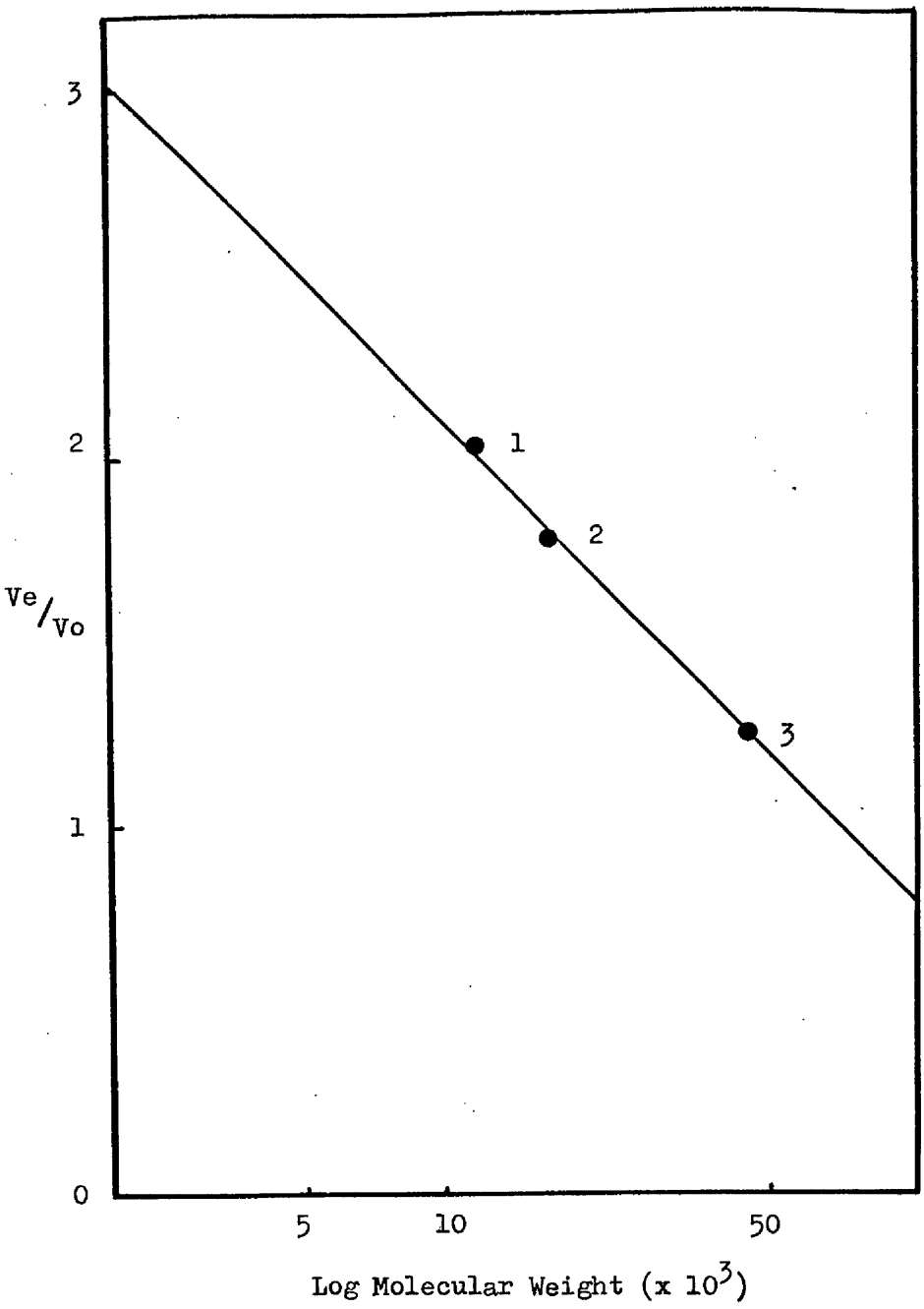

1
2
3
4
5
2
HEW
Lysozyme
Equivalent
(μg.ml-1)
1
0
0
50
100
150
Elution volume (ml)

Fig 17. Elution volume (Ve) to void volume (Vo) ratio on Bio-Gel P-60 of three proteins: (1) cytochrome c, (2) myoglobin and (3) ovalbumin.


3
2
1
0
Ve/Vo
1
2
3
5
10
50
Log Molecular Weight (x 10³)

A drawing of the results is shown in Figure 18. When the relative mobilities (Rf) of the protein standards were plotted against log molecular weight a linear relationship was found (Fig 19). For the major staining band in Mytilus lysozyme (A, Fig 18) (Rf = 0.46), a molecular weight of 18,000 daltons was indicated whilst the Rf of the minor band, 0.49, corresponded to a molecular weight of 16,500 daltons (Fig 19). The Rf of the major band in the HEW lysozyme sample (B, Fig 18), 0.59 corresponded to a molecular weight of 13,000 daltons (Fig 19). A second band, of Rf 0.42, corresponding to a molecular weight of 21,000 daltons was also obtained from HEW lysozymes samples.

## 5.3 Heat stability

While it was once considered that all lysozymes were heat stable, the isolation of a heat labile lysozyme from goose egg-white (Jolles et al, 1968) showed that heat stability might be used to classify certain lysozymes. The stability of Mytilus lysozyme at temperatures up to 100°C for periods up to 30 minutes was determined.

Initially the enzyme was suspended in distilled water but at 20°C 57% of the activity was lost within five minutes and 75% was lost in 30 min (Fig 20). At higher temperatures activity was lost more rapidly (Fig 20).

To minimise any apparent loss of activity which may have been due to the enzyme adhering to glass the Mytilus lysozyme was suspended in barbital-acetate buffer (pH 7.1, I = 0.006) containing gelatin (0.5% w/v). This treatment reduced the apparent thermal lability such that at 25°C, after 5 and 30 minutes, the activity loss was 39% and 54% respectively (Fig 21). The apparent lability at higher temperatures was also reduced (Fig 21).

Fig 18. SDS-PAGE of Mytilus and HEW lysozymes and standard proteins on 11% gels.

Where: A = Purified Mytilus lysozyme

B = HEW lysozyme (M.Wt. - 14,300 daltons)

C = Standards i - human serum albumin (67,000 daltons)

ii - ovalbumin (45,000 daltons)

iii - trypsin (23,300 daltons)

iv - myoglobin (17,000 daltons)

v - cytochrome c (11,700 daltons)

vi - insulin (5,733 daltons)

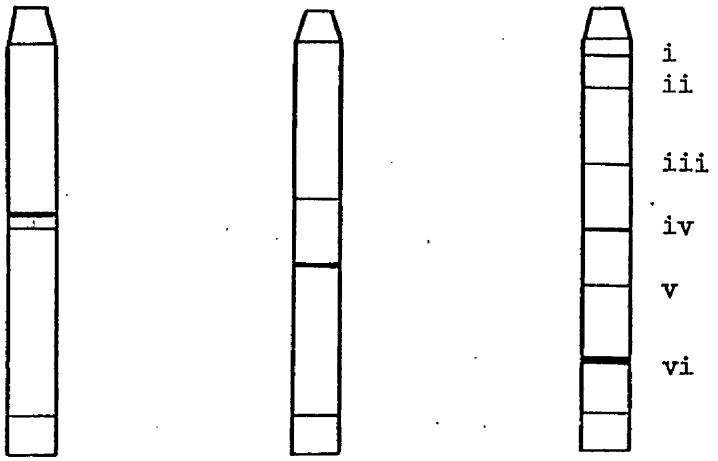

i
ii
iii
iv
v
vi
A
B
C

Fig 19. Relationship between relative mobility and log molecular weight of proteins on SDS-PAGE.

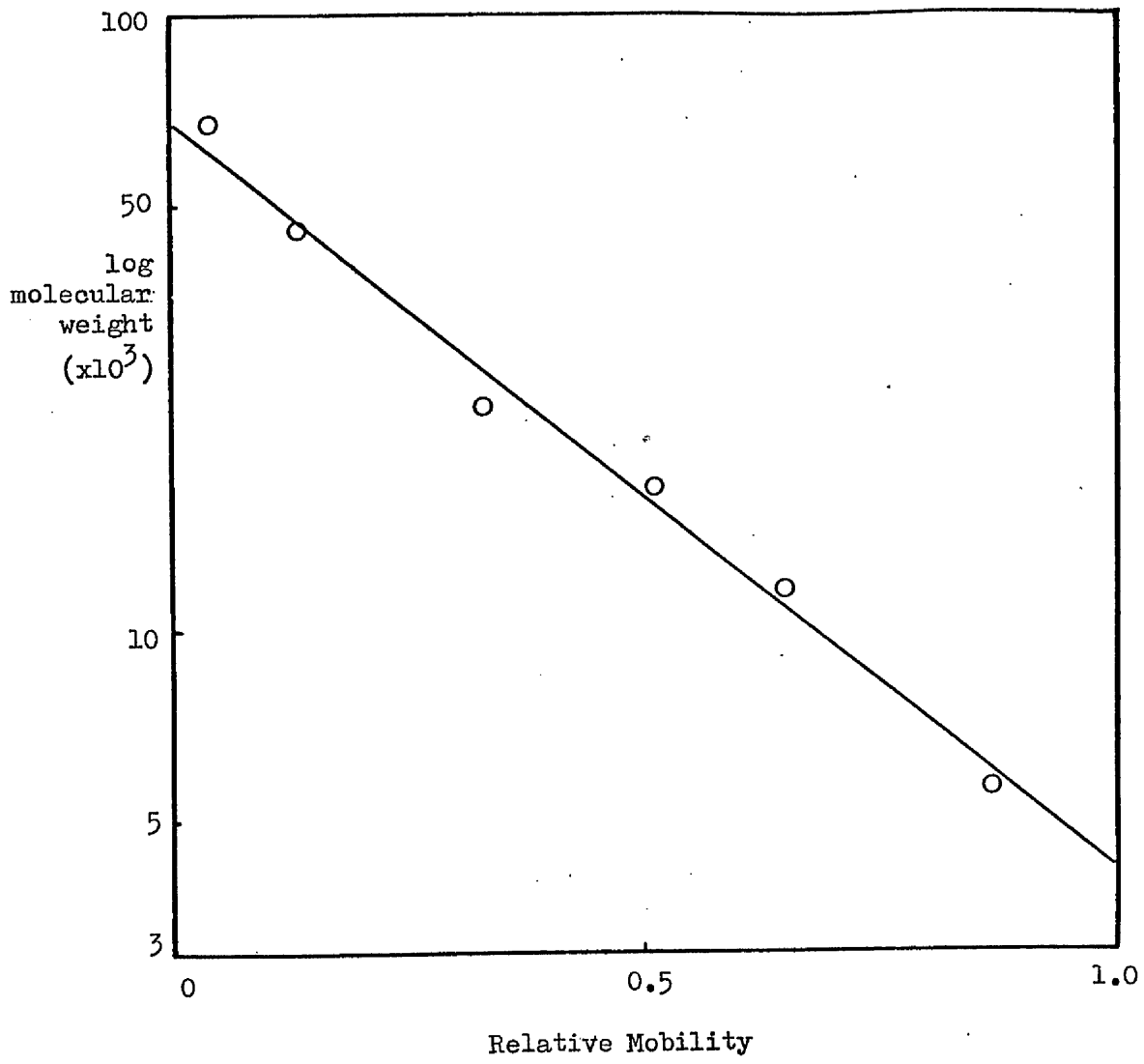
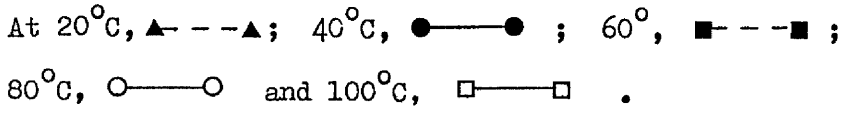

100
50
log
molecular
weight
(x10$^3$)
10
5
3
0
0.5
1.0
Relative Mobility

Fig 20. Thermal stability of Mytilus lysozyme in distilled water.



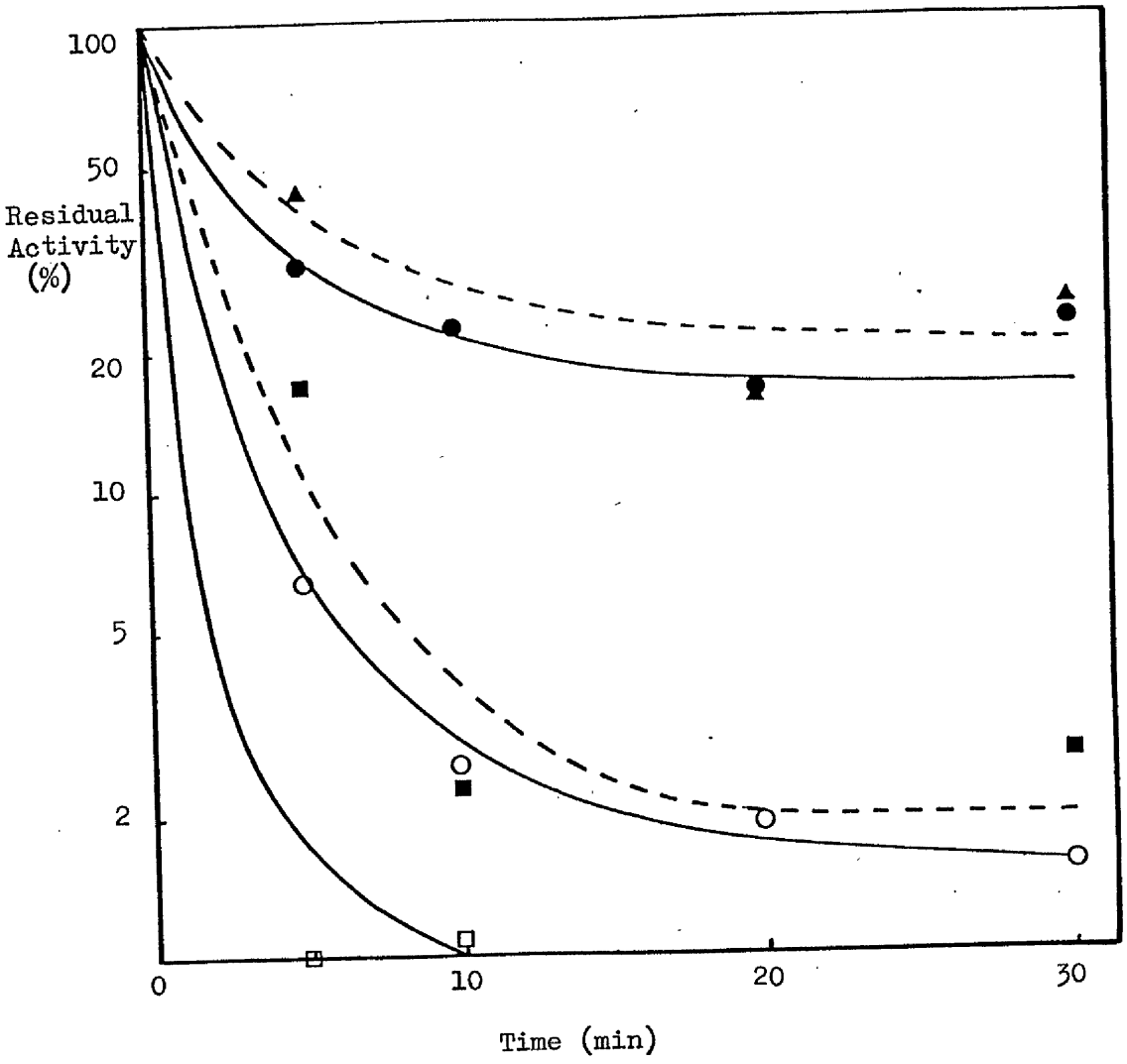
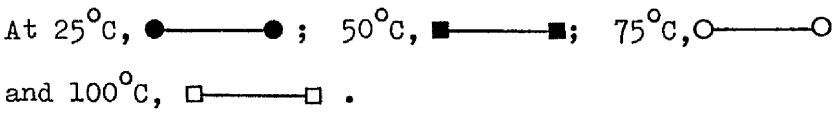
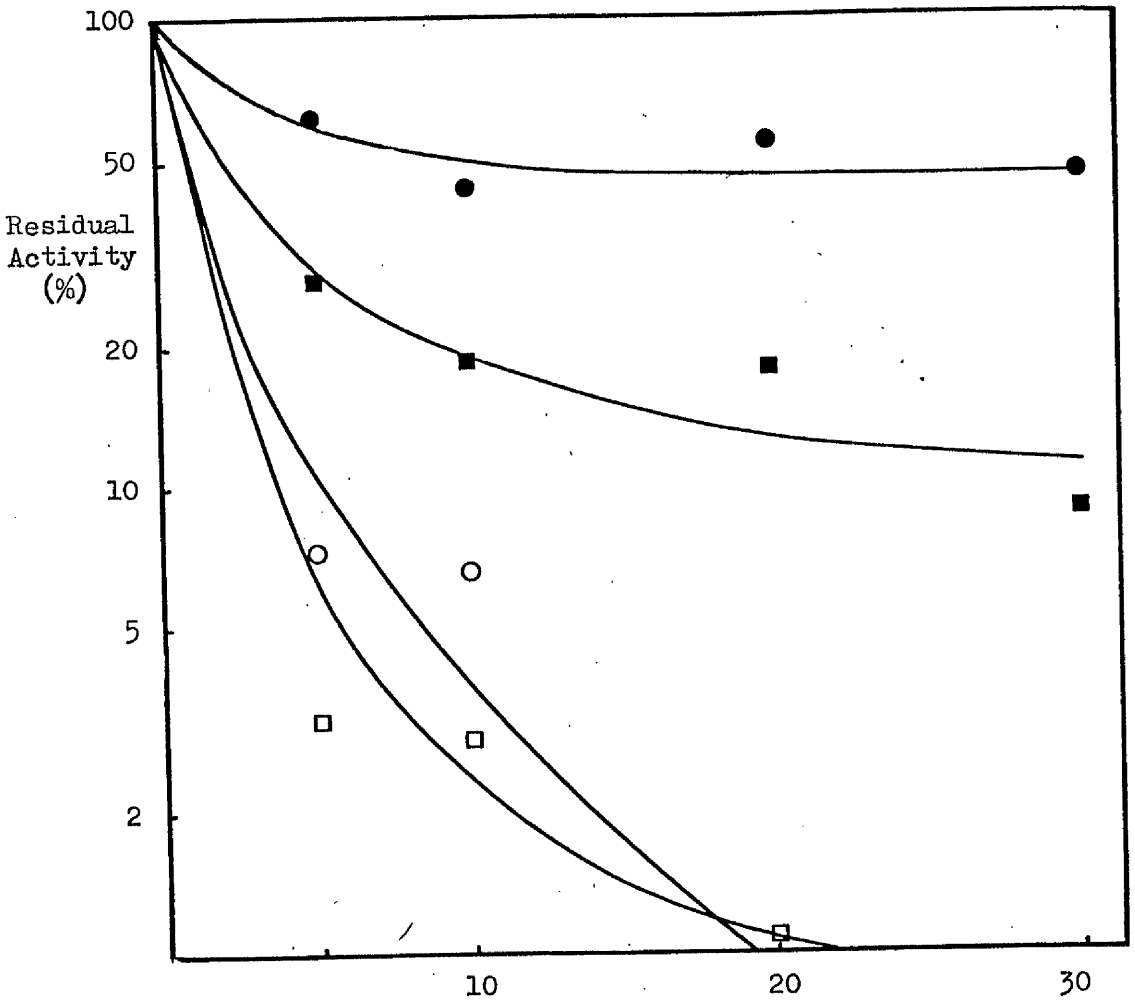

100
50
Residual Activity (%)
20
10
5
2
0
10
20
30
Time (min)

Fig 21. Thermal stability of Mytilus lysozyme in buffer containing gelatin.




100
50
20
10
5
2
Residual Activity (%)
10
20
30
Time (min)

The experimental procedure was further modified by the inclusion of sodium chloride (0.2M) in the above gelatin containing buffer. This was done to minimise self-aggregation by the purified enzyme. A further reduction in the apparent lability was noted; after 30 min at 25°C, 80% of the initial activity remained (Fig 22). Increased stability was found at all temperatures (Fig 22). At 100°C the half life of Mytilus lysozyme was 2.5 min.

## 5.4 Polyacrylamide gel electrophoresis of active and inactive enzyme preparations

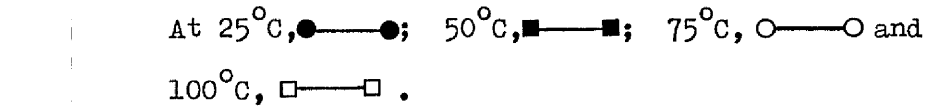
To determine what changes had occurred in Mytilus lysozyme preparations which had lost activity duplicate samples of active and inactive enzyme preparations were electrophoresed into 15% polyacrylamide gels (pH 4.3) which were subsequently stained or assayed for lysozyme activity for 20 h.

In the enzymically active sample (A, Fig 23), lysozyme activity was associated with the major staining band. In the inactive sample (B, Fig 23) the major band was of reduced intensity and migrated only as far as the second band in the active sample. The second band may also have been present in the inactive sample, masked by the larger band which retained slight activity (B, Fig 23). Three further bands of low mobility were present in the gel containing the inactive sample, which were not present in the active sample; no detectable activity was associated with these bands (Fig 23).

## 5.5 Dialysis of Mytilus lysozyme

Since two dialysis steps were involved in preparation of salt-free Mytilus lysozyme from style homogenates it was important to

Fig 22. Thermal stability of Mytilus lysozyme in buffer containing gelatin and sodium chloride.



