Studies on the Analysis of Ecdysteroids and on the Seasonal Variation of the Moulting Hormones of the Barnacle, *Balanus balanoides*.

by

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A thesis submitted to the University of Keele
for the Degree of Doctor of Philosophy.

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ABSTRACT

The separation properties of a number of ecdysteroids were examined. The ecdysteroids themselves were separated by thin layer and reverse phase thin layer chromatography. Trimethylsilyl ethers of the ecdysteroids were prepared by reaction with trimethylsilyl-imidazole under varying conditions of time and temperature. The analysis of the trimethylsilyl ethers was examined under thin layer chromatography and gas chromatography with an electron capture detector. Using the data from the chromatographic analysis of the ecdysteroid derivatives, Makisterone A was selected as the most suitable internal standard to use in the extraction scheme.

An analytical scheme adequate to detect the very low levels of ecdysteroids in an average population of barnacles was devised. The method permitted the use of relatively small samples (500 g. or less) of barnacle Balanus balanoides L. The method incorporated the use of solvent partitions, sephadex gel column chromatography, thin layer chromatography and gas chromatography. Using this scheme it was found that the levels of the predominant ecdysteroid, 20-hydroxy-ecdysone, vary seasonally with peaks occurring in the spring and late autumn. This variation corresponded tc the variation in moulting frequency, and is fully discussed in connection with the sea temperature, availability of food and the breeding cycle of this species of barnacle.

In field experiments at Portsmouth, live barnacles were injected either with sea water, pure ecdysone, pure hydroxyecdysone and a crude ecdysteroid fraction isolated from barnacles. Ecdysteroids did produce an increase in moulting frequency, but the effect was not easy to analyse.

Unless otherwise stated, all the work reported in this thesis was carried out by the author under the supervision of Dr. E.D. Morgan.

ACKNOWLEDGEMENTS

I should like to acknowledge the following people whose contribution made this thesis possible.

Dr. E.D. Morgan for his patience, guidance and encouragement throughout the course of this study.

The Science Research Council for financial support.

Mr. D. Houghton and the staff of the Exposure Trials Station, Eastney, Portsmouth.

Dr. I.D. Wilson for his unfailing interest and constant supply of ecdysteroids.

Mr. R. Dix and Mr. P. Holbrook for technical assistance.

Mr. C. Cork for glassware. T. Bolam for photography.

Mr. R. Pattison and Mr. D. Oliphant for efficient and prompt service in the stores department.

Mr. K.T. Alston, Mr. D. Mountford, Mr. J. Clews and Mr. S. Hudson, for technical support above and beyond the call of duty.

Mrs. I. Jones and Mrs. M. Furnival for typing of this thesis.

The technical staff of the Department of Biology.

The academic staff and my colleagues present in the Department of Chemistry, during the course of this research, for their patience and understanding in the presence of a lowly 'biologist'.

The Senior Meterological Officer at R.A.F. Valley, Anglesey, for the monthly weather reports.

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INTRODUCTION

Antifouling Research

The problem of fouling of submerged surfaces arises from the settlement of larvae and spores of marine organisms that develop into sedentary adults. These fouling communities include species of algae, hydroids, tube worms, molluscs, sea squirts, polyzoans and barnacles. Apart from acceleration of corrosion and damage to coatings, their most deleterious effect is the increase in the frictional resistance in ships' hulls. This effect, which can result in the loss of several knots from designed top speeds, has been calculated to increase fuel consumption by up to 40% (Anon, 1952). Houghton (1970) estimated that this increase in fuel consumption plus the charges for docking and re-coating and the subsequent loss of earnings cost in the region of £50 million in the U.K. and $500-700 million in the United States. A more recent estimate by Gitlitz (1981) calculated that a very large cargo carrier running at 15 knots during a 300 day operational year consumes over $4 million of fuel. To maintain its speed when moderately fouled the fuel consumption is increased by 30% at a cost of over $1 million! Fouling, though, is not restricted to ships, industrial coastal installations, particularly sea water intakes, can have serious fouling problems.

The objective of antifouling research is to develop means to control and prevent the settlement and growth of marine organisms on surfaces exposed to the marine environment. Methods such as electrolytic release of chlorine, ultrasonic vibrations, air bubble release and radioactive coatings have had limited success but none have proved

as satisfactory as the use of toxic laden coatings or paints. Many such paints, although very effective, have a limited life or have in turn been deemed unacceptable due to their deleterious effect upon man and the environment. A wide range of candidates proposed as antifouling agents have been reviewed by De la Court and De Vries (1973), Aras (1980) and Gitlitz (1981) and it would appear that future paints will probably incorporate organometallic polymers, particularly organotin, to ensure that coatings will have a wide spectrum of activity and a longer life than current systems. Another new development reverts back to the removal of antifoulants by mechanical means by scrubbing the surface with the use of a robot. This robot is a small craft that magnetically clings to ships' hulls but can traverse the surface under its own power viewing the area using cameras situated at the front and rear of the machine. Any area located that is fouled is then 'scrubbed' clean with a rotating brush located on an extendable arm at the front of the machine (Anon, 1982). This method is economically viable as the ship need not be dry docked for scrubbing.

Of all the fouling species barnacles are the most ubiquitous and approximately forty species have been recorded from fouling communities on ships. Southward and Crisp (1963) have catalogued those species that are from European waters. Barnacles are one of the major fouling species as they are among the first hard shelled organisms to settle on any submerged surface denuded of antifouling coating and, although the cypris is the initial fouling stage, it is the calcareous shell of the adult barnacle that increases the drag on a ship's hull or blocks water intakes.

Studies on barnacles and other major fouling species were initially concerned with the conditions of the submerged surfaces that affected the selection and settlement of the organisms and the effects of climatic, geographical and seasonal factors. However, recent research has been more concerned with the study of the biochemistry and physiology of fouling species specifically their metabolism, development and modes of attachment throughout their life cycles. It was proposed that these studies might result in the development or discovery of a controlling factor capable of disrupting their life cycles.

In the Department of Chemistry at Keele University a study was conducted by Bebbington (1975) that resulted in the discovery of the presence of two moulting hormones, or ecdysteroids, in the barnacle Balanus balanoides (Linnaeus). This result and the study of Tighe-Ford (1974) on the hormone action in barnacles suggested the possibility of a hormonal control factor in the prevention of barnacle fouling. This present project was devised to determine any seasonal variation in ecdysteroid production and relate the results to the known variation in moulting and the breeding cycle of one particular barnacle, Balanus balanoides.

The Barnacle, Balanus balanoides (Linnaeus)

The barnacle, Balanus balanoides is a common intertidal species of the class Cirripedia, a diverse and abundant group of sedentary crustaceans that are found in virtually all marine environments. This particular barnacle is found all round the coast of the United Kingdom, except for a few small areas, and on both sides of the Atlantic Ocean. At low tide, a conspicuous white band or 'balanid zone' is often visible on rocks and cliffs where B. balanoides is found, sometimes in competition with the immigrant australasian barnacle, Elminius modestus (Darwin), or Chthamalus stellatus (Poli). This northern hemisphere species, B. balanoides, has been studied extensively primarily due to its economic importance as a member of the marine fouling communities and is the subject of a detailed monograph by Stubbings (1975).

(i) Classification

The Crustacea is a sub phylum of the phylum Arthropoda and all members are characterised as such by possessing a chitinous exoskeleton which is flexible at intervals along the limbs to form joints, a specific limb arrangement, a peri-visceral blood space or haemocoel and a ventral nervous system. The Cirripedes, a class of the Crustacea, are classified by their aquatic mode of respiration, the arrangement of antennae and mandibles and in possession of a nauplius larval stage. The Cirripedia is sub-divided into four orders one of which, the Thoracica, contains the genus Balanus and the species B. balanoides the common intertital acorn barnacle (McLaughlin, 1980).

(ii) Development and Growth

This barnacle is hermaphroditic, fully functional male and female reproductive organs being present in all individuals, and cross fertilisation is obligatory. Barnes and Crip (1956) stated that there is no evidence of self-fertilisation in isolated individuals of this species. In Great Britain B. balanoides has a single breeding season restricted to late autumn and early winter. The acting male copulates with a neighbouring female by inserting its extended penis into the mantle space and depositing a mass of sperm, fertilisation occurs once the sperm have penetrated the elastic sac and reached the ova. Most individual mature adult barnacles at one locality are fertilised within a few days of one another. The actual time of fertilisation is dependent upon the position of the barnacles on the shore, the degree of exposure and latitude of that shore and can vary from August to November (Pyefinch, 1948). In North Wales fertilisation occurs in early November according to Crisp (1959).

The eggs are incubated throughout the winter in two compact masses in the mantle, below and at the sides of the body or prosoma (Barnes, 1957), for a period lasting three to four months depending upon the latitude, site, and shore position of the barnacle relative to mean tide level. The eggs and already hatched larvae are released from the mantle cavity in response to the activity of the contained larvae (Stubbings, 1975) from March onwards. The larvae are stimulated by a 'hatching substance' liberated from the adult after it has fed on plankton from the spring bloom (Crisp, 1956). This ensures that an adequate food supply is available for the newly released barnacle larvae and the hatch is generally synchronous with the diatom spring bloom (Barnes, 1957).

The barnacle embryo hatches into a dispersal larval form, the free swimming, planktonic stage I nauplius larva which, by a succession of moults or ecdyses, passes through a series of six naupliar stages, each being larger and more complex than the preceding one, before metamorphosing into the cypris larva. It is the free swimming cyprid that actively seeks out a suitable site for permanent settlement, becomes attached and undergoes the final metamorphoses into the sedentary, juvenile barnacle (see figure 1). The larvae of several species of barnacles, including B. balanoides, have been reared in the laboratory with varying degrees of success according to Tighe-Ford (1970) and Cheung (1973).

The cyprid, or settlement stage larva, adheres onto the substratum by exuding a quantity of 'cyprid cement' from the paired cement glands to embed the attachment organs, the terminal parts of the two antennules, to form permanent attachment (Walker, 1981). After metamorphosis to the juvenile, attachment is still initially by cyprid cement and it is some forty days or more, before the developing adult cement apparatus first releases its adhesive cement. Biochemical analysis has shown that the major component of this adhesive, in excess of 70% w/w of the adult cement, is protein (Walker, 1981).

Four days after the attachment of the cyprid the six shell plates and four opercular plates of the adult barnacle develop (Stubbings, 1975) and can be clearly seen in figure 2. These structures, which give the adult its characteristic appearance, are retained throughout the life of the barnacle, the calcareous shell growing continuously by deposition of material. Internally growth occurs by the periodic moulting and ecdysis of the exoskeleton. The moulting process involves the secretion of a new cuticle beneath the old, which are then separated

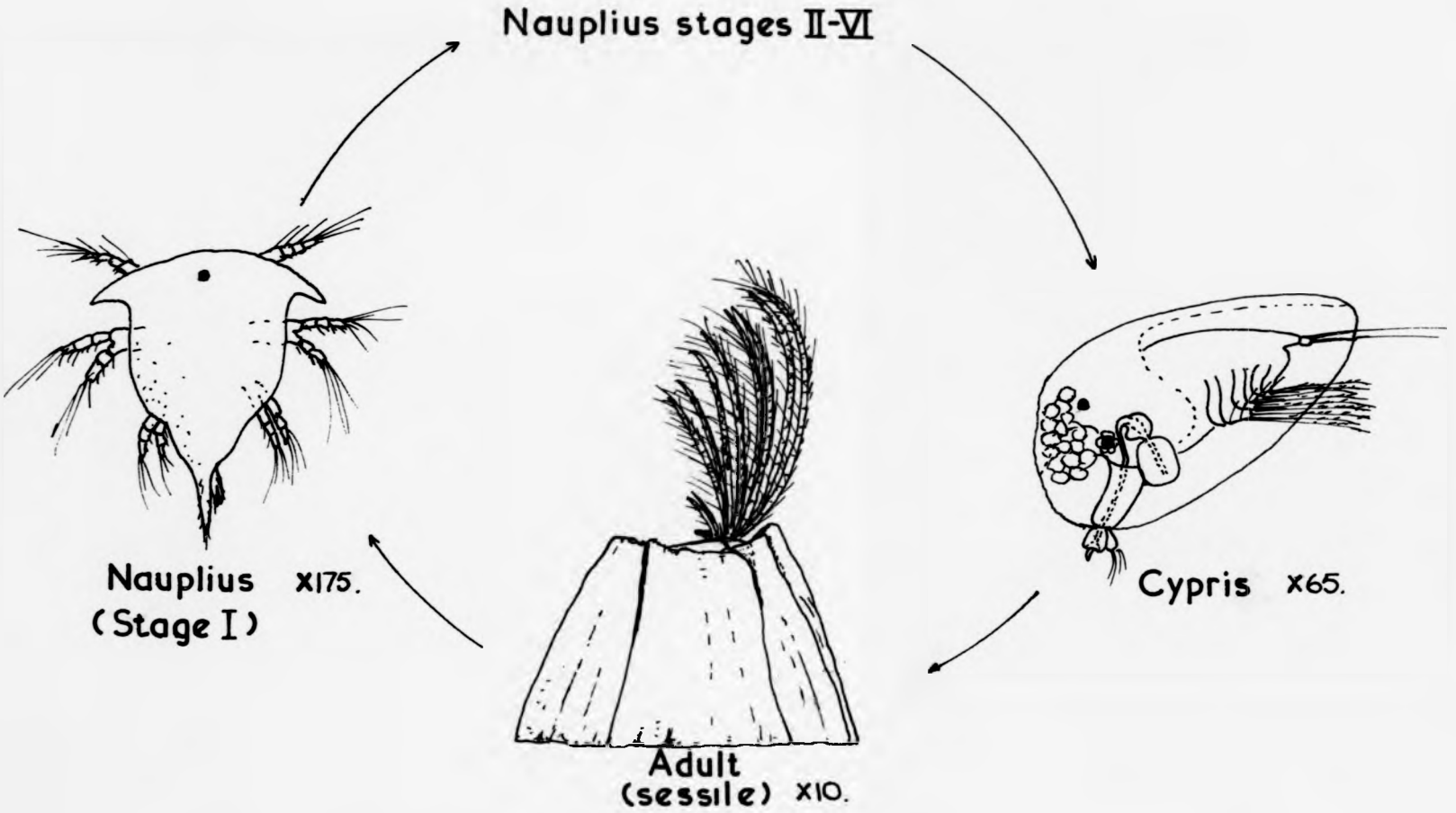

Figure 1. Life Cycle of the Barnacle, Balanus balanoides (L)

Figure 2. The adult barnacle, Balanus balanoides

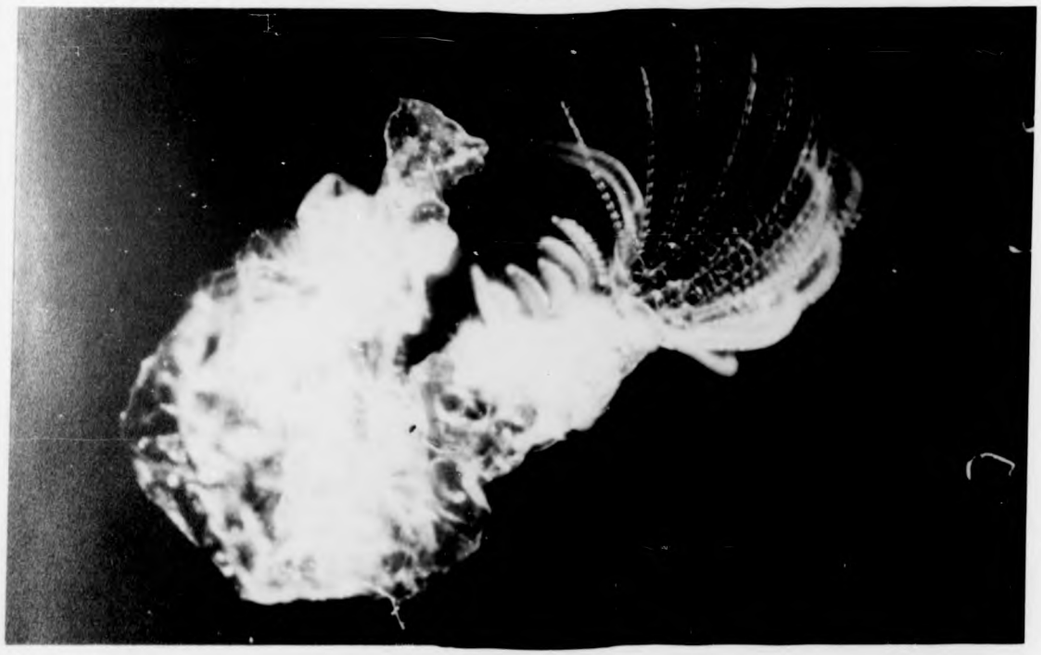

Figure 3. The cast or exuvia of an adult barnacle

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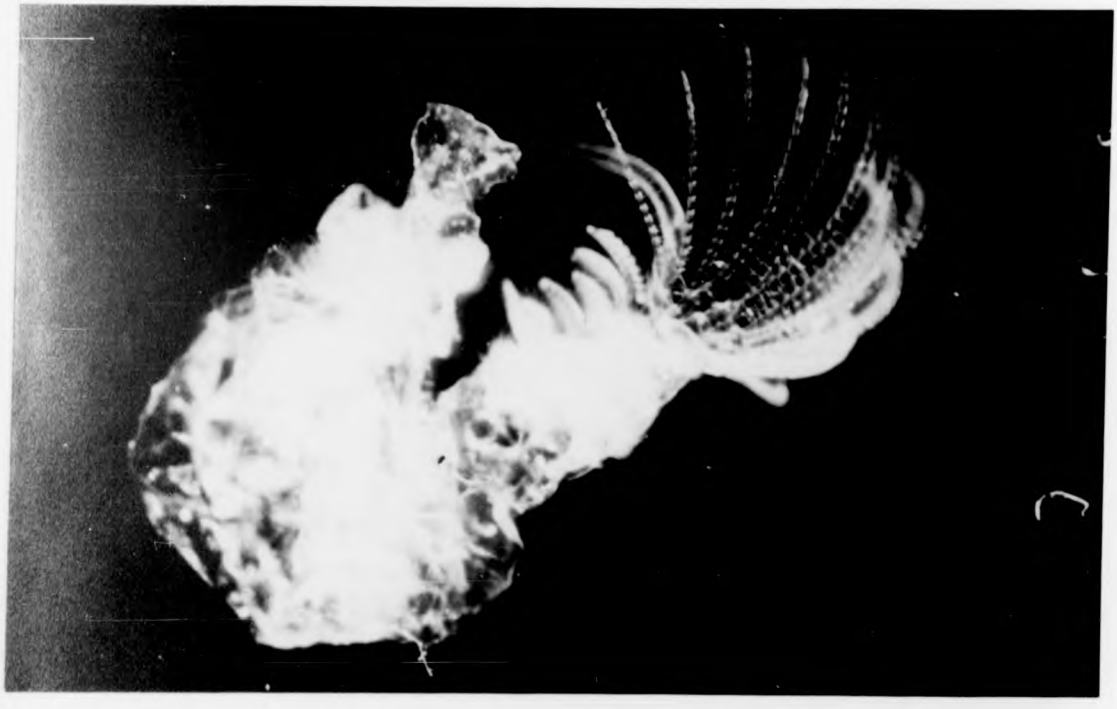

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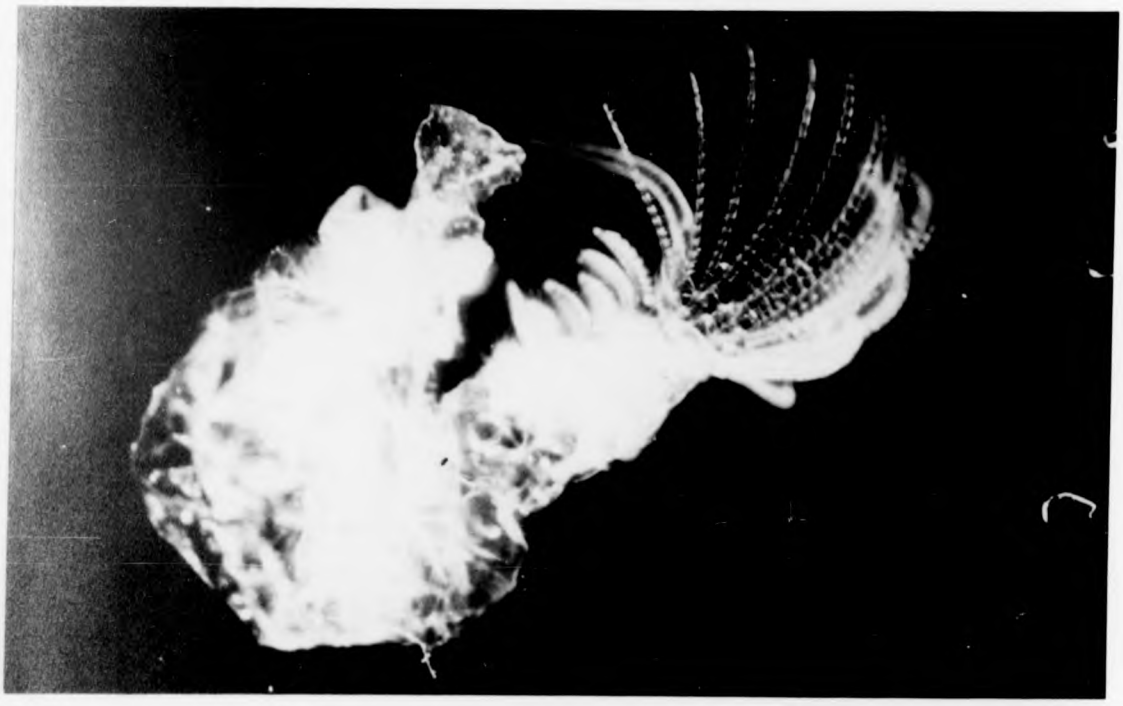


Figure 3. The cast or exuvia of an adult barnacle

by apolysis (Jenkins, 1966) and the old cuticle is finally shed at ecdysis by hydraulic pressure generated by the pumping action of the barnacle (Cheung, 1973). The new cuticle expands and hardens later. The cast consists of the ecdysed cuticle from the mantle lining, the gut, the prosoma, penis and thoracic appendages. It is the exoskeleton of the thoracic appendages or cirri that give the distinct fan-like structures of the exuviae that can be seen in aquaria containing barnacles, one such cast is shown in figure 3.

The moulting frequency of the barnacle, B. balanoides, has been shown by Crisp and Patel (1960) to be influenced by temperature, feeding and the season but not by lunar cycles or the tidal level. As with typical crustaceans there is also a close relationship in this barnacle between the moulting and breeding cycle (Crisp and Patel, 1958 and Patel and Crisp, 1961). Crisp and Patel (1960) studied the seasonal variations in the frequency of moulting of B. balanoides for three years by monitoring groups of barnacles kept in the laboratory and described an annual moulting rhythm in this species. Their results show that there was a sharp decrease in moulting frequency after fertilisation in late autumn followed by a period of little or no moulting, anecdysis, for at least two months during the winter (this period has been retitled as 'reproductive anecdysis' by Barnes, 1962). The moulting frequency increased throughout spring to a maximum by May of 8-12 moults/100 barnacles/day, followed by a slight decrease during the summer and then a rise to the second peak in October-November. This seasonal variation in moulting frequency and the relationship with the breeding cycle and the levels of free ecdysteroids found in the barnacle is dealt with in depth in the discussion.

(iii) Ecdysteroids in Barnacles

Ecdysteroids, or moulting hormones, were first isolated from the barnacle B. balanoides by a large scale extraction procedure by Bebbington (1975) who modified the technique used by Horn et al. (1968) for the extraction of moulting hormones from crustacea. From 1500 kg wet weight of adult barnacles Bebbington isolated and characterised two ecdysteroids, ecdysone and 20-hydroxyecdysone, at levels of 6 ng kg^{-1} and 1 $\mu g\ kg^{-1}$ respectively.

One of the aims of this project was to develop a simple but efficient extraction procedure to analyse the levels of ecdysteroids in small barnacle samples, of between 500 g and 1 kg wet weight, collected at regular intervals, to determine any seasonal variation in ecdysteroid production and to determine the presence of any conjugated ecdysteroids. If successful in determining any seasonal variation it was hoped that this could be related to the annual moulting cycle and possibly find some use in the field of antifouling research.

Arthropod Exoskeleton

A definitive feature of all members of the phylum Arthropoda, which includes the sub-phyla Insecta (insects), Arachnida (spiders, scorpions and mites), Merostomata (horseshoe crabs) and Crustacea (crayfish, lobsters, shrimps, crabs and barnacles), is the presence of an external covering or exoskeleton composed of a chitinous cuticle. By development of an organised cuticle, the arthropods have acquired a relatively rigid exoskeleton which provides a framework for the soft tissues within and an attachment surface for muscles allowing for the development of complex movements and specialised limbs. The cuticle also provides a degree of protection both as armour and in the prevention of dessication, specifically in terrestial arthropods.

The cuticle is a non-living layer secreted by the underlying epithelium and in general is composed of three layers; the inner endocuticle or flexible layer; the rigid exocuticle which can be hardened to varying degrees; and the outer epicuticle, a thin waxy layer. The cuticle is primarily a complex of a mucopolysaccharide, chitin, and proteins. It is found in its simple flexible, but non--elastic, form in insect larvae but more often the cuticle is hardened by the process of scelerotisation where the protein molecules are cross-linked to give a tough, inflexible and darkened cuticle. To maintain flexibility at joints the cuticle is neither hardened or reinforced at these points.

In contrast to most other arthropods, the exoskeleton of crustaceans is usually hardened by the deposition of calcium carbonate. All gradients of thickness can occur from the flexible non-calcified parts of the body, or prosoma, of cirripede barnacles to the thick calcified armour plate of some of the decapods, crabs and lobsters.

In crustaceans, as with all arthropods, some of the internal organs may be lined with the exoskeleton and this occurs in the fore and hind gut, the stomadeum and protodaeum.