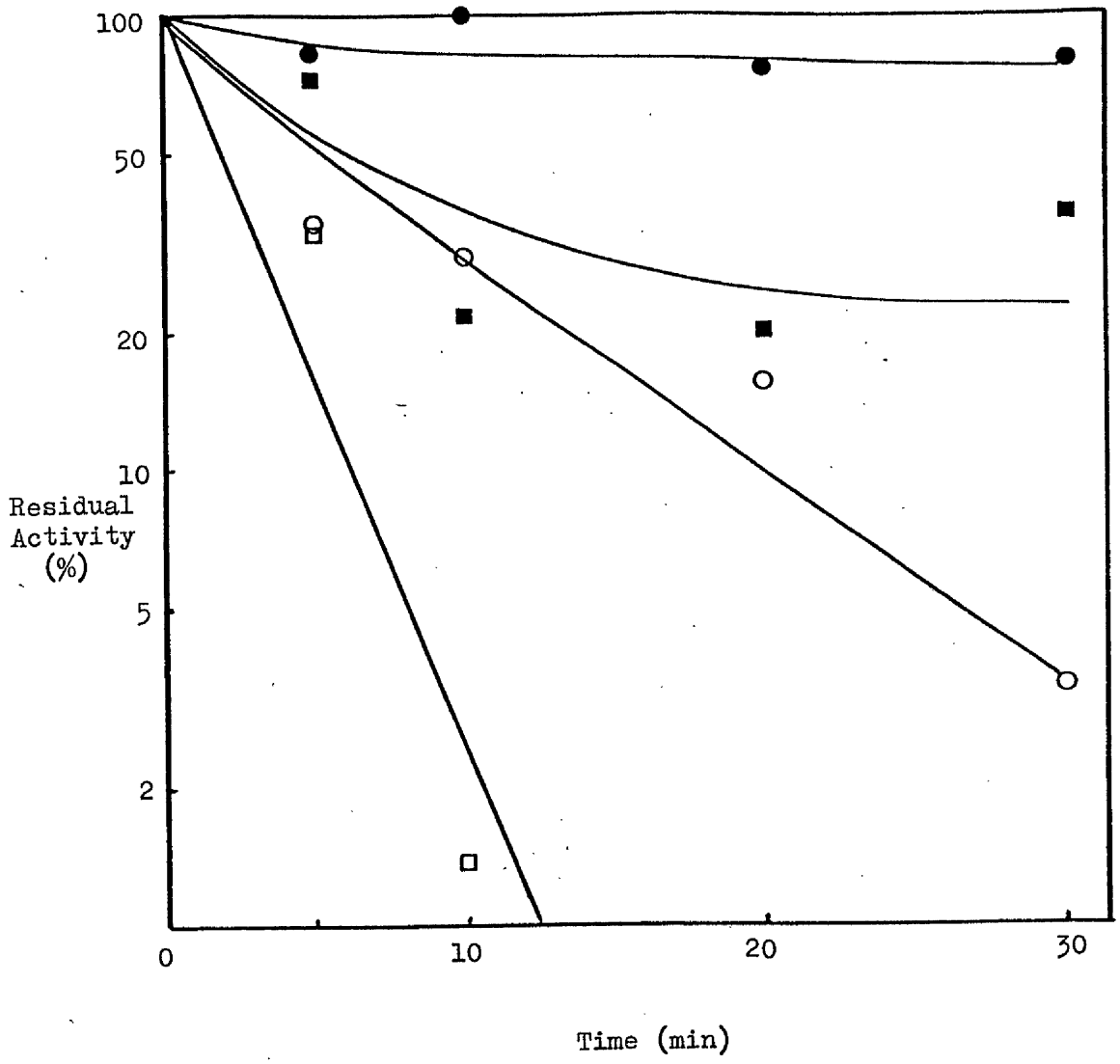
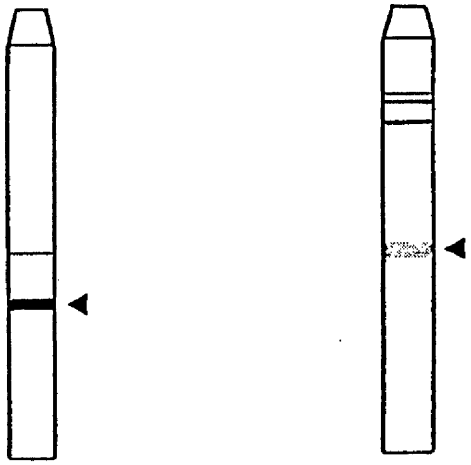

100
50
20
10
5
2
Residual Activity (%)
0
10
20
30
Time (min)

Fig 23. Examination of active and inactive Mytilus lysozyme preparations by disc electrophoresis.

Disc electrophoresis in 15% polyacrylamide gels (pH 4.3), of active (A) and inactive (B) Mytilus lysozyme preparations. The direction of protein migration is from anode (top) toward cathode (bottom). The location of activity is denoted thus: ◀



A B

determine whether activity was lost during dialysis. A crude style homogenate was dialysed against distilled water and samples assayed for lysozyme activity at various times.

After an initial drop in enzyme activity by 4 h (Fig 24) the activity remained relatively constant for up to 24 h (80.5% of original activity). No activity was detected in the dialysate. The lysozyme activity in the undialysed control samples apparently increased during the experiment and after 24 h contained 127% of the original activity (Fig 24).

## 6. Enzymic characteristics of *Mytilus* lysozyme

In addition to the differences in amino-acid sequence, molecular weight and immunological identity, the enzymatic properties of the lysozymes from different classes vary (Jolles and Jolles, 1975). The characteristics of *Mytilus* lysozyme were determined to compare it with the four established classes. Also knowledge of the optimal assay conditions for *Mytilus* lysozyme was desirable to ensure that the limited stocks of the enzyme available might be used most economically.

### 6.1 Optimum pH and ionic strength (I)

The sensitivity of lysozymes to changes in pH and I is well established (Davies *et al*, 1969; Saint-Blancard *et al*, 1970), with certain lysozymes exhibiting distinctive optima. In this experiment the optima for *Mytilus* and HEW lysozymes were determined.

The optimum conditions for *Mytilus* lysozyme were at I = 0.011, pH 7.1, and the activity remained high up to pH 7.5 but dropped sharply on either side of these points (Fig 25). As the ionic strength was lowered to 0.005 the activity was greatest at pH 8. When the ionic

Fig 24. Effect of dialysis of *Mytilus* lysozyme against distilled water at $4^{o}C$.

Dialysed sample (O——O), undialysed control (●——●).

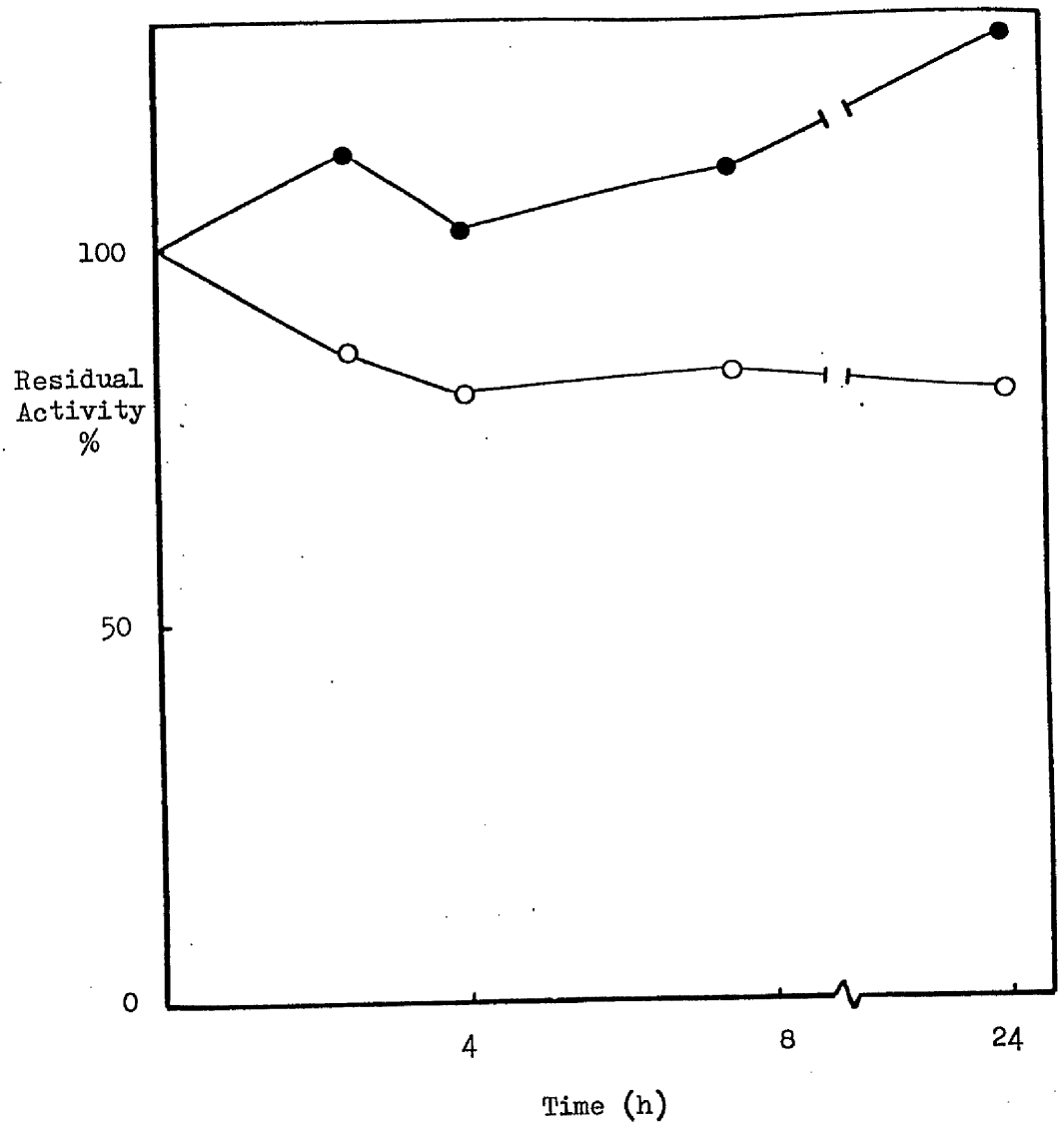
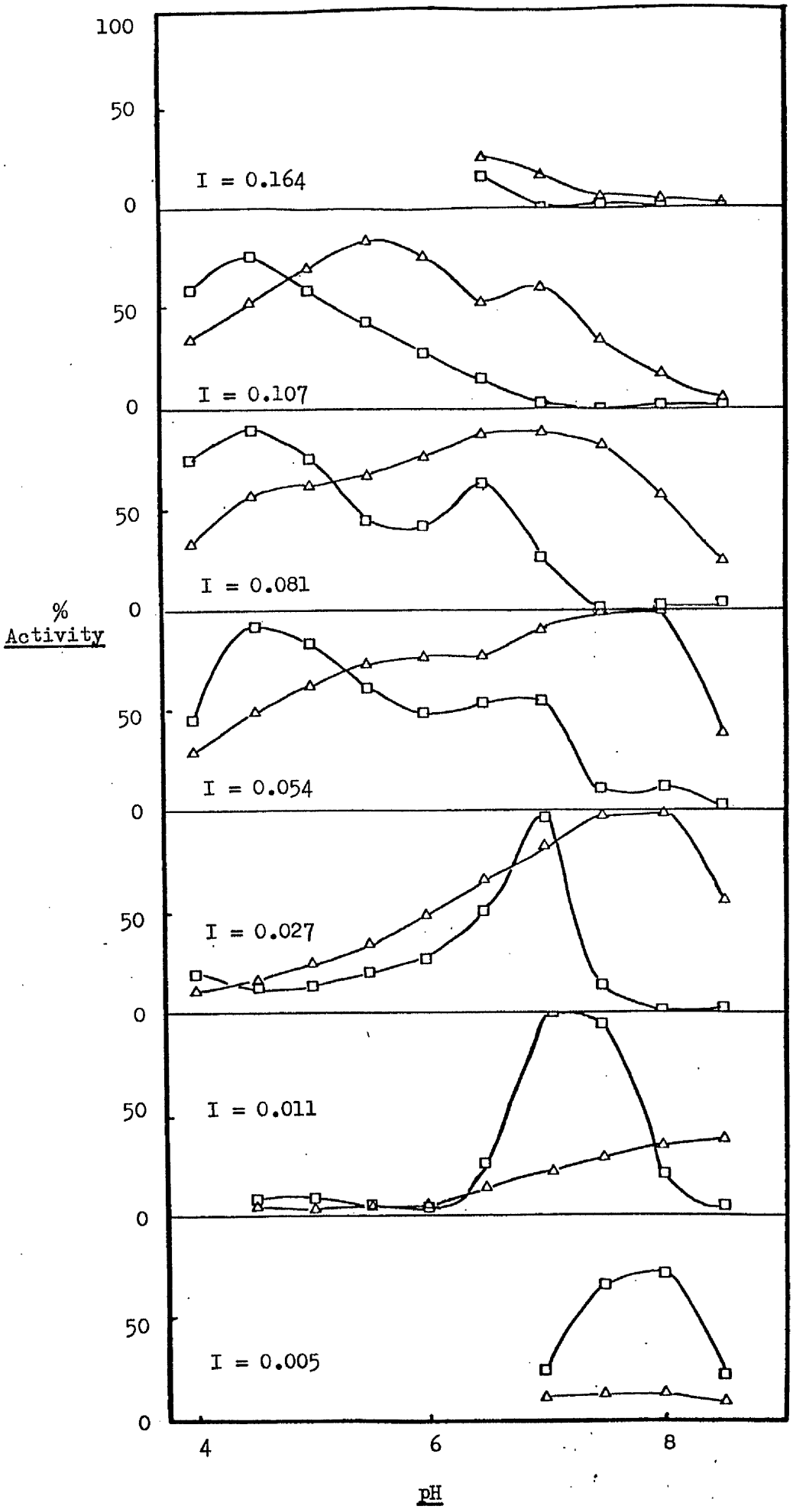

100
Residual
Activity
%
50
0
4
8
24
Time (h)

Fig 25. Effect of pH and I on lysozyme activity. The activities of *Mytilus* (□——□) and HEW lysozymes (△——△) are plotted relative to the maximum activity obtained in each case, *Mytilus* lysozyme at pH 7.1 and I = 0.011 and HEW lysozyme at pH 8 and I = 0.054.


100
50
0
I = 0.164
50
0
I = 0.107
50
0
I = 0.081
50
0
I = 0.054
50
0
I = 0.027
50
0
I = 0.011
50
0
I = 0.005
4
6
8
pH
% Activity

strength was increased the activity at acidic pH rose while that at pH ∿ 7 decreased such that at I = 0.054 maximum activity was at pH 4.6. As the ionic strength was further increased the activity at pH ∿ 4.6 remained high with a secondary activity peak being found at I = 0.081 at pH 6.5. Above this ionic strength the secondary peak was lost (Fig 25).

Very little activity was found for HEW lysozyme at low ionic strengths. However, activity increased with ionic strength such that at I = 0.054 maximum activity was found between pH 7.5 and 8, with slightly lower activities being found in the same pH range at I = 0.027 (Fig 25).

6.2 Optimum temperature

As Mytilus lysozyme was optimally active at pH 7.1, I = 0.011 with high activity also being found at pH 4.6, I = 0.054 (Fig 25), the optimum temperature for activity was determined under both conditions over the temperature range 8.5°C to 60.5°C. Hen egg-white lysozyme was assayed under identical conditions for comparison between lysozymes from poikilothermic and homeothermic origins.

At pH 7.1, I = 0.011 the activity of Mytilus lysozyme was maximal at 46°C with a sharp decline in activity above this temperature (Fig 26). At pH 4.6, I = 0.054 lower activity was found with a maximum at 39.5°C (Fig 26).

At pH 7.1 the activity of the HEW lysozyme rose over the range 8.5°C to 60.5°C with no maximum being apparent (Fig 27). However at pH 4.6 a maximum at 55°C was found (Fig 27).

Fig 26. Effect of temperature on Mytilus lysozyme activity.

In barbital acetate buffers pH 7.1 (I = 0.011) (□——□) and pH 4.6 (I = 0.054) (△——△).

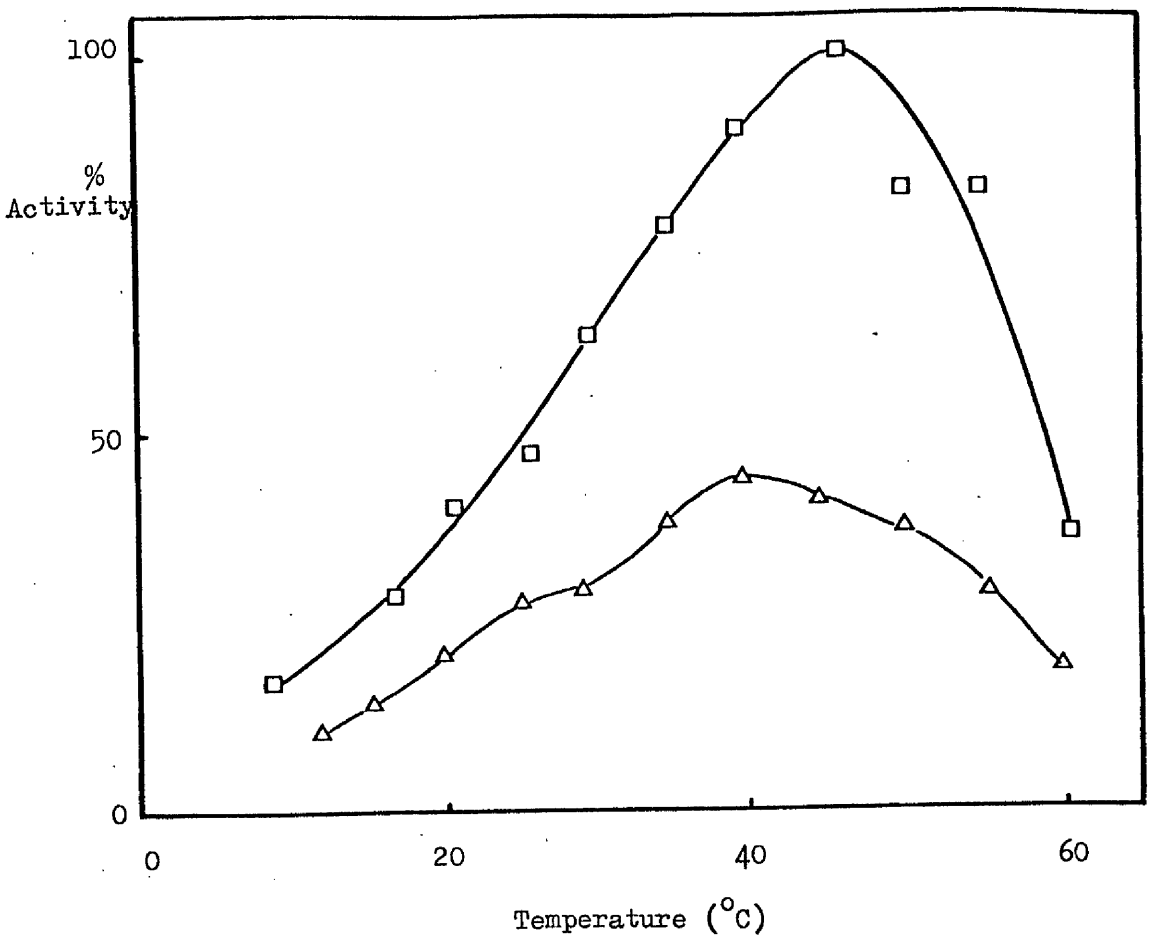
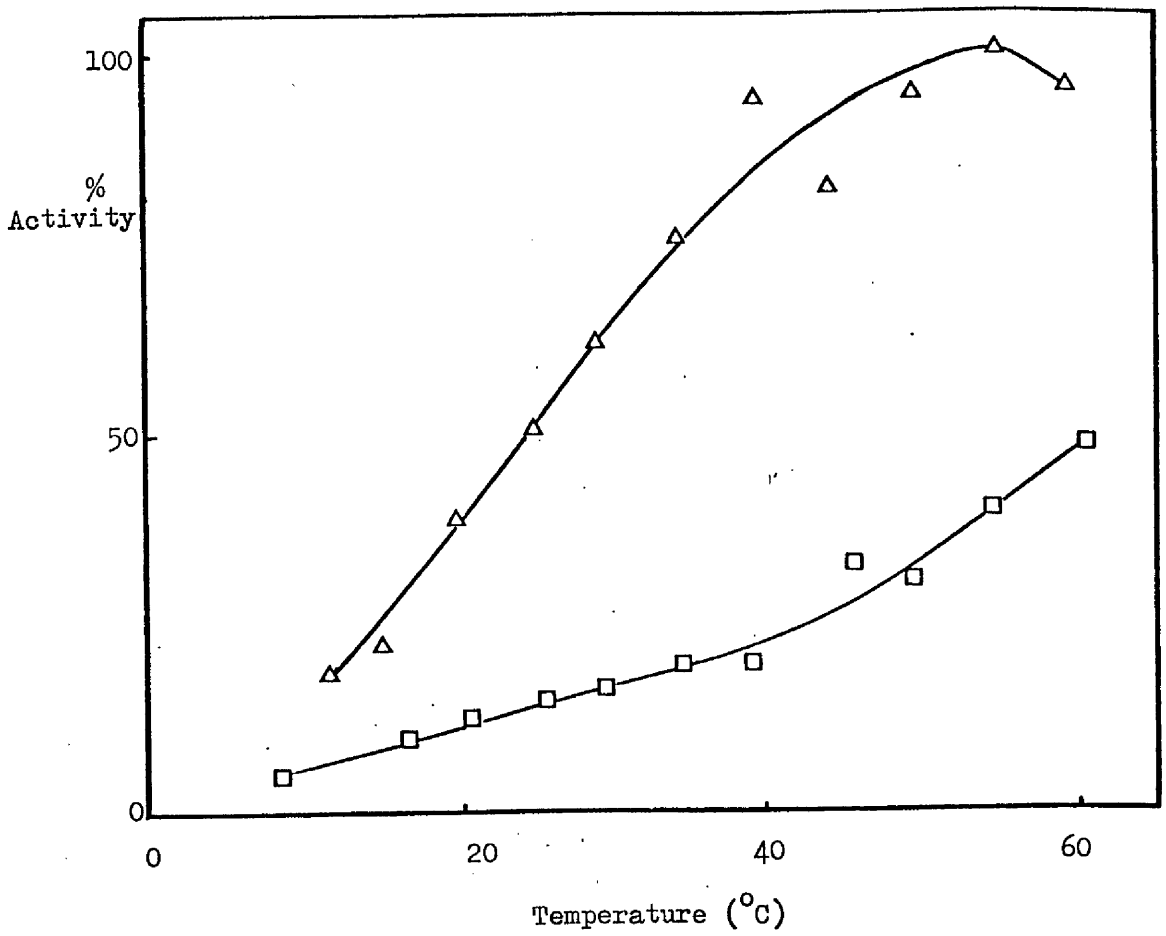

100
%
Activity
50
0
0
20
40
60
Temperature (°C)

Fig 27. Effect of temperature on HEW lysozyme activity.

In barbital acetate buffers pH 7.1 (0.011) (□——□)
and pH 4.6 (0.054) (△——△).


100
%
Activity
50
0
0
20
40
60
Temperature (°C)

## 6.3 Apparent affinity constants (Ka app.) for bacterial substrates

Whilst lysozymes from different sources may exhibit qualitatively the same biological activity they may possess different chemical structures which, in conjunction with other differences might influence their reaction velocities. This may be expressed in terms of the apparent affinity constant (Ka app.) which provides another parameter by which lysozymes may be grouped.

### a) Ka app. for M. luteus cells

The problem posed by an insoluble substrate was overcome by a method based on that of Locquet et al (1968). "Units of substrate (mg $l^{-1}$)" was substituted for molarity and over the concentration range 100-450 mg $l^{-1}$ Beer-Lamberts Law was valid in the spectrophotometric assay.

The results were analysed by the method of Locquet et al (1968) and the plotted line intercepted the $\frac{1}{[S]}$ axis at -0.00198 (Fig 28) indicating a Ka app. of 505 mg $l^{-1}$.

### b) Ka app. for M. luteus cell walls

Since cell walls may more accurately show the affinity of the enzyme for its natural substrate, peptidoglycan, cell walls were substituted for whole cells in the above experiment.

Linear regression analysis of the results over the concentration range 56 to 1000 mg $l^{-1}$ indicated that the line intercepted the $\frac{1}{[S]}$ axis at -0.0023 indicating a Ka app. of 435 mg $l^{-1}$ (Fig 29). At the highest concentration tested, 1,000 mg $l^{-1}$, the velocity was less than that at slightly lower concentrations (Fig 29).

Fig 28. Variation of $\frac{1}{V}$ vs $\frac{1}{[S]}$ for *Mytilus* lysozyme and *M. luteus* cells.

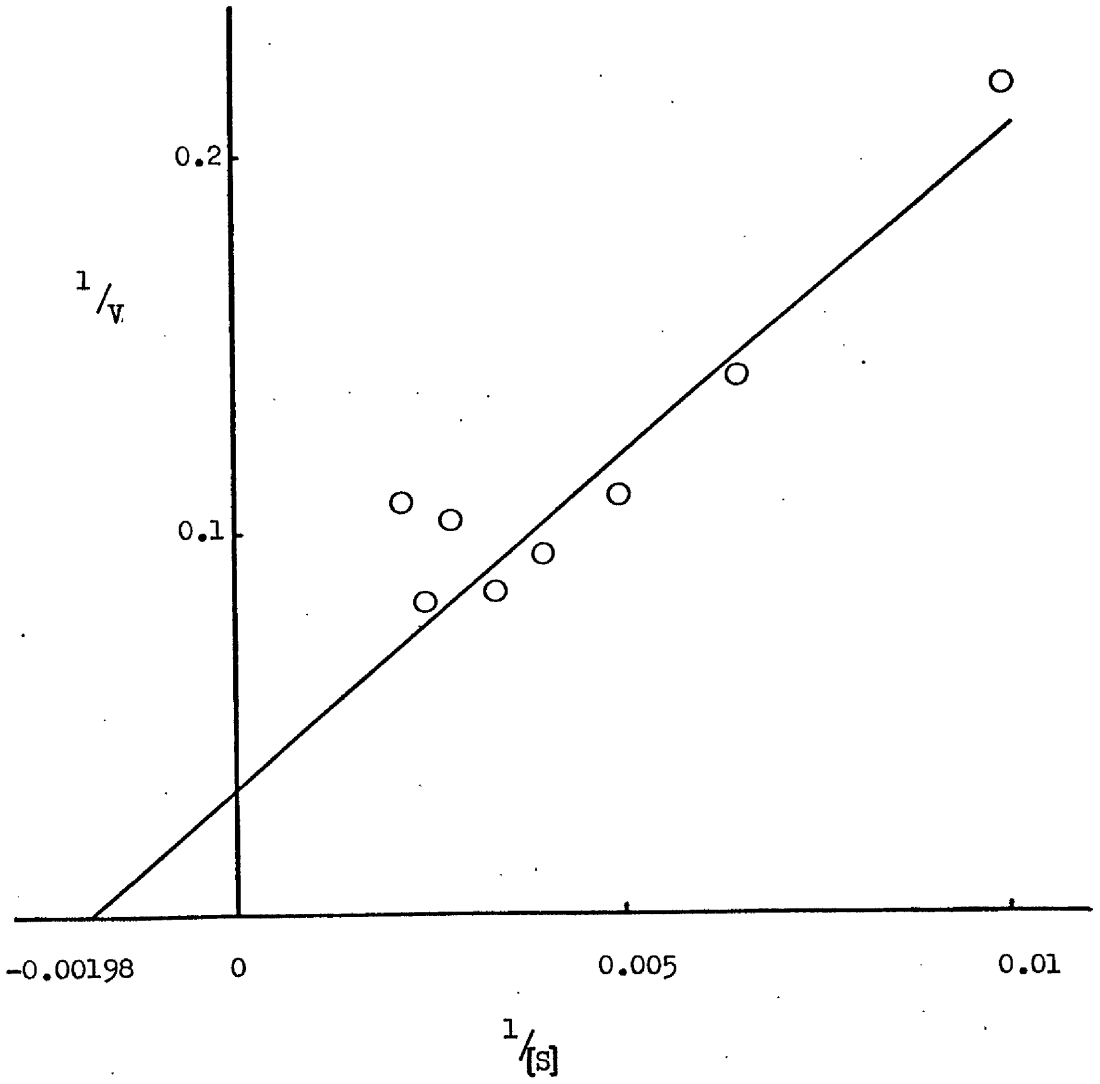
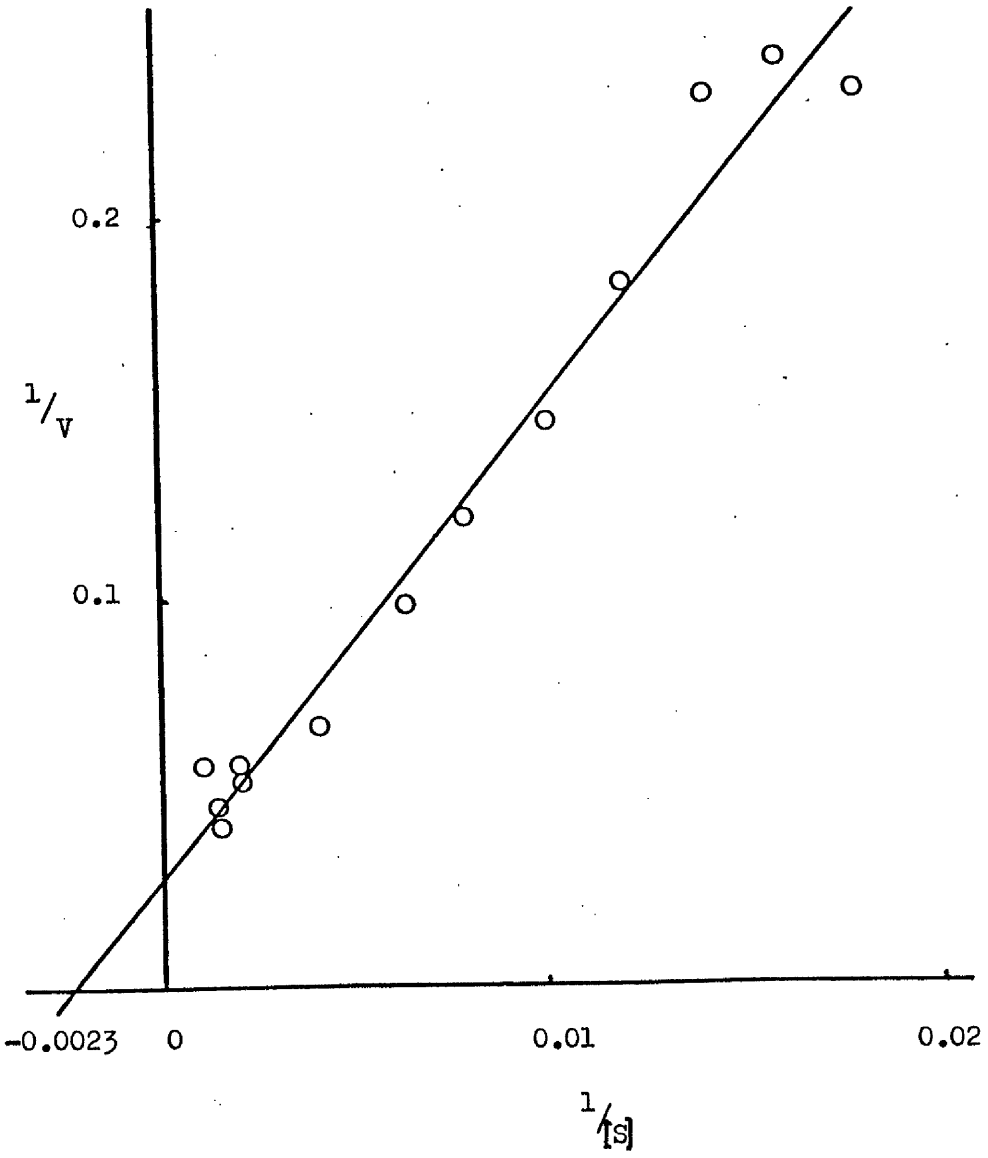

0.2
0.1
1/V
-0.00198
0
0.005
0.01
1/[S]

Fig 29. Variation of $\frac{1}{V}$ vs $\frac{1}{[S]}$ for *Mytilus* lysozyme and *M. luteus* cell walls.


0.2
0.1
1/V
-0.0023
0
0.01
0.02
1/[S]

## 6.4 Effect of cations on activity against M. luteus cells

The inhibitory effect of cations on vertebrate lysozymes is well established (Smolelis and Hartsell, 1952) and the sensitivity to cations of Mytilus lysozyme is critically important when one considers the chemical composition of the marine environment of Mytilus. The effect of three cations found in sea water were investigated. A 0.01M Tris-maleate buffer (pH 6.4) was used in these experiments and it contained sodium hydroxide at a concentration of 0.004M.

The addition of $Na^+$ (as sodium chloride) initially elevated enzyme activity to a maximum at 0.044M but at higher concentrations enzyme activity decreased. At 0.129M $Na^+$ activity was reduced to 50% of the maximum and at $\geq$ 0.4M no activity was detected (Fig 30). Thus at the concentration of sodium in the sea, 0.55M (12.65‰), the lytic activity of Mytilus lysozyme was not detected.

Inhibition by divalent cations was greater at low concentrations than that caused by $Na^+$ (Fig 31). Addition of $Mg^{++}$ increased activity to a maximum at 0.01M but at 0.020M $Mg^{++}$ the activity was reduced by 50% and at the concentration of $Mg^{++}$ in the sea, 0.05M (1.2‰) the activity was reduced to 4.7% of that initially detected (Fig 31).

Calcium ions were less inhibitory than $Mg^{++}$ (Fig 31). Unlike the other cations tested low levels of $Ca^{++}$ did not stimulate activity but produced a steady decrease with 50% inhibition by 0.029M $Ca^{++}$; at the concentration of calcium in sea water, (0.01M, 0.4‰), 96% of the initial activity remained.

Replotting the results such that the activity was plotted against the ionic strength of the added salts (Fig 32), showed that the differences between the monovalent and divalent cation results were not merely due to ionic strength.

Fig 30. Effect of $Na^+$ on Mytilus lysozyme activity.

Activity against M. luteus cell walls (O——O) and cells (●——●) related to activity without added salt.

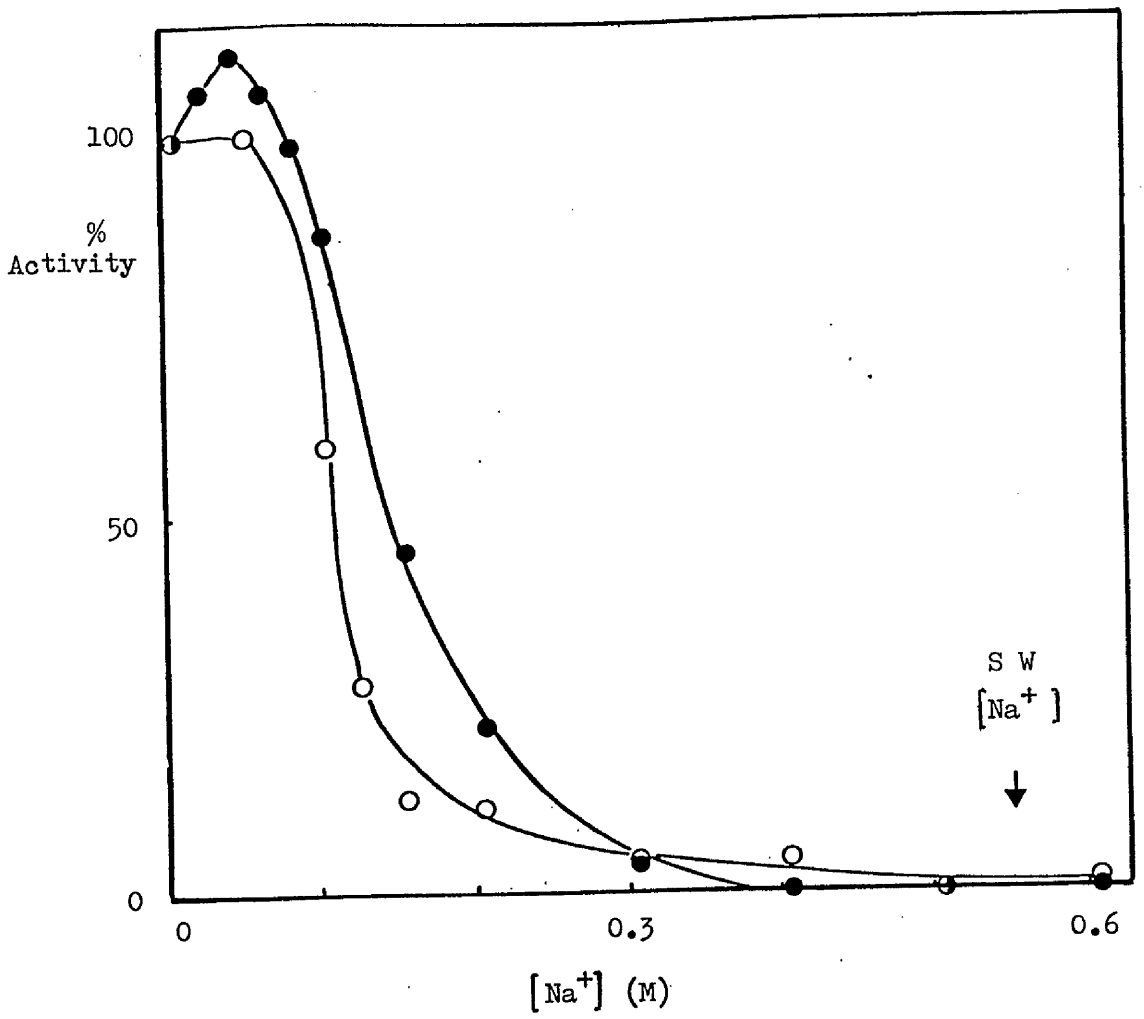

100
%
Activity
50
0
0
0.3
0.6
[Na+] (M)
S W
[Na+]

Fig 31. Effect of cations on the lytic activity of Mytilus lysozyme.

Where: $Na^{+}$, O——O; $Ca^{++}$, □——□ and $Mg^{++}$, ■——■ .

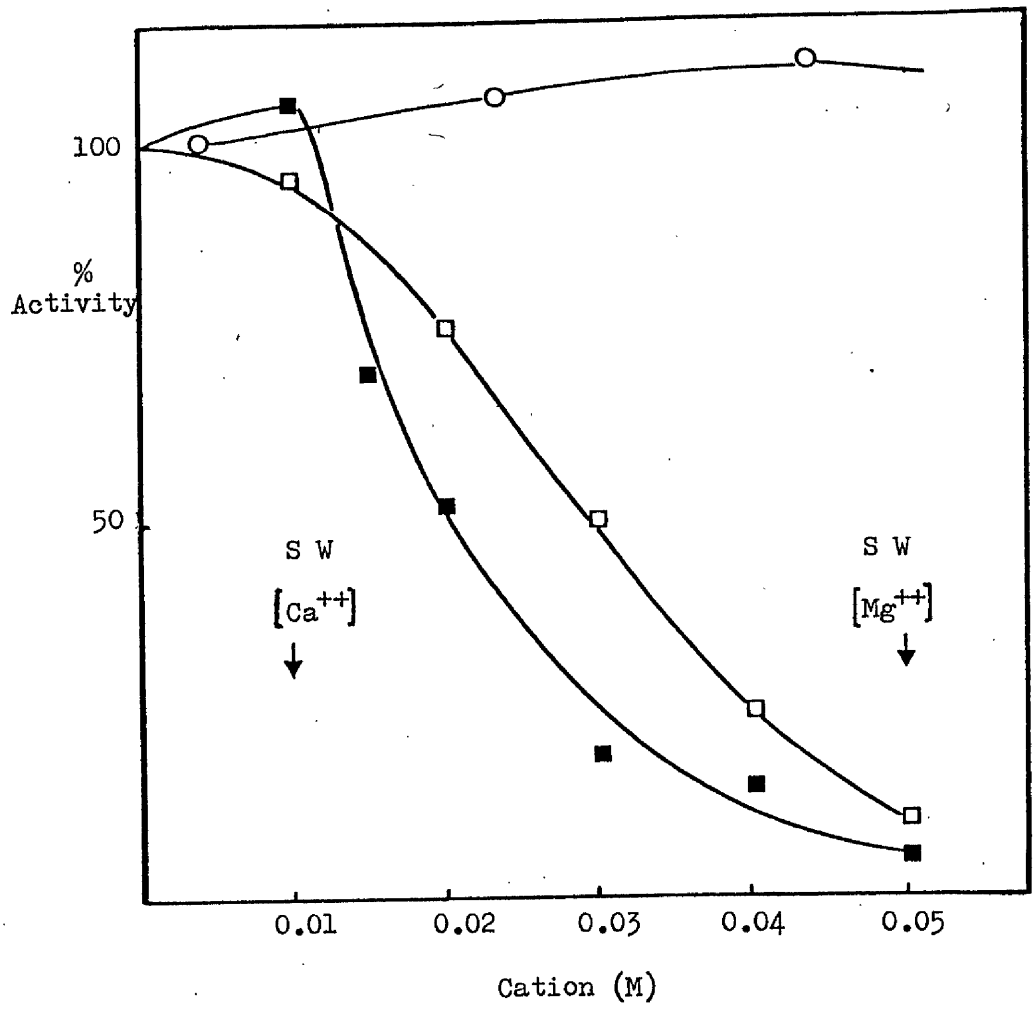
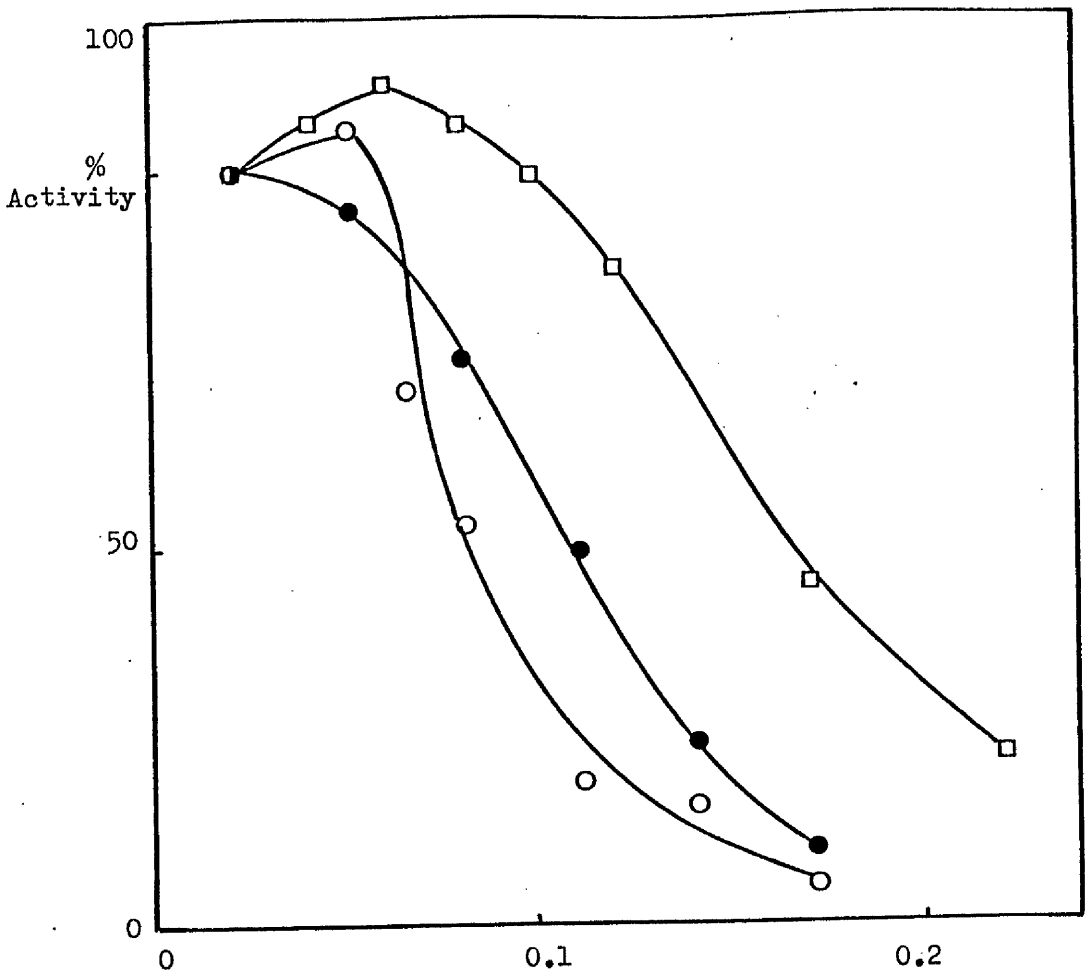

100
%
Activity
50
S W
[Ca++]
S W
[Mg++]
0.01
0.02
0.03
0.04
0.05
Cation (M)

Fig 32. Effect of ionic strength on Mytilus lysozyme activity against M. luteus cells.