The Moulting Process

Growth cannot occur continuously throughout life in arthropods because of the confinement of the rigid exoskeleton and any change in form or increase in size can only occur after the old cuticle is shed or ecdysed and before the underlying new cuticle is hardened. This is the moulting process in which two essential features are involved; the dissolution of the inner layers of the exoskeleton with the reabsorption of some of the components, and the growth of a new exoskeleton in the region between the living cells of the epidermis and the remaining cells of the old cuticle. At ecdysis this old exoskeleton is shed.

The term 'moulting' refers to all the processes of preparation up to, and including, the withdrawal from the old integument at ecdysis, and have been described most fully in the crustacea for brachyuran decapods, the crabs. Passano (1960) presented a modification of Drach's (1939) scheme that classifies the moult cycle into five stages, A to E with subdivisions at each stage, and relates changes in the layers of the integument with the progression of the moult cycle. These stages have been adopted in studies of moulting in crustacea and can be generalised as follows: Stage E, or ecdysis, is the short period when the old cuticle is shed; Stages A and B, the early and late postecdysis, metecdysis or postmoult during which the newly exposed exoskeleton is hardened; Stage C, interecdysis or intermoult which is generally referred to as the period of normality and; Stage D, the proecdysis or premoult during which the epidermal

cells separate from the cuticle, apolysis, and the new exoskeleton is laid down beneath the old one. Davis et al., (1973) also used the criteria of Drach (1939) to stage the moulting cycle of the barnacle, Balanus amphitrite by analysing the rigidity of the cirri and noting, from staining, the integumental changes in the two layers found in the dissected rami that were assumed to be homologous with the endocuticle and exocuticle in other arthropods. The results confirmed that cirripedes follow the general moulting pattern found in other crustaceae.

Moulting Frequency

Although many of the primitive apterygote insects (e.g. silver fish) moult frequently as adults, in the pterygotes (grasshoppers, ants, butterflies, etc.) moulting is generally associated with metamorphic development through a series of instars from the larvae to the imago and ceases on attaining the adult stage. Similarly in crustaceans some species cease moulting after the puberty moult such as the spider crab, Maia squinado (Carlisle 1957) and others undergo a fixed number of moults then remain in the intermoult stage as does the lined shore crab, Pachygrapsus crassipes (Marshall and Williams, 1975). The shore crab, Carcinus maenas continues moulting for three years after its puberty moult whereas the edible crab, C. pagurus, and the lobster, Homarus vulgaris, continue growing and moulting until their death (Highnam and Hill, 1969). Cirripede barnacles, after metamorphosing through naupliar and cyprid larval stages, likewise moult continuously throughout their adult life although some variation in moulting frequency may occur seasonally (Crisp and Patel, 1960). In barnacles the moulting process is different from

that found in decapods as the calcified shell grows continuously throughout the adult's life and is not shed during ecdysis.

Endocrinology of Insect Development

The first results of experiments that inferred that moulting in insects is under hormonal control were published by Kopec (1922) who, by ligating caterpillars of the gypsy moth, Lymantria dispar, halfway along their length, noted that the anterior half pupated but the posterior half remained unchanged in the larval form. Also extirpation of the brains of these caterpillars resulted in the failure of continued metamorphosis to the pupal stage, but if the nerve cord was cut just behind the head pupation and further development was not affected. Kopec concluded that the brain was a hormone source. Similar results were obtained by Fraenkel (1935) using the blowfly Calliphora erythrocephala. The concept of hormones governing growth and moulting arose from the work by Wigglesworth on the blood sucking bug Rhodnius (for a full review see Wigglesworth, 1970).

Although researchers had studied moulting and the effects arising from ligation, extirpation and implantation, Fraenkel (1935) was the first to induce moulting by injection of a substance with proposed moulting activity. Tanning of the cuticles of ligated Calliphora abdomens was induced by the injection of haemolymph from pupariating fly larvae. This test, in fact, was developed as the first bioassay to monitor the activity of proposed moulting hormones or ecdysteroids.

From these and many other studies the current view on the endocrine control of insect development has arisen and can be summarised in general terms as follows. The neurosecretory cells of the brain are stimulated to produce a hormone (thoracotrophic

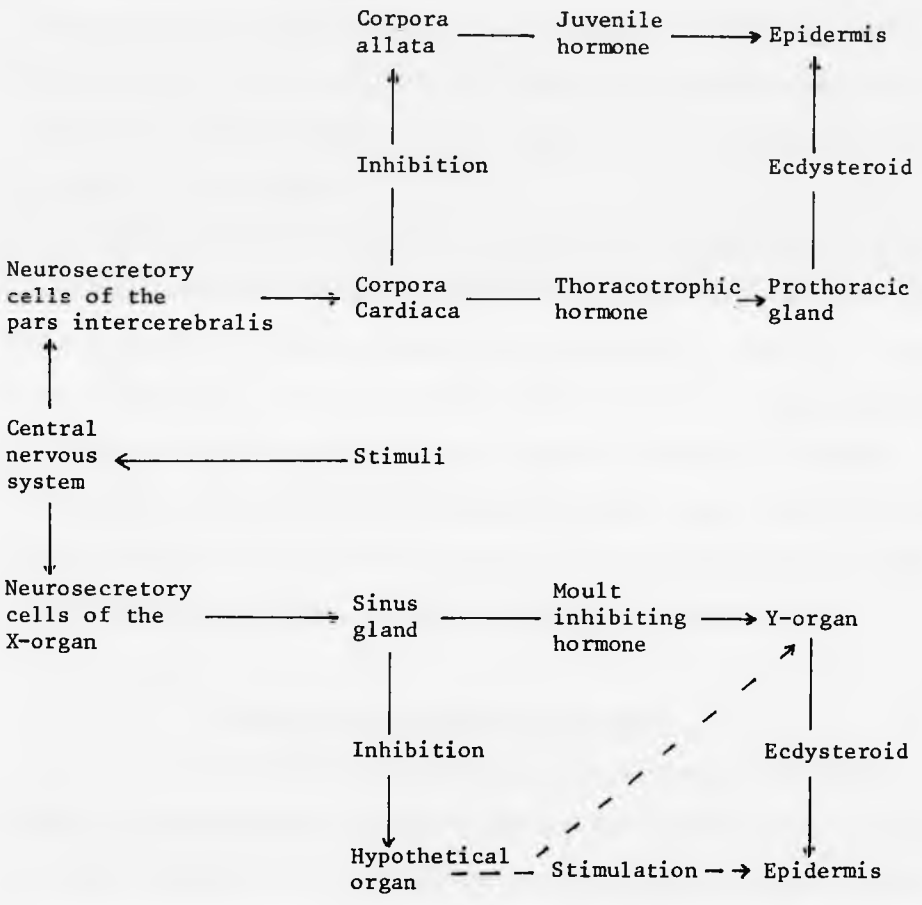
hormone) that via the neurosecretory cell axons is carried to the corpora cardiaca from where it is released into the blood. This stimulates the thoracic glands (or their equivalent) to produce a moulting hormone (an ecdysteroid) which causes the epithelial cells to begin the processes which lead to ecdysis. At each moult, except the last, another hormone (a juvenile hormone or JH) is secreted by the corpora allata to ensure the development of another larval instar. The corpora allata are inactive in the last moult and in the presence of the moulting hormone alone an adult is produced (see figure 4). In species where moulting ceases once the adult form is achieved, the prothoracic glands degenerate (Hoffmann et al., 1980).

Endocrinology of Crustacean Development

In crustaceans most of the research concerning the moulting processes has been conducted using adult decapods, crabs and lobsters, and little is known concerning the endocrinology of other orders and of larval stages. The initial interest in moulting in crustacea arose when several authors recorded that eyestalk removal often had a moult-inducing effect and it was proposed that a region of the eyestalk was the source of a moult-inhibiting hormone (see Passano, 1960). From results of selective destruction, or implantation, of tissues in the eyestalk it was deduced that the eyestalk neurosecretory system, composed of the X-organ and the neurohaemal organs, the sinus glands, produces a moult inhibiting hormone (MIH). This MIH, however, did not act directly upon the tissues to delay moulting. Results of removal or reimplantation of a pair of endocrine glands, the Y-organs found in the head of decapods, indicated that these were the site of production of a moulting hormone that acted at the tissue level.

Figure 4. Control of moulting in Crustaceans and Insects (after Highnam and Hill, 1969; Cheung, 1973 and Gilbert et al., 1980).

(a) Insects



(b) Crustaceans

(Broken lines - hypothetical or unconfirmed)

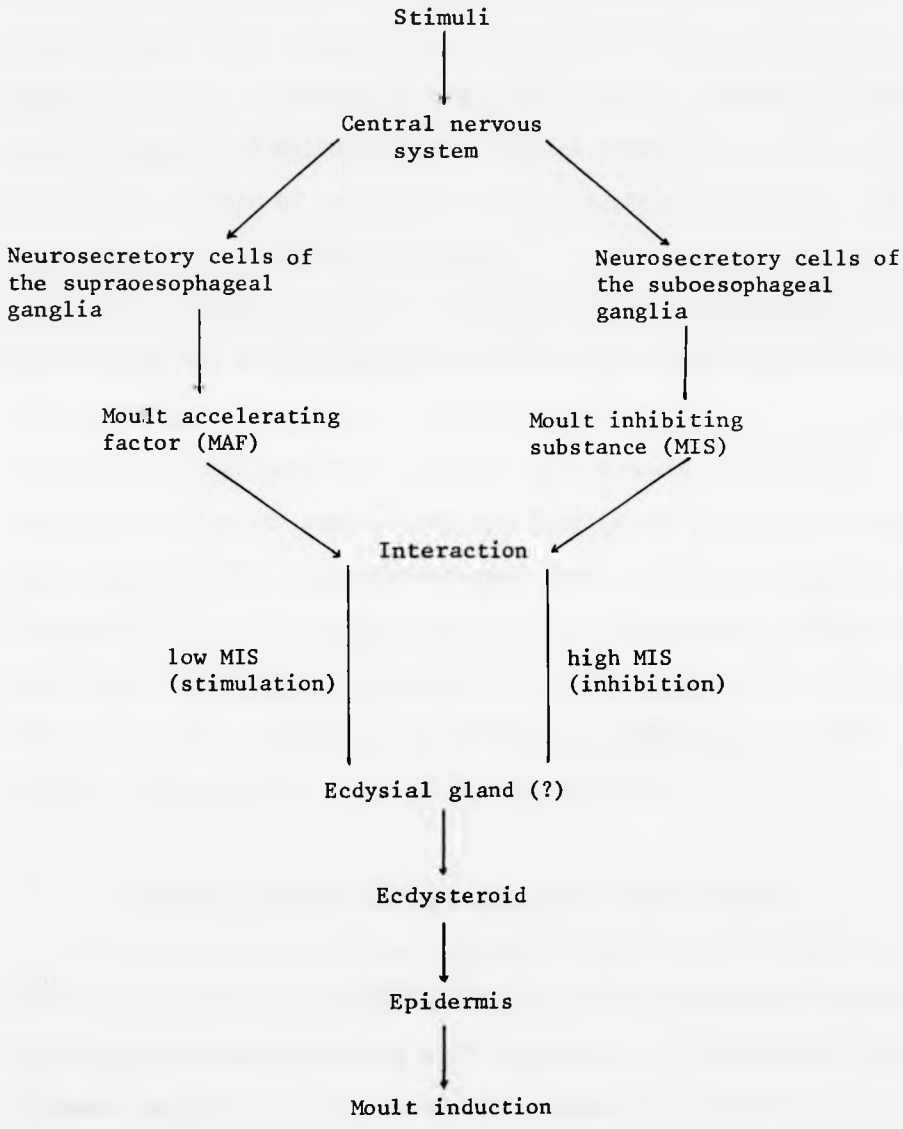
Thus the present view is that a MIH is produced and released from the X-organ-sinus gland complex that regulates the synthesis and release of a moulting hormone from the Y-organ. This inhibition maintains the level of ecdysteroid in the haemolymph at a low titre which keeps the crustacean in an intermoult state. Conversely a decrease in production and secretion of a MIH, by environmental stimuli or eye-stalk ablation, allows the Y-organ to produce and secrete a moulting hormone into the haemolymph to such a level that the onset of moulting is initiated (see figure 4).

It is clear that the endocrine systems controlling moulting in insects and crustaceans are comparable, the thoracic glands and Y-organs being analogous as sites of ecdysteroid production. However, in insects the thoracotrophic hormone stimulates the production of ecdysteroids in the thoracic gland in preparation for the moult, whereas in decapod crustaceans the neurosecretory hormone from the X-organ complex inhibits the activity of the ecdysteroid producing Y-organ during the intermoult period (Highnam and Hill, 1969 and Spindler et al., 1980).

Control of Moulting in Cirripedes

In cirripedes the X-organ complex and the Y-organ that are present in decapods have not been found but the presence of neurosecretory cells has been demonstrated. Barnes and Gonor (1958, a and b) first demonstrated the presence of possible neurosecretory cells in cirripedes using a paraldehyde-fuchsin (PAF) stain and found two types of cells containing stained granules in the suboesophageal and supraoesophageal ganglia of the central nervous system (CNS) of several species of barnacles. These results were confirmed by McGregor (1967) and Cheung (1973) using general histochemical stains for neurosecretory cells, PAF and chrome-haemotoxylin-phloxine B (CHP).

Figure 5. Proposed mechanism for the moulting process in cirripedes (after Cheung, 1973)



Evidence of neurosecretory activity was also obtained by Sandeen and Costlow (1961) who demonstrated that extracts of CNS of various barnacles dispersed the pigment in melanophores of eyestalkless fiddler crabs, Uca pugilator. This work was expanded by Costlow (1963) who found that using aqueous extracts of the CNS taken from the barnacle, Balanus eburneus, at different times after ecdysis resulted in differential expansion of melanophores in fiddler crabs during intermoult. This cyclic pattern of chromatophototropin activity suggested a cyclic production of the factor(s) involved.

Cheung (1973) studied the cyclic changes of the neurosecretory cells in the CNS of B. eburneus and correlated the moulting process with cyclic neurosecretions. Results from histochemical and implantation techniques have suggested that a moult accelerating factor (MAF) from the supraoesophageal ganglia and a moult inhibiting substance (MIS) from the suboesophageal ganglia interact to affect the production of an ecdysteroid from an, as yet undiscovered, ecdysial gland (see figure 5). The presence of an MIS has been confirmed by Davis and Costlow (1974) in the barnacle, B. improvisus, but they found no evidence for a moult accelerating factor.

Isolation and Characterisation of Moulting Hormones

The levels of moulting hormones present in arthropods in general is very low and particularly low in crustaceans and the initial extraction procedures required very large amounts of biological material. Butenandt and Karlson (1954) devised a scheme for isolating and concentrating the active substance from insects and obtained 25 mg of a crystalline compound, which they named ecdysone (α-ecdysone), from 500 kg of silkworm, Bombyx, pupae. They also extracted a second,

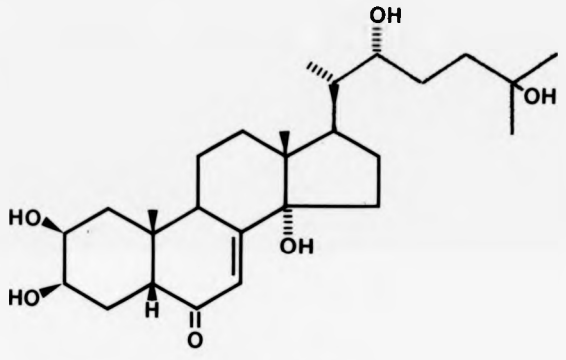
more polar and more active substance in smaller quantities which they named β-ecdysone. The quantities extracted were too small for accurate chemical analysis and ten years later, from a further 1000 kg dry weight of Bombyx pupae, 250 mg of ecdysone was obtained. The compound was found to have a molecular weight of 464 with an empirical formula of $C_{27}H_{44}O_6$ and x-ray crystallography showed that the fundamental structure was that of a steroid (Karlson et al., 1963). The elucidated structure of ecdysone (2β, 3β, 14α, 22R, 25-pentahydroxy--5β-cholest-7-en-6-one) is shown in figure 6. The more polar β-ecdysone was found to have an additional hydroxyl group on carbon 20 and was named 20-hydroxyecdysone (see figure 6).

Moulting hormones extracted from insects were found to be active in crustaceans by Carlisle (1965). Extracts from locusts, and other non-decapod crustaceae, were used to induce renewed moulting in Y-organ ablated juvenile shore crabs, Carcinus maenas. The activity of the extracts varied and it was suggested that a family of active substances existed with similar structures.

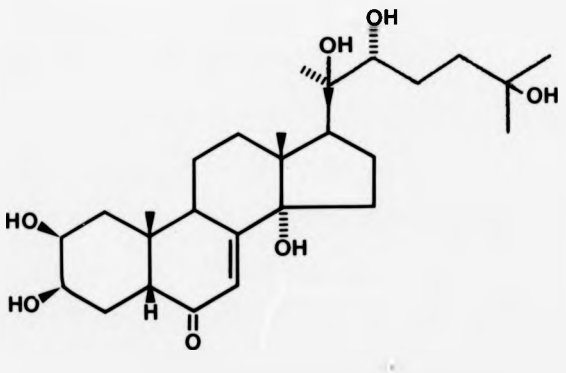
In crustaceans the levels of moulting hormones are much lower so that the losses that occurred in insect analysis could not be tolerated and a suitable procedure for crustaceans was developed by Hampshire and Horn, (1966). From 1000 kg of frozen waste of the australian rock lobster, Jasus lalandei, 2.3 mg of an active substance was obtained and designated crustecdysone. Comparison of this substance with β-ecdysone confirmed that they were both 20-hydroxy-ecdysone (Hampshire and Horn, 1966 and Horn et al., 1968).

Over sixty compounds with moulting hormone activity have now been characterised (Horn, 1971 and Horn and Hetru, 1981) and have been given the generic name ecdysteroids; those which have been

Figure 6. The structure of ecdysone and 20-hydroxyecdysone



Ecdysone



20-Hydroxyecdysone

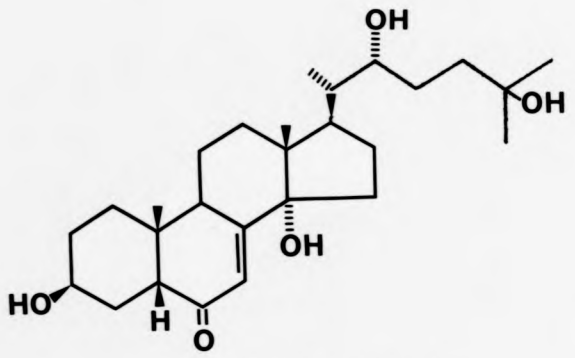
isolated from plants have been designated phytoecdysteroids and those from animals, zooecdysteroids (Goodwin et al., 1978).

To date, seven free ecdysteroids have been isolated and characterised from crustaceae; inokosterone or callinecdysone A (Faux et al., 1969) makisterone A or callinecdysone B (Faux et al., 1969); 2-deoxy-20-hydroxyecdysone (Galbraith et al., 1968); ponasterone A (McCarthy, 1979; Lachaise et al., 1981 and Lafont et al., 1982); 20,26-dihydroxyecdysone (McCarthy and Skinner, 1979 and Lachaise et al., 1981); α-ecdysone and 20-hydroxyecdysone, previously known as β-ecdysone, crustecdysone and ecdysterone, (Lachaise et al., 1981 and for a list of previous extractions see Table 1). The structure of these seven ecdysteroids are shown in figure 7. Table 1 lists the various crustacean species that have been analysed and the resultant titre levels of the ecdysteroid(s) present. In studies that analysed the titres with respect to the physiological moulting process, the titre range is also listed, the lowest and highest values generally represent the levels during the C-stages of the intermoult cycle and the D-stages of the premoult respectively.

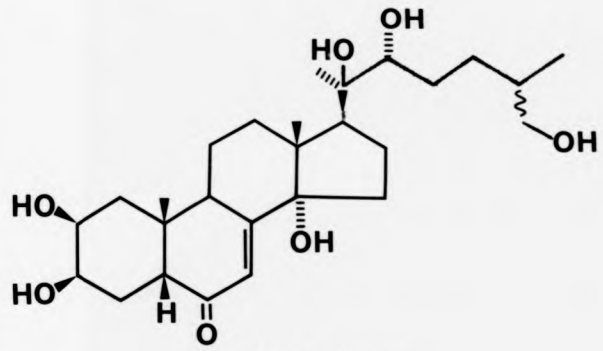
The predominant ecdysteroid found in crustaceans is 20-hydroxyecdysone and, to date, two zooecdysteroids have been found that are unique to crustaceans, inokosterone (Faux et al. 1969) and ponasterone A (McCarthy, 1979 and Lachaise et al., 1981).

Conjugated Ecdysteroids

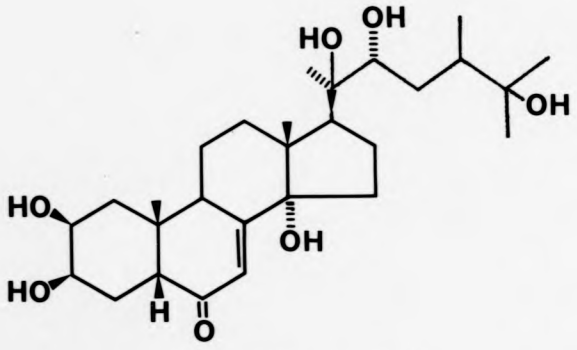
Highly polar derivatives of ecdysteroids have been found in insects; these are poorly immunoreactive and relatively inactive in bioassays. Hydrolysis of these 'ecdysteroid conjugates' using crude enzyme preparations (see Experimental) has yielded a variety of



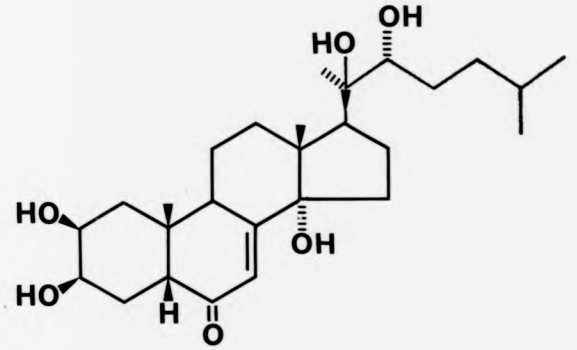
2-Deoxyecdysone



Inokosterone



Makisterone A



Ponasterone A

Figure 7. The structure of ecdysteroids (other than ecdysone and 20-hydroxyecdysone) isolated from crustaceae

commonly occurring ecdysteroids. Based on experiments in insects with hydrolytic enzymes the conjugated chemical moieties have been reported as α-glucoside, sulphate esters, glucuronides and phosphates (see Koolman, 1980). In insects conjugated ecdysteroids are found predominantly in newly laid and developing eggs. The function of these conjugates has been suggested to be as transport or storage compounds or inactivation products (Wilson and Morgan, 1978 and Gande and Morgan, 1979).

In crustaceans, McCarthy and Skinner (1979a, b) have discovered conjugates of 20-hydroxyecdysone in the embryos of the blue crab, Callinectes sapidus, and conjugates of ecdysone, 20-hydroxyecdysone and 20,26-dihydroecdysone in intermoult stage land crabs, Gecarcinus lateralis. In developing ovaries of the shore crab, Carcinus maenas, Lachaise et al., (1981) have found conjugates of ecdysone, 20-hydroxy-ecdysone and ponasterone A but all at very low levels compared to the titres of the corresponding free ecdysteroid. This is in contrast with insects where the ovaries and newly laid eggs contain up to 90% of their ecdysteroids in the conjugated form (see Dinan and Rees, 1981 and Scalia and Morgan, 1982).

As yet no-one has reported finding conjugated ecdysteroids in cirripede crustaceans.