Ionic strength was increased by the addition of NaCl (□———□), $MgCl_2$ (○———○) and $CaCl_2$ (●———●).


100
%
Activity
50
0
0
0.1
0.2
Ionic strength

The addition of SDS or NaOH to M. luteus suspensions containing inhibitory concentrations of NaCl or $MgCl_2$ which had been treated with Mytilus lysozyme failed to cause any reduction in the absorbance at 600 nm of the suspensions (Table 16). Thus, it was concluded that the enzyme did not give rise to sphaeroplasts which might have been stabilised by the salts present.

## 6.5 Effect of cations on activity against M. luteus cell walls

To determine whether Mytilus lysozyme could act against its natural substrate, cell walls, in the presence of high concentrations of cations, M. luteus cell walls were substituted for cells in the above experiment.

The increase in activity caused by low concentrations of $Na^+$ (0.054M) was less than that found with whole cells (Fig 30) and 50% inhibition of the activity against cell walls occurred at 0.109M compared to 0.129M for whole cells (Fig 30). At higher concentrations of $Na^+$ ( >0.3) activity was slightly greater than was found for whole cells (Fig 30). When a sample containing 0.604M $Na^+$ was incubated at 22°C overnight with Mytilus lysozyme the absorbance of the sample suspension fell by 0.24 from 0.816 whilst the absorbance of the control dropped by less than 0.01.

The inhibition of activity by $Mg^{++}$ was more marked with cell walls than with cell suspensions, there being no initial activation; 50% inhibition occurred at 0.008M compared to 0.020M required for 50% inhibition with cells (Fig 33).

As with cells, the greater inhibition of activity against cell walls found with divalent cations was not merely due to the differences in ionic strength (Fig 34).

Table 16. Effect of NaOH and SDS on *M. luteus* suspensions treated with *Mytilus* lysozyme, in the presence or absence of salts.

| Suspending buffer | NaOH | | SDS | |
|---|---|---|---|---|
| | $\Delta E \frac{1cm}{600}$[1] | | $\Delta E \frac{1cm}{600}$[1] | |
| | Before treatment | After treatment | Before treatment | After treatment |
| 0.01M Tris maleate buffer (pH 6.4) | 0.444 | 0.473 | 0.445 | 0.612 |
| " + 0.55M NaCl | 0.043 | 0.039 | 0.030 | 0.020 |
| " + 0.05M $MgCl_2$ | 0.030 | ppt[2] | 0.010 | 0.010 |

1. The difference between the $E \frac{1cm}{600}$ of the control and sample cuvettes after 10 minutes.

2. A precipitate was formed in the sample and control cuvettes when NaOH was added.

Fig 33. Effect of $Mg^{++}$ on Mytilus lysozyme activity.

Activity against M. luteus cell-walls (O———O) and cells (●———●) related to the activity without added salt.

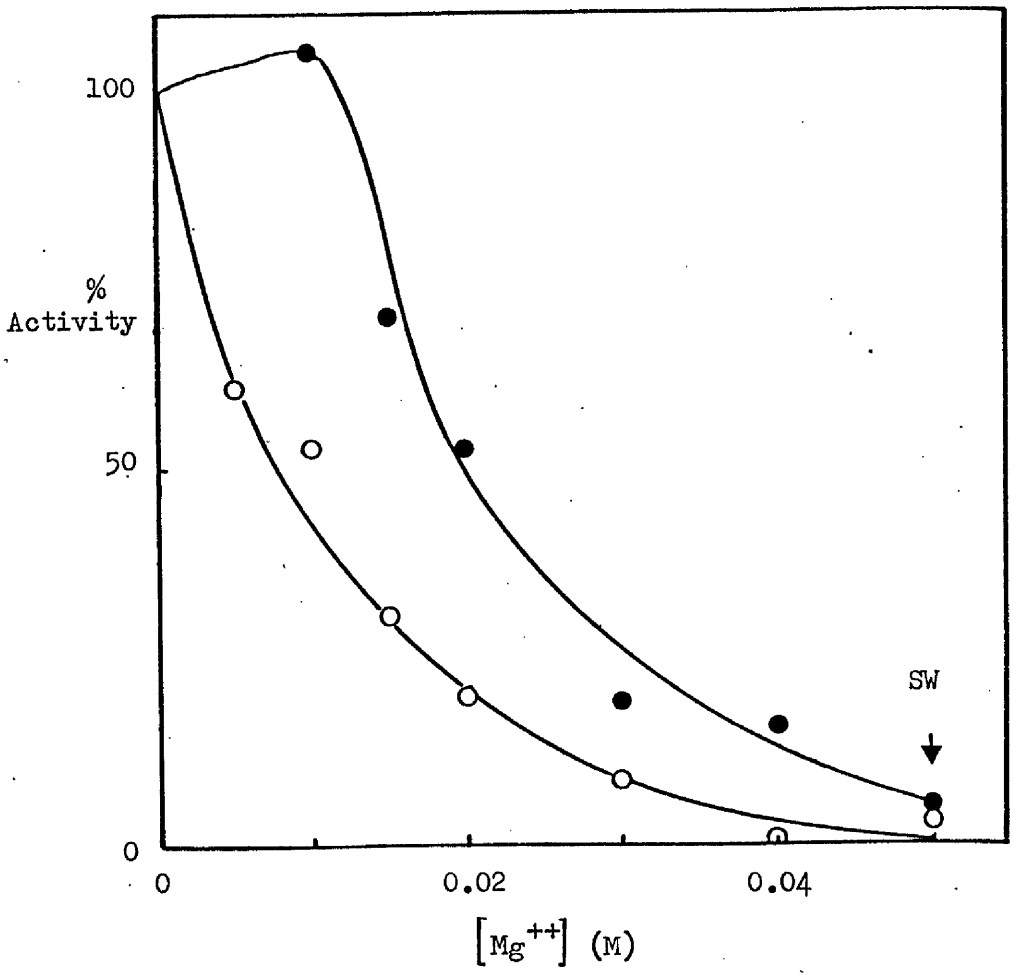
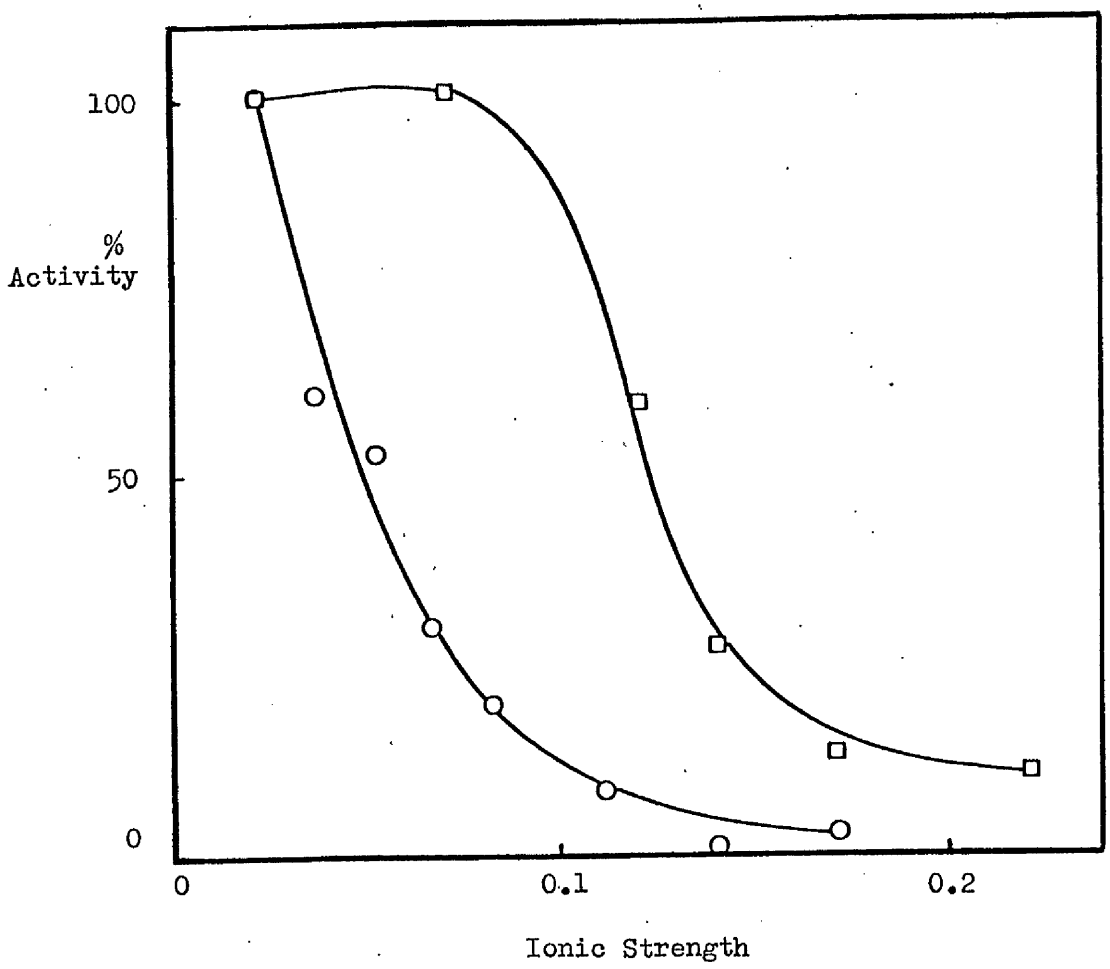

100
%
Activity
50
0
0
0.02
0.04
SW
[Mg++] (M)

Fig 34. Effect of ionic strength on Mytilus lysozyme activity against M. luteus cell walls.

Ionic strength was increased by the addition of NaCl (□———□) and $MgCl_2$ (○———○).


100
%
Activity
50
0
0
0.1
0.2
Ionic Strength

## 6.6 Effect of sea water on activity

Sea water is a complex mixture of which the three above ions ($Na^+$, $Ca^{++}$ and $Mg^{++}$) are but a small sample. A series of dilutions of sea water in distilled water was prepared and M. luteus cells used as substrate for Mytilus lysozyme assay.

Sea water markedly inhibited enzyme activity and only 50% activity occurred with 10% (v/v) sea water (Fig 35). However, at 40% sea water and above there was residual activity; in undiluted sea water 4% activity remained (Fig 35). In the buffer used in the spectrophotometric assay (0.06M phosphate, pH 6.4 containing 0.1% NaCl) activity was 70% of that obtained in distilled water.

# 7. Action of Mytilus lysozyme on marine bacteria

## 7.1 Characteristics of marine bacteria

Bacteria were isolated from the marine environment to determine whether Mytilus lysozyme was active against them. Some characteristics of the isolates were determined before the sensitivity to lysis was checked. These were:

(1) colony morphology (Table 17),

(2) microscopic morphology and gram stain characteristics (Table 18),

(3) growth at three temperatures (4°C, 22°C and 37°C) on marine agar (Table 19),

(4) growth on nutrient agar at 22°C (Table 19),

(5) growth on McConkey agar at 22°C (Table 19).

Of the 27 original isolates only three were gram positive. All isolates grew best at 22°C with the exception of JG22 which grew

Fig 35. Effect of sea water on Mytilus lysozyme activity.

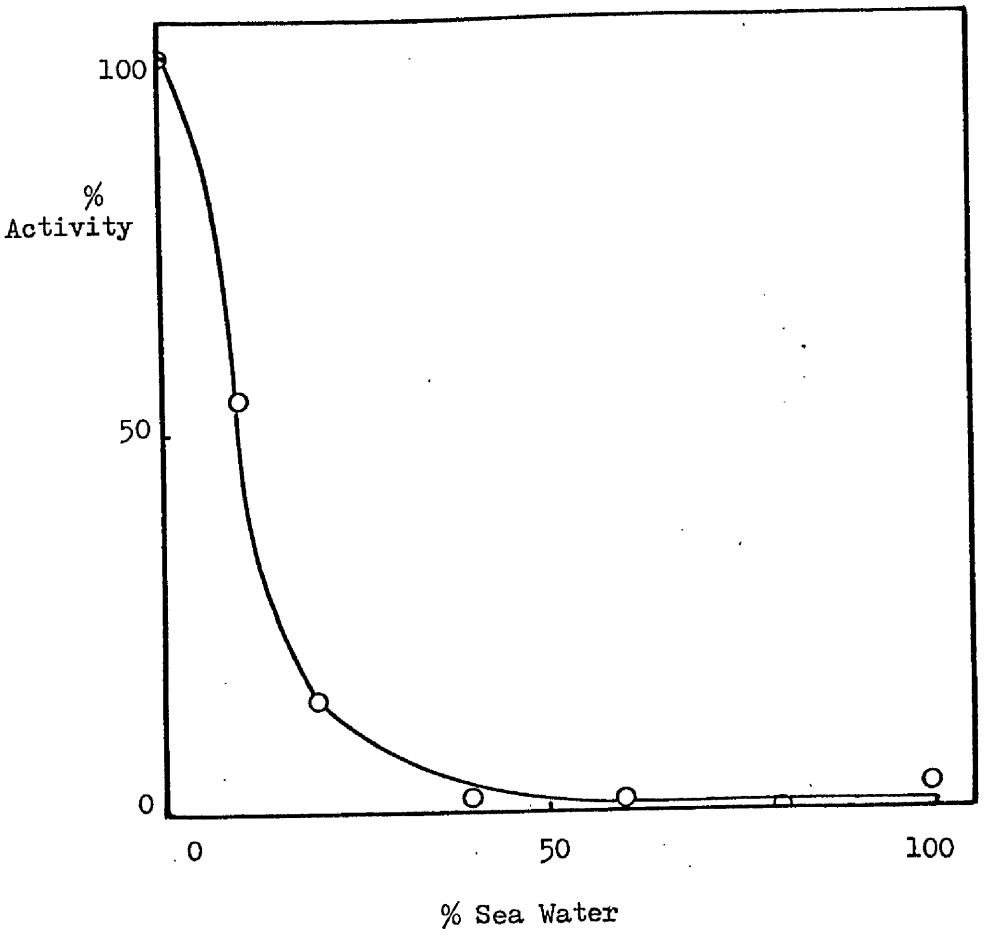

100
%
Activity
50
0
0
50
100
% Sea Water

Table 17. Colony morphology of marine isolates on marine agar.

| Isolate | Diameter (mm) | Shape | Edge | Surface appearance | Colour | Agar digester | Source* |
|---|---|---|---|---|---|---|---|
| JG1 | 1.5 | round | entire | smooth, glistening | yellow | - | OS |
| JG2 | 1.5 | rounded | " | " | off-white | - | OS |
| JG3 | 1.0 | round | " | " | orange | - | OS |
| JG4 | 1.7 | " | " | " | off-white | - | OS |
| JG5 | 1.5 | " | " | " | yellow | - | OS |
| JG6 | 2.5 | rounded | " | " | cream | - | OS |
| JG6B | V.small | round | " | " | red | - | OS |
| JG7 | 1.2 | " | " | " | orange | - | G |
| JG8 | V.small | " | " | moist | translucent white | + | G |
| JG9 | 0.5 | " | " | smooth, glistening | pale yellow | - | G |
| JG10 | 2.2 | rounded | " | moist | orange pink | - | G |
| JG11 | 3.5 | irregular | indented | smooth, glistening | cream | - | G |
| JG12 | 0.5 | round | entire | " | pale white | - | G |
| JG13 | 2.0 | rounded | " | " moist | " orange | - | OS |
| JG14 | 0.7 | round | " | " glistening | " white | - | OS |
| JG15 | 2.0 | " | " | " moist | " orange | - | G |
| JG16 | 1.5 | " | " | " | grey-brown | + | OS |
| JG17 | 2.2 | " | " | " | " " | + | G |
| JG18 | 1.6 | " | " | " glistening | dark-grey | + | F |
| JG19 | 1.5 | " | " | " | cream | + | G |
| JG20 | 2.0 | " | " | " | grey-brown | + | S |
| JG21 | 2.5 | " | " | " | black | + | S |
| JG22 | 0.7 | " | " | " dry | yellow | - | OS |
| JG24 | 0.5 | " | " | " glistening | white | - | G |
| JG26 | 2.1 | irregular | indented | rough, dry | cream | - | G |
| JG27 | 0.1-0.2 | round | entire | smooth, glistening | pale white | - | G |
| JG29 | 0.7 | " | " | " | " " | - | G |

*Sources OS : Mytilus outer shell
G : " gill
F : " foot
S : " stomach

Isolates incubated at 22°C for two days followed by two days at 4°C.

Table 18. Microscopic morphology of marine isolates.

| Isolate | Gram | Shape | Size Distribution | Arrangement |
|---|---|---|---|---|
| JG1 | - | long thin, curved rods | pleomorphic | short chains |
| JG2 | - | short rounded rods | " | random |
| JG3 | - | small rods | uniform | short chains |
| JG4 | - | " slightly curved rods | pleomorphic | random, some pairs |
| JG5 | - | " rods | " | chains |
| JG6 | - | " | " | random |
| JG6B | + | small cocci | uniform | 2's, 4's |
| JG7 | - | long thin rods | pleomorphic | long curved chains |
| JG8 | - | rods | " | random, short chains |
| JG9 | - | short rods | " | random, pairs |
| JG10 | - | cocco-bacillus | " | " " |
| JG11 | - | rods with rounded ends | " | " |
| JG12 | - | cocco-bacillus | " | " |
| JG13 | - | short, rounded rods | " | " |
| JG14 | + | cocco-bacillus | " | " pairs |
| JG15 | - | rods with rounded ends | " | " |
| JG16 | - | " " " " polar staining | " | " |
| JG17 | - | " " " " | " | " |
| JG18 | - | " " " " | " | short chains |
| JG19 | - | " " " " | " | pairs |
| JG20 | - | " | " | random pairs |
| JG21 | - | rods with rounded ends, capsulated | " | " " |
| JG22 | + | cocci | uniform | 4's |
| JG24 | - | short cocco-bacillus | pleomorphic | random pairs |
| JG26 | - | large rods with rounded ends | " | " |
| JG27 | - | small cocco-bacillus | " | random pairs |
| JG29 | - | long thin rods | " | random, pairs short chains |

Table 19. Growth of marine isolates on marine, nutrient and McConkey agar

| Agar | Marine (2 days) | | | Nutrient (4 days) | McConkey (4 days) | |
|---|---|---|---|---|---|---|
| Temperature (°C) / Isolate | 4° | 22° | 37° | 22° | 22° | Lactose fermentation |
| JG1 | ± | ++ | – | – | – | |
| JG2 | + | ++ | – | – | – | |
| JG3 | + | ++ | – | – | – | |
| JG4 | + | ++ | – | – | – | |
| JG5 | + | ++ | – | – | – | |
| JG6 | ± | ++ | – | + | – | |
| JG6B | ± | + | ± | + | + | – |
| JG7 | – | ++ | – | – | – | |
| JG8 | – | + | – | – | – | |
| JG9 | + | ++ | – | – | – | |
| JG10 | + | ++ | – | – | – | |
| JG11 | + | ++ | + | + | – | |
| JG12 | ± | + | – | + | – | |
| JG13 | + | ++ | – | + | – | |
| JG14 | ± | + | – | – | – | |
| JG15 | + | ++ | ± | + | – | |
| JG16 | ± | ++ | – | + | – | |
| JG17 | ± | ++ | ± | + | – | |
| JG18 | + | ++ | ± | + | – | |
| JG19 | + | ++ | + | + | – | |
| JG20 | + | ++ | ± | + | – | |
| JG21 | + | ++ | + | + | – | |
| JG22 | – | ++ | ++ | + | + | – |
| JG24 | + | ++ | – | + | + | – |
| JG26 | + | ++ | ± | + | – | |
| JG27 | – | ++ | + | – | – | |
| JG29 | – | ++ | – | – | – | |

equally well at 37°C. Fifteen isolates grew on nutrient agar but only three grew on McConkey agar, including two of the gram positive isolates, JG6B and JG22.

## 7.2 Sensitivity to Mytilus lysozyme

If the Mytilus lysozyme is to have a function in the marine environment, then it might be expected to be active against bacteria isolated from that environment. The activity of Mytilus lysozyme against 14 of the above isolates suspended in 0.06M sodium phosphate buffer (pH 6.4) containing 0.1% (w/v) sodium chloride was compared with the activity against M. luteus. In addition the sensitivity of some bacteria to HEW lysozyme was determined.

The results for the experiment are listed in Table 20, where all of the results relate to the change in absorbance at 600 nm obtained with M. luteus and the relevant lysozyme over 1 minute.

One gram-positive organism, JG6B, was quite resistant to lysis by both Mytilus and HEW lysozymes, and only one gram-negative isolate, JG10, and M. roseus (which is known to be resistant to lysozyme and was included as a control) were more resistant. Of the gram-negative isolates, 3 were particularly sensitive to the lysozymes: JG24, JG27 and JG29, with the Mytilus lysozyme having a greater effect in each case.

Table 20. Activity of *Mytilus* and HEW lysozymes against a range of bacteria.

| Bacteria | Lysozyme | % Activity* |
|---|---|---|
| *M. luteus* | *Mytilus* | 100 |
| " " | HEW | 100 |
| *M. roseus* | *Mytilus* | 0 |
| " " | HEW | 0 |
| JG3 | *Mytilus* | 26 |
| JG5 | " | 4.3 |
| JG6 | " | 32 |
| JG6B | " | 2.3 |
| " | HEW | 0.9 |
| JG8 | *Mytilus* | 39 |
| JG10 | " | 0 |
| JG11 | " | 17 |
| JG13 | " | 15 |
| JG14 | " | 21 |
| JG15 | " | 16 |
| JG20 | " | 11 |
| JG24 | " | 49 |
| JG24 | HEW | 21 |
| JG27 | *Mytilus* | 117 |
| JG27 | HEW | 35 |
| JG29 | *Mytilus* | 90 |
| JG29 | HEW | 33 |

*The drop in the $E_{600}$ of the *M. luteus* suspension caused by the relevant lysozyme is taken as 100% activity.

# DISCUSSION

Factors affecting the assay of lysozyme on lysoplates

Several procedures employed in the study of Mytilus lysozyme gave rise to large numbers of fractions for enzyme assay and this necessitated use of the lysoplate assay. The principal disadvantage of this assay found by Osserman and Lawlor (1966) was the discrepancy between the results obtained with lysoplates and those from the spectrophotometric assay. In the present investigation, when lysoplates prepared by the method of Osserman and Lawlor (1966) were used to assay Mytilus lysozyme the values obtained were more than two thousand times greater than those obtained for the same samples in the spectrophotometric assay. Similar values were obtained with both assays when the sodium chloride content of the lysoplate agarose base was increased to 1% (w/v); under these conditions the diameters of the zones of clearance produced by both Mytilus and HEW lysozymes were proportional to the log of the lysozyme activity, as determined in the spectrophotometric assay.