Table 1. Ecdysteroids found in crustaceans

Species	Source	Ecdysteroid isolated	Concentration†		reference
Acanthonyx lunulatus (spider crab)	haemolymph	n.i.	0-232 ng ml^{-1}		Chaix et al., 1981
Callinectes sapidus (blue crab)	total	inokosterone	5 ng g^{-1}	early premoult	
			20 ng g^{-1}	late premoult	
		20-hydroxyecd.	4 ng g^{-1}	late premoult	
			280 ng g^{-1}	postmoult	
		makisterone A	24 ng g^{-1}	postmoult	Faux et al., 1969
	developing embryos	20-hydroxyecd.	0-160 ng g^{-1}	inc. w. embryogenesis	
		ponasterone A	85-475 ng g^{-1}	max. before prehatch	
		n.i.	7.5.-75 ng g^{-1}	inc. w. embryogenesis	McCarthy 1979 and
		conjugates	n.d.	n.d.	McCarthy & Skinner, 1979b.
Carcinus maenas (shore or green crab)	total	n.i.	5-110 ng g^{-1}	max. just before moult	Adelung, 1969
		n.i.	5-120 ng g^{-1}		Spindler et al., 1974
	haemolymph	n.i.	62-470 ng ml^{-1}	max. D_2-D_4	Lachaise et al., 1976
		n.i.	30-15,000 ng ml^{-1}	min. A-D_o, max D_3-D_4	
	Y-organ	n.i.	90-550 pg $gland^{-1}$	max. D_2-D_3	Andrieux et al., 1976
	ovary	ecdysone	185-742 ng g^{-1}	during ovarian	
	haemolymph	n.i.	14 ng ml^{-1}	maturation	
	eggs	ecdysone	418-603 ng g^{-1}	vitellogenesis	Lachaise & Hoffmann, 1977

Table 1 (continued)

	♀ haemolymph	ecdysone	0.46-4.6 ng ml^{-1})	max. just	
		20-hydroxyecd.	2.9-12)	before egg laying	
		ponasterone A	0.46-18.6	max. C_4	
	ovary	ecdysone	1.4-48.3 ng g^{-1}	max. C_3-C_4	
		20-hydroxyecd.	16.3-109.4	C_4	
		conjugates	very low	C_4	Lachaise et al., 1981
	eggs	ponasterone A	n.d.	n.d.	Lafont et al., 1982
Gecarcinus lateralis (land crab)	haemolymph	20-hydroxyecd.	6.1-120 ng ml^{-1}		
		ecdysone	> 1 ng ml^{-1}	max.	
		I-III, n.i.	0-19 ng ml^{-1}	D_1-D_2	McCarthy & Skinner,
		IV, n.i.	2-82 ng ml^{-1}		1977 and 1978.
		ponasterone A	n.d.	max. D_2-D_3	McCarthy 1979.
Helleria brevicornis	total ♀	n.i.	0.5-3 µg g^{-1} dry wt.		
	total ♂	n.i.	0.2-1.3 µg g^{-1} " "		
	haemolymph ♀	n.i.	50 ng - 2.8 µg ml^{-1}		
	haemolymph ♂	n.i.	20 ng - 0.7 µg ml^{-1}		Hoarau and Hirn, 1978
Homarus americanus (american lobster)	total ♀	20-hydroxyecd.	6 ng g^{-1}	postmoult	Gagosian et al., 1974 & Gagosian & Bourbonniere,
	haemolymph, juveniles	n.i.	35-350 ng ml^{-1}		Chang & Bruce, 1980. 1976.
Jasus lalandei (australian rock lobster)	total	20-hydroxyecd.	2 ng g^{-1}		Hampshire & Horn, 1966
		2-deoxy-20-hydroxyecd.	0.06 ng g^{-1}		Galbraith et al., 1968

Table 1 (continued)

Ligia oceanica	total	ecdysone, 20-hydroxyecd.	n.d.	absent A-C_3 present C_2, C_4-D_2	Maissiat & Maissiat, 1976
Orchestia gammarellus	total ♂	n.i.	12-63 ng g^{-1}	max. D_2	Blanchet *et al.*, 1976
	total ♀	20-hydroxyecd.	4-70 ng g^{-1}	max. D_2	
	haemolymph	20-hydroxyecd.	0-167 ng ml^{-1}	max. D_2	
	ovaries	20-hydroxyecd., + n.i.	8-77 pg $ovary^{-1}$	max. D_2	Blanchet *et al.*, 1979
Orconectes limosus	total	n.i.	0.3-57 ng g^{-1}	max. D_2	Willig and Keller, 1973
	Y-organs	ecdysone	125 pg $gland^{-1}$		
	haemolymph	20-hydroxyecd.	3-120 ng ml^{-1}	max. D_2-D_3	Keller and Scmid, 1979.
O. propinquus	total	ecdysone, 20-hydroxyecd.	n.d.		Carlisle & Connick, 1973
O. sanborni	haemolymph	n.i.	4-30 mg ml^{-1}	max. D_1-D_2	Stevenson *et al.*, 1979
Pachygrapsus crassipes (lined shore crab)	haemolymph	ecdysone, 20-hydroxyecd.	25-120 ng ml^{-1}		Chang and O'Connor, 1976.
	Y-organ	ecdysone	very low		Chang and O'Connor, 1977.
P. marmoratus	haemolymph	n.i.	0-163 µg ml^{-1}	max. D_3-D_4	Charmamtier *et al.*, 1980
Sphaeroma serratum	total ♂	20-hydroxyecd.	5-80 ng g^{-1}	max. D_2	Charmantier *et al.*, 1976a.

Table 1 (continued)

Artemia salina (brine shrimp)	total dormant cysts nauplii	ecdysone, 20-hydroxyecd.	n.d.	Spindler *et al.*, 1980
Balanus balanoides (acorn barnacle)	total	20-hydroxyecd.	1 ng g^{-1}	Bebbington, 1975.
		ecdysone	6 pg g^{-1}	
	total	20-hydroxyecd.	> 100 pg - 2 ng g^{-1}*	
		conjugate, 20-hydroxyecd.	> 40 pg - 174 pg g^{-1}*	

n.i. - not identifed

n.d. - not determined

* - seasonal variation

† - fresh weight unless specified otherwise

Methods of Detection used in the Analysis of Ecdysteroids in Crustaceans

(i) Bioassays

The first method used to assess the presence of an active substance that induced moulting, a moulting hormone or ecdysteroid, from a biological extract was a bioassay using ligated abdomens of the blowfly, Calliphora erythrocephala. The "Calliphora bioassay" was devised by Fraenkel (1935) and has been used successfully in the past in the analysis of ecdysteroids and was recently re-evaluated by Fraenkel and Zdarek (1970). This assay was first used to demonstrate the presence of moulting hormone in extracts from crustaceae by Karlson (1956) and was also employed by Hampshire and Horn (1966), Horn et al. (1968), and Galbraith et al. (1968) to determine the activity of the first identified ecdysteroid, 20-hydroxyecdysone, extracted from Jasus lalandei, the Australian rock lobster. Adelung (1969) used the Calliphora bioassay to determine the total ecdysteroid content of the shore crab, Carcinus maenas, throughout a moult cycle and Carlisle and Connick (1973) determined the presence of two ecdysteroids, ecdysone and 20-hydroxyecdysone, in the crayfish, Orconectes propinquus, using this assay in conjunction with thin layer chromatography.

Kaplanis et al. (1966) successfully developed a new and more sensitive bioassay for ecdysone using the housefly, Musca domestica, and this 'Musca bioassay' was used by Willig and Keller (1973) to analyse the moulting hormone content of the crayfish, Orconectes limosus, throughout its moult cycle. The insect bioassay has been used most recently in the analysis of ecdysteroids in C. maenas by Spindler et al. (1974).

These bioassays, however, are now seldom used as there is the possibility of misleading results arising from ecdysteroid metabolism

in the ligated abdomens, because they lack specificity for a particular compound, and because of the increased sensitivity of modern analytical techniques. Nevertheless the larvae of a merostome Limulus polyphemus, the horseshoe crab, have been studied as candidates for a new bioassay for ecdysteroids (Jegla and Costlow, 1979). Using first stage larvae at the point of cuticle formation they have found the assay to be sensitive to both ecdysone and 20-hydroxyecdysone at 20 pg, which is far more sensitive than the two insect bioassays. An advantage of this bioassay, apart from its sensitivity, is the tolerance of the Limulus larvae to relatively impure samples, Jegla and Costlow's results indicate that the larvae accept injections of ethanolic solutions without apparent ill effect. As yet this bioassay has not been used in a detailed study of extraction of ecdysteroid from a biological source.

(ii) Radioimmunoassays

The initial research into ecdysteroids involved the large scale extraction of a compound whose activity as a moulting hormone was determined with a bioassay, and whose structure was characterised by chemical and spectroscopic techniques; ultraviolet, infrared, nuclear magnetic resonance and mass spectrometry. Once these ecdysteroids had been isolated and characterised from insects, crustaceans and plants, the next step was to quantify accurately the levels present and conduct in vivo studies to relate the presence of a circulating moulting hormone to the physiological moulting cycle. For this to be achieved more sensitive methods of detection were required.

The sensitivity of the Calliphora bioassay is in the order of 5-50 ng ecdysteroid per ligated abdomen (Thompson et al., 1970) and

in the Musca test 5-6 ng (Kaplanis et al., 1966). A radioimmunoassay (RIA) specific for ecdysteroids was developed by Borst and O'Connor (1972 and 1974) that could detect accurately down to 200 pg, the sensitivity of the method resulting in its widespread use in ecdysteroid analysis. The advantages of this technique are that it requires little expertise, no sophisticated equipment as does gas chromatography (GC) and high performance liquid chromatography (HPLC) (see later). Only a relatively simple extraction and purification procedure is required and due to the low level of detection, measurements on small samples and even individual animals are possible. However, RIA is a very non-specific technique that will detect both biologically active and inactive ecdysteroids. Richards (1980) has shown how conflicting results have arisen in eight studies of ecdysteroid titres during larval and pupal development in Drosophila melanogaster. Hirn and Delaage (1980), who estimate that the absolute sensitivity of the best ecdysone RIA is 1.6 pg, conclude that if RIA is to be used, "it is preferable to use a moderately specific antibody in combination with a chromatographic step that easily separates the ecdysteroids according to their polarity".

In the determination of ecdysteroids in crustaceae RIA has been used as the sole detection method by the following authors; Lachaise et al. (1976) and Andrieux et al. (1976) in the analysis of Y-organs and haemolymph of C. maenas; by Blanchet et al. (1976) during the intermoult of male Orchestia gamarella; by Chang et al. (1976) during the course of the moult cycle in the crab, Pachygrapsus crassipes; by Hoarau and Hirn (1978) during the intermoult cycle of males and females of the terrestial isopod, Hellaria brevicornis; by Stevenson et al. (1979) during the course of the moult cycle of the crayfish, Orconectes

sanborni; by Chang and Bruce (1980) during the moult cycle of juvenile lobsters, Homarus americanus; and by Chaix et al. (1981) during the moult cycle of the spider crab, Anthonyx lunulatus.

Other research groups have employed RIA in combination with one or more chromatographic technique, GC, HPLC or thin layer chromatography (TLC), to precisely determine not only the specific ecdysteroid(s) present but also the titre of each. A combination of techniques was used to determine the presence of; ecdysone and 20-hydroxyecdysone in P. crassipes by Chang et al. (1976) and Chang and O'Connor (1977 and 1978); ecdysone (Lachaise and Hoffmann, 1977), 20-hydroxyecdysone and ponasterone A in C. maenas by Lachaise et al. (1981); ecdysone, 20-hydroxyecdysone, 20,26-dihydroxyecdysone and ponasterone A in the land crab Gecarcinus lateralis by McCarthy and Skinner (1977 and 1979a) and McCarthy (1979); ecdysone, 20-hydroxyecdysone and ponasterone A in the blue crab, Callinectes sapidus by McCarthy and Skinner (1979b) and McCarthy (1979); 20-hydroxyecdysone and a less polar ecdysteroid in the amphipod Orchestia gammarellus by Blanchet et al. (1979); ecdysone and 20-hydroxyecdysone in O. limosus by Keller and Schmid (1979) and Spindler et al. (1980) and ecdysone and 20-hydroxyecdysone in the brine shrimp, Artemia salina, by Spindler et al. (1980).

(iii) High Performance Liquid Chromatography

High performance liquid chromatography (HPLC), which has been used in ecdysteroid analysis in combination with RIA, is a relatively recent technique which is particularly useful for high molecular weight and polar substances such as ecdysteroids. Formation of a derivative of the ecdysteroid, as for gas chromatography, is not a requisite and the effluent can be collected for subsequent analysis by other means.

The method suffers from lacking a universal and sensitive method of detection. For ultraviolet absorbing compounds the most useful detector is an ultraviolet cell. Fortunately all the ecdysteroids possess an unsaturated ketone group which absorbs strongly at approximately 240 nm and so they are ideal for this means of detection and can be detected accurately down to 10 ng. The early use of HPLC analysis for ecdysteroids was reviewed by Morgan and Poole (1976a) and more recently by Lafont et al. (1980 and 1982) and Wilson et al. (1982). HPLC has been used in the analysis of ecdysteroids in crustaceae by Gagosian and Bourbonniere (1974 and 1976), Keller and Schmid (1979), Blanchet et al. (1979), McCarthy and Skinner (1977, 1979a, b) and Lachaise et al. (1981). A comparison of the use of HPLC and gas chromatography in the analysis of ecdysteroids in locusts and barnacles was made by Wilson et al. (1981).

(iv) Gas Chromatography

One of the most sensitive, selective and specific means now available for measurement of ecdysteroids is by gas chromatography with an electron capture detector (GC-ECD). The main disadvantage of this technique, apart from the sophisticated equipment, is that the heat sensitive, non-volatile ecdysteroids must first be converted to stable volatile derivatives. This has been achieved using a variety of reagents (see Morgan and Poole 1976a) but the one most commonly used is trimethylsilyl imadazole (TMSI) which protects some, or all, of the hydroxyl groups to produce trimethylsilyl (TMS) ethers. The derivative formed depends upon the reaction time and temperature (discussed later in the thesis). Another disadvantage is the difficulty in obtaining conversion to a single derivative.

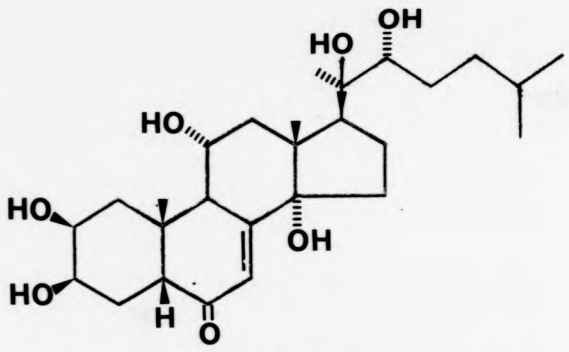
Initially a flame ionisation detector (FID) was used. Katz and Lensky (1970) reported the first derivatisation of ecdysone using bis(trimethylsilyl)acetamide (BSA) and subsequently analysed the unidentified derivative by GC-FID. Morgan and Woodbridge (1971), using the same silylating reagent, first identified the resulting derivatives. A series of ecdysones were then derivatised by Ikekawa et al. (1972) using trimethylsilyl imadazole (TMSI) and heptafluorobutyrate (HFB), the silylated derivatives were analysed by GC-FID and clearly defined by mass spectrometry. Analysis of ecdysteroids by silylation, and subsequent determination by GC-FID, was used in the determination of ecdysteroid levels present in crustaceans by Gagosian et al. (1974) and Gagosian and Bourbonniere (1976).

As the limit of sensitivity of the FID is only 50 ng large scale extractions were necessary to obtain sufficient quantities of ecdysteroids from insects and crustaceans for analysis and quantification by GC-FID. Therefore, for analysis with the more sensitive electron capture detector (ECD), a suitable derivative was required for conversion of the ecdysteroids. Ikekawa et al. (1972) reported that HFB derivatives had ideal GC properties and could be detected with either a FID or an ECD, but these derivatives could not be prepared directly and their formation was achieved by silylation followed by an exchange reaction between TMS and HFB moieties. Independently, Poole et al. (1975) and Borst and O'Connor (1974) found that the ECD was sensitive to TMS ethers of ecdysteroids down to 20-50 pg. The GC-ECD has since been used in the analysis of ecdysteroids from insects (Morgan and Poole, 1976b; Wilson and Morgan, 1978; Wilson, 1979; Gande and Morgan, 1979 and Gande et al., 1979) and from crustaceans (Poole et al., 1975; Bebbington, 1975 and Wilson et al., 1981).

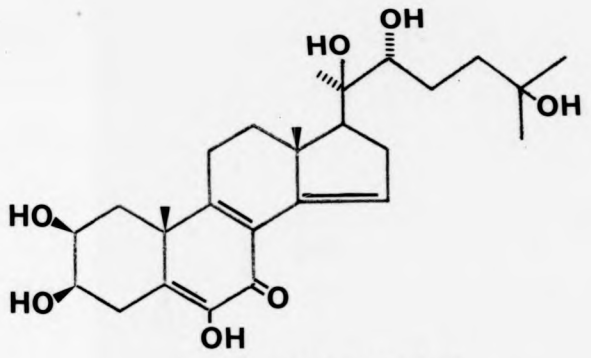
The use of gas chromatography and spectroscopic methods in the analysis of ecdysteroids has been reviewed most recently by Morgan and Wilson (1980) and the use of fluorometry has been discussed by Koolman (1980).

The prime aim of this project was to devise a simple and efficient analytical scheme to determine the seasonal variation, if any, of ecdysteroid production in the barnacle Balanus balanoides.

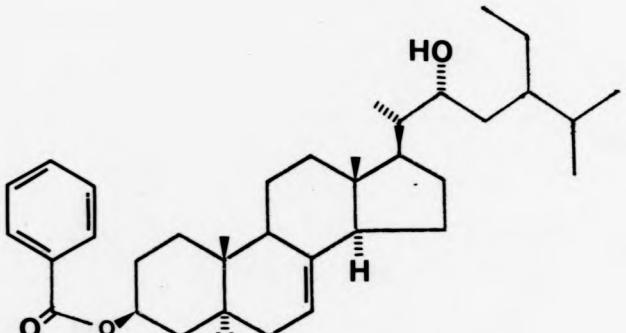
During the course of this study a variety of ecdysteroids were obtained, predominantly as gifts. These ecdysteroids were chromatographically analysed by TLC and GC-ECD after derivatisation to their TMS ethers under a range of conditions. From the results the feasibility of these ecdysteroids as possible internal standards in the analysis of ecdysone and 20-hydroxyecdysone was determined. In conjunction with this study the underivatised ecdysteroids were also analysed on RPTLC (see Discussion) and HPLC (Wilson et al. 1982). The structure of these ecdysteroids are shown in figures 8-10.



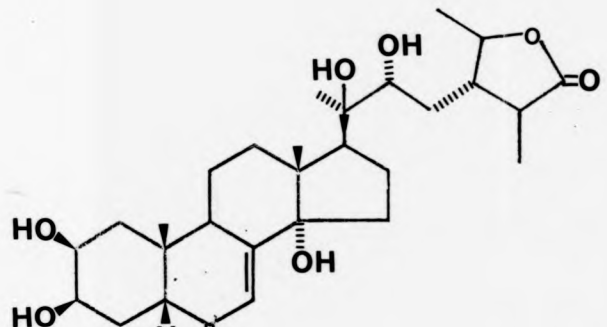
Ajugasterone C



Calonysterone

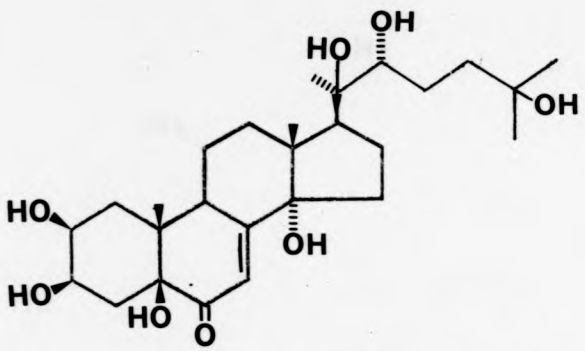


Carpesterol

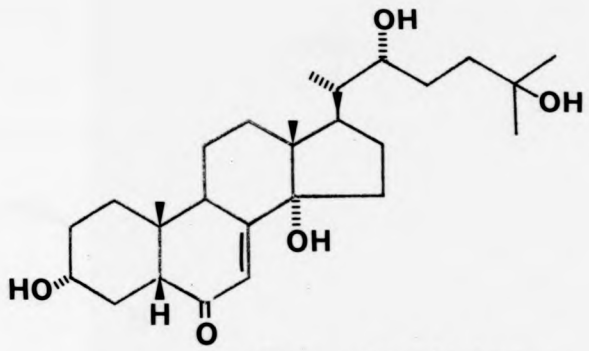


Cyasterone

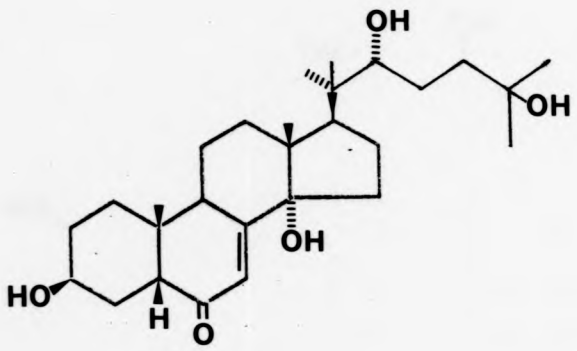
Figure 8. The structure of other ecdysteroids analysed in this study.



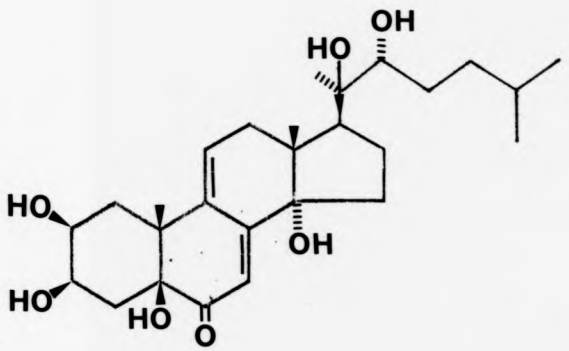
Dacrysterone



2-Deoxy 3-epiecdysone

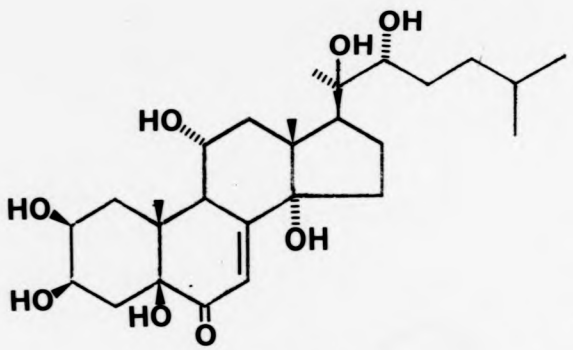


2-Deoxy-20-hydroxyecdysone

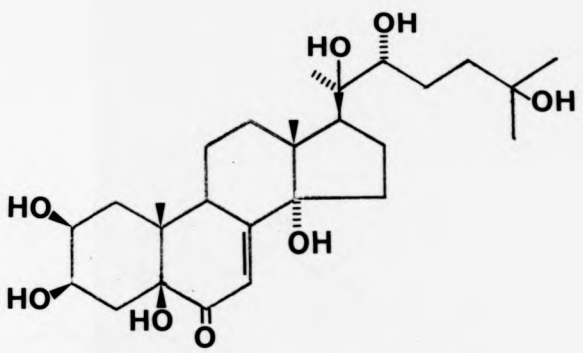


Kaladasterone

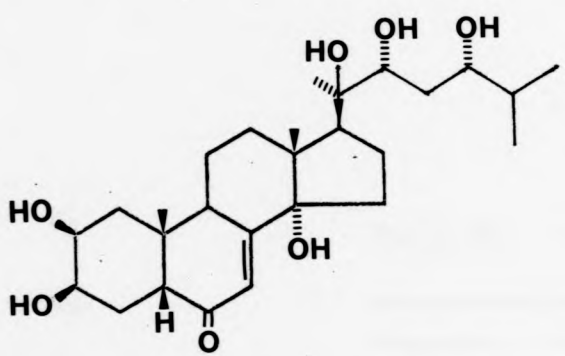
Figure 9. The Structure of further ecdysteroids analysed in this study.



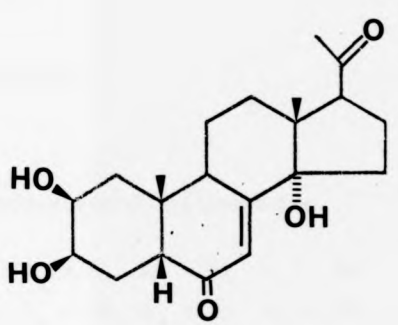
Muristerone



Polypodine B

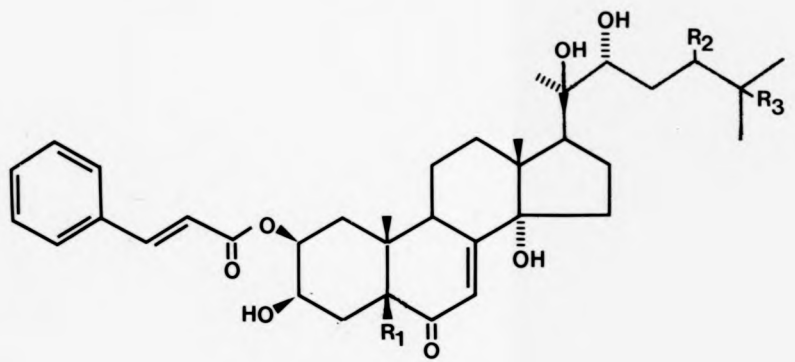


Ponasterone C



Poststerone

Figure 9. - continued



Ecdysteroid 2-cinnamate

Figure 10. The structure of ecdysteroid 2-cinnamates.

20-hydroxyecdysone 2-cinnamate;	R_1 = H, R_2 = H, R_3 = OH
Polypodine B 2-cinnamate;	R_1 = OH, R_2 = H, R_3 = OH
Ponasterone C 2-cinnamate;	R_1 = H, R_2 = OH, R_3 = H

DISCUSSION

Reverse Phase Thin Layer Chromatography

Reverse phase thin layer chromatography (RP-TLC) is an alternative to the use of thin layer chromatography (TLC) for the isolation and separation of ecdysteroids, as polar compounds such as these may become irreversibly adsorbed onto the normal phase silica plates and give poor recovery results. RP-TLC plates have a non-polar stationary phase and use polar eluents, so that, in comparison with TLC, the mobile phase is more polar than the stationary phase.

A number of RP-TLC plates are commercially available (see Sander et al., 1981) but they can also be prepared in the laboratory by physically or chemically coating a normal phase TLC silica plate with a non-polar phase. Wilson et al. (1981) compared chemically bonded commercial and 'homemade' RP-TLC for the separation of some commonly occurring ecdysteroids in a variety of developing solvents. The 'homemade' RP-TLC plates were prepared by coating a silica TLC plate with trichlorooctadecylsilane and capping with trimethyl--chlorosilane.

We determined the efficiency of a C_{12} bonded RP-TLC plate (OPTI-UP) and two other plates that were prepared by physically coating normal phase TLC plates with liquid paraffin ("Nujol"). The non-polar phase was merely chromatographed onto the TLC plates by development in dichloromethane containing liquid paraffin, a much simpler and less time-consuming method than coating chemically.

The two types of 'homemade' RP-TLC plates were prepared from high performance (HP-K) Whatman TLC plates and 0.6 mm preparative plates generally used for the purification of TMS ethers of ecdysteroids. The R_f values of some representative ecdysteroid developed on the three types of RP-TLC plates are given in table 2.

The time for development of these plates was found to be strongly dependent upon the particle size of the silica and the quantity of organic modifier in the solvent, but independent of the degree of coating. Thus the preparative 0.6 mm RP-TLC plates, made with relatively coarse grained silica, developed much faster. Using a developing solvent of 50% aqueous methanol the preparative 0.6 mm RP-TLC plates developed at 0.16 cm min^{-1}, the Whatman HP-K RP-TLC plates at 0.026 cm min^{-1} and the OPTI-UP plates at 0.082 cm min^{-1}. With 20% and 80% aqueous methanol the development times for the Whatman RP-TLC plates were 0.017 and 0.13 cm min^{-1} respectively. Identical rates were obtained for all degrees of coating from 2½-10% v/v Nujol in dichloromethane.

Wilson et al. (1981) recorded recoveries of 80% of 100 ng of ecdysone from RP-TLC plates compared with 50% from normal phase adsorption silica TLC plates. The recoveries obtained from our 'Nujol' coated plates are comparable with these results and are shown in table 3. During the determination of the recoveries of ecdysone it became apparent that unless the Whatman HP-K plates were pre-washed, by chromatographing in dichloromethane before conversion to RP-TLC plates, they were of little use in quantitative analysis. This was due to the co-elution of compounds that interfered with the detection of ecdysone by the electron capture detector. These contaminants prevented quantification of recoveries

Table 2. R_f Values of Pure Ecdysteroids on RP-TLC Plates.

Plate	OPTI-UP C_{12}		Laboratory made from Whatman HPTLC plate		Laboratory made from 0.6 mm TLC plate	
Developing Solvent	MeOH:H_2O (40:60)		MeOH:H_2O (50:50)		MeOH:H_2O (50:50)	
Compound	R_f	R_e^*	R_f	R_e^*	R_f	R_e^*
Ecdysone	0.35	1	0.37	1	0.39	1
20-Hydroxyecdysone	0.44	1.26	0.52	1.41	0.52	1.33
Ajugasterone C	0.41	1.17	0.46	1.24	-	-
Cyasterone	0.43	1.23	0.57	1.54	-	-
2-Deoxyecdysone	0.25	0.71	0.24	0.65	0.18	0.46
Inokosterone	0.40	1.14	0.50	1.35	-	-
Makisterone A	0.39	1.11	0.43	1.16	0.42	1.07
Muristerone	0.39	1.11	0.46	1.23	0.43	1.1
Polypodine B	0.44	1.26	0.55	1.49	0.54	1.38
Ponasterone A	0.29	0.83	0.20	0.54	0.15	0.38
Poststerone	0.43	1.09	0.52	1.41	-	-

*R_e ≡ Relative to ecdysone

Table 3

Recovery (%) of Ecdysone from RP-TLC plates.

Amount applied (ng)	Average Recovery (%)		
	OPTI-UP C_{12}	Laboratory made from Whatman HPTLC plates.	Laboratory made 0.6 mm RP-TLC plates
500	-	-	95*
250	83	-	106*
200	85	82*	-
100	99	74	88
50	91	69	68
25	85*	-	-
10	69	-	-

* These results were obtained from single determinations.

The other results were obtained from triplicate experiments.

of ecdysone at less than 200 ng per plate. After being washed, the plates could be used satisfactorily. The problem is exemplified in figure 11, which shows gas chromatograms of ecdysone recovered from Whatman RP-TLC plates that were converted without and after pre-washing. This problem did not arise with the bonded OPTI-UP RP-TLC plates or the 0.6 mm preparative RP-TLC plates (where the silica was extensively washed before use - see experimental).

The recovery results indicate that these physically coated plates, which are simpler to prepare, give comparable results to those obtained for chemically coated RP-TLC plates.

These results also indicate another chromatographic technique that can be used to identify ecdysteroids. If a fluorescent indicator is incorporated into the plates the ecdysteroids, whose 7-en-6-one group strongly absorbs UV light at 240 nm, can be readily detected by ultra-violet illumination down to 100 ng. Conversely if the material is not to be recovered detection using a vanillin-sulphuric acid spray followed by heating at 110^{o}C or heating in an atmosphere of ammonium carbonate followed by visualising under ultra violet light at 366 nm are methods that have been suggested (Wilson *et al.*, 1981); (the latter technique apparently detects down to 50 ng).

The preparative 0.6 mm RP-TLC plates were also employed to determine their efficiency in separating ecdysteroids from pigmented impurities in the extraction and purification of these moulting hormones from barnacles (see later), but did not prove successful.

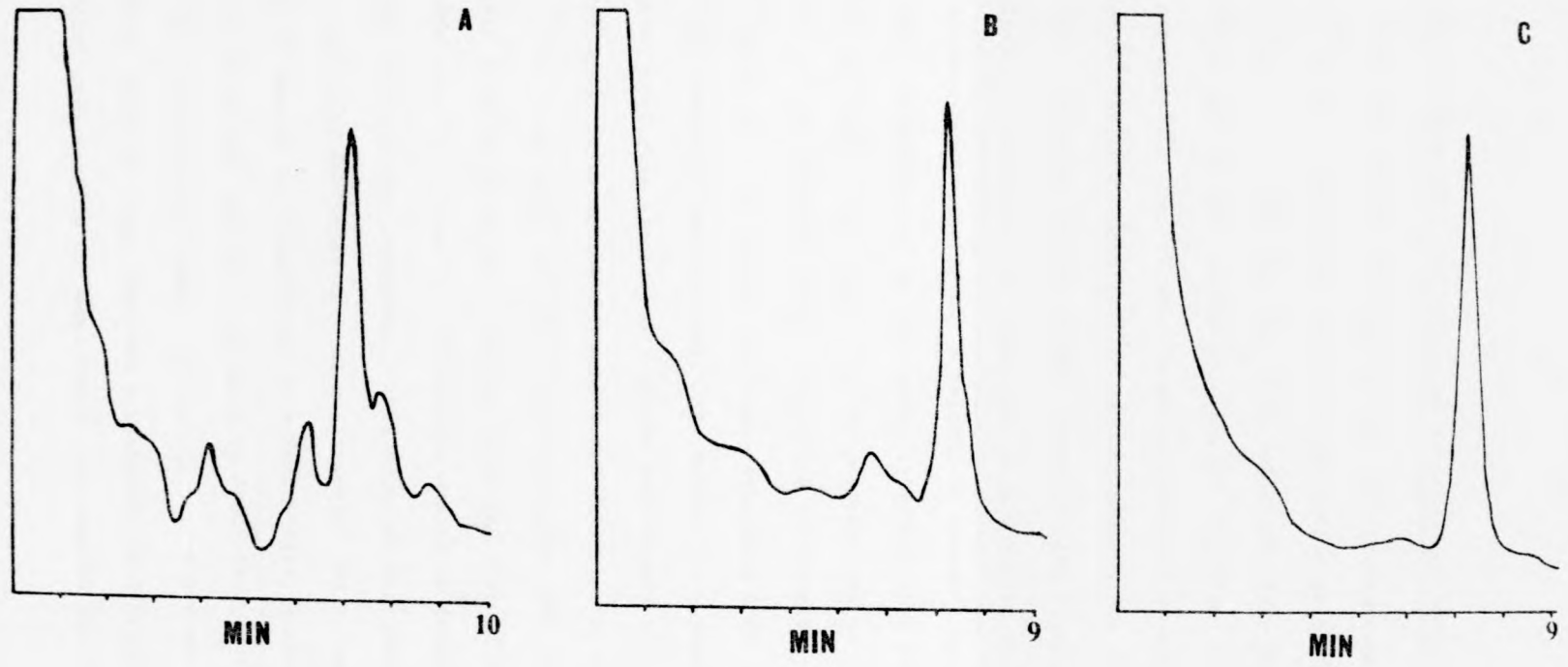


Figure 11. Gas chromatographic traces, with electron capture detector, of ecdysone (100 ng) recovered from Whatman HP-K silica plates which had been impregnated with paraffin. Trace A shows the result with no treatment. B shows the effect of washing the plate with dichloromethane before impregnation and use. C shows a sample which had been recovered from a laboratory prepared preparative TLC plate impregnated with paraffin.

The Preparation of Silyl Ethers of Ecdysone and 20-hydroxyecdysone

In the analysis of ecdysteroids from crustaceans, where the sample volume is small and therefore the levels of moulting hormone are likely to be very low, the final detection stage must be very sensitive and the only suitable technique available, other than the non-specific RIA, is gas chromatography with an electron capture detector. As this technique can only be used in the separation of volatile, thermally stable organic compounds the polar, polyhydroxyl ecdysteroids must first be converted to less polar, stable volatile derivatives before analysis. A variety of reagents are available for this conversion but in this study trimethylsilyl imadazole (TMSI) was used, which reacts with the ecdysteroids to protect some, or all, of the hydroxyl groups to produce trimethylsilyl (TMS) ethers (see figure 12), the derivative formed depending upon the duration and temperature of the reaction. The ecdysteroids posses a strongly electron capturing electrophore (Poole and Morgan, 1975) which confers a high sensitivity to the TMS ethers for detection by GC-ECD.

The reactivity of commercially available TMSI has been found to vary from batch to batch (Poole, 1975) and often did not produce satisfactory silylation of ecdysteroids and so we prepared our own reagent as described elsewhere. This material was consistent in its reactivity and separate 1 ml vials sealed for a long time, could be opened and transferred to a Reacti-vial, sealed with a rubber serum cap, and portions could be drawn from it to give reproducible reaction times. Nevertheless it was found advisable to assay separate vials from one preparation by silylation of standard ecdysteroids at least every three months and it was

found necessary to prepare fresh reagent annually.

As the rate of reactivity was known to show some variation, the silylation of two standards, ecdysone and 20-hydroxyecdysone, was analysed to determine the conditions required to form single derivatives. Ecdysone possesses five hydroxyl (-OH) groups on carbons 2,3,14,22 and 25 and an additional -OH group on carbon 20 occurs in 20-hydroxyecdysone. Poole (1975) reacted these two ecdysteroids with TMSI under a variety of conditions and analysed the resulting derivatives by mass spectrometry. He concluded that by reacting ecdysone with TMSI for 15 min at room temperature the four hydroxyl groups on C-2, C-3, C-22 and C-25 were rapidly silylated, to give the tetrakis TMS ether, and only by continuing the reaction for 40 h at 120°C could the 14α-OH group be fully derivatised. With 20-hydroxyecdysone the hydroxyl group on C-20 was only fully derivatised by silylation for 4 h at 100°C and, as with ecdysone a reaction of 40 h at 120°C was required to obtain silylation of the 14α-OH to give the fully derivatised hexakis TMS ether of 20-hydroxyecdysone. Using our laboratory prepared TMSI it was found that the hydroxyl groups on C-2, C-3, C-22 and C-25 were fully silylated after 30 min at room temperature, a reaction for 5 h at 120°C was required to derivatise the C-20 hydroxyl group and 60 h at 140°C was necessary to fully silylate the 14α hydroxyl.

The rate of reactivity of hydroxyl groups with TMSI was found to depend upon their chemical environment and the order in which they became completely reacted, as reported by Poole (1975) and Morgan and Poole (1976c), is 2,3,22,25>20>>14.

From these results it is apparent that the tetrakis TMS ethers of both ecdysone and 20-hydroxyecdysone, where the

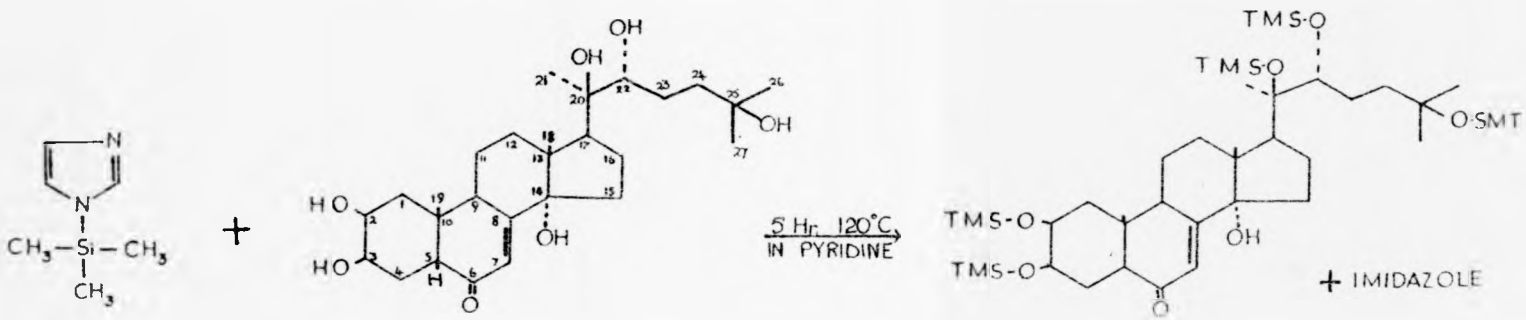
C-2, C-3, C-22 and C-25 hydroxyl groups are silylated, can be readily formed by silylation for 30 min at room temperature. Even when left overnight at room temperature no further derivatisation occurs. If, however, a sample is silylated longer at a higher temperature, 5 h at 120°C, then ecdysone still remains as the tetrakis TMS ether but 20-hydroxyecdysone is now fully converted to the pentakis TMS ether. Any further reaction time results in the gradual formation of the fully derivatised TMS ethers of both ecdysteroids but to obtain complete silylation to the pentakis and hexakis TMS ethers of ecdysone and 20-hydroxyecdysone respectively a mixture must be heated to 140°C for 60 h. The results of silylating 20-hydroxyecdysone under varying conditions are shown in figure 13, where the extent of the reaction is monitored by GC. Initially the tetrakis TMS ether is formed which is slowly converted to the pentakis and finally the hexakis TMS ether.

The results of silylating a mixture of these two ecdysteroids for 30 min at room temperature and 5 h at 120°C is shown in figure 14. From these gas chromatograms it is clear that both sets of silylating conditions can be used to separate ecdysone and 20-hydroxyecdysone. The first method has the advantage of speed of analysis whereas the longer reaction time, with heating, results in the formation of the pentakis, rather than the tetrakis, TMS ether of 20-hydroxyecdysone which is better resolved from the tetrakis TMS ether of ecdysone.

During the study of the rate of silylation with time it was found that using one sample and withdrawing aliquots at timed intervals was not satisfactory because of the unavoidable

Figure 12 . Gas chromatographic traces of silylation products of 20-hydroxyecdysone after varying periods of time. A = tetrakis ether (a) formed after 30 minutes. B = mixture of the tetrakis (a) and pentakis (b) ethers formed after 4h at 120 °C. C = the pentakis (b) ether after 5 h at 120 °C. D = a mixture of the pentakis (b) and hexakis (c) ethers after 16 h at 140 °C. Chromatographic conditions as described in text.

SILYLATION



TMSI (TRI-METHYL SILYL IMIDAZOLE)

20-HYDROXYECDYSONE

PENTAKIS TMS ETHER

Order of Derivitisation 2 , 3 , 22 , 25 > 20 >> 14 .

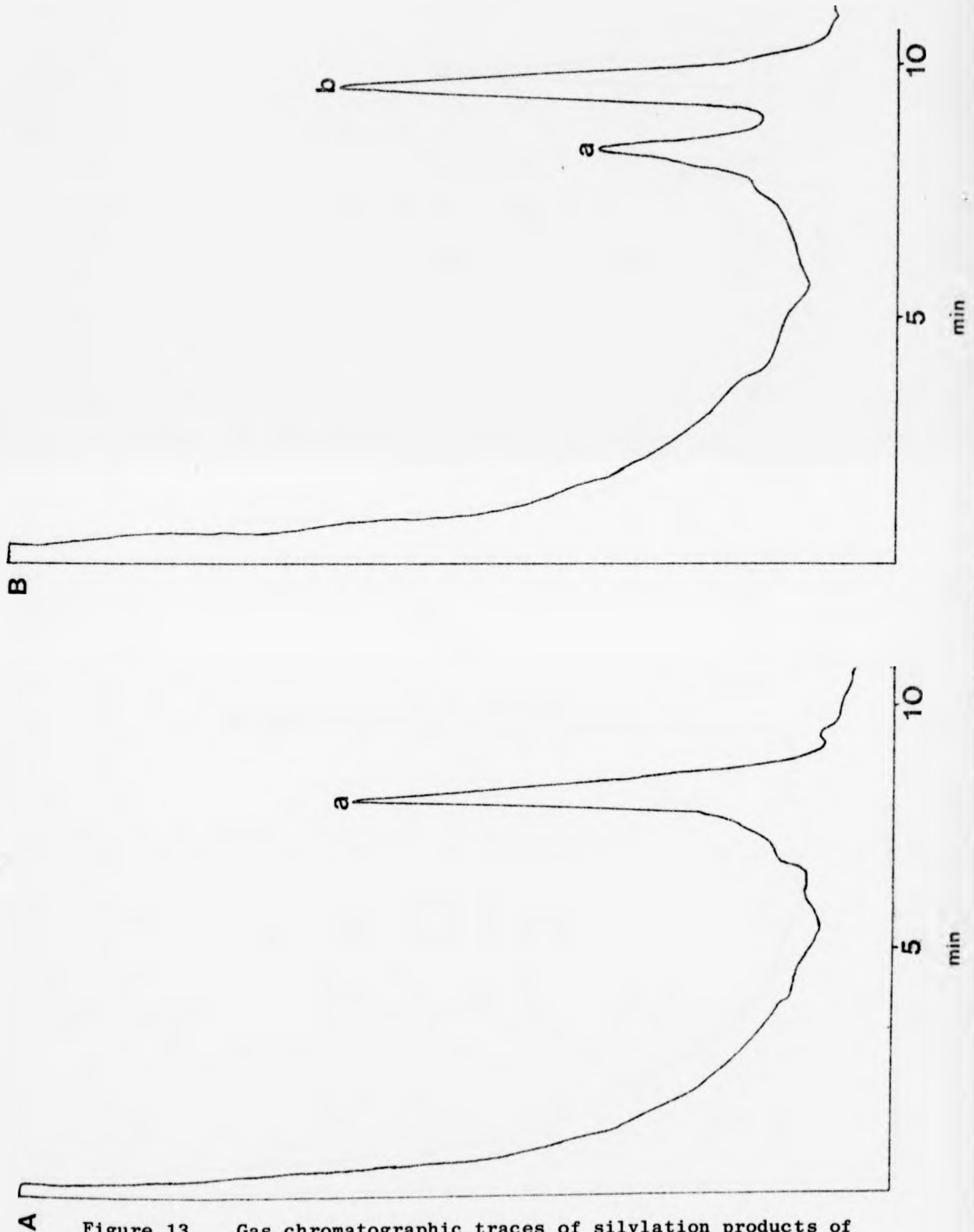


Figure 13. Gas chromatographic traces of silylation products of 20-hydroxyecdysone after varying periods of time.
A = tetrakis ether (a) formed after 30 minutes. B = mixture of the tetrakis (a) and pentakis (b) ethers formed after 4h at 120 °C. C = the pentakis (b) ether after 5h at 120 °C. D = a mixture of the pentakis (b) and hexakis (c) ethers after 16h at 140 °C. Chromatographic conditions as described in text.

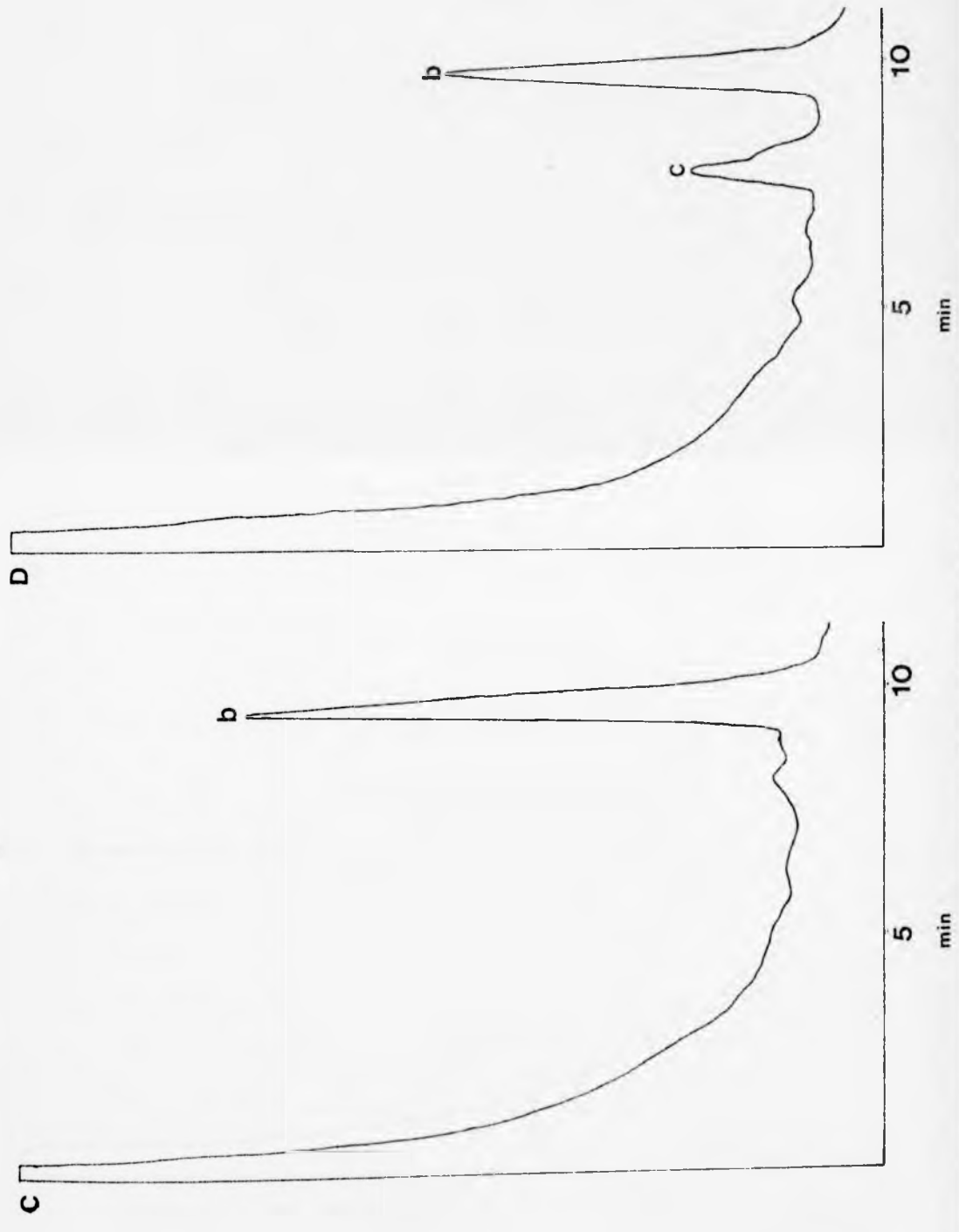


Figure 13 - continued

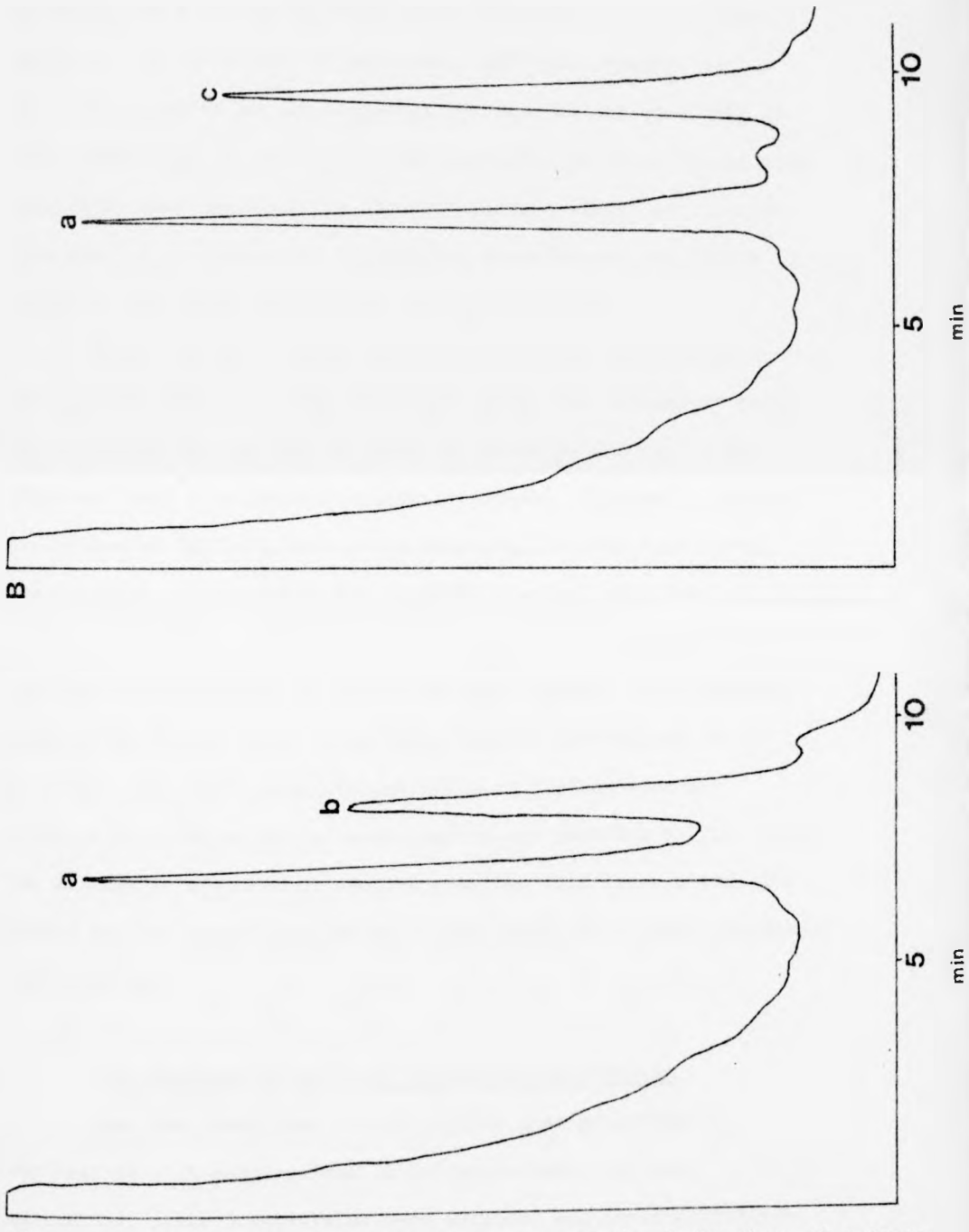


Figure 14. Gas chromatography traces of the derivatives of ecdysone and 20-hydroxyecdysone after silylation for 30 min (A) and 5 h (B). Peaks: a = tetrakis TMS ether of ecdysone b = tetrakis TMS ether of 20-hydroxyecdysone; c = pentakis TMS ether of 20-hydroxyecdysone. Chromatographic conditions; 285 ^{o}C oven temperature with detector at 300 ^{o}C, gas flow 50-60 ml min^{-1}, on an 0.9 m 1.5% OV0101 column.

exposure to moisture at each opening of the Reacti-vial. To overcome this a series of vials were prepared each containing identical concentrations of pyridine, TMSI and ecdysteroids and all were placed in an oven together but removed one at a time at fixed intervals. It was also found advisable to use an accurately controlled oven particularly when obtaining a single derivative from the 5 h silylation as fluctuating temperatures are liable to result in the formation of more than one product.