The observation that increasing the sodium chloride content of the lysoplate matrix increased the diameter of the zones of clearance obtained with HEW lysozyme was largely in agreement with the findings of Peeters and Vantrappen (1977) and Ensink and van Haeringen (1977) for HEW and human lysozymes. Mytilus lysozyme responded differently from HEW lysozyme to the presence of sodium chloride and the closeness of the assay results in the spectrophotometric and lysoplate assays depended on the unusual response of the Mytilus lysozyme to sodium chloride. It is probable that, with HEW lysozyme standards such results will only be obtained when the response of the unknown lysozyme to the addition of salt is identical to that of HEW lysozyme or similar to that of Mytilus lysozyme. Therefore, for each lysozyme the assay should be restandardized to obtain similar dose response curves.

Peeters and Vantrappen (1977) attributed the changes in the diameters of the zones of clearance caused by lysozymes under different salt concentrations to ionic strength effects; increasing the ionic strength first stimulated diffusion and, at higher concentrations inhibited the lytic activity. This however does not explain the changes found with the Mytilus lysozyme over the same concentration range, which may indicate differences in the charge distribution and/or structure of the different enzymes.

That gelatin increased the diameter of the zones of clearance produced by HEW lysozyme, most notably at low enzyme concentrations, was at variance with the effect reported for B.S.A. and human lysozyme, where B.S.A. reduced the zone size caused by lysozyme (Peeters and Vantrappen, 1977).

The preceding observations support the proposal of Ensink and van Haeringen (1977) that, in general unknowns should be standardized against the same type of lysozyme, under the same conditions in the lysoplate system, to permit valid comparisons of unknowns and standards.

The distribution of lysozyme in bivalves

In general, high concentrations of lysozyme occurred in tissues and structures associated with the digestive process, i.e. style and digestive gland. However, in Mya and Modiolus high concentrations were found in the gill and byssus gland respectively. The trace quantities detected in muscle, body fluid and kidney probably arose from contamination, due to the difficulty in completely separating the inter-digitating tissues. This may indicate that the haemolymph (body fluid) lysozyme of Mytilus edulis investigated by Hardy et al (1976) originated from organs of animals during the extraction process.

As the concentration of lysozyme did not differ between that part of the style in the style sac and that part in the stomach lumen it can be assumed that the lysozyme in the style is secreted by cells of the style sac epithelium and incorporated at the same time as the style is secreted by the glands associated with the major and minor typhlosoles. The low level of lysozyme in the style sac compared to the high levels in the styles of Mytilus, Modiolus and Chlamys suggests a high turnover of the lysozyme secreted by this organ.

The origin of the lysozyme within the digestive gland is less certain. It may be released from the head of the style and pass to the lumen of the gland with food particles destined for intracellular digestion, in which case 'extracellular' lysozyme from the style would be present in food vacuoles within the vacuolated cells of the digestive gland. Conversely, the acidophilic, vacuolated cells themselves might produce lysozyme, or it may be produced and secreted by the pyramidal, basophilic cells of the gland. In Mytilus and Modiolus the logarithms of the lysozyme concentrations in the style and digestive gland were proportional (Appendix 5) whereas in Mya and Chlamys, where the correlation was lower, the logs of the concentrations were inversely proportional (Appendix 5). A possible explanation is that in Mya, which had low style lysozyme levels, the animals dissected were in a non-digestive phase of the feeding process, or that the origin(s) of lysozyme and the digestive processes differ between species. This might explain why so little lysozyme was present in the digestive gland of Chlamys when there were high concentrations in the style. These differences in distribution were consistently found and suggest that lysozyme may act on bacteria in the stomach and digestive gland of Mytilus and Modiolus but principally in the stomach of Chlamys. Another

possibility is that lysozyme is secreted from the digestive gland into the stomach lumen in Mytilus and Modiolus.

Before any conclusions on the possible periodic variation in lysozyme content and distribution in bivalves can be drawn further studies are required. The differences in lysozyme concentration at different times of the year in both Mytilus and Chlamys do not necessarily reflect seasonal differences as, following disturbance, styles may dissolve and in littoral species influenced by tides, formation and dissolution of the style is cyclical and related to a feeding rhythm (Morton, 1971). This might also explain the considerable variation in the lysozyme content recorded for style, style sac and digestive gland in each species, samples being taken at different phases of a cyclical event.

High concentrations of lysozyme were not restricted to particular feeding types or bivalve groups, with high concentrations being found in the filibranch suspension feeders, Mytilus and Modiolus, the eulamellibranch deposit feeder Tellina and the suspension feeder Chlamys. In contrast, in the soft-deposit dwelling, suspension feeding eulamellibranch bivalve Mya, which takes in large quantities of particulate matter, relatively small amounts of lysozyme were found as with the protobranch deposit feeder Nucula. The difference (in concentration and distribution) in Mya may reflect a choice of food particle, lysozyme concentration being related to the proportion of bacteria utilized from the food, while the low concentration of lysozyme in Nucula may reflect differences in the physiology of digestion between protobranch and lamellibranch bivalves (Allen, 1978).

That gill and foot contain lysozyme may be significant as both

have large numbers of gland cells, particularly cells secreting muco-polysaccharides of various kinds. Lysozyme has been detected in mucus secreted from the mantle of the American oyster, C. virginica (McDade and Tripp, 1967b) and in the mucus covering the gill and skin of the plaice, Pleuronectes platessa (Fletcher and White, 1973). In bivalves the gill filters particles from the respiratory flow which are then passed toward the mouth; thus in Mya the initial action of the lysozyme on microorganisms may occur before food particles reach the mouth. Alternatively, lysozyme in external secretions may reflect an adaptation to the environment, the enzyme playing a role in the external defence of the animal (Tripp, 1974a). In the case of the lysozyme associated with the foot and byssus gland, this could be associated with the need to prepare a surface for the attachment of the byssus threads (Allen et al, 1976).

The distribution of lysozyme in the bivalves studied suggests that a host defence role (McDade and Tripp, 1967a; Hardy et al, 1976) is secondary to that of nutrition. A further role for lysozyme may be in maintaining a stable bacterial flora in the gut as has been suggested for lysozyme of the cockroach, Periplaneta americana (Mohrig and Messner, 1968b). In the hind gut of some deep water deposit feeding tellinacean bivalves of the genus Abra the bacterial flora assist in the breakdown of schleroproteins present in the ingested sediments (Allen and Sanders, 1966).

## Purification of Mytilus lysozyme

In whole animal homogenates the lysozyme content of Mytilus, 35.9 mg of HEW lysozyme equivalent per kilogram of animals (less shells), was approximately four times that detected in body fluid by Hardy et al

(1976). This higher value may be due to the distribution of lysozyme in Mytilus and the homogenisation step in this study, whereas Hardy et al (1976) merely centrifuged whole animals (less shells) and assayed the supernate. While the lysozyme content of Mytilus was of the same order as those of the sea star A. rubens and annelid N. hombergii it was lower than that of hen egg white (4.5 g/Kg; Jolles and Jolles, 1975) and papaya latex where lysozyme represents up to one third of the soluble protein (Smith et al, 1955).

The purification process was complicated by the high lipid content of the Mytilus homogenate which interfered with column chromatography. Ion exchange chromatography on Amberlite CG-50 resin proved to be a good initial purification step, providing a high degree of purification together with good recovery of the applied material, similar to that reported by Powning and Davidson (1973) using Galleria mellonella lysozyme. The recovery of enzyme activity from affinity chromatography on CC-cellulose was extremely low and while the purification appeared high it was less than was obtained using the same resin for plaice lysozyme (Fletcher and White, 1976).

The greater retention of the Mytilus lysozyme on CM-cellulose than on DEAE-cellulose was to be expected, the former being a cation exchange matrix to which the basic lysozyme would bind more strongly. The high recovery of activity from the CM-cellulose column compared favourably with the recoveries reported for plaice lysozyme (Fletcher and White, 1976) and tortoise lysozyme (Gayen et al, 1977) whilst the purification of the Mytilus lysozyme was almost five times greater than with the latter of the two.

The crystalline style provided a superior starting material to whole animal homogenates and did not appear to contain appreciable

amounts of lipid. Also, the specific activity of the lysozyme in styles was 220 times that in the whole animal homogenates. Many other enzymes have been demonstrated in the crystalline styles of marine bivalves (Reid, 1968; Kristensen, 1972a) and here β-glucuronidase and collagenase were detected in Mytilus style homogenates, and used as indicators of purification. The former enzyme was previously detected in vacuolated digestive cells, but not in the basophilic cells of the digestive gland (Sumner, 1969). Proteolytic activity has also been detected in Mytilus digestive gland (Rosen, 1949), though neither activity has previously been detected in Mytilus style.

The specific activity of the style lysozyme eluted from CG50 Amberlite was similar to that of the most pure preparation of lysozyme obtained from the whole animal homogenate. The purification was similar to that obtained by Powning and Davidson (1973) using G. mellonella lysozyme whilst the recovery was closer to that obtained by Fernandez-Sousa et al (1977). After dialysis of the eluted material, no β-glucuronidase or collagenase activity was detected in this or any subsequent fraction, which had elevated lysozyme activities.

Recovery of style lysozyme activity from CM-cellulose was, on average, less than half that found with the whole animal lysozyme and represented less than a quarter of the purification previously found. The lower purification was most likely due to the higher purity of the style material applied to the CM-cellulose and was of the same order as that found for tortoise lysozyme by Gayen et al (1977).

Affinity chromatography of Mytilus style lysozyme on CC-cellulose resulted in even lower recovery than was obtained with the whole animal homogenate lysozyme. While inclusion of gelatin in the

eluting buffers increased the recovery dramatically it was still lower than was found with A. rubens lysozyme subjected to affinity chromatography on Sepharose 4-B chitotetraoside (Jolles and Jolles, 1975). Although the degree of purification was similar it was some 30-fold less than that obtained by Fletcher and White (1976). The higher recovery from CM-cellulose and the need to add gelatin to obtain a satisfactory recovery from CC-cellulose favoured the retention of the second ion-exchange step in the purification process.

On PAGE in acidic gels, the relative mobility of Mytilus lysozyme was slightly lower than that of HEW lysozyme, and by inference was slightly less than that determined for Mytilus 'haemolymph' lysozyme by Hardy et al (1976). Both results were in contrast to the findings of Feng (1974a) that the lysozyme-like activity of C. virginica was associated with acidic proteins in PAGE. However, it is possible that a basic protein such as lysozyme, present in small quantities in haemolymph extracts, would associate with acidic proteins and thus be apparently acidic. Thus, purification of the C. virginica lysozyme is required before definitive statements can be made.

It was clear from the duplicate polyacrylamide gels that the lysozyme activity was associated with the major staining band in the gels containing purified enzyme. From these gels it was estimated by densitometry that this major staining band contained 90% of the protein compared to 98% for the HEW lysozyme sample.

The purified enzyme satisfied the criteria of Salton (1957) (Introduction, Page 11 ) cleaving the glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine in M. luteus cell walls; thus it was a mucopeptide N-acetylmuramylhydrolase (E.C.3.2.1.17), i.e. a lysozyme.

Physico-chemical characteristics of *Mytilus* lysozyme

Failure of *Mytilus* lysozyme to focus sharply on isoelectric focusing may have been due to interaction with ampholytes as has been shown with other proteins by Cann and Stimpson (1977). Whilst the isoelectric point of pH 9.2 is lower than those recorded for HEW lysozyme (11.18; Sophianopoulos and Sasse, 1965), human lysozyme (10.5-11; (Parry *et al*,1969) it is similar to that reported for the snail, *Helix pomatia* ( ∿pH 9; Takeda *et al*, 1966) to which *Mytilus* is phylogenetically closer.

The late elution of HEW lysozyme from Bio-Gel P-60 cast doubt on the suitability of gel filtration for the determination of the molecular weight of *Mytilus* lysozyme. While the late elution of HEW lysozyme from dextran and agarose columns has been attributed to a specific interaction between the active site of the enzyme and the polysaccharide matrices of the gels, which, it has been proposed, resemble the bacterial cell wall polysaccharide substrate (Fernandez-Sousa and Rodriguez, 1977), no such similarity is apparent in the polyacrylamide matrix of the Bio-Gel P-60. The low recovery of *Mytilus* lysozyme from Bio-Gel P-60 was similar to that found with HEW lysozyme on Bio-Gel P-2 (Bonilla, 1970) and suggests that the lysozyme was bound by electrostatic interaction with the gel matrix.

While the molecular weight of *Mytilus* lysozyme, 18,000 daltons, as determined by SDS-PAGE, is higher than the molecular weights of HEW, *N. hombergi* and *A. rubens* lysozymes it is lower than those of *C. capitata* and *H. pomatia* lysozymes (Tables 1 and 3) and appears to lie in the middle of the range of invertebrate lysozyme molecular weights (Table 3).

Inactivation of purified *Mytilus* lysozyme occurred at room

temperature in distilled water but the enzyme could be stabilised by gelatin and NaCl. Comparison of active and inactive lysozyme on acid polyacrylamide gels showed that either the charge on the Mytilus lysozyme had decreased or that the enzyme had aggregated into higher molecular weight polymers, as has been found for HEW lysozyme (Sophianopoulos and van Holde, 1964; Bruzzesi, Chiancone and Antonini, 1965). In HEW lysozyme the aggregation process involves the loss of one proton from each monomer, possibly from the glutamic acid residue (35) at the active site of HEW lysozyme and this might interfere with the enzymic activity. If a similar process affected the Mytilus lysozyme, it might explain the PAGE results with polymers giving rise to the more anodal bands which exhibited no activity. The more cathodal band may represent monomers which had lost protons (thus affecting activity) but which had not yet polymerised. The loss of protons might also reduce the basic nature of the enzyme and so reduce its migration towards the cathode.

Studies on the effect of dialysis suggested that the Mytilus lysozyme which was not recovered adhered to the visking tubing, due to charge effects as was suggested for HEW lysozyme by Fraenkel-Conrat (1950) rather than by passing through the membrane, as suggested for HEW lysozyme by Shugar (1952). The comparatively small losses of Mytilus lysozyme contrasts with the loss of the majority of C. virginica and Mya arenaria lysozyme activities during dialysis (Rodrick and Cheng, 1974b; Cheng and Rodrick, 1974). Comparison of dialysis and gel filtration for desalting supports the use of dialysis during the purification process.

Enzymic characteristics

Mytilus lysozyme responded similarly to alterations in pH and

ionic strength to the majority of vertebrate lysozymes studied by Saint-Blancard et al (1970) as did insect lysozymes (Powning and Davidson, 1973), but for Mytilus lysozyme the variation in the optimum pH with changing ionic strength was more marked than for HEW lysozyme. Here the optimum pH of HEW lysozyme was lower than previously reported by Saint-Blancard et al (1970) and Davies et al (1969). Mytilus lysozyme exhibited a higher pH optimum than other bivalve lysozymes (McDade and Tripp, 1967a; Rodrick and Cheng, 1974b) although the secondary optimum of the Mytilus lysozyme, at pH 4.6, was similar to the optimum of Mya arenaria lysozyme (Cheng and Rodrick, 1974). These differences may be due to the use of different buffer systems; Cheng and Rodrick (1974) observed that the pH optima varied between buffers, as would be expected if the ionic strengths were not constant.

The optimum temperature of the Mytilus lysozyme was lower than for HEW lysozyme, the results for the latter being in agreement with the findings of Smolelis and Hartsell (1952). The response of both lysozymes to temperature was influenced by the buffer pH and I, illustrating the difficulty in comparing results of different workers.

With the possible exception of goose lysozyme (Locquet et al, 1968), Mytilus lysozyme has a higher apparent affinity constant (Ka app.) for M. luteus cells than those of most vertebrate lysozymes. It is also higher than that of N. hombergi lysozyme but lower than that of A. rubens (Perin and Jolles, 1973, 1976). Whilst these results may reflect differences in the affinity for the M. luteus substrate it may be due to the use of different buffers, as the pH and I of the suspending buffer can effect the determined Ka app. value (Jolles et al, 1974; Perin and Jolles, 1976).

C. virginica lysozyme, the only other bivalve lysozyme for which the affinity constant for M. luteus has been determined has an affinity constant of 50 mg $l^{-1}$ (Rodrick and Cheng, 1974b). This is 10 times lower than that of Mytilus lysozyme. However, the value determined by the same workers for HEW lysozyme was considerably lower than that determined by Jolles and co-workers (Locquet et al, 1968; Jolles et al, 1974; Perin and Jolles, 1976). Again the differences between these affinity constants for HEW lysozyme may be due to differences in either the buffers or in the interpretation of results, in which case the affinity constant of C. virginica lysozyme might be considerably higher.

A further similarity between Mytilus and goose lysozymes, lies in the apparent reduction in the velocity of lysis at high concentrations of M. luteus by the Mytilus and goose lysozymes (Locquet et al, 1968) indicating substrate inhibition. That substrate inhibition did occur was supported by the reduction in the velocity of solubilisation of cell walls at the highest concentration of this substrate examined.

The inhibitory effect of cations on the Mytilus lysozyme activity against M. luteus cells was similar to that reported for HEW lysozyme, divalent cations being more inhibitory than monovalent ones, with initial increases in activity being found in the presence of low concentrations of sodium and magnesium ions. However, the cations became inhibitory to Mytilus lysozyme at lower concentrations than for HEW lysozyme (Smolelis and Hartsell, 1952). While the concentration of sodium chloride which inhibited the activity of Mytilus lysozyme against M. luteus cells was lower than that which inhibited C. virginica lysozyme (McDade and Tripp, 1967a), the inhibitory concentration of $Mg^{2+}$ was higher than that required for C. virginica lysozyme (Rodrick and Cheng, 1974b).

That Mytilus lysozyme was inhibited by the cations, and did not merely give rise to sphaeroplasts stabilised by the cations was confirmed by the reduced activity against cell walls with both monovalent and divalent cations. The inhibition of HEW lysozyme by cations was attributed by Davies et al (1968) to interference with the electrostatic binding of the enzyme to the bacterial cell wall at high ionic strengths. However, differences in the degree of inhibition of Mytilus lysozyme by the different cations cannot be accounted for by ionic strength alone. The divalent cations, particularly magnesium, may have a specific effect, possibly by linking two negatively charged sites on the cell wall preventing access of the enzyme to its substrate. Or, it may stop the polysaccharide from fitting into the active site of the enzyme.

The function of lysozyme in Mytilus

Activity against cell walls could still be detected with the Mytilus lysozyme at the concentrations of sodium and magnesium ions in the sea, suggesting that the lysozyme might function in the marine environment. Even diluted sea water inhibited Mytilus lysozyme but in 40% sea water and above there was residual activity. The problem posed by the inhibition of Mytilus lysozyme by sea water may be alleviated in the mussel by the lips of the palps, which are found at the mouths of bivalves and which may prevent sea water from entering the gut with food (Gilmour, 1974). If this is the case then the ion concentration in the gut may be lower than in sea water.

In view of the environment of Mytilus and the predominance of gram-negative bacteria in the sea (ZoBell and Feltham, 1934), factors in addition to lysozyme may be required for effective degradation of the

cell walls of most bacteria. Repaske (1956) has shown that for lysis of E. coli by HEW lysozyme prior treatment with a chelating agent, such as EDTA, is required to destabilise the cell wall. In view of the sensitivity of Mytilus lysozyme to cations and the nature of the marine environment, chelating agents might be required for Mytilus lysozyme to be active. Additionally, the bacteriolytic activity of Mytilus lysozyme may be enhanced by style associated surfactants (Kristensen, 1972b) which could disturb the integrity of the outer membrane of the gram-negative bacteria. The lysozyme may also be aided by other enzymes, such as β-glucuronidase, which may play a role in the degradation of bacteria in the haemolymph of C. virginica and Mercenaria mercenaria (Cheng, 1976). Ions which enter the stomach with the food may be bound to the non-enzymic proteins in the style which may act as ion chelators. Alternatively the cations may interact with the hexosamines released from the style as it grinds against the gastric shield and these may be re-absorbed by the animal. However, the potential problem posed by cations in the stomach may not be so significant if, as Kristensen (1972b) proposed, the digestion of bacteria occurs in the digestive gland and not in the stomach itself. Digestion in this organ is intracellular, occurring in phagosomes (Owen, 1972), the environment of which the cell could be expected to regulate. Thus, lysozyme from the style may be taken into the digestive cells adhering to bacteria and only start to function when the ionic environment becomes favourable (i.e. intracellularly).

That bacteria isolated from the marine environment exhibited similar sensitivities to Mytilus and HEW lysozyme further supported the evidence that the Mytilus enzyme was indeed a true lysozyme. It also showed that under suitable conditions, when the stability of the marine

bacteria was probably reduced, the Mytilus lysozyme could lyse many gram-negative bacteria.

C. virginica and Mercenaria mercenaria can filter the gram-negative bacteria E. coli, Salmonella typhimurium and Salmonella flexneri from sea water and subsequently kill them. It was proposed that some of these bacteria enter the digestive tract of the animal and are killed by enzyme action (Hartland and Timoney, 1979). If this is the case then bacteria entering the sea from sources such as sewers may provide an additional source of food for filter-feeding bivalves.

It has been proposed that the enzyme spectrum of a particular species reflects the composition of its diet (Yonge, 1937). If this is the case then the presence of at least two enzymes in the digestive tract of Mytilus which are capable of degrading bacterial substrates suggests that bacteria form part of the diet of Mytilus. However, although bacteria probably form an important part of the diet of Mytilus and lysozyme is probably involved in their digestion the principal function of lysozyme in Mytilus is not necessarily the killing of bacteria. It may, as has been suggested for vertebrate lysozymes (Chipman and Sharon, 1969), be involved in the digestion of glycopeptide debris from the cell walls of bacteria killed in other ways.

Further studies

While the distribution studies suggest that the principal function of lysozyme in Mytilus is in digestion the lysozymes in other parts of the animal may have different properties and characteristics from the style lysozyme. For example, byssus gland lysozyme may have a role in clearing sites for attachment of byssus threads and haemolymph lysozyme may indeed fulfil a role in host defence. Comparison of the

lysozymes from these different sources is required to ascertain how similar they are.