In all the silylation reactions the ratio of pyridine to TMSI was maintained at 2:1. Although it has been suggested that it is possible to use TMSI without any pyridine (Lafont et al., 1980) we found this procedure unsatisfactory. Wherever a greater proportion of TMSI was used for silylation, or when used alone, specifically in reactions that required heating, there was a tendency for the imadazole to crystallize from the reaction on cooling and to occlude the silylated ecdysteroids. This problem never arose if the ratio of pyridine:TMSI was maintained at 2:1 or more. The use of high concentration of TMSI also causes problems when the silylated ecdysteroids are purified by TLC. When the mixture is streaked or spotted onto the thin layer plate, the silica may be lifted from the glass and result in a poorly developed chromatogram.

The Preparation of Silyl Ethers of Ecdysteroids

When the conditions for silylation that gave single derivatives with ecdysone and 20-hydroxyecdysone had been determined, other ecdysteroids were obtained and their derivatives analysed by GC, after silylation under the same conditions. Each

ecdysteroid was silylated for 30 min at room temperature, 5 h at 120°C and 60 h at 140°C and the retention time of the derivatives determined by GC-ECD and compared with the values obtained for ecdysone which in this instance was used as a standard. These results are presented in table 4 and the structure of the free ecdysteroids discussed can be seen in figures 6-10.

From our present knowledge of the rate of silylation of hydroxyl groups on specific carbons (see earlier) we can make suggestions as to the nature of the derivatives formed from ecdysteroids that posses hydroxyl groups on C-2, C-3, C-14, C-20, C-22 and C-25. Those ecdysteroids that contain only hydroxyl groups on these carbons and their proposed derivatives are shown in table 5. This table also includes inokosterone and pterosterone which have an hydroxyl group on C-26 and C-24 respectively, both of which are in unhindered positions and are very likely to be readily silylated and have therefore been included in this list. These proposed derivatives are based on data obtained for ecdysone and 20-hydroxyecdysone but have yet to be confirmed by mass spectrometry. This confirmation was not possible to conduct with the very small samples at our disposal.

The remaining ecdysteroids either posses an additional chemical moiety or a hydroxyl group in such a position that it would be unwise to predict its rate of silylation without confirmation of the structure of the derivatives by mass spectrometry. In cyasterone a lactone ring occurs on the side chain on C-24 (Takemoto et al., 1969) which increases the molecular weight and the polarity of the silyl ether in comparison with other ecdysteroids. This causes the derivative to have a longer retention

Table 4. Gas chromatographic properties of ecdysteroid TMS ethers.

Conditions of silylation	30 min, RT		5h, 120 °C		60h, 140 °C	
Ecdysteroid	t_R	t_R relative to ecdysone	t_R	t_R relative to ecdysone	t_R	t_R relative to ecdysone
Ecdysone	6.9	1	6.9	1	5.85	1
20-Hydroxyecdysone	7.95	1.15	9.45	1.37	7.55	1.3
Ajugasterone C	6.55	0.95	7.8	1.13	6.15	1.05
Calonysterone	9.25	1.34	10.5	1.52	10.5	1.79
Carpesterol	-		27.5*	4	-	
Cyasterone	-		19.8†	2.87	19.5	3.3
Dacrysterone	12.7*	1.84	9.85	1.43	-	
2-Deoxyecdysone	5.9	0.85	5.9	0.85	5.3	0.9
2-Deoxy 3-epiecdysone	5.5	0.8	5.5	0.8	5.3	0.9
2-Deoxy 20-hydroxyecdysone	7.1	1.03	8.5	1.23	-	
20-Hydroxyecdysone 2-cinnamate	11.6	1.68	>30	-	8.6	
Inokosterone	9.9	1.43	10.8	1.57	8.0	
22-Isoecdysone	7.5→6.5δ	1.09	6.5	0.94	6.4	
Kaladosterone	6.8	0.98	6.3	0.91	4.1	
Makisterone A	10.0	1.45	11.4	1.65	9.15	

Table 4 continued

Muristerone	†	-	9.25†	1.34	5.1†
Polypodine B	9.1	1.32	10.7	1.55	7.5
Polypodine B 2-cinnamate	11.0*	1.59	~10.6*	-	†
Ponasterone A	5.9	0.85	6.2	0.9	4.4
Ponasterone C	11.0	5.9	9.0	1.3	-
Ponasterone C 2-cinnamate	†	-	~10.0	-	-
Poststerone	1.65	0.24	1.65	0.24	1.3
Pterosterone	8.7	1.26	9.1	1.32	8.5

* - very broad peak

† - mixed derivatives

δ - if left longer than 30 min. at room temperature.

GC conditions - 285 ^{o}C, 0.9 m 1.5% OV 101 with a gas flow at 50-60 ml min^{-1}.
Detector at 300 ^{o}C.

Table 5. Proposed derivatives of some ecdysteroid TMS ethers.

Parent Compound	CONDITIONS OF SILYLATION					
	30 min, RT		5h, 120 °C		60h, 140 °C	
	No. of silyl groups	Position of -OH's silylated	No. of sily groups	Position of -OH's silylated	No. of silyl groups	Position of -OH's silylated
Ecdysone	4	2,3,22,25	4	2,3,22,25	5	2,3,14,22,25
20-Hydroxyecdysone	4	2,3,22,25	5	2,3,20,22,25	6	2,3,14,20,22,25
2-Deoxyecdysone	3	3,22,25	3	3,22,25	4	3,14,22,25
2-Deoxy 3-epiecdysone	3	3epi,22,25	3	3epi,22,25	4	3epi,14,22,25
2-Deoxy 20-hydroxyecdysone	3	3,22,25	4	3,20,22,25	5	3,14,20,22,25
Inokosterone	4	2,3,22,26	5	2,3,20,22,26	6	2,3,14,20,22,26
Makisterone A	4	2,3,22,25	5	2,3,20,22,25	6	2,3,14,20,22,25
Ponasterone A	3	2,3,22	4	2,3,20,22	5	2,3,14,20,22
Poststerone	2	2,3	2	2,3	3	2,3,14
Pterosterone	4	2,3,22,24	5	2,3,20,22,24	6	2,3,14,20,22,24

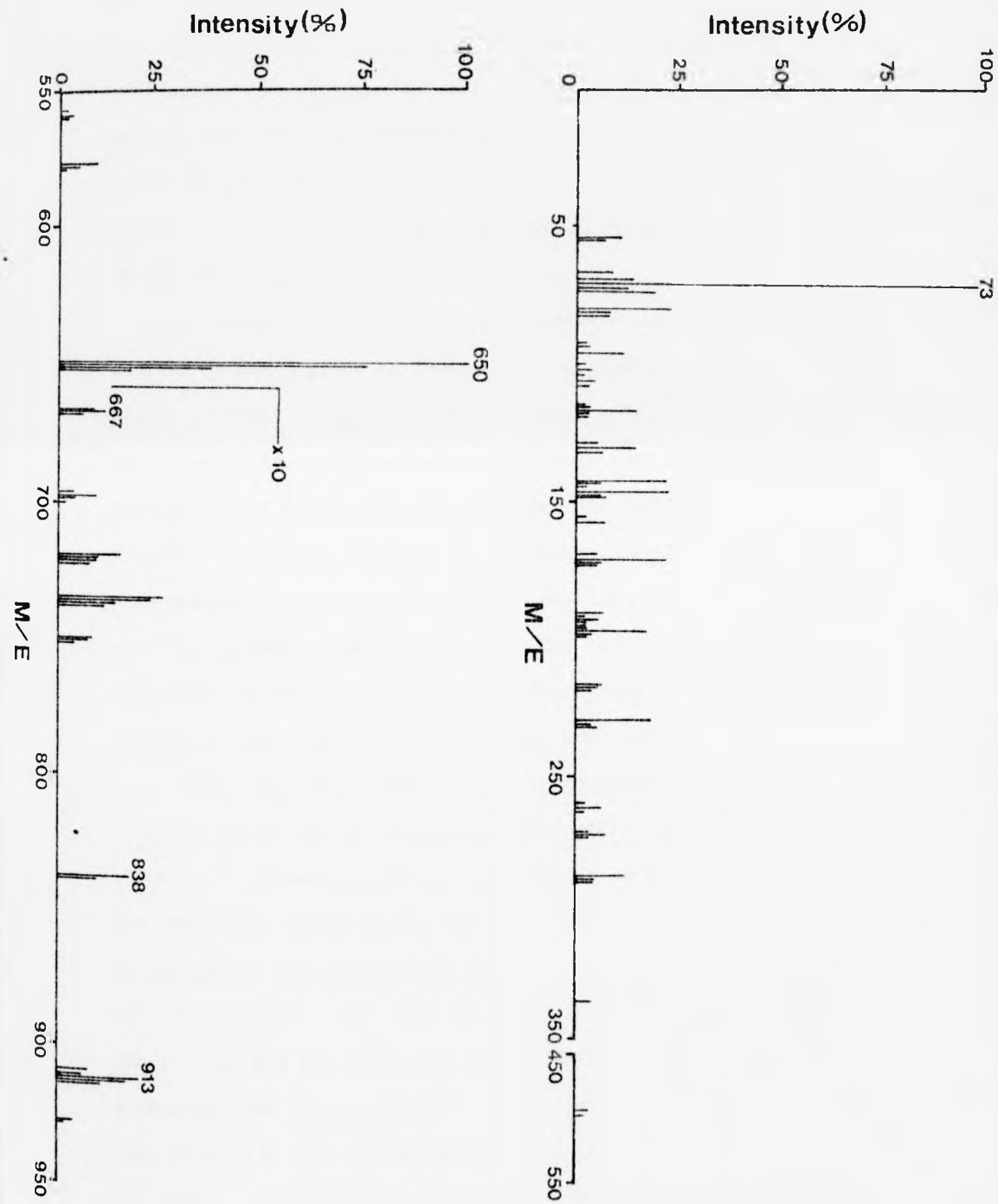
time, and poor peak shape at the temperature used for other ecdysteroid derivatives.

A group of plant ecdysteroids were also studied that have an additional chemical group, a cinnamate ester, attached to C-2 in place of a hydroxyl group. These ecdysteroids, which have been isolated from the bark of Dacrydium intermedium by Russell et al. (1972), are 20-hydroxyecdysone-2-cinnamate, polypodine B 2-cinnamate and ponasterone C 2-cinnamate. As with cyasterone, these compounds had poor GC characteristics and the broad peak shape and multiple product formation may have arisen from hydrolysis of the ecdysteroid into the parent compound and cinnamic acid on silylation. It has been noted elsewhere that this had in fact occurred when developing these ecdysteroids on silica TLC plates.

Four ecdysteroids studied possess a hydroxyl group in the β position on carbon 5. These were dacrysterone (5β-hydroxymakisterone A), a phytoecdysteroid isolated by Russell and Fraser (1973), kaladasterone (2β, 3β, 5β, 14α, 20, 22-hexahydroxycholest-7-en-6-one) and muristerone (11α-hydroxykaladasterone) both isolated by Canonica et al. (1975) and polypodine B (2β, 3β, 5β, 14α, 20, 22, 25-heptahydroxycholest-7-en-6-one) a phytoecdysteroid first extracted from a fern by Jizba et al. (1967a, b). Fortunately we were able to obtain sufficient quantities of polypodine B to determine the mass spectrum of the derivative formed after 5 h silylation at 120°C. This is shown in figure 15 and indicates that six of the seven hydroxyl groups have been silylated to form the hexakis TMS ether. As we know that the 14α hydroxyl group is not silylated under these conditions we can deduce that the 5β-OH has been derivatised. As yet though we do not know whether this hydroxyl group is silylated

Figure 15. Mass Spectrum of the Hexakis TMS ether of Polypodine B.

Conditions; Temperature of block at 300 °C, multiplier voltage at 2.5 kV and pre-amplifier sensitivity at x 10.



readily at room temperature and so we can only conclude what the 5 h and 60 h derivatives actually are. We can extrapolate this result and speculate that after 5 h silylation at 120°C the following derivatives of ecdysteroids with a 5β-OH group are formed; the hexakis TMS ether of dacrysterone and the pentakis TMS ether of kaladasterone, and after 60 h at 140°C, when the 14α-OH has been fully silylated, the heptakis and hexakis TMS ethers of dacrysterone and kaladasterone are formed respectively.

In ajugasterone C (11α-hydroxyponasterone A) and muristerone a novel hydroxyl group is found on the C ring in an α position on carbon 11 (Imai et al., 1969 and Canonica et al., 1975). As only small quantities of these two ecdysteroids were available no mass spectra of the derivaties were obtained and it is therefore not possible to propose the rate of silylation for this group. Also with muristerone multiple peaks were obtained when the derivatives were gas chromatographed which indicated that either the compound was impure or that some of the hydroxyl groups were only partially silylated under the sets of conditions employed.

The last phytoecdysteroid to be analysed was calonysterone (2β, 3β, 6, 20, 22, 25 -hexahydroxycholesta-5,8(9),14-trien-7-one) which is an unusually modified ecdysteroid as it is more unsaturated, has the ketone group on C-7, not on C-6 on which there is an hydroxyl group and the 14α hydroxyl is absent. Silylation yielded two products, one after derivatisation for 30 min at room temperature and the other after 5 h at 120°C. No further derivatisation occurred by increasing the silylation time or temperature and it was concluded that after 5 h the ecdysteroid was fully derivatised as the hexakis TMS ether. The derivative formed by silylation at room temperature was assumed to be the pentakis TMS ether where all but the hydroxyl

group on C-20 had been derivatised. As the hydroxyl group on C-6 is in such an unhindered position we propose that this group is readily silylated as are those hydroxyl groups on C-2, C-3, C-22 and C-25. Unfortunately a comparison of the response of these derivatives to the ECD, with ecdysone and other ecdysteroids, could not be made as the sample was too small to allow for any solutions of accurate concentrations to be prepared. Had this been possible we could have assessed the effect of the 5,8(9),14-trien-7-one as an electrophore in comparison with the 5β-7-en-6-one found in most ecdysteroids. The silyl ethers of calonysterone however gave sharp peaks when gas chromatographed and were readily detected.

The final ecdysteroid analysed was one that had been prepared synthetically by Harrison et al. (1966), 22-isoecdysone, and has been shown to have between one quarter and one eighth of the moulting hormone activity of ecdysone in bioassays. This ecdysteroid differs from ecdysone only in its orientation of the hydroxyl group on C-22 which is in the iso position. If this difference has no effect on the rate of silylation we would expect two derivatives to be formed as with ecdysone. In fact three derivatives were made. After a 30 min silylation at room temperature a single derivative was made which had a retention time of 7.5 min when gas chromatographed. If the mixture was silylated in excess of 30 min but still at room temperature a second peak gradually developed with a shorter retention time. This peak with the shorter retention time was obtained as a single derivative by silylation for 5 h at 120°C. From these results it was speculated that the hydroxyl group on C-22 did not silylate as readily as C-22 hydroxyl on ecdysone. Hence the first derivative was the tris TMS ether where the C-2,

C-3 and C-25 hydroxyl groups were silylated and the derivative formed after 5 h at 120°C was the tetrakis TMS ether where the C-22 group had also been fully derivatised.

The data provided by this study, even without the mass spectra that could establish the exact structures of the silyl ethers, is useful in providing a wider range of standard substances to help with identification of unknown ecdysteroids and understanding the range of properties they may possess.

Thin Layer Chromatography of Silyl Ethers of Pure Ecdysteroids

The formation of derivatives by silylation to give the trimethylsilyl (TMS) ethers, reduces the polarity of the ecdysteroids and these derivatives can be separated by TLC without the complications of poor recovery associated with the TLC of the polar parent compounds on silica.

The TLC procedure was used primarily to purify the reaction mixture after silylation to remove excess silylating reagent and imidazole, before injection onto the gas chromatograph, to prevent contamination of the electron capture detector. When pure ecdysteroids, however, were silylated they were generally at such a high concentration that dilution with toluene was adequate to prevent detector contamination. Only where the levels present are very low, as in a biological sample, is this TLC clean up procedure necessary. In this instance the sample is streaked along the origin of a specially prepared 0.6 mm thick silica TLC plate and developed in toluene:ethyl acetate (7:3). After development, a band of silica containing the derivatives is scraped from the plate and any silyl ethers are eluted with diethyl ether. To determine the appropriate R_f band to remove from the plate the R_f values for standard ecdysteroids must first be found.

By applying at least 500 ng of derivatised pure ecdysteroid onto a TLC plate the R_f of the compound, after development, can be determined by visualising under ultra violet illumination. Using this procedure the R_f values of derivatives, formed under varying conditions, for a number of ecdysteroids were obtained and are listed in table 6.

To determine the recoveries of tetrakis TMS ether of ecdysone and pentakis TMS ether of 20-hydroxyecdysone the method used by Wilson (1979) was employed. A sample of ecdysteroid was divided into two and both portions were then silylated, for 5 h at 120°C to give the appropriate derivative, but only one sample was used for TLC. After recovery of this half from the silica both samples were diluted to the same volume with toluene and analysed by gas chromatography. Their resultant peak areas were compared and recoveries of 80-90% down to 10 ng were recorded which confirm the results obtained by Wilson (1979) who cites 85% recoveryof 1×10^{-8} g or 10 ng.

An initial problem arose from impurities that were extracted from the plates, along with the silyl ethers by the elution of diethyl ether, that contaminated the electron capture detector giving a very broad solvent peak. This hampered the quantification of ecdysteroids at low levels that were likely to be encountered in a biological sample. The impurity was found to occur in the silica and the problem was resolved by extensive washing of the support with both methanol and diethyl ether before use in the preparation of TLC plates. This extensive washing may also have prevented any hydrolysis of silyl ethers which Wilson (1979) reported, but here was not found to occur.

Table 6

R_f Values of the TMS Ethers of Ecdysteroids on Silica

PARENT COMPOUND	R_f Value of the Derivative Formed After Silylation for:		
	30 min at room temperature	5 h at 120°C	60 h at 140°C
Ecdysone	0.72	0.72	0.82
20-Hydroxyecdysone	0.65	0.69	0.75
Ajugasterone C	0.63	0.8	0.8
Calonysterone	0.7	0.76	0.76
Carpesterol	0.7	0.7	0.7
Cyasterone	-	-	0.72
Dacrysterone	0.54	0.77	0.81
2-Deoxyecdysone	0.74	0.74	0.78
2-Deoxy 3-epiecdysone	0.74	0.74	0.77
2-Deoxy-20-hydroxyecdysone	0.74	0.68	-
20-Hydroxyecdysone 2-cinnamate	0.64	0.81	0.75
Inokosterone	0.65	0.72	0.8
22-Isoecdysone	-*	0.77	0.79
Kaladasterone	0.7	0.73	0.75
Makisterone A	0.67	0.72	0.78
Muristerone	-*	0.47*	0.59
Polypodine B	0.65	0.74	0.76
Polypodine B 2-cinnamate	0.4	0.76	0.83
Ponasterone A	0.67	0.78	0.71
Ponasterone C	0.34	0.71	-
Ponasterone C 2-cinnamate	0.38	0.75	0.78
Poststerone†	0.6-0.8	0.6-0.8	0.6-0.8
Pterosterone	0.71	0.71*	0.73

* - silylation of these ecdysteroids under these conditions formed mixed derivatives.

† - too small a sample to be viewed under UV illumination, so 1 cm bands of silica were removed and analysed for the presence of silyl ethers.

Evaluation of Some Phytoecdysteroids as Internal Standards for the Chromatographic Analysis of Ecdysone and 20-hydroxyecdysone from Barnacles

In the analysis of trace constituents such as ecdysteroids some losses during sample preparation are probable. Where an extensive extraction and isolation procedure is employed the use of an internal standard ensures that the method is both reproducible and efficient by determining the percentage recovery of that standard.

Results from analysis of levels of 20-hydroxyecdysone extracted from barnacles over a period of one year (see later) indicated that a considerable variation occurred seasonally. At this point it was considered essential to introduce an internal standard into the extraction procedure to ensure that this variation was not merely due to the irreproducibility and inefficiency of the analytical scheme. Ideally such a standard would co-elute with the ecdysteroid throughout the chromatographic stages but would be readily separable at the final detection stage. The most likely candidate would probably be another ecdysteroid and so a further consideration was that the standard had not been isolated from barnacles, ideally not from other crustaceans either. A phytoecdysteroid of similar structure and retention times would be most suitable.

Miyazaki et al. (1973) have used cyasterone as a GLC internal standard, as have Lafont et al. (1974 and 1980). More recently Lafont et al. (1980) has also used makisterone A. The properties of these two, plus a number of other ecdysteroids, were studied on a variety of chromatographic techniques. Wilson et al., (1982) studied their properties on HPLC, and after derivatisation their retention times on TLC and GLC-ECD were determined. The derivatives

were prepared by silylation with TMSI under the conditions described elsewhere for the preparation of silyl ethers of ecdysone and 20-hydroxyecdysone for detection by TLC and GLC-ECD.

From the large number of pure ecdysteroids studied earlier, five candidates were selected as possible compounds for use as an internal standard; these were calonysterone, inokosterone, makisterone A, polypodine B and ponasterone A. Unfortunately makisterone A and more recently ponasterone A have been detected in crustacean extracts (Faux et al., 1969; McCarthy, 1979 and Lachaise et al., 1981) as well as in plants and other arthropods (see Horn and Hetru, 1980) and inokosterone has also been isolated from crustaceans (Faux et al., 1969) but calonysterone has only been extracted from Kaladana seeds of a plant of the Ipomoea species (Canonica et al., 1973) and polypodine B, a phytoecdysteroid, also occurs only in plants.

As ecdysone had been detected in barnacles in addition to 20-hydroxy ecdysone (Bebbington, 1975) the most useful derivatisation of these two ecdysteroids was silylation for 5 h at 120^{o}C with TMSI to yield the tetrakis TMS and pentakis TMS silyl ethers respectively which could be readily resolved by GLC-ECD. This was therefore the silylation procedure used for analysis of ecdysteroids extracted from barnacle and the retention times of the derivatives of the internal standard candidates formed under these conditions were compared with them. The results are shown in table 7. The ECD response is also included and it can be seen that, relative to ecdysone, they all show a similar response excepting polypodine B where the response is only about a fifth of that of ecdysone presumably due to the presence of the 5β hydroxyl group. No value is given for calonysterone as the sample we were given was very small

Figure 16. Gas Chromatogram of TMS ethers of some Ecdysteroids derivatised by silylation for 5h at 120 °C.

1. Tetra-TMS Ponasterone A
2. Tetra-TMS Ecdysone
3. Penta-TMS 20-Hydroxyecdysone
4. Hexa-TMS Polypodine B
5. Penta-TMS Inokosterone
6. Penta-TMS Makisterone A.
7. Hexa-TMS Calonysterone

GC conditions; 285 °C, 0.9 m 1.5% OV101 with a gas flow of 50-60 ml min^{-1}. Detector at 300 °C.

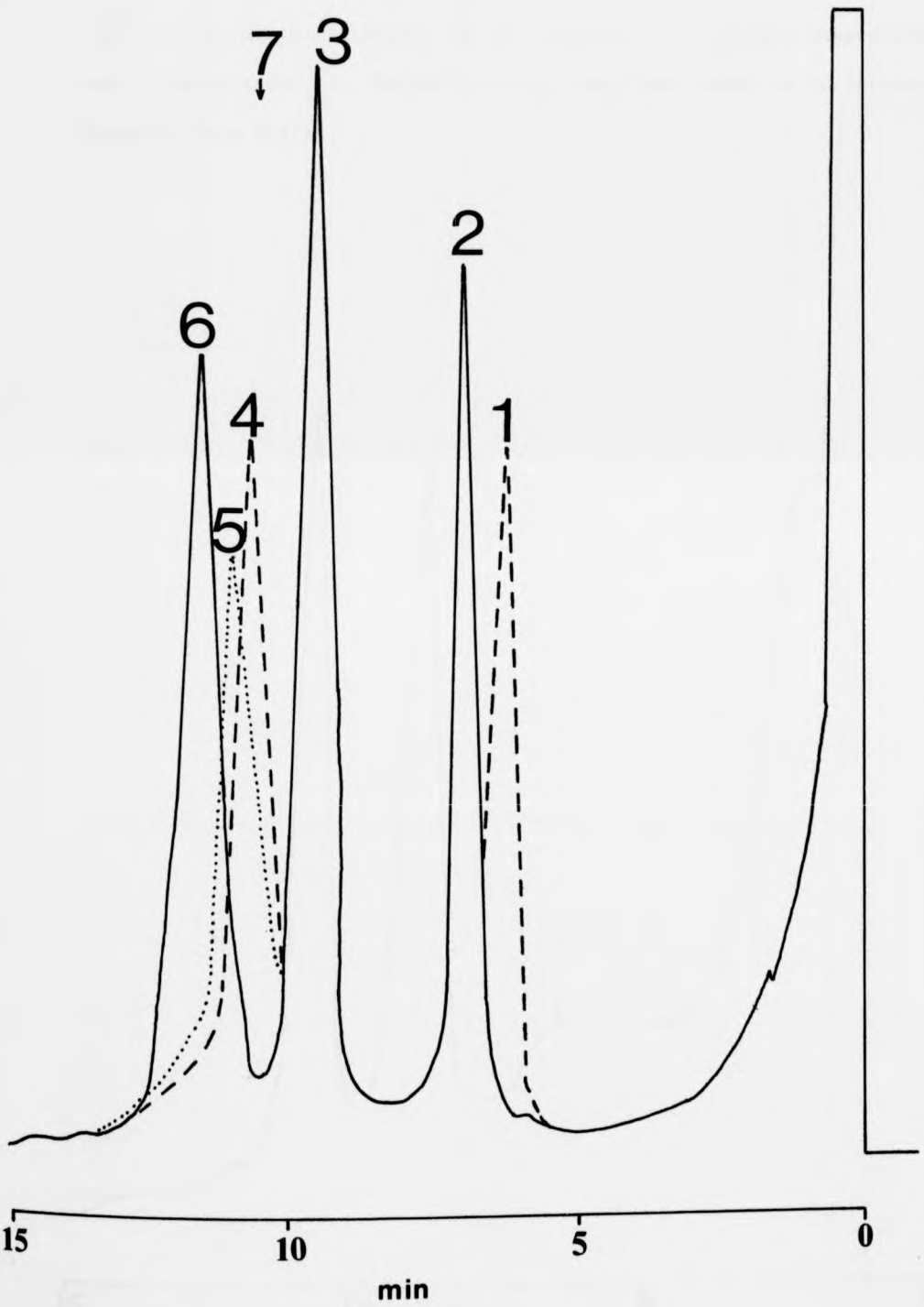

7
3
2
6
4
1
5
15
10
5
0
min

Figure 16(a). Gas chromatogram of Ecdysteroids Extracted from Barnacles.

Conditions as for figure 16. 1 = ecdysone, 2 20-hydroxyecdysone and 3 makisterone A. The makisterone A had been added as an internal standard (see text).

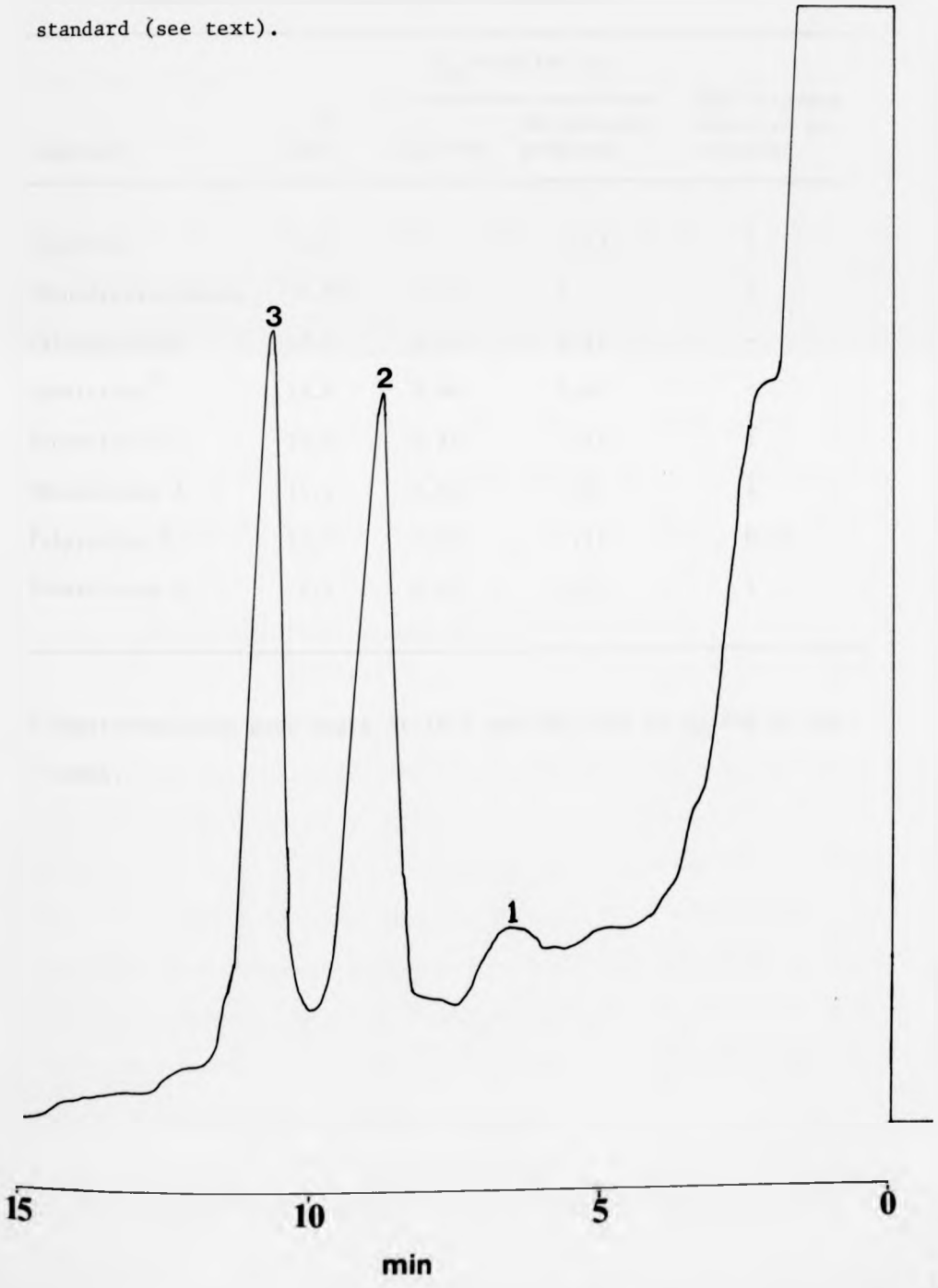


Table 7

GLC Retention Data for TMS Ethers of Ecdysteroids Prepared by Silylation for 5 h at 120°C.

Compound	t_R (min)	t_R relative to Ecdysone	t_R relative to 20-hydroxyecdysone	ECD response relative to ecdysone
Ecdysone	6.9	1	0.73	1
20-hydroxyecdysone	9.45	1.37	1	1
Calonysterone	10.5	1.52	1.11	-
Cyasterone*	19.8	2.86	2.07	-
Inokosterone	10.8	1.57	1.14	1
Makisterone A	11.4	1.64	1.2	1
Polypodine B	10.7	1.55	1.12	0.22
Ponasterone A	6.2	0.89	0.65	1

* Cyasterone also gave peaks at 16.3 and 28.4 min to ca 20% of the total.

and some insoluble material appeared to be present so that sample weighing was not reliably accurate. From the results of the retention times it was apparent that derivaties of calonysterone, inokosterone and polypodine B did not separate adequately from the pentakis TMS ether of 20-hydroxyecdysone and that only makisterone A and ponasterone A gave base line separation. However, if ecdysone is also present in the sample, ponasterone A will interfere with the detection of this ecdysteroid and hence the only suitable candidate as an internal standard in the analysis of ecdysteroids from barnacles was makisterone A. A composite chromatogram of the results is shown in figure 16 which further confirms this conclusion.

It is unfortunate that makisterone has been found previously in crustaceans, nevertheless, no reports have been made of its occurrence in cirripedes and it has not been detected in the barnacle, Balanus balanoides.

Thin Layer Chromatography of Pure Ecdysteroids.

Although the recovery of ecdysteroids from normal silica thin layer chromatography (TLC) plates is inefficient, this method of separating ecdysteroids and identifying them from their R_f values is still very popular. The ecdysteroids we studied could be readily detected by visualising them under ultraviolet illumination but the limit of detection was approximately 500 ng. This confirms the detection levels given by Morgan and Poole (1976a) but Wilson et al. (1981) have recently reported that, using TLC plates incorporating a fluorescent marker, 100 ng could be detected as visible dark spots against a fluorescent background.

A variety of solvent systems have been employed in the analysis of ecdysteroids by TLC and have been reviewed by Morgan and Poole

(1976a) and Morgan and Wilson (1980). The R_f values of a number of ecdysteroids, developed on 0.6 mm silica TLC plates in three solvent systems, are presented in table 8.

In this study these results were obtained merely to assist other research workers in their identification of unidentified extracted ecdysteroids. The possibility of using TLC in the extraction and isolation of ecdysteroids from barnacles was discounted because of the poor recovery results due to irreversible adsorption of the polar ecdysteroids onto the silica. The most recent evaluation of percentage recovery of an ecdysteroid from a TLC plate was made by Wilson et al. (1981) who reported 50% recoveries of 1 µg, 500 ng and 100 ng of ecdysone. The barnacle samples we were analysing were expected to contain in the region of 500 ng - 1 µg of ecdysteroid at the peak at the seasonal variation of ecdysteroid production. Therefore such poor recoveries prohibited the use of this technique for quantification studies.

It was also apparent that this technique is not suited to the study of those ecdysteroids which have a cinnamate group on carbon number 2. After running in the developing solvent, three spots were visible under the UV illumination. The most concentrated spot was the ecdysteroid 2-cinnamate but a spot, albeit very faint, was also present that gave an R_f value corresponding to the parent ecdysteroid. The third spot occurred just below the solvent front and was deemed to be due to the hydrolysed cinnamic acid. These results were obtained with all three compounds, 20-hydroxyecdysone 2-cinnamate, polypodine B 2-cinnamate and ponasterone C 2-cinnamate. Analysis of these phytoecdysteroids by silylation and GLC-ECD (see earlier) suggested multiproduct formation and so two possibilities arose. The samples may have been contaminated with a low concentration of the free parent

Table 8. R_f Values of Pure Ecdysteroids on 0.6 mm Silica TLC Plates

Ecdysteroid	Solvent System				
	CH_2Cl_2:MeOH (4:1)	$CHCl_3$:95% EtOH(85:15)		CH_2Cl_2:MeOH (85:15)	
	R_f	R_f	R_e*	R_f	R_e*
Ecdysone	0.31	0.10	1	0.10	1
20-Hydroxyecdysone	0.26	0.07	0.7	0.07	0.7
Ajugasterone C	0.29	-	-	0.09	0.9
Carpesterol	0.91	-	-	0.78	7.8
Cyasterone	0.44	-	-	0.14	1.4
Dacrysterone	-	0.13	1.3	0.14	1.4
2-Deoxyecdysone	-	-	-	0.23	2.3
2-Deoxy-3-epi-ecdysone	-	0.23	2.3	0.23	2.3
2-Deoxy 20-hydroxyecdysone	0.43	-	-	0.17	1.7
20-Hydroxyecdysone 2-cinnamate	-	0.35	3.5	0.30	3
Inokosterone	0.24	0.10	1	0.08	0.8
Makisterone A	0.28	0.08	0.8	0.09	0.9
Maristerone	0.31	-	-	0.14	1.4
Polypodine B	0.29	0.10	1	0.10	1
Polypodine B 2-cinnamate	-	0.40	4	0.38	3.8
Ponasterone A	0.49	0.25	2.5	0.23	2.3
Ponasterone C	-	0.26	2.6	0.20	2
Ponasterone C 2-cinnamate	-	0.55	5.5	0.52	5.2
Poststerone	0.49	-	-	0.16	1.6
Pterosterone	-	0.17	1.7	0.16	1.6

* R_e - Relative to ecdysone

ecdysteroid with the original hydroxyl group on carbon 2, or some hydrolysis of the compound may have occurred. This second hypothesis is supported by the TLC data and the fact that a similar problem has been recorded previously by Wilson (1979) where some hydrolysis of TMS ethers occurred when silylated ecdysteroids contacted dry silica on a TLC plate.

Collection of Samples of Balanus balanoides

The prime consideration was to locate a suitable collection site from where small samples of barnacles could be obtained regularly over a two to three year period. The site had to be readily accessible from the laboratory and the barnacle Balanus balanoides had to be present in sufficient numbers to enable samples to be collected frequently without denuding or adversely effecting the area. After a preliminary survey a site at Menai Bridge, North Wales, was found that met these requirements; the area was easily accessible from the laboratory at Keele University and the species of barnacle was present in abundance. As there is a large tidal range at this site, in excess of ten metres from low to high water during the equinoctial tides, there is a very wide balanid zone. Thus it was possible to collect small samples regularly from this zone from a fixed tidal position by always collecting barnacles from the same height from a north facing bridge support.

Another consideration when choosing the site concerned the species of barnacles present. Three species of barnacles commonly occur in the inter tidal region around the British coastline; Chthamalus stellatus (Poli), the australasian immigrant Elminius modestus (Darwin) and B. balanoides. C. stellatus and B. balanoides are difficult to distinguish from external appearances as they both

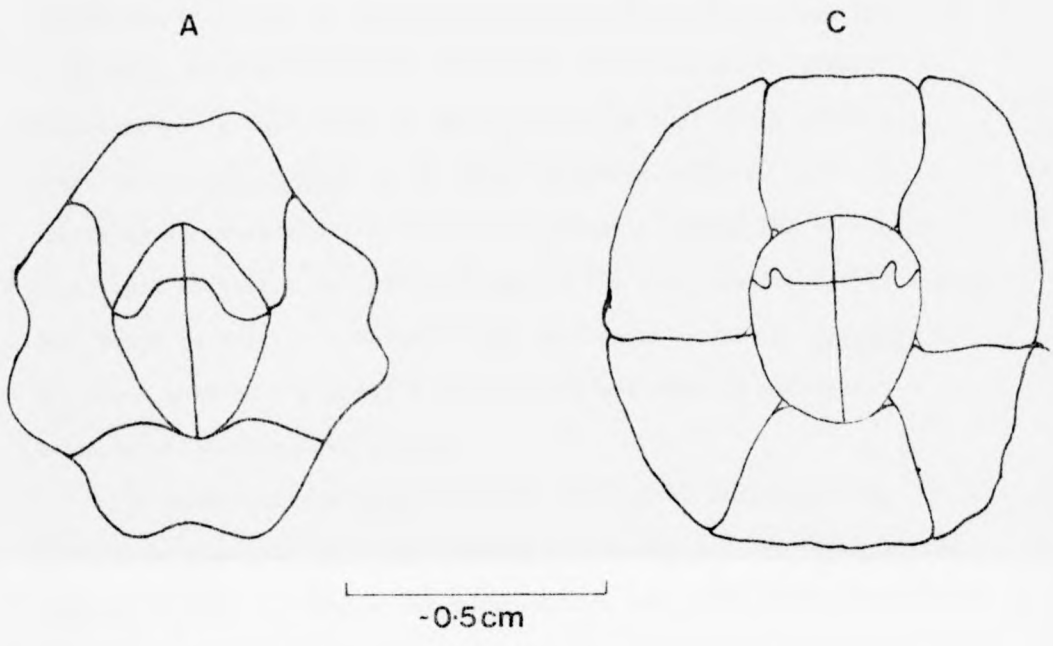


Figure 17. Diagrammatic Representation of Three Common Intertidal Barnacles (After Southward, 76).

A - Elminius modestus
B - Balanus balanoides
C - Cehthalamus stellatus

c = carina; c-l = carino-lateral shell plate;
l = lateral plate; o.v = opercular valves;
r = rostrum.

posses six shell plates unlike E. modestus which only posses four and is readily distinguished (see figure 17 after Southward, 1976). Fortunately, at this site, E. modestus is the only other barnacle, apart from B. balanoides, to be found in great numbers. The australasian immigrant was found predominantly around the mean high water mark above the intertidal range so few individuals of this species were found in the lower balanid zone, but whenever any E. modestus did occur they were readily distinguished and removed before any barnacle collection took place.

In addition to removal of other species of barnacles the collection area was carefully cleared of seaweed and any other marine organism before the sample was scraped from the stone face. Whenever possible barnacles of approximately the same size were collected ensuring, particularily during the late spring and summer, that newly settled spat were not included. The barnacles were carefully prised off the stone to ensure that the membranous base of the individuals suffered little or no damage. This was achieved most successfully by prising tightly packed groups or settlements of barnacles, as opposed to individuals, from the surface.

The collection was made as rapidly as possible, the barnacles being killed immediately by complete submersion in methanol. This solvent causes protein denaturation and subsequently halts enzyme activity and prevents any possible loss of hormone by autolysis (Bebbington, 1975).

Introduction of An Internal Standard Into the Analytical Scheme

After analysis of barnacle sample for levels of 20-hydroxy-ecdysone for one year, and after the chromatographic analysis of ecdysteroids recorded earlier, it was apparent that a wide range of

levels occurred seasonally. To ensure this was an accurate result and not the result of irreproducibility of the scheme, an internal standard was introduced into the system. This was makisterone A and in all future samples 1 µg of this compound was added to the barnacle sample before grinding to determine the percentage recovery of both this standard and the extracted 20-hydroxyecdysone.

Extraction of Ecdysteroids from Barnacles.

With the small sample of barnacles the one quarter h.p. grinder proved adequate to completely break down the hard calcareous shells but the resultant semi-solid mass was difficult to filter through a Buchner funnel and filter paper and this was substituted with an 11 cm sintered glass funnel. By increasing the volume of methanol to a total amount of four times the volume of barnacles, and using a vacuum line, the methanolic filtrate was rapidly obtained free from any particulate matter. Care was taken to ensure that the residue was thoroughly washed with methanol so that no loss of ecdysteroid occurred by adsorption onto the finely ground calcareous particles.

(i) Solvent Partition Systems.

The viscous dark green residue obtained by the evaporation of the methanolic filtrate was first partitioned between aqueous methanol (80%) and light petroleum (b.p. 40-60) with 1% iso-propanol. The colour of the methanol from the preliminary extraction indicated that pigments from the algae encrusted on the barnacle shell (Parke, 1935) would be a major source of impurities. Although these pigments along with lipids and non-polar steroids, passed predominantly into the light petroleum phase when a simple aqueous methanol:light petroleum system was used, this purification was enhanced by the

addition of 1% isopropanol to the ether phase. To ensure that the polar ecdysteroids were still retained in the aqueous methanol the recovery of three pure ecdysteroids in this partition system was found. Standard of makisterone A and the two ecdysteroids known to occur in barnacles, ecdysone and 20-hydroxyecdysone were analysed to determine their partition coefficients in the solvent mixture. In separate tests each ecdysteroid was added to the aqueous methanol phase of an aqueous methanol : light petroleum with 1% isopropanol partition system. The two phases were then analysed for ecdysteroid by evaporating to a residue, reacting with trimethylsilyl imadazole to form the trimethylsilyl ether of the ecdysteroid, and the quantity present determined using gas chromatography with an electron capture detector. With all three ecdysteroids no appreciable amount was found in the non-polar petroleum phase. Full recovery of the ecdysteroids, after two backwashes of the petroleum, was obtained from the aqueous phase and this modified partition was deemed acceptable.

No problems with formation of emulsions were encountered with this quarternary mixture.

The solid residue obtained from evaporation of the aqueous phase from the first partition was then partitioned between equal volumes of distilled water and butanol. In the analysis of ecdysteroids from insect material it was found that polar ecdysteroid conjugates when present (from faeces, eggs or adults), were partitioned into the lower aqueous phase whereas only free polar ecdysteroids were partitioned into the upper butanolic phase (Wilson, 1979). This separation therefore provides a convenient method for the separation of free and conjugated ecdysteroids in one sample and was retained unaltered in this analysis to determine the presence, if any, of

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both free and conjugated ecdysteroids in B. balanoides. After two backwashes with butanol the aqueous phase was retained at $4^{o}C$, after being reduced to a small volume, for enzymatic analysis of conjugates at a later date.

The ethyl acetate-water partition system that was used next by Morgan and Poole (1976) and Wilson (1979) did not reduce the total amount of material or remove the coloured impurities from the sample when analysing barnacle material. Various solvent systems were examined, the most useful was found to be similar to the first partition system using a non-polar and an aqueous phase; hexane and aqueous iso-propanol. This new system proved adequate in further removal of the brown pigmented material and determination of recovery of standard ecdysone, 20-hydroxyecdysone and makisterone A as before, showed that these ecdysteroids partitioned exclusively into the lower aqueous phase. To ensure maximum recovery the hexane was backwashed twice with aqueous iso-propanol and the combined aqueous extracts evaporated to give a brown residue.

In the analysis of ecdysteroids from insect material, Wilson (1979) found that three solvent partition systems were satisfactory for purification of the ecdysteroids present and at this stage the residue from the final partition was thoroughly dried and silylated to convert any ecdysteroids present to their trimethylsilyl ether derivatives. These derivatives were then purified by thin layer chromatography and recovered from the TLC plate by scraping the appropriate R_f band of silica into a glass column, from which the derivatives were then eluted with diethyl ether. After evaporating the ether, the residue was taken up in toluene and samples were injected onto a gas chromatograph, fitted with an electron capture detector, to determine the presence and concentration of any

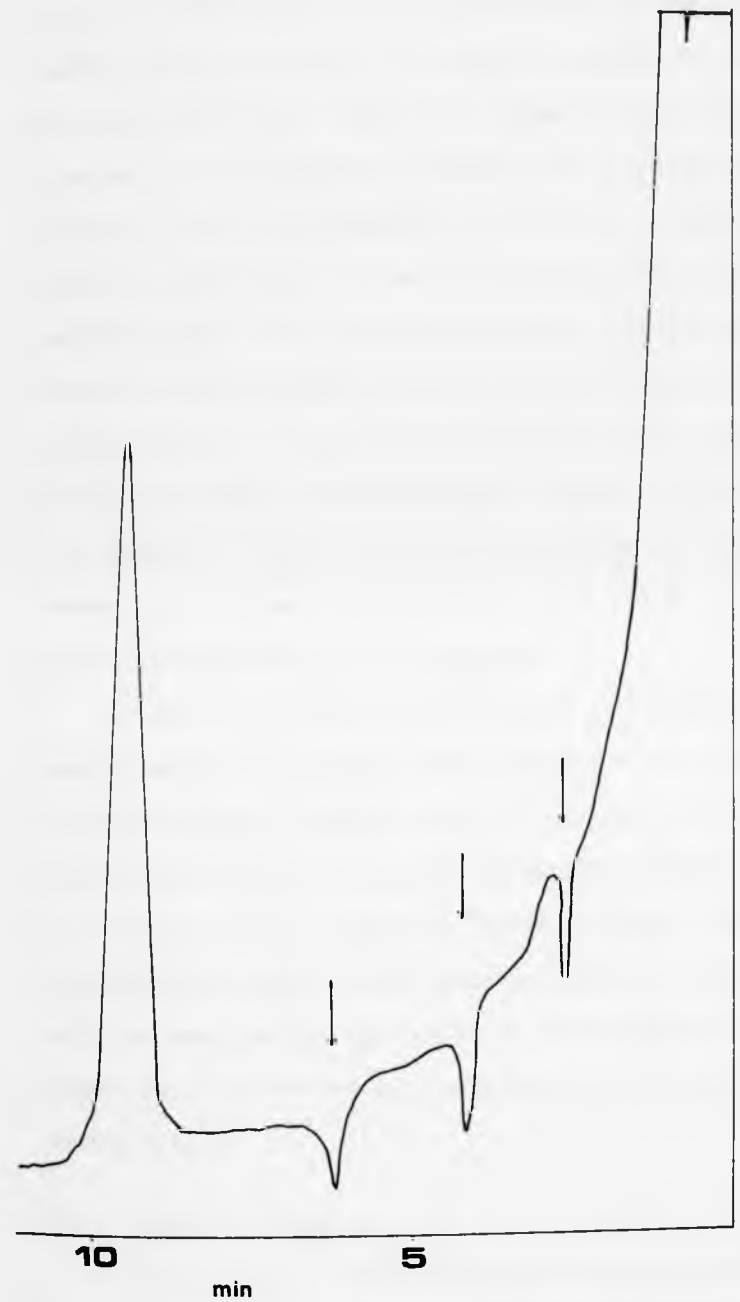


Figure 18. A Gas Chromatogram showing Contamination of the Electron Capture Detector.

Trace shows the pentakis TMS ether of 20-hydroxy-ecdysone and the presence of 'negative' peaks (arrowed) due to contamination. Note also the broad solvent front.

silylated ecdysteroids. This procedure was attempted with a barnacle sample. After drying and silylating the sample was developed on a TLC plate where it was noted that pigmented impurities had similar R_f values with any silylated derivatives of ecdysone or 20-hydroxy-ecdysone. When the appropriate R_f band was scraped from the plate, placed in a small glass column, and eluted with ether these impurities were eluted with any derivatised ecdysteroid. Subsequently, when the ether had been evaporated and the resultant residue taken up in toluene, and samples injected onto the gas chromatograph, gross contamination was observed with formation of negative peaks (see figure 18). The impurities were present in a high enough concentration to mask the detection of the relatively low levels of ecdysteroids present in B. balanoides.

From this analysis it was clear that a further purification step, in addition to three solvent partition systems, was required before the barnacle sample would be ready for silylation. The considerations for an additional analytical technique were that; it would need to be a relatively rapid technique using readily accessible equipment, as barnacles were collected approximately once every two weeks and as the levels of ecdysteroids present in small samples was so low (>500 ng) the recovery would need to be high and reproducible.

(ii) Sep-Pak Cartridges.