Although Mytilus lysozyme can be grouped with the hen type lysozymes with regard to its response to pH and I, its response to salt in lysoplates contrasts sharply with those of the two members of this group which have been examined (Ensink and van Haeringen, 1977; Peeters and Vantrappen, 1977). The Ka app. of Mytilus lysozyme for M. luteus cells is closer to that of goose lysozyme than to hen type lysozymes (Locquet et al, 1968) and both Mytilus and goose lysozyme exhibit substrate inhibition. Therefore, Mytilus lysozyme may be grouped with the lysozyme of the annelid N. hombergi, in that it exhibits properties of both the hen and goose type lysozymes (Perin and Jolles, 1973). However its Ka app. is closer to that of the sea star, A. rubens, lysozyme, which may represent a separate invertebrate type lysozyme (Perin and Jolles, 1976).

Clearly, before the Mytilus lysozyme can be classified into any of the four types proposed by Jolles et al (1974), if indeed it can be fitted into any of them, further investigations will be required. Such studies would include an examination of the activity of Mytilus lysozyme against chitin and synthetic substrates, such as the 3,4-dinitrophenyl tetra-N-acetyl-β-chitotetraoside of Ballardie and Capon (1972). Also, the inhibition of Mytilus lysozyme by N-acetylglucosamine and its polymers has yet to be examined.

While Mytilus lysozyme lyses both gram-positive and gram-negative bacteria the action of the enzyme on Cyanobacter is unknown. If the enzyme is involved in digestion it may well act against members of this order. It has yet to be determined whether Mytilus lysozyme

can bind to bacteria in sea water and, if bound, whether the enzyme can cause lysis when the salt concentration is reduced.

The presence of bacteriolytic enzymes in Mytilus does not necessarily mean that all bacteria will be dissolved by these animals in vivo. Although it has been established by ZoBell and Landon (1937) and ZoBell and Feltham (1938) that bacteria may act as a food source for marine invertebrates the range of bacteria utilised by Mytilus remains to be established. Experiments in which the uptake of radiolabelled bacteria by Mytilus could be monitored and the fate of the bacteria determined might show what types of bacteria were digested and the fate of the various bacterial components.

It is unlikely that lysozyme alone can effectively degrade most marine bacteria in Mytilus and the role of other agents, such as β-glucuronidase, style-associated surfactants (Kristensen, 1972b) and haemolysins (Hardy et al, 1976) as accessory factors to lysozyme should be investigated. Of particular interest are the haemolysins; in addition to the $Ca^{2+}$ dependent haemolysin detected in body fluid (Hardy et al, 1976) two further distinct haemolysins have been demonstrated in digestive gland and style in Mytilus (Birkbeck and McHenery, unpublished observation). Such studies would provide an insight into the mechanisms by which bacteria were digested and the role of bacteria in nutrition of marine bivalves.

# REFERENCES

Abraham, E.P. & Robinson, R. (1937). Crystallization of lysozyme. Nature (London), 140: 24.

Allen, J.A. (1978). Evolution of the deep sea protobranch bivalves. Phil. Trans. R. Soc. Lond. B, 284: 387-401.

Allen, J.A., Cook, M., Jackson, D.J., Preston, S. & Worth, E.M. (1976). Observations on the rate of production and mechanical properties of byssus threads of Mytilus edulis. J. Moll. Stud., 42: 279-289.

Allen, J.A. & Sanders, H.C. (1966). Adaptations to abyssal life as shown by the bivalve Abra profundurum (Smith). Deep-Sea Res., 13: 1175-1184.

Allen, W.R. (1921). Studies on the biology of fresh-water mussels. Biol. Bull., 40: 210-241.

Allison, A.C. & Sandelin, K. (1963). Activation of lysosomal enzymes in virus-infected cells and its possible relationship to cytopathic effects. J. Exp. Med., 117: 879-887.

Ames, G.F.L. (1974). Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem., 249: 634-644.

Anderson, R.S. & Cook, M.L. (1979). Induction of lysozyme-like activity in the haemolymph and haemocytes of an insect Spodoptera eridania. J. Invertebr. Pathol., 33: 197-203.

Araki, Y., Nakatani, T., Nakayama, K. & Ito, E. (1972). Occurrence of N-nonsubstituted glucosamine residues in peptidoglycan of lysozyme-resistant cell walls from Bacillus cereus. J. Biol. Chem., 237: 6312-6322.

Arimura, H. (1973). Effect of lysozyme from human placenta on ectromelia virus. Acta Virol. (Prague), 17: 130-137.

Arnheim, N., Inouye, M. & Laudin, A. (1973). Chemical studies on the enzymatic specificity of goose egg-white lysozyme. J. Biol. Chem., 248: 233-236.

Ballardie, F.W. & Capon, B. (1972). 3,4-Dinitrophenyl tetra-N-acetyl-β-chitotetraoside, a good chromophoric substrate for hen's egg-white lysozyme. J. Chem. Soc. Chem. Commun., 14: 828-829.

Bang, F.B. (1961). Reaction to injury in the oyster (Crassostrea virginica). Biol. Bull., 121: 57-68.

Bayne, B.L., Thompson, R.J. & Widdows, J. (1976). Physiology. I. In: Marine Mussels: 121-206. Editor: B.L. Bayne, Cambridge University Press, Cambridge.

Bayne, B.L., Widdows, J. & Thompson, R.J. (1976). Physiology. II. In: Marine Mussels: 207-260. Editor: B.L. Bayne, Cambridge University Press, Cambridge.

Berger, L.R. & Weiser, R.S. (1957). The β-glucusaminidase activity of egg-white lysozyme. Biochim. Biophys. Acta, 26: 517-521.

Berkeley, C. (1959). Some observations on Cristispira in the crystalline style of Saxidomus giganteus Deshayes and in that of some other lamellibranchiata. Can. J. Zool., 37: 53-58.

Bernier, I., van Leemputten, E., Horisberger, M., Bush, D.A. & Jolles, P. (1971). The turnip lysozyme. FEBS Lett., 14: 100-104.

Blake, C.C.F., Koenig, D.F., Mair, G.A., North, A.C.T., Phillips, D.C. & Sarma, V.R. (1965). Structure of hen egg-white lysozyme. A three-dimensional Fourier synthesis at two angstrom resolution. Nature (London), 206: 757-761.

Blake, C.C.F., Johnson, L.N., Mair, G.A., North, A.C.T., Phillips, D.C. & Sarma, V.R. (1967). Crystallographic studies of the activity of hen egg-white lysozyme. Proc. Roy. Soc. Lond. B, 167: 378-388.

Boasson, E.H. (1938). On the bacteriolysis by lysozyme. J. Immunol., 34: 281-293.

Bonilla, C.A. (1970). Adsorption of lysozyme to polyacrylamide gel (Bio-Gel P-2). J. Chromatog., 47: 499-501.

Braun, V., Rehn, K. & Wolf, H. (1970). Supramolecular structure of the rigid layer of the cell wall of Salmonella, Seratia, Proteus and Pseudomonas fluorescens. Number of lipoprotein molecules in a membrane layer. Biochem., 9: 5041-5049.

Brown, J.R. (1964). Disulphide bridges of lysozyme. Biochem. J., 92: 13p.

Brumfitt, W. & Glynn, A.A. (1961). Intracellular killing of Micrococcus lysodeikticus by macrophages and polymorphonuclear leucocytes. A comparative study. Brit. J. Exp. Pathol., 42: 408-423.

Brumfitt, W., Wardlaw, A.C. & Park, J.T. (1958). Development of lysozyme-resistance in Micrococcus lysodeikticus and its association with an increased O-acetyl content of the cell wall. Nature, 181: 1783-1784.

Bruzzesi, M.R., Chiancone, E. & Antonini, E. (1965). Association-dissociation properties of lysozyme. Biochem., 4: 1796-1800.

Buchanan, R.E. & Gibbons, N.E. (Editors) (1974). In: Bergey's Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore.

Canfield, R.E. (1963). The amino acid sequence of egg-white lysozyme. J. Biol. Chem., 238: 2698-2707.

Canfield, R.E., Collins, J.C. & Sobel, J.H. (1974). Human leukemia lysozyme. In: Lysozyme: 63-70. Editors: E.F. Osserman, R.E. Canfield, S.Beychock, Academic Press, New York and London.

Canfield, R.E., Kanmerman, S., Sobel, J.H. & Morgan, F.J. (1971). Primary structure of lysozymes from man and goose. Nature New Biology, 232: 16-17.

Cann, J.R. & Stimpson, D.L. (1977). Isoelectric focusing of interacting systems. I. Carrier ampholyte-induced macromolecular isomerisation. Biophys. Chem., 7: 103-114.

Chadwick, J.S. (1970). Relation of lysozyme concentration to acquired immunity against Pseudomonas aeruginosa in Galleria mellonella. J. Invertebr. Pathol., 15: 455-456.

Cheng, K.J., Ingram, J.M. & Costerton, J.W. (1970). The release of alkaline phosphatase from cells of Pseudomonas aeruginosa by manipulation of cation concentration and pH. J. Bacteriol. 104: 748-753.

Cheng, T.C. (1976). Beta-Glucuronidase in the serum and haemolymph cells of Mercenaria mercenaria and Crassostrea virginica (Mollusca:Pelecypoda). J. Invertebr. Pathol., 27: 125-128.

Cheng, T.C., Chorney, M.J. & Yoshino, T.P. (1977). Lysozyme-like activity in the haemolymph of Biomphalaria glabrata challenged with bacteria. J. Invertebr. Pathol., 29: 170-174.

Cheng, T.C. & Rodrick, G.E. (1974). Identification and characterization of lysozyme from haemolymph of the soft-shelled clam, Mya arenaria. Biol. Bull., 147: 311-320.

Cheng, T.C. & Rodrick, G.E. (1975). Lysosomal and other enzymes of Crassostrea virginica and Mercenaria mercenaria. Comp. Biochem. Biophys., 52b: 443-447.

Cheng, T.C., Rodrick, G.E., Foley, D.A. & Koehler, S.A. (1975). Release of lysozyme from haemolymph cells of Mercenaria mercenaria during phagocytosis. J. Invertebr. Pathol., 25: 261-265.

Cheng, T.C. & Rudo, B.M. (1976). Distribution of glycogen resulting from degradation of $^{14}C$-labelled bacteria in the American oyster Crassostrea virginica. J. Invertebr. Pathol., 27: 259-262.

Chipman, D.M., Pollock, J.J. & Sharon, N. (1968). Lysozyme-catalysed hydrolysis transglycosylation reactions of bacterial cell wall oligosaccharides. J. Biol. Chem., 243: 487-496.

Chipman, D.M. & Sharon, N. (1969). Mechanism of lysozyme action. Science, 165: 454-465.

Cooper, E.L. (1976). Immunity and neoplasia in mollusks. Israel J. Med. Sci., 12: 479-494.

Crespi, H.J., Mandeville, S.E. & Katz, J.J. (1962). The action of lysozyme on several blue-green algae. Biochem. Biophys. Res. Commun., 96: 569-573.

Davies, R.C., Neuberger, A. & Wilson, B.M. (1969). The dependence of lysozyme activity on pH and ionic strength. Biochim. Biophys. Acta, 178: 294-305.

Day, D.F., Marceau-Day, M.L. & Ingram, J.M. (1978). Protein-lipopolysaccharide interactions. 1. The reaction of lysozyme with Pseudomonas aeruginosa LPS. Can. J. Microbiol., 24: 196-199.

Doyle, J. (1966). Studies on the chemical nature of the crystalline style. In: Some contemporary Studies in Marine Science: 253-263. Editor: H. Barnes, Allen and Unwin, London.

Ensink, F.T.E. & van Haeringen, N.J. (1977). Pitfalls in the assay of lysozyme in human tear fluid. Opthal. Res., 9: 366-373.

Fange, R., Lundbland, G. & Lind, J. (1976). Lysozyme and chitinase in blood and lymphomyeloid tissues of marine fish. Marine Biol., 36: 277-282.

Fenchel, T. (1972). Aspects of decomposer food chains in marine benthose. Verk. Dtch. Zool. Ges. 65: 14-23.

Feng, S.Y. (1974a). Lysozyme-like activities in the haemolymph of Crassostrea virginica. Contemp. Topics. Immunobiol., 4: 225-231.

Feng, S.Y. (1974b). Communication cited by Cheng et al (1975).

Feng, S.Y. & Canzonier, W.J. (1970). Humoral responses in the American oyster (Crassostrea virginica) infected with Bucephalus and Minchinia nelsoni. Symposium on diseases of fishes and shellfishes. Special publication 5 : 497-510. American Fisheries Society.

Fernandez-Sousa, J.M., Gavilanes, J.G., Municio, A.M., Perez-Aranda, A. & Rodriguez, R. (1977). Lysozyme from the insect Ceratitis capitata eggs. Eur. J. Biochem., 72: 25-33.

Fernandez-Sousa, J.M. & Rodriguez, R. (1977). Lysozyme-agarose interaction. Biochem. Biophys. Res. Commun., 74: 1426-1431.

Fleming, A. (1922). On a remarkable bacteriolytic element found in tissues and secretions. Proc. Roy. Soc. Lond. B, 93: 306-317.

Fletcher, T.C. & Grant, P.T. (1968). Glycoproteins in the external mucous secretions of the plaice, Pleuronectes platessa and other fishes. Biochem. J., 106: 12P.

Fletcher, T.C. & White, A. (1973). Lysozyme activity in the plaice (Pleuronectes platessa L). Experentia 29: 1283-1285.

Fletcher, T.C. & White, A. (1976). The lysozyme of the plaice Pleuronectes platessa L. Comp. Biochem. Physiol., 55B: 207-210.

Fletcher, T.C., White, A. & Baldo, B.A. (1977). C-reactive protein-like precipitin and lysozyme in the lump sucker Cyclopterus lumpus L during the breeding season. Comp. Biochem. Physiol., 57B: 353-357.

Foley, D.A. (1974). Ph.D. thesis cited by Cheng et al (1975).

Fraenkel-Conrat, H. (1950). The essential groups of lysozyme, with particular reference to its reaction with iodine. Arch. Biochem., 27: 109-124.

Fulco, L., Karfankel, P. & Aaronson, S. (1967). Effect of lysozyme (muramidase) on marine and fresh water blue green algae. J. Phycol., 3: 51-52.

Gayen, S.K., Som, S., Sinha, N.K. & Sen, A. (1977). Lysozyme in egg-white of tortoises and turtle: purification and properties of egg-white lysozyme of Trionyx gangeticus Cuvier. Arch. Biochem. Biophys., 183: 432-442.

George, W.C. (1952). The digestion and absorption of fat in lamellibranchs. Biol. Bull., 102: 118-127.

Ghuysen, J.M., Tipper, D.J. & Strominger, J.L. (1966). Enzymes that degrade bacterial cell walls. In: Methods in Enzymology, 8: 685-699. Editors: S.P. Colwick, N.O. Kaplan, Academic Press, New York and London.

Gilmour, T.H.J. (1974). The structure, ciliation and function of the lips of some bivalve molluscs. Can. J. Zool., 52: 335-343.

Giusti, F. (1970). The fine structure of the style sac and intestine in Mytilus galloprovinciallis Lam. Proc. Malacol. Soc. London, 39: 95-104.

Gomori, G. (1948). Histochemical demonstration of sites of choline esterase activity. Proc. Soc. Exp. Biol. Med., 68: 354-358.

Greenwald, R.A. & Moy, W.N. (1976). Effect of agarose variability on the measurement of lysozyme activity. Clin. Chim. Acta, 73: 299-305.

Hanschke, R. & Hanschke, M. (1977). Untersuchungen uber die aktivierung humoraler abwehrfaktoren gegen bakterien bei larven der wachsmotte Galleria mellonella L. Acta Biol. Med. Germ., 36: 267-273.

Hansen, N.E., Karle, H., Andersen, V. & Olgaard, K. (1972). Lysozyme turnover in man. J. Clin. Invest., 51: 1146-1155.

Hardy, S.W., Fletcher, T.C. & Gerrie, L.M. (1976). Factors in the haemolymph of the mussel Mytilus edulis L of possible significance as defense mechanisms. Biochem. Soc. Trans., 4, 473-475.

Hartland, B.J. & Timoney, J.F. (1979). In vivo clearance of enteric bacteria from the haemolymph of the hard clam and the American oyster. Appl. Env. Microbiol., 37: 517-520.

Hash, J.H. (1963). Purification and properties of staphylolytic enzyme from Chalaropsis sp. Arch. Biochem. Biophys., 102: 379-388.

Hash, J.H. (1974). Lysozyme Chalaropsis. In: Lysozyme: 95-104. Editors: E.F. Osserman, R.E. Canfield, S. Beychock, Academic Press, New York and London.

Hirsch, J.G. (1960). Antimicrobial factors in tissues and phagocytic cells. Bact. Rev., 24: 133-140.

Howard, J.B. & Glazer, A.N. (1967). Studies on the physiochemical and enzymatic properties of Papaya lysozyme. J. Biol. Chem., 242: 5715-5723.

Imoto, T., Johnson, L.N., North, A.C.T., Phillips, D.C. & Rupley, J.A. (1972). Vertebrate lysozymes. In: The Enzymes, 3rd edition, 7: 665-868. Editor: P.D. Boyer, Academic Press, New York and London.

Imoto, T. & Yagishita, K. (1973). Chitin coated cellulose as an adsorbent of lysozyme-like enzymes. Preparation and properties. Agric. Biol. Chem., 37: 465-470.

Jeanloz, R.W., Sharon, N. & Flowers, H.M. (1963). The chemical structure of a disaccharide isolated from Micrococcus lysodeikticus cell walls. Biochem. Biophys. Res. Commun., 13: 20-25.

Johansson, J. (1945). Uber oxydasen und peroxydasen im kristallstiel der muscheln. Ibid., 36A: 1-7. Cited by Kristensen (1972b).

Jolles, J., Dianoux, A.-C., Hermann, J., Niemann, B. & Jolles, P. (1966). Relationship between the cystine and tryptophan contents of five different lysozymes and their heat stability and specific activity. Biochim. Biophys. Acta, 128: 568-570.

Jolles, J., Jaurequi-Adell, J., Bernier, J. & Jolles, P. (1963). La structure chimique du lysozyme de blanc d'oeuf de poule. Etude detaillee. Biochim. Biophys. Acta, 78: 668-689.

Jolles, J. & Jolles, P. (1975). The lysozyme from Asterias rubens. Eur. J. Biochem., 54: 19-23.

Jolles, P. (1964). Recent developments in the study of lysozymes. Angew. Chemie. Int. Edn., 3: 28-36.

Jolles, P. (1967). Relationship between chemical structure and biological activity of hen egg-white lysozyme and lysozymes of different species. Proc. Roy. Soc. B, 167: 350-364.

Jolles, P. (1969). Lysozymes: A chapter of molecular biology. Angew. Chemie. Int. Edn. 8: 227-239.

Jolles, P. (1976). A possible physiological function of lysozyme. Biomed., 25: 275-276.

Jolles, P., Bernier, I., Berthou, J., Charlemagne, D., Faure, A., Hermann, J., Jolles, J., Perin, J.-P. & Saint-Blancard, J. (1974). From lysozymes to chitinases: structural, kinetic and crystallographic studies. In: Lysozyme: 31-54. Editors: E.F. Osserman, R.F. Canfield, S. Beychock, Academic Press, New York and London.

Jolles, P., Jaurequi-Adell, J. & Jolles, J. (1964). Le lysozyme de blanc d'oeuf de poule: disposition des points disulfures. C.R. Acad. Sci., 258: 3926-3928.

Jolles, P., Jolles-Thaureaux, J. & Fromageot, C. (1957). Recherche de lysozymes chez quelques invertebres lysozymes de Nephthys hombergi. C.R. Soc. Biol., 151: 1368-1369.

Jolles, P., Saint-Blancard, J., Charlemagne, D., Dianoux, A.C., Jolles, J. & Le Baron, J.-L. (1968). Comparative behaviour of six different lysozymes in the presence of an inhibitor. Biochim. Biophys. Acta, 151: 532-537.

Jolles, P. & Zuilli, S. (1960). Purification et etude comparee de nouveaux lysozymes; extraits du poumon depoule et de Nephthys hombergi. Biochim. Biophys. Acta, 39: 212-217.

Kotani, S., Watanabe, Y., Kimoshita, F., Kato, K. & Perkins, H.R. (1977). Immunoadjuvant activities of the enzymatic digests of bacterial cell walls lacking immunoadjuvancy by themselves. Biken J., 20: 87-90.

Kravchenko, N.A. (1967). Lysozyme as a transferase. Proc. Roy. Soc. B, 167: 429-430.

Kravchenko, N.A. & Maksimov, V.I. (1964). Izvestia Academie Nauk. SSSR Seriya Khimicheskaya 1964: 584. Cited by Chipman and Sharon (1969).

Kristensen, J.H. (1972a). Carbohydrases of some marine invertebrates with notes on their food and on the natural occurrence of the carbohydrates studied. Marine Biol., 14: 130-142.

Kristensen, J.H. (1972b). Structure and function of crystalline styles of bivalves. Ophelia 10: 91-108.

Krizencky, J. & Podhradsky, J. (1927). Studien XI. Ist die bakterienflora der vermittler zwischien den hieren und den aufgelosten nahrsubstanzien. E. Verg. 1. Physiol., 6: 431-444. Cited by ZoBell (1946).

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Li, C.P. (1960). Antimicrobial activity of certain marine fauna. Proc. Soc. Exp. Biol. Med., 104: 366-368.

Litwack, G. (1955). Photometric determination of lysozyme activity. Proc. Soc. Exp. Biol. Med., 89: 401-403.

Locquet, J.-P., Saint-Blancard, J. & Jolles, P. (1968). Apparent affinity constants of lysozymes from different origins for Micrococcus lysodeikticus cells. Biochim. Biophys. Acta, 167: 150-153.

Lowry, O.H., Rosebrough, W.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

MacGinitie, G.E. (1932). The role of bacteria as food for bottom animals. Science, 76: 490.

Malke, H. (1965). Uber das vorkommen von lysozym in inskten. E. Allg. Mikrobiol., 4: 88-91. Cited by Chadwick (1970).

McDade, J.E. & Tripp, M.R. (1967a). Lysozyme in the haemolymph of the oyster Crassostrea virginica. J. Invertebr. Pathol., 9: 531-535.

McDade, J.E. & Tripp, M.R. (1967b). Lysozyme in oyster mantle mucus. J. Invertebr. Pathol., 9: 581-582.