Wilson et al., (1980) had used disposable Sep-Pak cartridges for the successful purification of locust haemolymph before ecdysteroid analysis by high performance liquid chromatography. These cartridges provide a simple and rapid purification step by simply passing a solution through the support, using a syringe, and

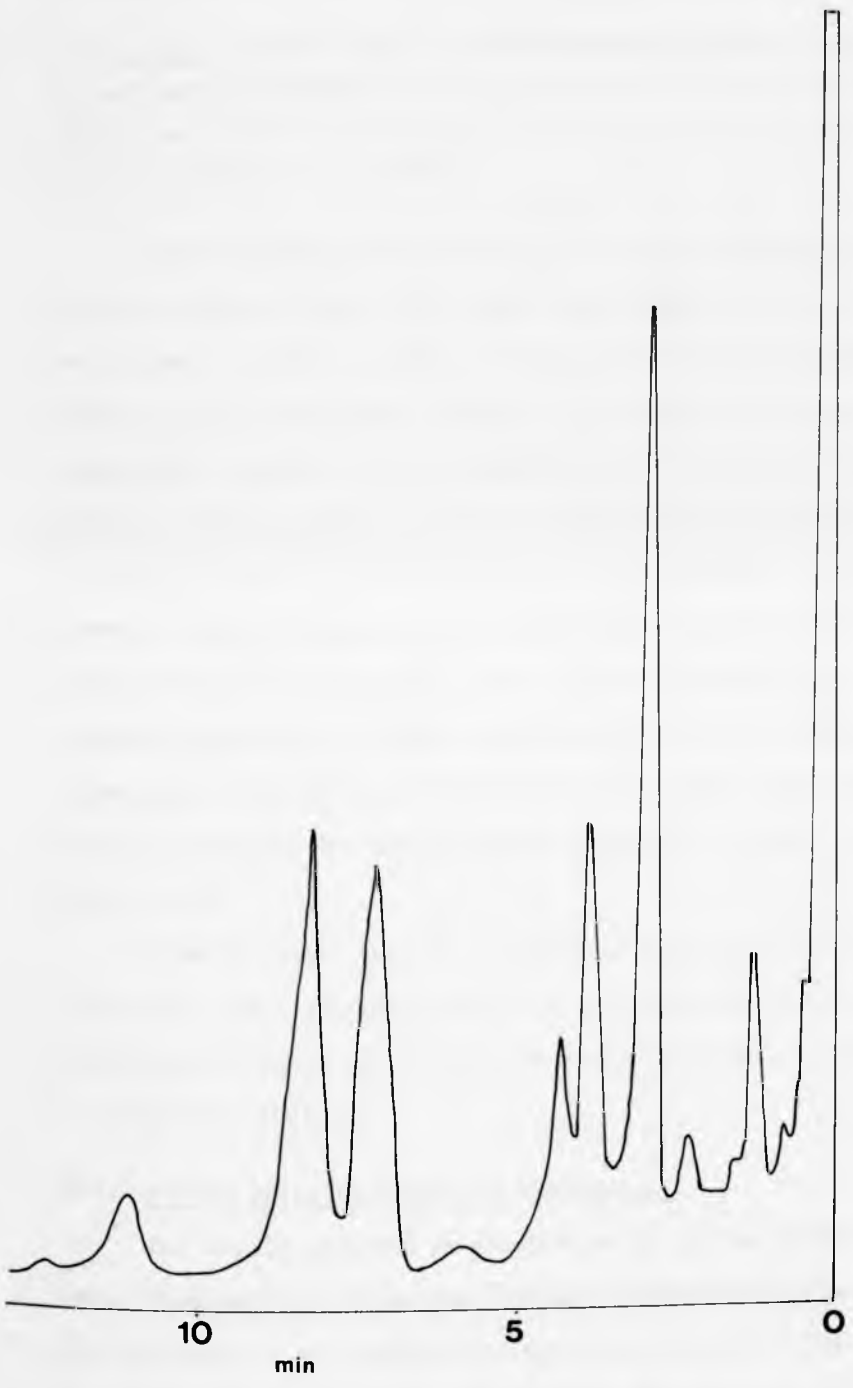


Figure 19. A gas chromatographic trace showing impurities from a Sep-Pak. See text for details.

are available with either a silica or a C_{18} reverse-phase packing.

Wilson et al., (1980) found that after chromatographing a sample on a C_{18} reverse phase Sep-Pak eluting with aqueous methanol, the resultant solution was relatively free of UV absorbing contaminants and therefore ideal for detection by HPLC/UV.

Barnacle samples were analysed both before and after silylation on reverse phase C_{18} and silica cartridges respectively in methanol and toluene, but these organic solvents extracted, what has been assumed to be, plasticizers from the cartridges which subsequently caused gross contamination and negative peak formation with the electron capture detector. Even after pre-washing the cartridges with these solvents the impurities were still present at levels which prevented any accurate quantification of the extracted ecdysteroids from barnacles (see figure 19). More recently Lafont et al., (1981) have used Sep-Paks in a simple and efficient scheme for sample processing in preparation for HPLC, where the plasticizers apparently caused no problems, and have recorded recoveries of 85% of 1 µg of makisterone A.

It would appear that for analysis of biological fluids or tissues with relatively high levels of ecdysteroids present these cartridges will be of great value for their simplicity and rapidity in ecdysteroid analysis.

(iii) Reverse Phase Thin Layer Chromatography.

The success achieved in preparation of reverse phase thin layer chromatography plates (see earlier) prompted us to determine the efficiency of this method as a purification step in the isolation of ecdysteroids from barnacles. The recovery results obtained by us and Wison et al. (1981) indicated that this method was suitable even with the low levels of ecdysteroids likely to be

present in barnacles. The plates used in this method were prepared by development in dichloromethane containing 7½% v/v liquid paraffin "Nujol".

The 0.6 mm RPTLC plates had a greater loading capacity and developed more rapidly than the commercially available plates because of the larger grain size of silica used in their preparation. Nevertheless, it can be seen that this did not impair the recovery of the ecdysteroids, and as the levels in the barnacle samples would be of the order of 100-500 ng, the use of these plates was considered.

The barnacle residue, after the solvent partition systems, was taken up in a small known volume of methanol. An aliquot of this was evaporated to reduce the volume and streaked along the origin of an equilibrated RPTLC plate. After development in 50% aqueous methanol, and by comparing known R_f values for ecdysteroids in this solvent system, it was apparent that the sample contained many pigmented impurities, some of which co-chromatographed with any ecdysteroids present. However, an attempt to recover any ecdysteroids was made by scraping the appropriate R_f band of silica into a small glass column and eluting with methanol. The pigmented contaminants eluted off the silica immediately as would any ecdysteroids, further emphasising that this scheme was of no assistance at this stage in the analysis of barnacle samples. The possible use of RPTLC in the analysis of ecdysteroid, however, must not be discounted as the technique is simple, the recoveries are excellent and samples can be loaded on the inexpensive laboratory prepared plate in aqueous solutions.

Thin layer chromatography is a suitable purification technique for a compound in large quantities (micrograms or milligrams) where the small loss, of say 100 ng, would still result in a high percentage

recovery. With samples at a much lower level of several hundred nanograms such a loss would result in a very low percentage recovery and render the technique useless for quantification studies. These small amounts are also difficult to visualise by non-destructive means such as UV fluorescence, and it is necessary to scrape a wide band of silica to ensure maximum recovery particularly if more than one compound present is to be recovered. This scraped silica is then generally placed into a small glass column and eluted with a suitable solvent to obtain the desired compound. Obviously in the case of small samples it would be preferable to use column chromatography directly, where manipulative losses are liable to be less and the procedure more rapid.

For these reasons the next technique considered as a further step in the purification of ecdysteroid from barnacles was column chromatography. It was deemed inadvisable to use silica or alumina, where recoveries are known to be low due to irreversible adsorption of the highly hydroxylated ecdysteroids onto the support (Miyazaki et al., 1973), or ion exchange resins as ecdysteroids are sensitive to both acid and alkaline conditions. A less polar adsorbant was required and microcrystalline cellulose, Floridin earth (Florex) and Sephadex were proposed as possible candidates.

(iv) Column Chromatography on Microcrystalline Cellulose.

Gilgan and Farquharson, (1973) had used descending paper chromatography in the analysis of ecdysteroids using various solvents composed of benzene saturated with aqueous isopropanol. The most successful developing solvent they labelled BiP45 which consisted of equal volumes of benzene, and 45% iso-propanol in distilled water being shaken together and then left to equilibrate and separate for 24 h. The benzene phase was then used as the developing solvent.

This developing solvent plus BiP50, BiP60, benzene saturated with distilled water only and petroleum ether (b.p. 40-60): diethyl ether (90:10) were assayed for development of ecdysteroids on pre-prepared cellulose TLC plates as a preliminary to microcrystalline cellulose column chromatography. After determination of the R_f value of ecdysone on a cellulose TLC plate in each of the solvent systems a barnacle sample was partially purified using the partition systems and the final residue was taken up in methanol. To this solution sufficient ecdysone was added so that a corresponding spot could be readily visualised under an ultra violet lamp (254 nm) after the solution had been chromatographed on the cellulose TLC plates. A sample of this solution was then streaked along the origin of a cellulose TLC plate which was then developed in one of the solvent systems. The results of these assays are shown in table 9, where the R_f of ecdysone is compared with the R_f values of visible pigmented impurities. From these results BiP50 (benzene saturated for 24 h with an equal volume of 50% aqueous isopropanol) was chosen as the eluting solvent for analysis of a barnacle sample by microcrystalline cellulose column chromatography.

Initially a barnacle sample with 10 µg of standard ecdysone added was placed on a column of microcrystalline cellulose and eluted with BiP50, fractions were collected and evaporated to dryness and analysed for the presence of ecdysone by silylation and gas chromatography. Unfortunately visible impurities still co-chromatographed with the ecdysteroid and on silylation caused contamination on the gas chromatograph to such an extent that it was impossible to quantify the recovery of ecdysone. As the levels of ecdysteroids in a barnacle sample would be far lower than used in this test case, the method was deemed unacceptable in this instance.

Table 9. Development of a barnacle sample with added ecdysone on cellulose TLC plates.

Solvent System	R_f value	
	Ecdysone*	Pigmented Impurities
BiP45	0.36 - 0.58	0.73 - 0.96
BiP50	0.41 - 0.51	0.74 - 1.00
BiP60	0.57 - 0.81	0.71 - 0.95
Benzene (wet)	0	0 - 0.8
Petroleum ether : diethyl ether (90:10)	0	0

* Using aqueous solvent it was difficult to obtain a concentrated spot and these values give the range over which the ecdysone could be seen under UV light.

(v) Column Chromatography on Floridin Earth (Florex)

The second support analysed was the relatively non-polar Floridin Earth (now marketed as Florex) which had previously been used by Woodbridge (1971) while studying the moulting hormones present in locusts. Woodbridge eluted a pre-washed column with a graded solvent system using ether and acetone, gradually increasing the content of the latter from 0-100% and then finally eluting any ecdysteroids with aqueous (15%) methanol.

The first problem that arose on adoption of this method was due to the use of methanol as the solvent for taking up the final barnacle residue after the partitions. If the sample was introduced onto the column in methanol and then developed according to the scheme used by Woodbridge (1971), some of the ecdysteroid present was eluted with the initial ether fractions because of the presence of this methanol. As no other less polar solvent was found suitable to take the final barnacle residue into solution, the problem was overcome by reducing the initial sample volume and including a less polar solvent than diethyl ether as the first eluting solvent, hexane. The column was then developed with hexane, diethyl ether, acetone containing ethanol (0,5,10 and 20%), and finally ethanol. Using a barnacle sample (known to contain a negligible amount of ecdysone), to which 10 µg of pure ecdysone was added, the ecdysteroid was found to elute with the acetone containing ethanol (5 and 10%). This solvent was used in place of the aqueous methanol (Woodbridge,1971) in the original scheme in order that the eluant could be readily evaporated to dryness in preparation for silylation.

After silylation of the active fraction it was apparent from the number of peaks obtained from the gas chromatogram that the

derivatisation had been catalysed by an impurity extracted from the support. An attempt at preventing this recurring was made by extensively washing the Floridin Earth before use with a solution of saturated sodium bicarbonate (to remove any acidic residues), distilled water, and dry distilled ethanol. The support was then dried over calcium chloride granules overnight. (Drying in an oven proved unsatisfactory as this over-activated the support and a large percentage ($\sim$ 50%) of the ecdysteroid was then found to be irreversibly adsorbed onto the phase when used in a column). After packing into a column and before introduction of the sample the column was pre-washed with hexane. Under these conditions and using a barnacle sample with ecdysone added (1.25 µg) a 100% recovery was obtained. This result, however, was not found to be reproducible in subsequent samples. Where ecdysone had been added, the ecdysteroid co-eluted from the column with green pigmented material. After silylation and whilst cooling this material appeared to precipitate out and occlude any ecdysteroid derivative present. This could only be prevented by dilution of the silylating solution, with toluene, while still hot and injecting the sample onto the gas chromatograph immediately but in this case the impurities now interferred with the electron capture detector and it was not possible to quantify any ecdysteroids present.

Due to the lack of reproducibility of this method, and the time involved in operating a gradient elution system, the technique was discarded in favour of Sephadex gel column chromatography.

(vi) Column Chromatography on Sephadex Gel.

In the first large scale extraction of ecdysteroids from crustaceans, Horn et al., (1968) incorporated Sephadex G-25 and CM-Sephadex, a cation exchanger, in the extraction scheme.

CM-Sephadex was also used by Woodbridge (1971) in his study of locust moulting hormone.

A Sephadex grade was also modified to enable gel filtration in organic solvents (Joustra et al., 1967) and marketed as Sephadex LH-20. This is prepared by hydroxypropylation of Sephadex G-25, the hydroxypropyl groups being attached by ether linkages to glucose units of the dextran chains. The resultant gel has both hydrophilic and lipophilic properties and separations on this support depend not only on molecular sieving but also on effects of solvent polarity, adsorption and partition if a mixed eluant is used.

Initially acetone was used as the developing solvent and in preparation of this a sample of the LH-20 Sephadex was swollen overnight in dry, distilled acetone. The first column prepared was 16 cm x 100 mm i.d. and the gel was packed as a slurry ensuring that no air bubbles or discontinuity layers occurred. The column was then pre-washed with acetone.

A barnacle sample, with 10 µg of ecdysone added, was then evaporated to a small volume (500 µl) and shaken with acetone (3 ml) which yielded a flocculent precipitate that was removed by bench centrifugation. The supernatant was retained and the residue was redissolved in a small volume of methanol and then re-precipitated with a further volume of acetone (3 ml). This was repeated once more and then the combined supernatants were evaporated to a small volume (1 ml). Analysis of an aliquot of this solution confirmed that no ecdysteroid had been lost in the precipitate and this result concurs with the results obtained by McCarthy and Skinner (1979a) in a similar experiment. The remainder of the acetone solution was chromatographed on the Sephadex column and, after introduction of

the sample, fractions (4 ml) were collected. These fractions were analysed by evaporation to dryness followed by silylation and determination of any ecdysone and its quantity by GC-ECD. The results showed that yellow and brown pigmented impurities were eluted from the column in the first four fractions and the ecdysone was eluted, as a pale green solution, in the following four fractions. This pigment gave little interference with the analysis and a recovery of ecdysone of 107% was recorded.

A larger column was now prepared using a sealed system so that the column would be used repeatedly (see figure 20). Using 10 µg of ecdysone and 20-hydroxyecdysone in acetone the column was developed and fractions were collected and analysed. From this the elution profile of the acetone eluted LH-20 Sephadex column was obtained (see figure 21) and the active fraction containing the ecdysteroids found to be 75-150 ml after sample introduction. Recoveries of both standard ecdysone and 20-hydroxyecdysone were then determined and the results of these experiments are shown in table 10.

Although this appeared to be an adequate technique to further purify the barnacle sample before silylation, the fraction containing ecdysteroids was still partially contaminated and it was considered likely that in barnacle samples with very low levels of ecdysteroids present this contamination would inhibit accurate quantification. This, and the possibility of some loss from small samples in the precipitation of the methanolic solution in acetone, prompted us to consider the use of a methanol eluted Sephadex LH-20 column.

Thus a second column was prepared, identical with the acetone eluted one, except that the dextran swells more in methanol and as

Table 10

Recoveries (%) of Ecdysteroids from Sephadex Chromatography Columns

Ecdysteroid and amount (µg)		% Recovery	
		Acetone eluted	MeOH eluted
Ecdysone	2	96.5	94
	1	92	95
	0.5	102	89.5
20-hydroxyecdysone			
	2	97	-
	1	93	86.5
	0.5	93.5	92.5

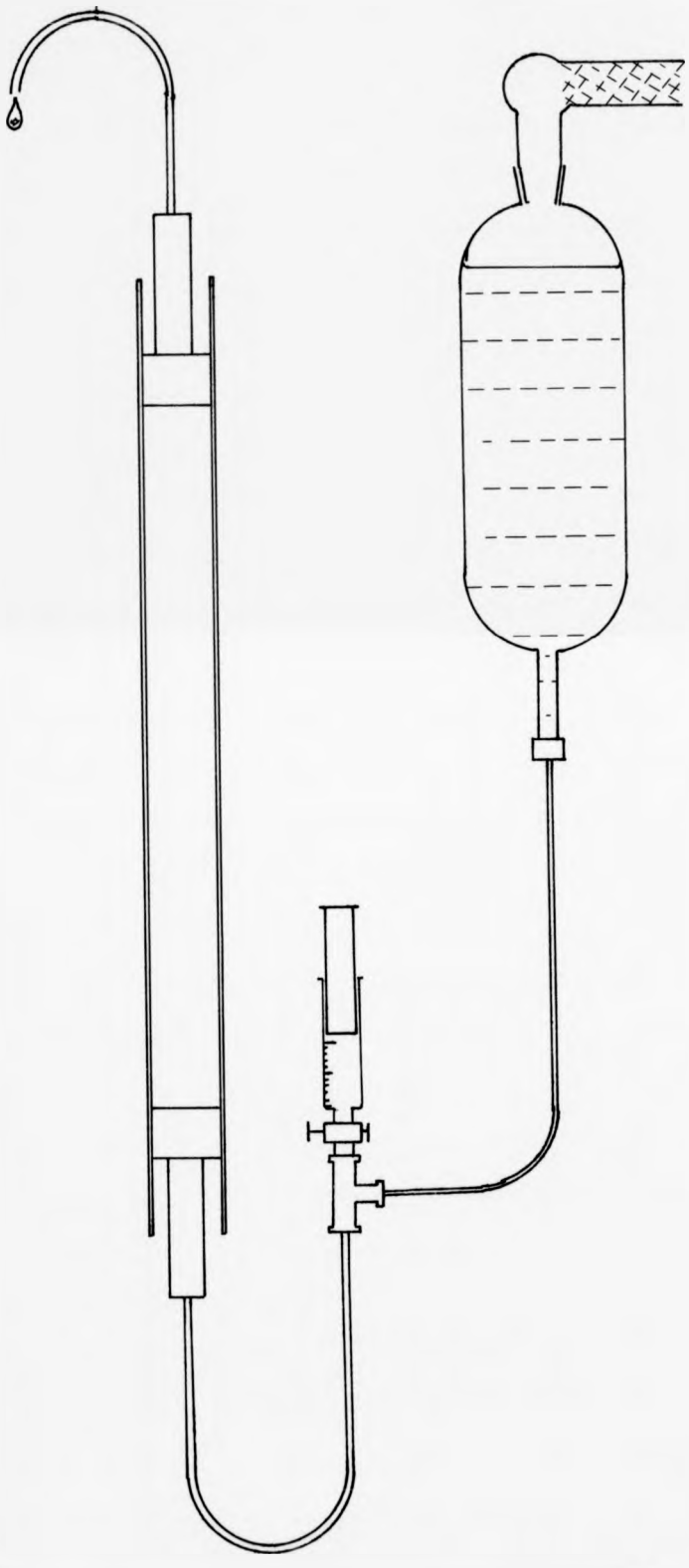


Figure 20. Sephadex Gel Column Chromatography Apparatus (see experimental for a full description).

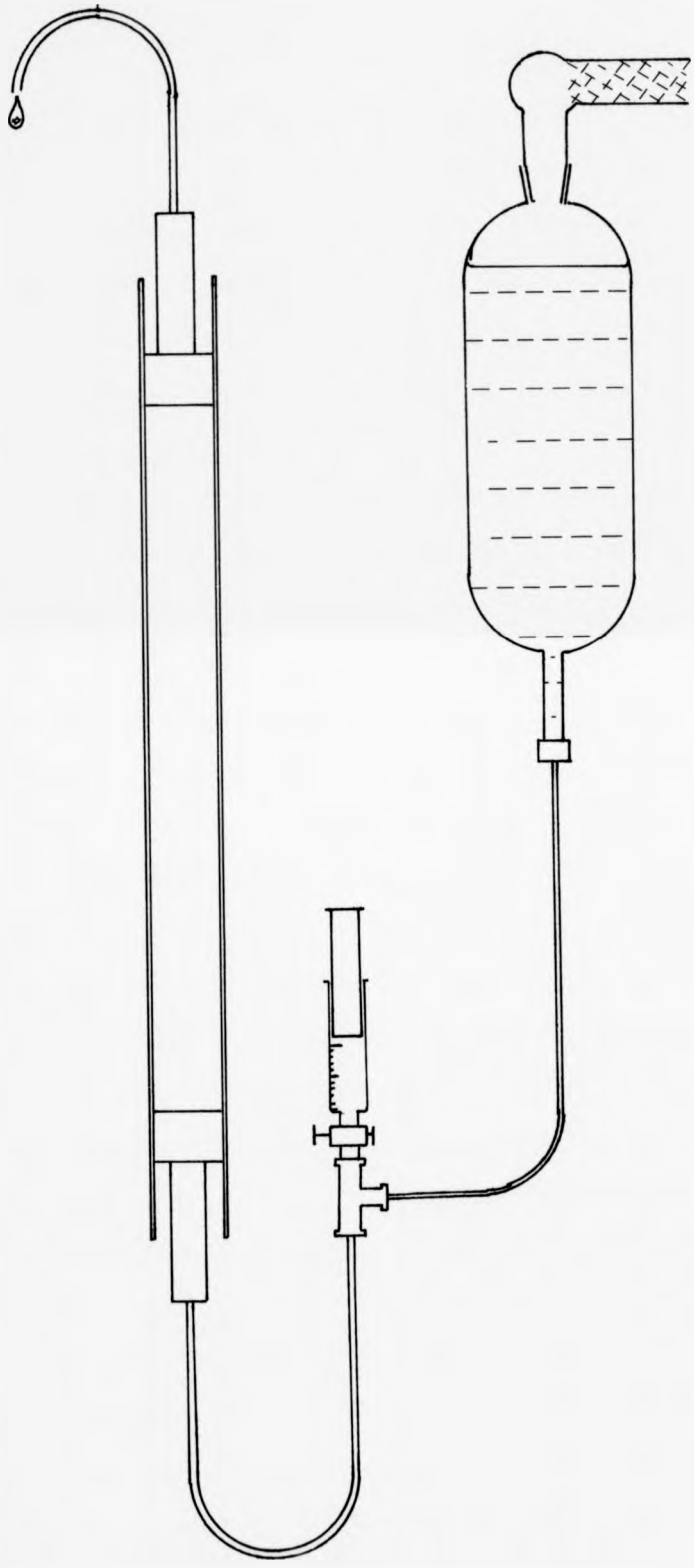


Figure 20. Sephadex Gel Column Chromatography Apparatus (see experimental for a full description).

a result, although the same weight of dry LH-20 was used, the dimensions of the methanol column are larger (see experimental). As before, this column was used first to study standards of ecdysone and 20-hydroxyecdysone to obtain an elution profile (see figure 21) and percentage recoveries (see table 10). The active fraction containing the ecdysteroids eluted 45-75 ml after sample introduction.

A barnacle sample was introduced onto the methanol eluted Sephadex column and developed, the first 31 ml was collected as fraction one and thereafter, as pigmented compounds eluted from the column, smaller fractions (3 ml) were collected until 100 ml had been retained. These fractions were then analysed on an ultra-violet spectrometer which revealed that the active fractions in which ecdysone and 20-hydroxyecdysone (55-80 ml) would be found, contained an orange pigment with a λ_{max} 430-440 nm but the methanol column, almost completely separated the ecdysteroids from a green pigment (λ_{max} 662 nm) which eluted before the active fractions (see figure 22).

The fractions that would contain any ecdysteroids were then combined and prepared for development on the acetone eluted Sephadex column. By analysis of fractions eluted from this column in a similar manner it transpired that the orange pigment that co-chromatographed with the ecdysteroids on the methanol eluted Sephadex column, eluted before the ecdysteroids on the acetone developed column (see figure 23). Unfortunately, data from the UV spectrometer indicated that traces of the green pigment (λ_{max} 662-665 nm) were still present in the active fractions. Nevertheless, by using these two Sephadex columns in conjunction the most reproducible results and the best purification of samples of ecdysteroids from a barnacle sample

Figure 21. Elution profiles of ecdysteroids on Sephadex Gel Chromatography

Elution of ecdysone (▲——▲) and 20-hydroxyecdysone (●– –●) on an acetone swollen and eluted column (A) and a methanol swollen and eluted column (B). Makisterone elutes between these two ecdysteroids on both columns. Conditions as described in text.