Messner, V.B. & Mohrig, W. (1969). Zum lysozym-vorkommen bei muscheln (Anodonta anatina L). Zool. Jb. Physiol., 74: 427-435.

Michaelis, L. (1931). Der acetat-veronal-puffer. Biochem. Z., 234: 139-141.

Mikhailova, I.G. & Prazduckar, E.V. (1961). On the problem of reactions of the tissues of Mytilus edulis. Trudy Murmanskogo biologicheskogo Instituta, 3: 125-130. Cited by Bayne et al (1976).

Mikhailova, I.G. & Prażduckar, E.V. (1962). Inflamatory reactions in the Barents sea mussel (Mytilus edulis). Trudy Murmanskogo biologicheskogo Instituta, 4: 208-220. Cited by Bayne et al (1976).

Miller, T.C. (1969). Killing and lysis of gram negative bacteria through the synergistic effect of hydrogen peroxide, ascorbic acid and lysozyme. J. Bacteriol., 98: 949-955.

Mohrig, W. & Messner, V.B. (1968a). Immunoreaktionen bei insekten. I. Lysozym als grundegender antibakterieller faktion im humoralen akwermechanismus der insekten. Biolog. Z., 87: 439-470.

Mohrig, W. & Messner, V.B. (1968b). Immunoreaktionen bei insekten. II. Lysozym als antimikrobielles agens im darmtrakt von insekten. Biolog. Z., 87: 705-718.

Moore, M.N. & Lowe, D.M. (1977). Cytology and cytochemistry of the haemocytes of Mytilus edulis and their responses to experimentally injected carbon particles. J. Invertebr. Pathol., 29: 18-30.

Morgan, F.J. & Arnheim, N. (1974). Preliminary biochemical studies of the lysozymes of the black swan, Cygnus atratus. In: Lysozyme: 81-88. Editors: E.F. Osserman, R.E. Canfield & S. Beychock, Academic Press, New York and London.

Morita, I., Hara, S. & Matsushima, Y. (1978). Purification and characterisation of lysozyme produced by Streptomyces erythraeus. J. Biochem., 83: 893-903.

Morton, B. (1971). The diurnal rhythm of feeding and digestion in Ostrea edulis. Biol. J. Linn. Soc., 3: 329-342.

Muschel, L.H., Carey, W.F. & Baron, L.S. (1959). Formation of bacterial protoplasts by serum components. J. Immunol., 83: 38-42.

Nace, G.W. & Ostrovsky, D.S. (1977). Frog lysozyme. IV. Isozymes of lysozyme and the Lucke renal adenocarcinoma. J. Natl. Cancer Inst., 58: 453-454.

Nace, G.W., Suyama, T. & Iwata, T. (1965). The relationship between a lysozyme-like enzyme and frog adenocarcinoma. Ann. N.Y. Acad. Sci., 126: 204-221.

Newell, R. (1965). The role of detritus in the nutrition of two marine deposit feeders, the prosobranch Hydrobia ulvae and the bivalve Macoma balthica. Proc. Zool. Soc. Lond., 144: 25-45.

Nikaido, H. (1976). Outer membrane of Salmonella typhimurium. Transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta, 433: 118-132.

Ochi, N., Azegami, M. & Ishii, S. (1978). Bacteriolytic enzyme induced from pyocinogenic Pseudomonas aeruginosa. J. Biochem., 83: 727-736.

Orton, J.H. (1910). The mode of feeding of Crepidula, with a current producing mechanism in the mouth cavity, and some remarks on the mode of feeding in Gastropods and Lamellibranchs. J. Mar. Biol. Assoc., 9: 444-478.

Osserman, E.F., Canfield, R.E. & Beychock, S. (Editors) (1974). In: Lysozyme, Academic Press, New York and London.

Osserman, E.F. & Lawlor, D.P. (1966). Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J. Exp. Med., 124: 921-952.

Ostrovsky, D.S., Snyder, J.A., Iwata, T., Izaka, K.-I., Maglott, D.S. & Nace, G.W. (1976). Frog lysozyme. I. Its identification, occurrence as isozymes, and quantitative distribution in tissues of the leopard frog, Rana pipiens. J. Exp. Zool., 195: 279-290.

Ostroy, F., Gams, R.A., Glickson, J.D. & Lenkinski, R.E. (1978). Inhibition of lysozyme by polyvalent metal ions. Biochim. Biophys. Acta, 527: 56-62.

Owen, G. (1955). Observations on the stomach and digestive diverticula of the Lamellibranchia. I. The Anysomyaria and Eumellibranchia. Quarterly J. Microscop. Sci., 96: 517-537.

Owen, G. (1966). Digestion. In: Physiology of Mollusca. II: 53-96. Editors: K.M. Wilbur & C.M. Yonge, Academic Press, New York and London.

Owen, G. (1970). The fine structure of the digestive tubules of the marine bivalve Cardium edule. Phil. Trans. R. Soc. Lond. B, 258: 245-260.

Owen, G. (1972). Lysosomes, peroxisomes and bivalves. Sci. Prog., 60: 299-318.

Owen, G. (1974). Feeding and digestion in the Bivalvia. Adv. Comp. Physiol. Biochem., 5: 1-35.

Parry, R.M., Chandon, R.C. & Shahani, K.M. (1969). Isolation and characterization of human milk lysozyme. Arch. Biochem. Biophys., 103: 59-65.

Peeters, T.L. & Vantrappen, G.R. (1977). Factors influencing lysozyme determinations by the lysoplate method. Clin. Chim. Acta, 74: 217-225.

Perin, J.-P. & Jolles, P. (1972). The lysozyme from Nephthys hombergi (annelid). Biochim. Biophys. Acta, 263: 683-689.

Perin, J.-P. & Jolles, P. (1973). The lysozyme of Nephthys hombergi (annelid). II. Study of some enzymatic properties. Molec. Cell. Biochem., 2: 189-195.

Perin, J.-P. & Jolles, P. (1976). Enzymatic properties of a new type of lysozyme isolated from Asterias rubens: comparison with the Nephthys hombergii (annelid) and hen lysozymes. Biochim., 58: 657-662.

Perkins, H.R. (1967). Inhibition of lysozyme by positively charged groups in the mucopeptide of the bacterial cell wall. Proc. Roy. Soc. B., 167: 443-445.

Peterson, R.G. & Hartsell, S.E. (1955). Lysozyme spectrum of the gram-negative bacteria. J. Infec. Dis., 96: 75-81.

Phillips, D.C. (1966). The three-dimensional structure of an enzyme molecule. Sci. Amer., 215: 78-90.

Phillips, D.C. (1967). The hen egg-white lysozyme molecule. Proc. Nat. Acad. Sci. U.S., 57: 484-495.

Pollock, J.J., Chipman, D.M. & Sharon, N. (1967a). Glycosyl transfer to acceptor saccharides catalysed by lysozyme. Arch. Biochem. Biophys., 120: 235-238.

Pollock, J.J., Chipman, D.M. & Sharon, N. (1967b). The active site of lysozyme. Some properties of subsites E and F. Biochem. Biophys. Res. Commun., 28: 779-784.

Powning, R.J. & Davidson, W.J. (1973). Studies on insect bacteriolytic enzymes. I. Lysozyme in haemolymph of Galleria mellonella and Bombyx mori. Comp. Biochem. Physiol., 45B: 669-686.

Powning, R.J. & Davidson, W.J. (1976). Studies on insect bacteriolytic enzymes. II. Some physical and enzymatic properties of lysozyme from the haemolymph of Galleria mellonella. Comp. Biochem. Physiol. 55B: 221-228.

Powning, R.F. & Irzykiewicz, H. (1967). Lysozyme-like action of enzymes from the cockroach Periplaneta americana and from some other sources. J. Insect. Physiol., 13: 1293-1299.

Primosigh, J., Pelzer, H., Maass, D. & Weidel, W. (1961). Chemical characterization of mucopeptides released from E. coli B cell wall by enzymic action. Biochim. Biophys. Acta, 46: 68-80.

Reid, R.G.B. (1965). The structure and function of the stomach in bivalve molluscs. J. Zool., 147: 156-184.

Reid, R.G.B. (1968). The distribution of digestive tract enzymes in Lamellibranchiate bivalves. Comp. Biochem. Physiol., 24: 727-744.

Reid, R.G.B. & Reid, A. (1969). Feeding processes of members of the genus Macoma (Mollusca:Bivalvia). Can. J. Zool., 47: 253-262.

Reisfeld, R.A., Lewis, U.J. & Williams, D.E. (1962). Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature, 195: 281-283.

Repaske, R. (1956). Lysis of gram-negative bacteria by lysozyme. Biochim. Biophys. Acta, 22: 189-191.

Rodrick, G.E. & Cheng, T.C. (1974a). Activities of selected haemolymph enzymes in Biomphalaria glabrata. J. Invertebr. Pathol., 24: 374-375.

Rodrick, G.E. & Cheng, T.C. (1974b). Kinetic properties of lysozyme from the haemolymph of Crassostrea virginica. J. Invertebr. Pathol., 24: 41-48.

Rosen, B. (1949). Proteases in the digestive gland of lamellibranchs. Arkiv. Kemi., 1: 205-211.

Rubin, M.L., Ar, D.B. & Nace, G.W. (1966). The virus inhibiting action of a lysozyme present in normal frogs but lacking in tumour frogs and eggs. Amer. Zool., 6: 29.

Saint-Blancard, J., Chuzel, P., Mathieu, Y., Perrot, J. & Jolles, P. (1970). Influence of pH and ionic-strength on the lysis of Micrococcus lysodeikticus cells by six human and four avian lysozymes. Biochim. Biophys. Acta, 220: 300-306.

Salton, M.R.J. (1952). Cell wall of Micrococcus lysodeikticus as the substrate for lysozyme. Nature (London), 170: 746-747.

Salton, M.R.J. (1957). The properties of lysozyme and its action on microorganisms. Bact. Rev., 21: 82-99.

Salton, M.R.J. & Ghuysen, J.M. (1959). The structure of the di- and tetra-saccharides released from cell walls by lysozyme and Streptomyces $F_1$ enzyme and the beta (1-4) N-acetylhexosaminidase activity of these enzymes. Biochim. Biophys. Acta, 36: 552-554.

Schubert, V.T. & Messner, B. (1971). Untersuchungen uber das vorkommen von lysozym bei anneliden. Zool. Jb. Physiol. Bd., 76s: 36-50.

Seed, R. (1976). Ecology. In: Marine Mussels: 13-66. Editor: B.L. Bayne, Cambridge University Press, Cambridge.

Selsted, M.E. & Martinez, R.J. (1978). Lysozyme: Primary bactericidin in human plasma serum against Bacillus subtilis. Infect. Immun. 29: 782-791.

Sharon, N. (1964). Third International Lysozyme Symposium, Milan, 1964. Cited by Jolles (1969).

Sharon, N. (1967). The chemical structure of lysozyme substrates and their cleavage by the enzyme. Proc. Roy. Soc. B, 167: 402-415.

Sharon, N. & Jeanloz, R.W. (1964). A procedure for the preparation of gram quantities of bacterial cell walls. Experentia, 20: 253-254.

Sharon, N. & Seifter, S. (1964). A transglycosylation reaction catalysed by lysozyme. J. Biol. Chem., 239: PC 2398-2399.

Shinitzky, M., Grisaro, V., Chipman, D.M. & Sharon, N. (1966). Influence of inhibitory sugars on the fluorescence of lysozyme. Arch. Biochem. Biophys., 115: 232-233.

Shugar, D. (1952). The measurement of lysozyme activity and the U.V.-inactivation of lysozyme. Biochim. Biophys. Acta, 8: 302-308.

Smith, E.L., Kimnel, J.R., Brown, D.M. & Thompson, E.O.P. (1955). Isolation and properties of a crystalline mercury derivative of a lysozyme from papaya latex. J. Biol. Chem., 215: 67-89.

Smolelis, A.N. & Hartsell, S.E. (1949). The determination of lysozyme. J. Bacteriol., 58: 731-736.

Smolelis, A.N. & Hartsell, S.E. (1952). Factors affecting the lytic activity of lysozyme. J. Bacteriol., 63: 665-674.

Smyth, C.J. & Arbuthnott, J.P. (1974). Properties of Clostridium perfringens (welchii) type A α-toxin (phospholipase C) purified by electrofocusing. J. Med. Microbiol., 7: 41-66.

Snyder, J.A. & Harrison, J.H. (1977). Frog lysozyme. V. Isolation and some physical and immunological properties of lysozyme isozymes of the leopard frog, Rana pipiens. J. Exp. Zool., 202: 89-96.

Sophianopoulos, A.J. & van Holde, K.E. (1964). Physical studies of muramidase (lysozyme). II. pH-dependent dimerization. J. Biol. Chem., 239: 2516-2524.

Sophianopoulos, A.J. & Sasse, E.A. (1965). Isoelectric points of proteins by differential conductimetry. J. Biol. Chem., 240: PC1864-1866.

Sorensen, S.P.L. (1909). Enzymestudien. II. Mitteilung. Uber die messung und die Bedeutung der wasserstoffionenkonzentration bei enzymatischen progressen. Biochem. Z. 21: 131-200.

Strominger, J.L. & Tipper, D.J. (1974). Structure of bacterial cell walls: The lysozyme substrate. In: Lysozyme: 169-184. Editors: E.F. Osserman, R.E. Canfield & S. Beychock. Academic Press, New York and London.

Sumner, A.T. (1966). The cytology and histochemistry of the digestive gland cells of freshwater lamellibranchs. J. Roy. Microscop. Soc., 85: 201-211.

Sumner, A.T. (1969). The distribution of some hydrolytic enzymes in the cells of the digestive gland of certain lamellibranchs and gastropods. J. Zool., 158: 277-291.

Takatsuki, S. (1934). On the nature and functions of the amoebocytes of Ostrea edulis. Quarterly J. Microscop. Sci., 76: 379-431.

Takeda, H., Strasdine, G.A., Whitaker, D.R. & Roy, C. (1966). Lytic enzymes in the digestive juice of Helix pomatia: Chitinases and muramidases. Can. J. Biochem., 44: 509-518.

Talbot, P. & Caie, I.S. (1975). A horizontal trough apparatus for isoelectric focusing. In: Isoelectric Focusing: 74-77. Editors: J.P. Arbuthnott and J. Beeley, Butterworths, London.

Thompson, A.R. (1951). Destruction of dinitrophenol amino acids by tryptophane. Nature (London), 168: 390-391.

Thompson, A.R. (1952). The C-terminal residue of lysozyme. Nature (London), 169: 495-496.

Thompson, J.S. & Shockman, G.D. (1968). A modification of the Park and Johnson reducing sugar determination suitable for the assay of insoluble materials; its application to bacterial cell walls. Analyt. Biochem., 22: 260-268.

Thompson, R.J., Ratcliffe, N.A. & Bayne, B.L. (1974). Effects of starvation on structure and function in the digestive gland of the mussel (Mytilus edulis L). J. Mar. Biol. Assoc., 54: 699-712.

Tomcsik, J. (1956). Bacterial capsules and their relation to the cell wall. In: Bacterial Anatomy: 41-67. Editors: E.T.C. Spooner and B.A.D. Striker, Cambridge University Press, Cambridge.

Tripp, M.R. (1960). Mechanisms of removal of injected microorganisms from the American oyster Crassostrea virginica. Biol. Bull., 119: 273-282.

Tripp, M.R. (1961). The fate of foreign materials experimentally introduced into the snail Australorbis glabratus. J. Parasitol., 47: 745-751.

Tripp, M.R. (1974a). Humoral factors and molluscan immunity. In: Invertebrate Immunity: 201-223. Editors: K. Maramorosch and R.F. Shope, Academic Press, New York and London.

Tripp, M.R. (1974b). Oyster haemolymph proteins. Ann. N.Y. Acad. Sci., 234: 18-20.

Tsugita, A., Inouye, M., Terzaghi, E. & Streisinger, G. (1968). Purification of bacteriophage T4 lysozyme. J. Biol. Chem., 243: 391-397.

Turner, G.A., Ghneim, H. & Freeman, J. (1979). Evaluation of 3,4-dinitrophenyl tetra-N-acetyl-β-chitotetraoside as a substrate for the measurement of lysozyme in normal and pathological sera. Ann. Clin. Biochem., 16: 51-53.

Upadhyay, J.M., Mares, B.A., Hemelt, D.M. & Rivet, P.G. (1977). Purification and specificity of bacteriolytic enzyme I from Hartmannella glebae. Appl. Environ. Microbiol., 33: 1-5.

Vernon, C. (1967). The mechanisms of hydrolysis of glycosides and their relevance to enzyme catalysed reactions. Proc. Roy. Soc. B, 167: 389-401.

Vladimirov, V.L. (1968). Immunity in fish. Bull. Off. int. Epizoot., 69: 1365-1372.

Wagge, L.E. (1955). Amoebocytes. In: International Review of Cytology, 4: 31-78. Editors: G. Bourne and J.F. Danielli, Academic Press, New York and London.

Waksman, S.A. & Carey, C.L. (1935). Decomposition of organic matter in sea water by bacteria. II. Influence of addition of organic substances upon bacterial substances. J. Bacteriol., 29: 545-561.

Walpole, G.S. (1914). Hydrogen potentials of mixtures of acetic acid and sodium acetate. J. Chem. Soc., 105: 2501-2521.

Wardlaw, A.C. (1962). The complement-dependent bacteriolytic activity of normal human serum. The effect of pH and ionic strength and the role of lysozyme. J. Exp. Med., 115: 1231-1249.

Weber, K. & Osborn, M. (1969). The reliability of molecular weight determination by dodecyl sulphate polyacrylamide gel electrophoresis. J. Biol. Chem., 244: 4406-4412.

Whitaker, J. (1963). Determination of molecular weights of proteins by gel filtration on Sephadex. Anal. Chem., 35: 1950-1953.

Wilkinson, B.J. (1977). Cell envelope of Paracoccus denitrificans outer membrane permeability to lysozyme and hydrophobic antibiotics. FEMS Mikrobiol. Lett., 2: 285-288.

Work, E. (1969). Muralytic enzymes. In: Methods in Microbiology, 5A: 408-418. Editors: J.R. Norris and D.W. Ribbons, Academic Press, New York and London.

Wright, D.G. & Malawista, S.E. (1973). Mobilization and extracellular release of granular enzymes from human leukocytes during phagocytosis: Inhibition by colchicine and cortisol but not by salicylate. Arthrit. Rheumat. 16: 749-758.

Yonge, C.M. (1926a). The digestive diverticula in the lamellibranchs. Trans. Roy. Soc. Ed., 54: 703-718.

Yonge, C.M. (1926b). Structure and physiology of the organs of feeding and digestion in Ostrea edulis. J. Mar. Biol. Assoc., 14: 295-386.

Yonge, C.M. (1937). Evolution and adaptation in the digestive system of the Metazoa. Biol. Revs. Cambridge Phill. Soc., 12: 87-115.

Yonge, C.M. (1971). The Sea Shore. Collins, London and Glasgow.

Zachs, S.I. & Welsh, J.H. (1953). Cholinesterase and lipase in the amoebocytes, intestinal epithelium and heart mussel of the quahog Venus mercenaria. Biol. Bull., 105: 200-211.

ZoBell, C.E. (1946). Relation of marine bacteria to flora and fauna. In: Marine Microbiology, chapt. 14: 170-176. Chronica Botanica Co., Waltham, Mass., U.S.A.

ZoBell, C.E. & Feltham, C.A. (1934). Preliminary studies on the distribution and characteristics of marine bacteria. Bull. Scripps Inst. Oceanogr., 3: 279-294.

ZoBell, C.E. & Feltham, C.A. (1938). Bacteria as food for certain marine invertebrates. J. Mar. Res. Sears Foundn., 1: 312-327.

ZoBell, C.E. & Landon, W.A. (1937). Bacterial nutrition of the California mussel. Proc. Soc. Exp. Biol. Med., 36: 607-609.

Zucker, S., Hanes, D.J., Vogler, W.R. & Eanes, R.Z. (1970). Plasma muramidase: A study of methods and clinical applications. J. Lab. Clin. Med., 75: 83-92.

# APPENDICES

## Appendix I Media

### Peptone Yeast Extract Water (P.Y.E.W.)

P.Y.E.W. was prepared by mixing:

| | | |
|---|---|---|
| Bactopeptone (Difco; Michigan, USA) | | 50 g |
| Yeast extract (Difco; Michigan, USA) | | 1 g |
| Sodium chloride | | 5 g |
| Distilled water | to | 1 l |

The mixture was steamed for 20 min to dissolve the ingredients and, when cooled, the pH was adjusted to 7.2 with 40% (w/v) NaOH. The medium was dispensed and autoclaved at 121°C for 15 min.

### Peptone Yeast Extract Agar (P.Y.E.A.)

P.Y.E.W. was prepared as above and 15 g Bacto-agar (Difco; Michigan, USA) added as a solidifying agent, pH adjusted to 7.2, steamed to dissolve agar, dispensed into 200 ml aliquots and autoclaved at 121°C for 15 min.

### Marine Broth

Marine broth (Difco; Michigan, USA), 37.4 g, was rehydrated in distilled water, 1 l, by boiling for one to two minutes after which it was dispensed and autoclaved at 121°C for 15 min.

### Marine Agar

Marine agar (Difco; Michigan, USA), 55.1 g, was added to cold distilled water, 1 l, dissolved by boiling for one to two minutes, dispensed as required and autoclaved at 121°C for 15 min.

McConkey Agar

McConkey agar (Oxoid; Basingstoke), 52 g, was added to cold distilled water, 1 l, dissolved by boiling, dispensed as required and autoclaved at 121°C for 15 min.

Nutrient Agar

Nutrient agar (Oxoid; Basingstoke), 28 g, was added to cold distilled water, 1 l, dissolved by boiling, dispensed as required and autoclaved at 121°C for 15 min.

## Appendix II Buffers and Diluents

Sodium acetate buffer, 0.01M (pH 5) (Walpole, 1914)

Stock solutions:

A: 0.2M solution of acetic acid (11.55 ml in 1 l)

B: 0.2M solution of sodium acetate (27.2 g $CH_3O_2Na.3H_2O$ in 1 l)

Buffer was prepared by mixing 14.8 ml A and 35.2 ml B and diluting to a total of 1 l.

Tris-maleate buffer, 0.01M (pH 6.4) (Gomori, 1948)

Stock solutions:

A: 0.02M solution of Tris acid maleate (2.42 g tris + 2.32 g maleic acid in 1 l)

B: 0.02M NaOH.

Buffer was prepared by mixing 50 ml A and 37 ml B and diluting to a total of 200 ml.

Sodium phosphate buffer, 0.06M (pH 6.4) (Sorensen, 1909)

Stock solutions:

A: 0.2M solution of monobasic sodium phosphate (27.8 g in 1 l)

B: 0.2M solution of dibasic sodium phosphate (71.7 g of $Na_2HPO_4.12H_2O$ in 1 l).

Buffer was prepared by mixing 44.1 ml A and 15.9 ml B and diluting to a total of 200 ml.

Potassium phosphate buffer

Stock solutions:

A: 1M solution of monobasic potassium phosphate (136.09 g in 1 l)

B: 1M solution of dibasic potassium phosphate (174.18 g in 1 l)

Stock buffers were prepared thus:

pH 6.5 : 68.5 ml A + 31.5 ml B

pH 6.8 : 51.0 ml A + 49.0 ml B

pH 7 : 39.0 ml A + 61.0 ml B

Stock buffers were diluted to the desired molarity thus:

| Molarity | Parts by volume | |
|---|---|---|
| | Stock buffer | Distilled water |
| 0.05 | 0.5 | 9.5 |
| 0.1 | 1 | 9 |
| 0.2 | 2 | 8 |
| 0.4 | 4 | 6 |
| 0.6 | 6 | 4 |
| 1.0 | 10 | 0 |

Barbital sodium-acetate (Michaelis, 1931)

Stock solutions:

A: 0.21M sodium acetate in 0.21M barbital sodium (29.15 g $C_2H_3O_2.3H_2O$ + 44.15 g barbital sodium in 1 l)

B: 0.15N HCl

C: 12.75% (w/v) NaCl solution.

Composition of stock buffers: 50 ml A + x ml B + 20 ml C made up to 250 ml with distilled water. Buffers of the desired pHs were prepared with the following volumes of B :

| pH | ml B | pH | ml B |
|---|---|---|---|
| 3.0 | 154.0 | 6.5 | 67.5 |
| 3.5 | 143.0 | 7.0 | 60.5 |
| 4.0 | 125.5 | 7.5 | 46.5 |
| 4.5 | 105.5 | 8.0 | 26.0 |
| 5.0 | 88.0 | 8.5 | 11.0 |
| 5.5 | 76.5 | 9.0 | 4.0 |
| 6.0 | 71.0 | | |

The stock buffers were of I = 0.177 and were diluted as below to give the desired range of I values. Upon dilution the found pH values of some of the buffers deviated from those above.

| Ionic strength | Parts by volume | |
|---|---|---|
| | Stock buffer | Distilled water |
| 0.177 | 30 | 0 |
| 0.118 | 20 | 10 |
| 0.089 | 15 | 15 |
| 0.059 | 10 | 20 |
| 0.030 | 5 | 25 |
| 0.012 | 2 | 28 |
| 0.006 | 1 | 29 |

Glycine buffer (pH 10.7) (Allison and Sandelin, 1963)

A 0.4M solution of glycine was prepared by dissolving 30.02 g in 1 l distilled water and the pH was adjusted to 10.7 with N NaOH.

Collagenase assay - Diluent A

| | |
|---|---|
| 0.05M Tris | 6.05 g |
| 0.05M Maleic acid | 5.80 g |
| 1N NaOH | 0.48 ml |

The ingredients were dissolved in 750 ml dissolved water and the pH adjusted to 7 with 1N NaOH and the volume made up to 1 l.

## Appendix III Disc-Gel Electrophoresis

ABBREVIATIONS:

| | |
|---|---|
| BIS | N,N-methylenebisacrylamide (BDH, Poole) |
| Tris | Tris (hydroxymethyl) aminoethane (BDH, Poole) |
| TEMED | N,N,N,'N'-tetramethylenediamine (BDH, Poole) |

1. Acid system (Reisfeld et al., 1962)

Separating gel.

| Reagent/100 ml water | | | Parts by volume |
|---|---|---|---|
| Acrylamide (BDH, Poole) | 60 g | | 8 |
| BIS | 0.4 g | | |
| 1N KOH | 48 ml | pH 4.3 | 4 |
| Acetic acid (glacial) | 17.2 ml | | |
| TEMED | 4.0 ml | | |
| $(NH_4)_2S_2O_8$ | 0.28 g | | 16 |
| Water | | | 4 |

Stacking gel.

| Reagent/100 ml water | | | Parts by volume |
|---|---|---|---|
| Acrylamide | 10.0 g | | 4 |
| BIS | 2.5 g | | |
| 1N KOH | 48 ml | pH 6.8 | 2 |
| Acetic acid (glacial) | 2.87 ml | | |
| TEMED | 0.46 ml | | |
| Riboflavin | 0.004 g | | 2 |
| Water | | | 8 |

Tank buffer (pH 4.5)

| | |
|---|---|
| β-alanine | 31.2 g |
| Acetic acid glacial | 8.0 ml |

Made up to 1 l in distilled water

Buffer was diluted ten fold for use.

2. SDS system (Laemmli, 1970 and Ames, 1974)

Stock solutions

| | | |
|---|---|---|
| 1. | Acrylamide | 30 g |
| | BIS | 0.8 g |
| | | in 100 ml $H_2O$ |

Filtered and stored in brown bottle at 4°C.

2. 1M Tris/HCl buffer pH 8.8

| | |
|---|---|
| 1M Tris | 50 ml |
| 1N HCl | 8.1 ml |
| $H_2O$ | 49.9 ml |

3. 1M Tris/HCl buffer pH 6.8

| | |
|---|---|
| 1M Tris | 50 ml |
| 1N HCl | 45 ml |
| $H_2O$ | 5 ml |

4. Tris/glycine buffer pH 8.3 (running buffer).

| | |
|---|---|
| Glycine | 144.13 g |
| Tris | 30.28 g |
| | per litre |

Buffer was diluted ten fold for use and SDS added at a final concentration of 0.1%.

| | | |
|---|---|---|
| 5. | Ammonium persulphate | 0.8% w/v |
| 6. | S.D.S. | 20% w/v |

7. Bromophenol blue 0.1% w/v

| Solubilizing buffer | Stock solns | Volume (ml) |
|---|---|---|
| | 3 | 2.0 |
| | 6 | 3.2 |
| | β-mercaptoethanol | 1.6 |
| | glycerol | 3.2 |
| | 7 | 0.32 |
| | $H_2O$ | 5.68 |
| Separating gel | | |
| | 1 | 14.7 |
| | 2 | 15 |
| | 6 | 0.2 |
| | 5 | 4.0 |
| | TEMED | 10 µl |
| | $H_2O$ | 6.1 |
| Stacking gel | | |
| | 1 | 1.7 |
| | 3 | 1.25 |
| | 6 | 0.05 |
| | 5 | 1.0 |
| | TEMED | 2.5 µl |
| | $H_2O$ | 6.0 |

3. Fix-stain solution (Weber and Osborn, 1969)

| | |
|---|---|
| Coomassie blue | 1.25 g |
| 50% methanol | 454 ml |
| Acetic acid (glacial) | 46 ml |

Destain solutions:

| | |
|---|---|
| Methanol | 50 ml |
| Acetic acid (glacial) | 75 ml |
| $H_2O$ | 875 ml |

## Appendix IV Statistical Analysis

### Regression analysis

All calculations were performed on a Novus 6030 Statistician calculator (National Semiconductor Corporation, California employing the following formulae:-

correlation coefficient (r)

$$= \frac{\Sigma xy - \frac{\Sigma x \, \Sigma y}{n}}{\sqrt{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right]\left[\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right]}}$$

gradient (m)

$$= \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

y-axis intercept (c)

$$\frac{\Sigma y - m \, \Sigma x}{n}$$

### t-Test

All calculations were performed on the above calculator employing the formla:

$$t = \frac{\left|\bar{x}_A - \bar{x}_B\right|}{\sqrt{\frac{S^2_A}{n_A} - \frac{S^2_B}{n_B}}}$$

Where $\bar{x} = \frac{\Sigma x}{n}$

$S = \sqrt{\frac{\Sigma(x - \bar{x})^2}{(n-1)}}$

$n$ = the number of observations in a sample.

The found t statistic was compared with the tabulated values of t for $n_A + n_B - 2$ degrees of freedom.

Mann-Whitney U-test

The data in each sample were arranged in ascending order and to each was assigned its rank in the joint ordering of the two samples. The ranks for each sample were totalled, giving $R_1$ and $R_2$. $U_1$ and $U_2$ were calculated by the formulae:

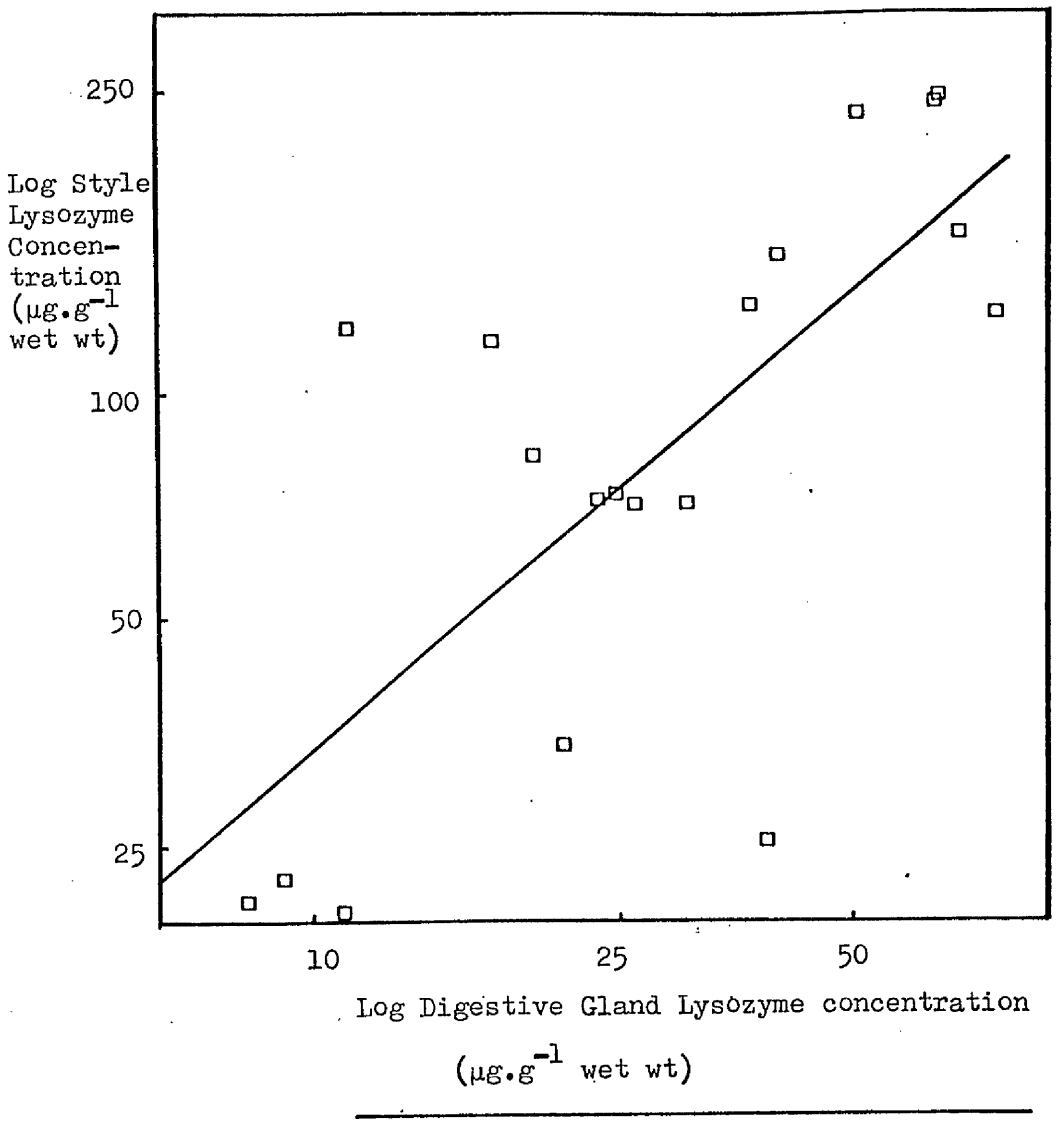
$$U_1 = n_1 n_2 + \frac{1}{2} n_2 (n_2 + 1) - R_2$$

$$U_2 = n_1 n_2 + \frac{1}{2} n_1 (n_1 + 1) - R_1$$

The significance test was performed by noting whether either $U_1$ or $U_2$ was less than or equal to the tabulated value U; if not, it was concluded that the differences were not significant.

# Appendix V

## Correlation of Lysozyme Concentrations in Style and Digestive Gland



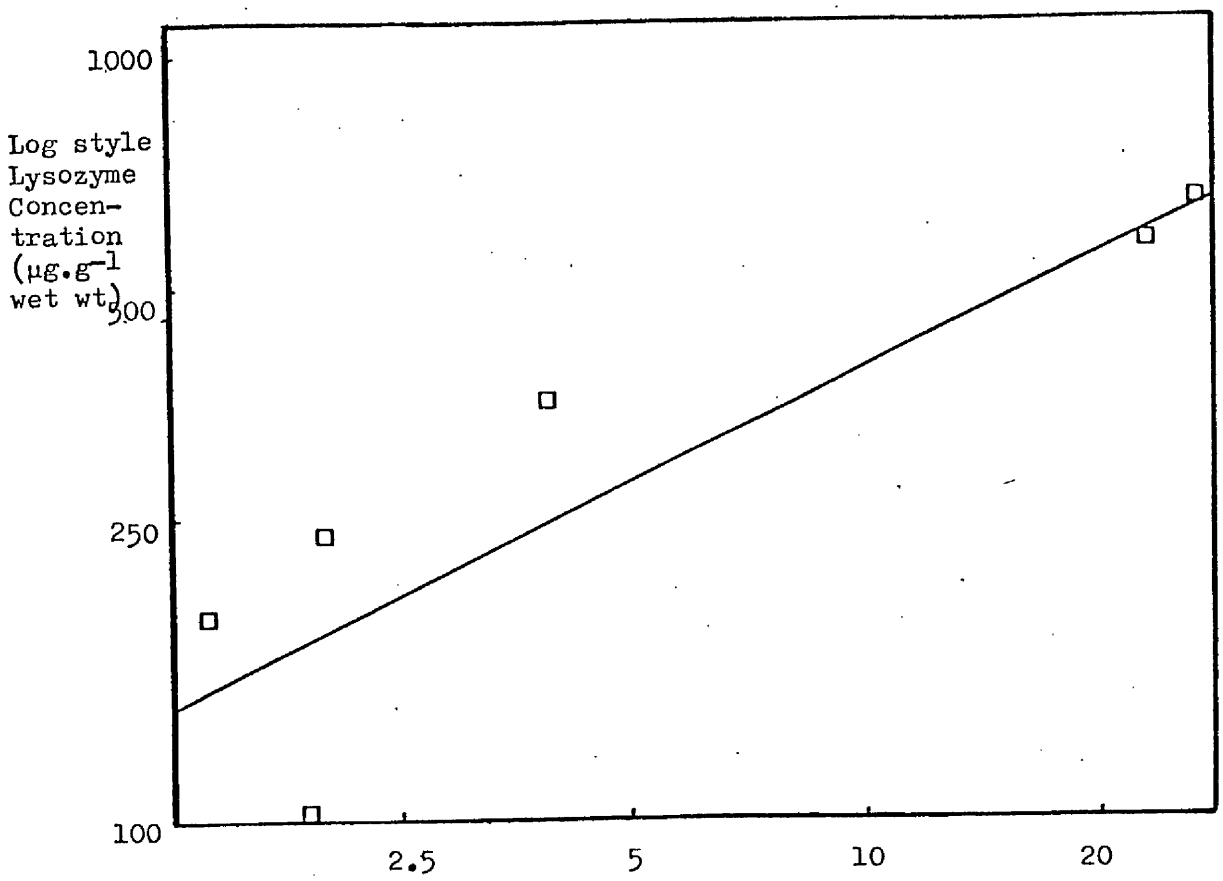

Mytilus edulis

Regression analysis parameters:

r = 0.7194

m = 0.8587

C = 0.6716.




---

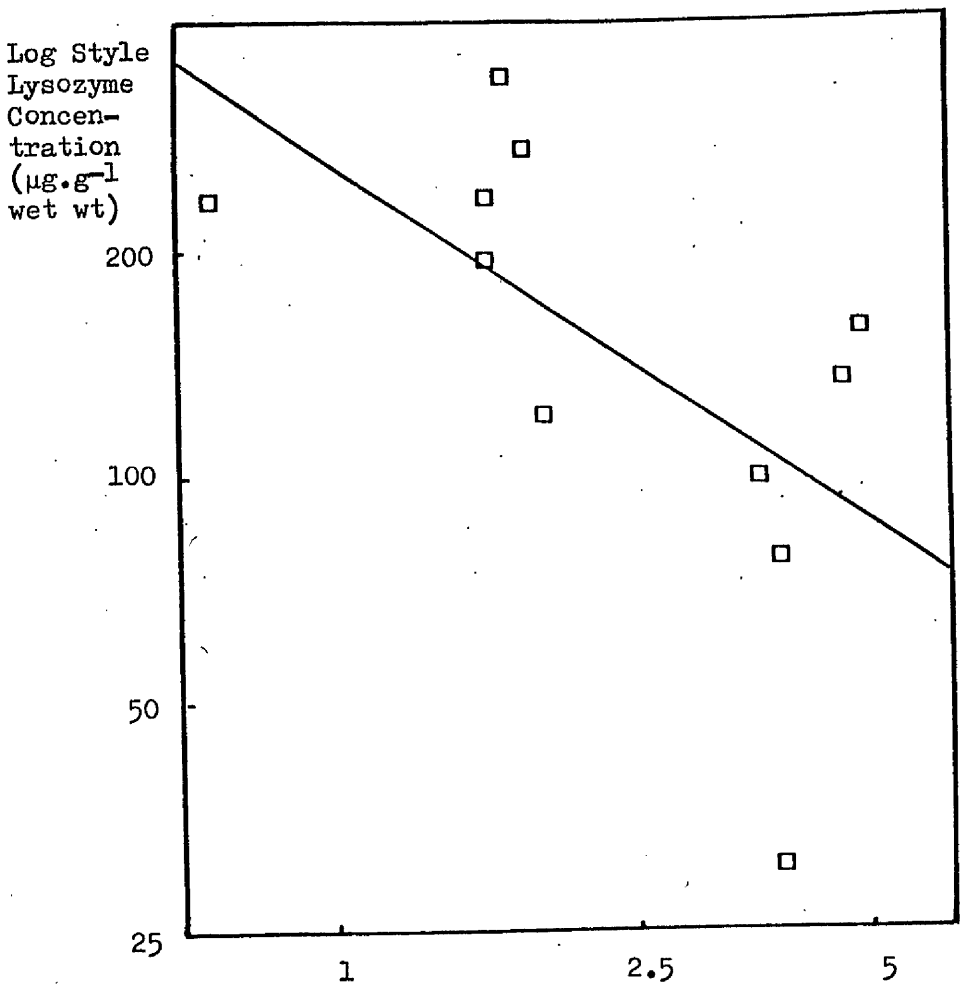
Modiolus modiolus

Regression analysis parameters:

r = 0.9152

m = 0.4867

c = 2.0998




---

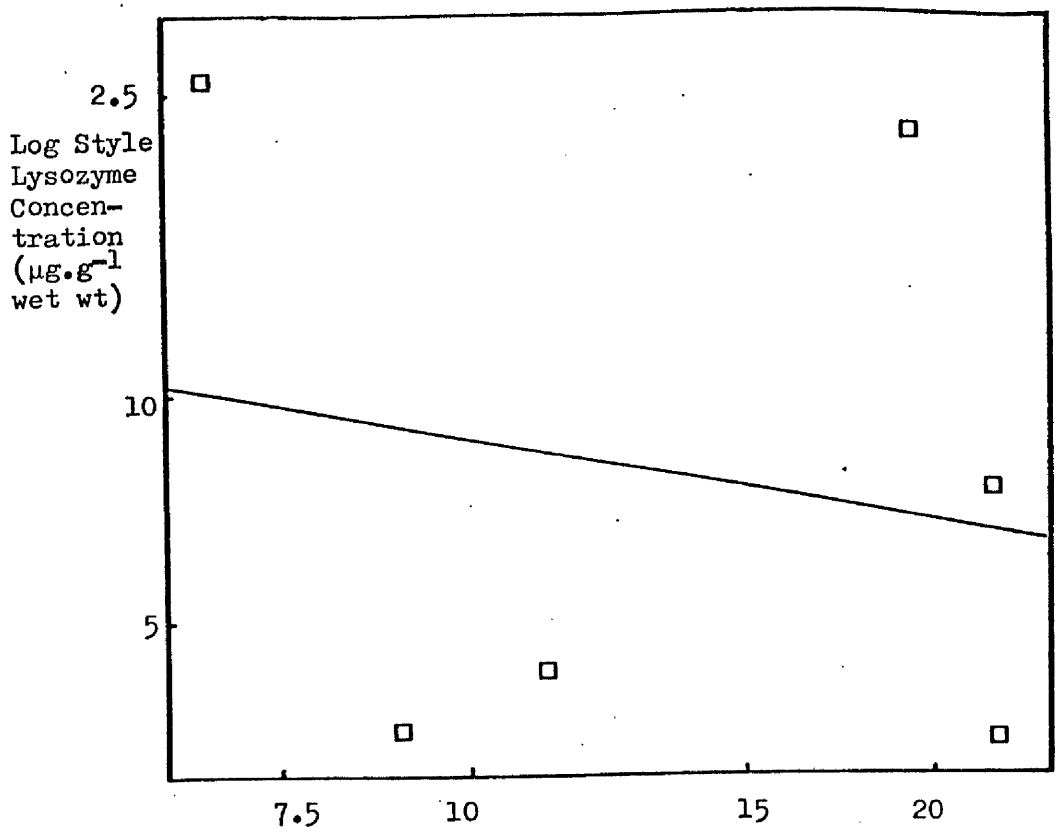
Chlamys opercularis

Regression analysis parameters:

r = 0.6016

m = -0.6864

c = 2.4127




---

Mya arenaria

Regression analysis parameters:

r = 0.2000

m = -0.3592

c = 1.301