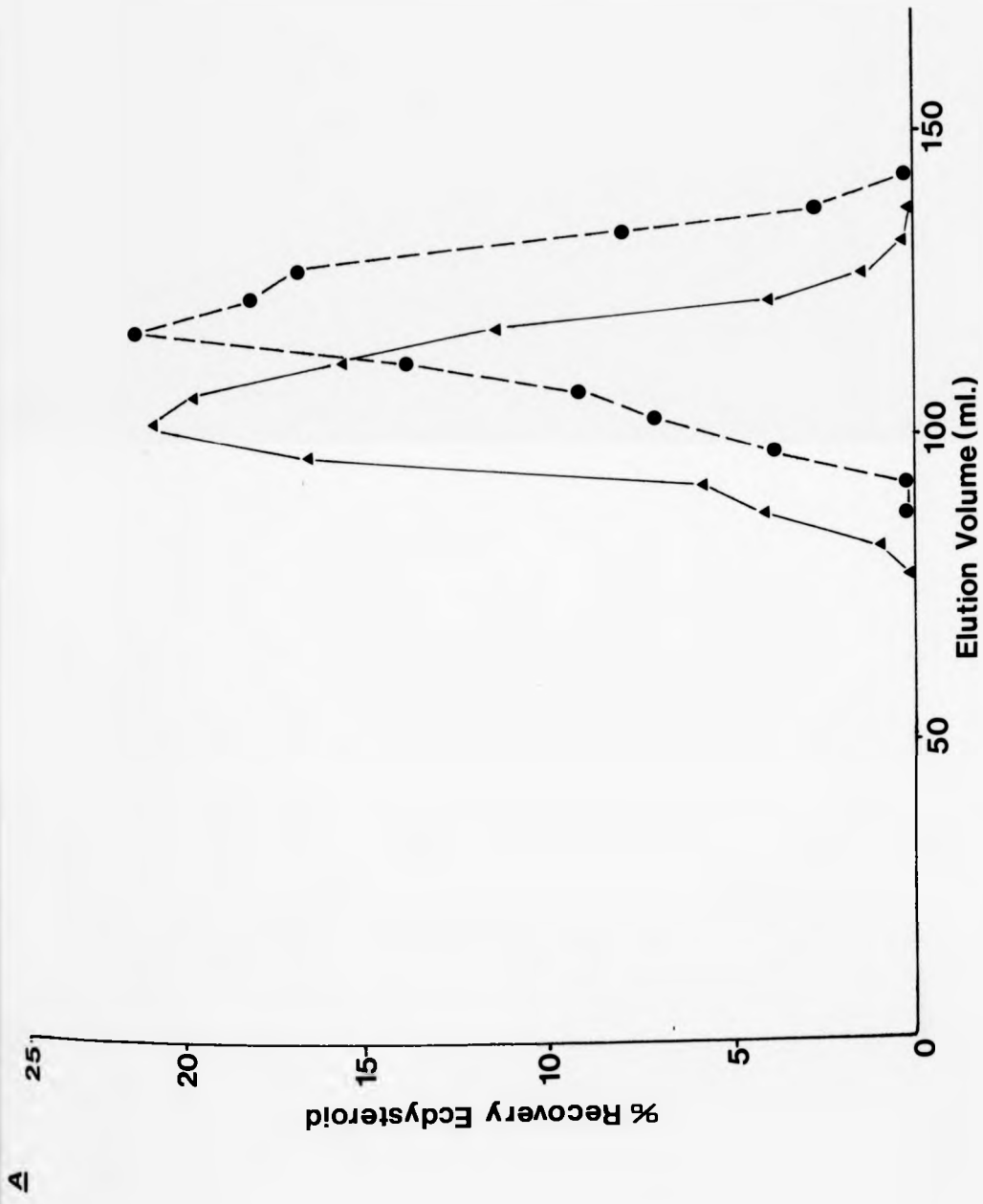
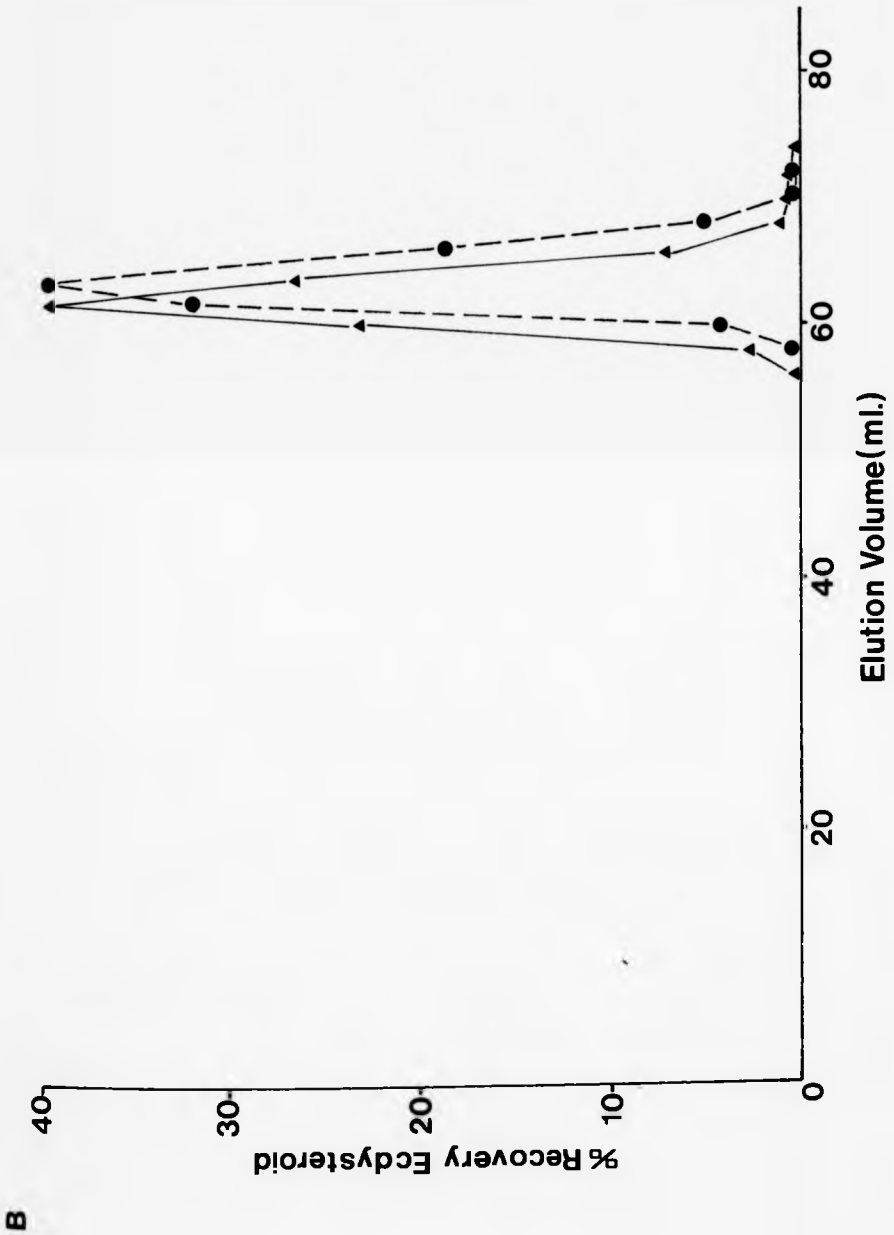


Figure 21. Elution profiles of ecdysteroids on Sephadex Gel Chromatography

Elution of ecdysone (▲——▲) and 20-hydroxyecdysone (●- -●) on an acetone swollen and eluted column (A) and a methanol swollen and eluted column (B). Makisterone elutes between these two ecdysteroids on both columns. Conditions as described in text.



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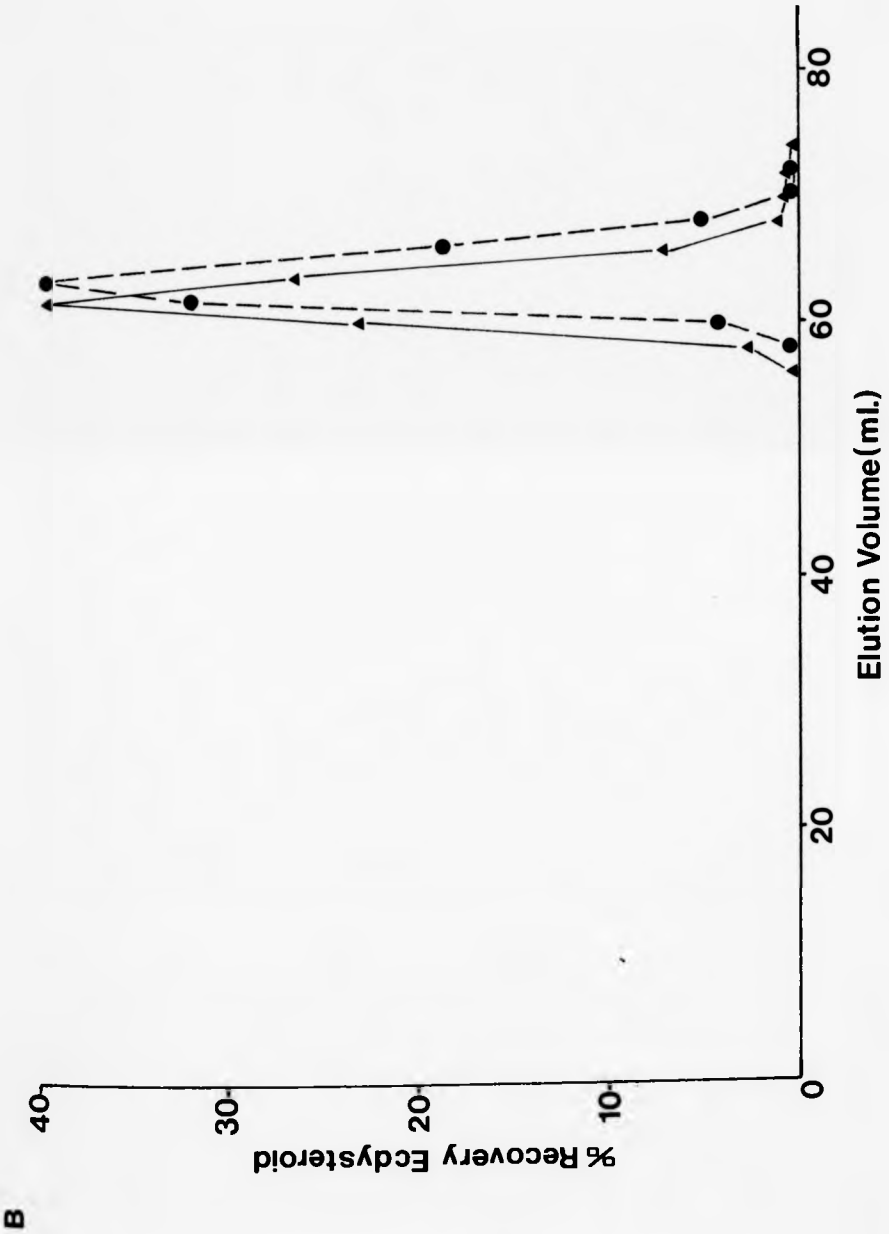


Figure 22. Elution profile of a barnacle sample on a methanol eluted sephadex gel column.

A; pigmented impurity with λ_{max} at 662 nm (▲——▲)

B; 20-hydroxyecdysone (●- -●)

C; pigmented impurity with λ_{max} at 430-440 nm (△——△)

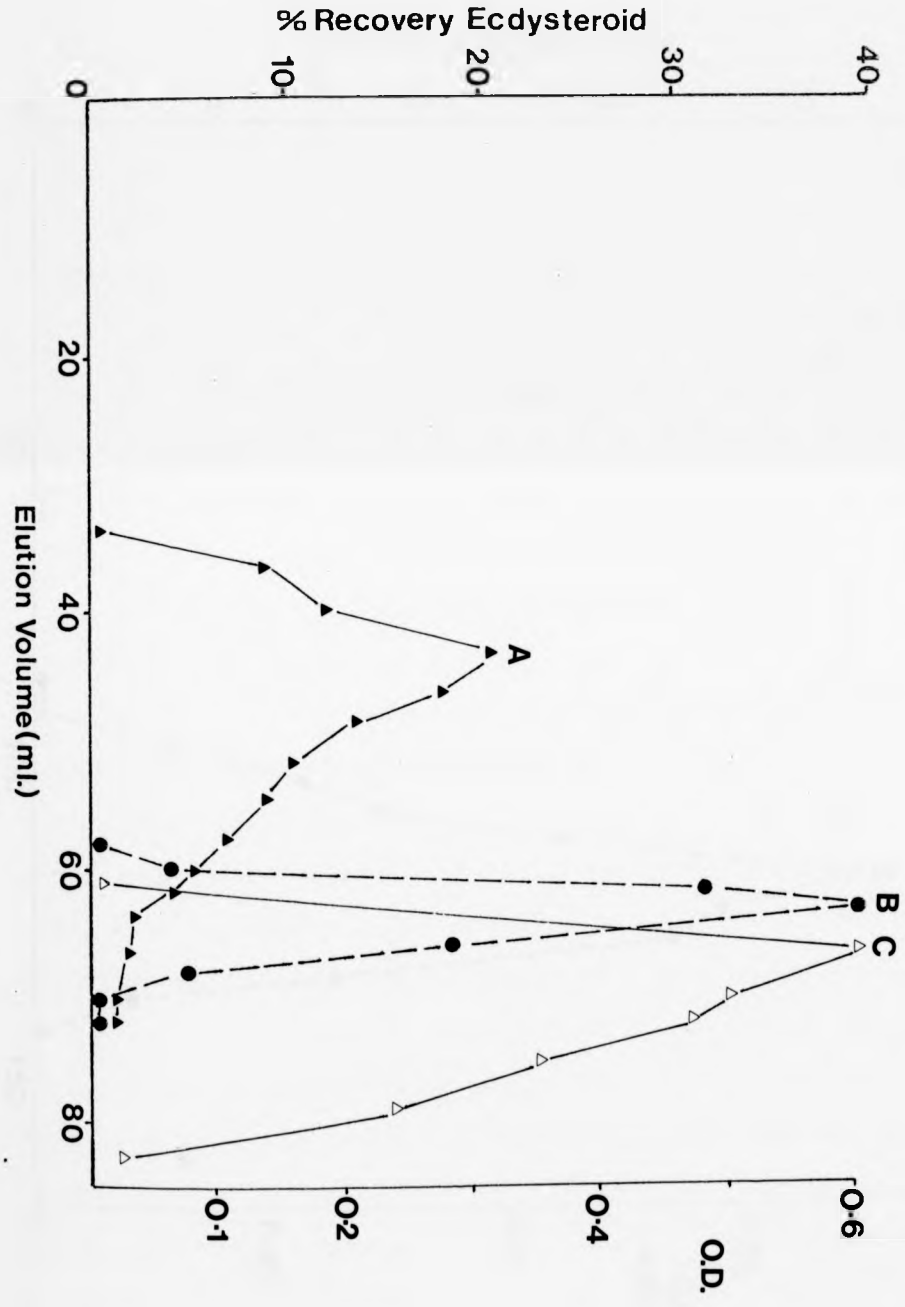
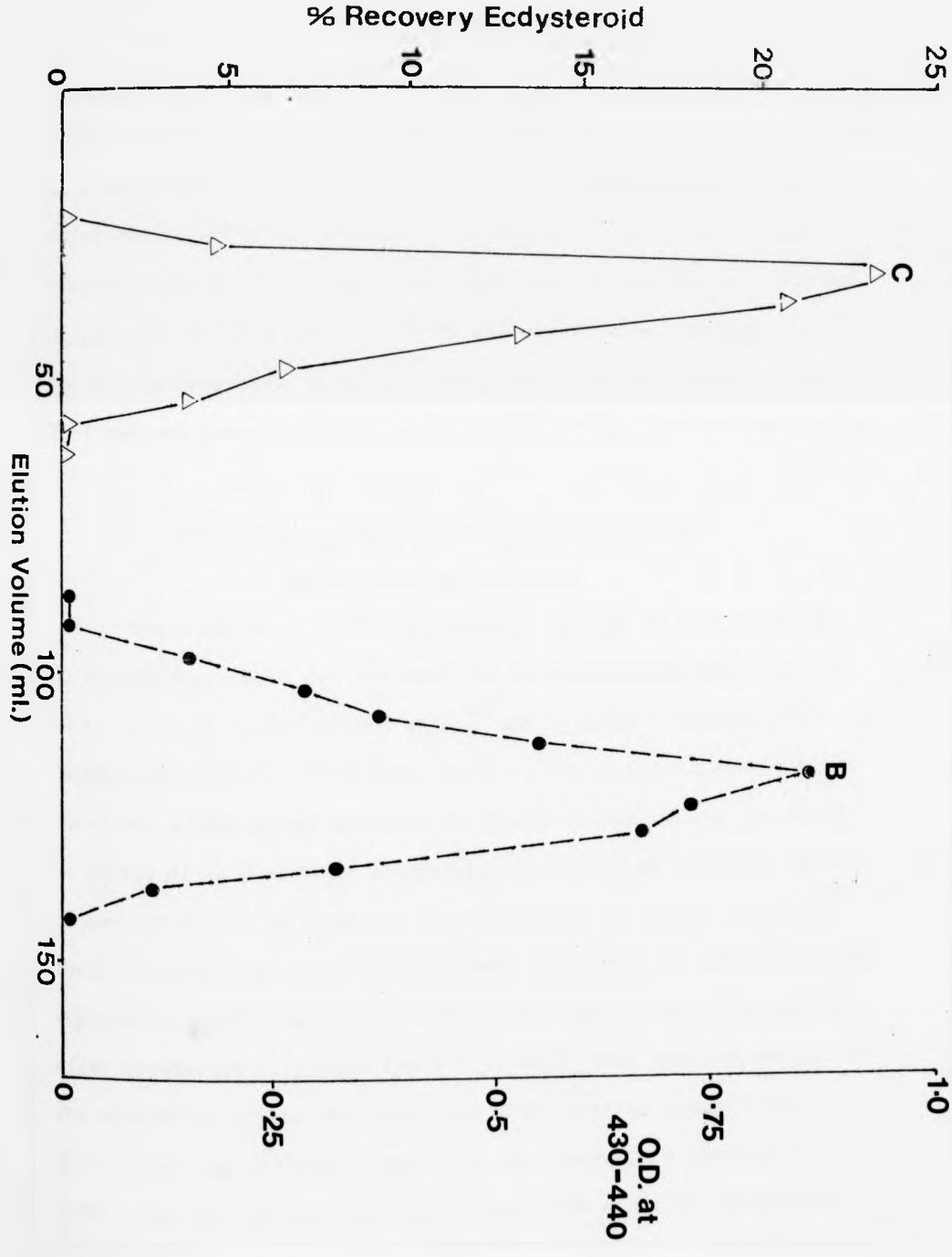


Figure 23. Elution profile of a barnacle sample on an acetone eluted sephadex gel column.

B; 20-hydroxyecdysone (●- -●)

C; pigmented impurity with λ_{max} at 430-440 nm. (△——△)



were obtained.

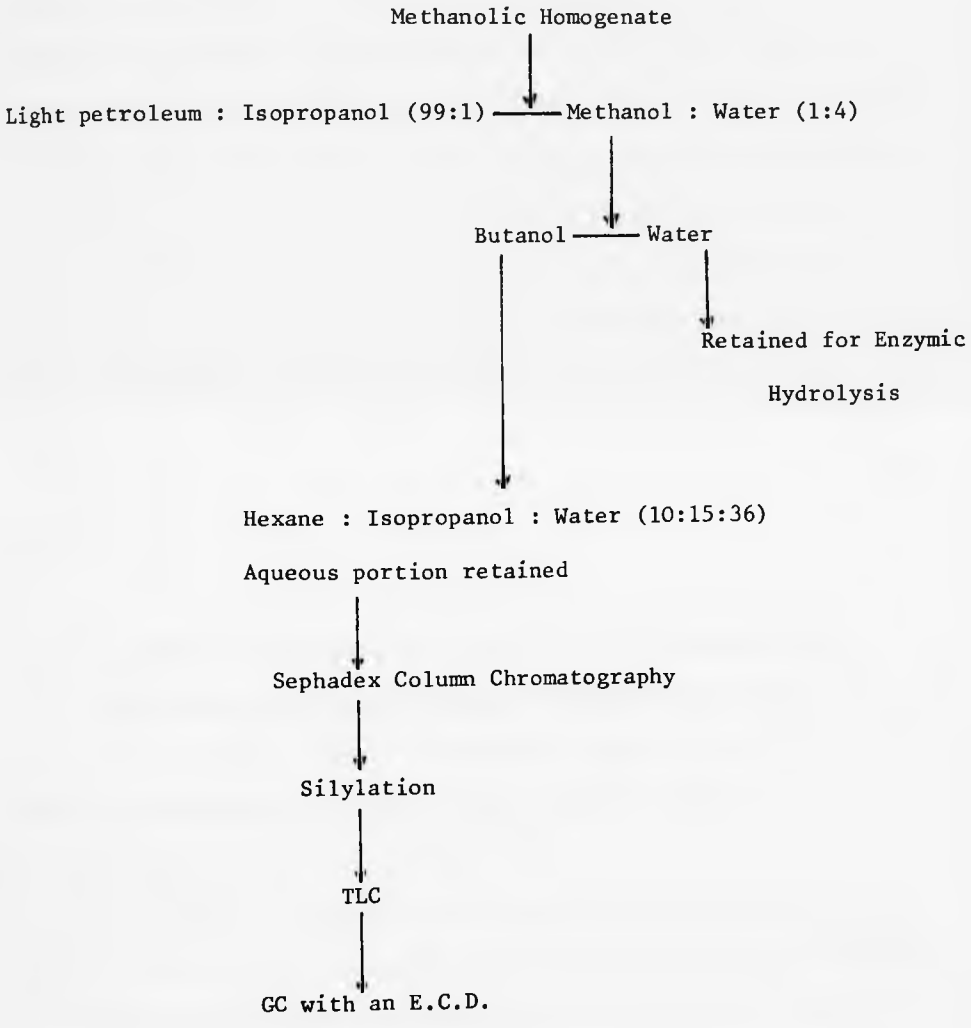
The final scheme used for the extraction and determination of ecdysteroids from barnacles is shown in the flow diagram, figure 24.

After development on the second acetone eluted Sephadex column, the active fraction (75-150 ml after introduction of the sample) was reduced to a small volume by evaporation and then transferred serially to a 1 cm^3 Reacti-vial. With the aid of nitrogen gas and a hair drier the solution was reduced to dryness to give a pale orange viscous residue. This was dried completely by placing in a drying pistol over boiling acetone (56^{o}C) and placed under vacuum for 1 h. Failure to completely dry the sample can cause hydrolysis of the TMSI and may interfere with the formation of silylated ecdysteroids.

Formation of Silyl Ethers of Ecdysteroids Extracted from Barnacles

Two ecdysteroids were known to be present in the barnacle B. balanoides, these are ecdysone and 20-hydroxyecdysone expected to be found at levels near 6 ng Kg^{-1} and 1 µg Kg^{-1} respectively (Bebbington, 1975). From this result it was apparent that, unless there was a very great seasonal variation in the levels, it would be difficult to determine accurately the amount of ecdysone present in our small, 0.5 Kg samples. Nevertheless, to ensure that both these ecdysteroids could be determined accurately if they did occur together in sufficient quantities in one sample, the final residue after drying was silylated for 5 h at 120^{o}C (see earlier section of the discussion on the determination of silylating conditions). This silylating procedure would give the tetrakis trimethylsilyl (TMS) ether of ecdysone and the pentakis TMS ether of 20-hydroxy-

Figure 24. Extraction and Analysis of Ecdysteroids from Biological Material.



ecdysone which are readily distinguished on the gas chromatograph. But, although silylating for 5 h at 120°C gave these single derivatives with standard solutions of these two ecdysteroids, when ecdysone and 20-hydroxyecdysone extracted from barnacles were derivatised under these conditions over-silylation occurred. This resulted in some of the ecdysone and 20-hydroxyecdysone being converted to their fully derivatised TMS ethers, pentakis and hexakis respectively, and the mixture of peaks seen on the gas chromatogram prevented accurate quantification of the ecdysteroids present. By using a barnacle sample with added ecdysone and 20-hydroxyecdysone it was apparent that some impurity, that was extracted along with the ecdysteroids, catalysed the silylation reaction. By reducing the duration of the silylation from 5 h to 4½ h, but maintaining the temperature at 120°C, this problem was solved.

Thin Layer Chromatography of Silyl Ethers of Ecdysteroids Silylated after Extraction from Biological Material

The conditions used for the purification, by TLC, of silyl ethers of ecdysteroids extracted from biological material were as described previously.

The problems associated with this purification procedure arose from the fact that the levels of ecdysteroid present were so low that even after silylation and TLC they could not be detected simply by visualising under UV illumination. To ensure full recovery of any derivatives from the TLC plate a wide band of silica incorporating the appropriate R_f band had to be scraped from the plate. This procedure relied upon the R_f values of the silyl ethers of the ecdysteroids from biological material being identical, or at least

ecdysone which are readily distinguished on the gas chromatograph. But, although silylating for 5 h at 120°C gave these single derivatives with standard solutions of these two ecdysteroids, when ecdysone and 20-hydroxyecdysone extracted from barnacles were derivatised under these conditions over-silylation occurred. This resulted in some of the ecdysone and 20-hydroxyecdysone being converted to their fully derivatised TMS ethers, pentakis and hexakis respectively, and the mixture of peaks seen on the gas chromatogram prevented accurate quantification of the ecdysteroids present. By using a barnacle sample with added ecdysone and 20-hydroxyecdysone it was apparent that some impurity, that was extracted along with the ecdysteroids, catalysed the silylation reaction. By reducing the duration of the silylation from 5 h to 4½ h, but maintaining the temperature at 120°C, this problem was solved.

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very similar, to those values for standard compounds. To ensure that contaminants, apart from excess silylating reagents, did not affect these R_f values a concentration of ecdysone and 20-hydroxy-ecdysone was added to a barnacle sample, known to contain negligible levels of ecdysteroids, before silylation in such quantities to be visible under UV illumination after TLC. Analysis indicated that the R_f values of these two ecdysteroids when present in a biological extract showed little variation and full recovery of the material was achieved by extraction of silica from a R_f band 0.55-0.85. In subsequent analyses of barnacle samples this band was always retained and any silyl ethers of ecdysteroids extracted with diethyl ether.

Gas Chromatography

After elution of the TMS ethers from the silica, the diethyl ether was evaporated and the residue taken up in ECD grade toluene. Samples of this solution were then subjected to gas chromatography. The identity of any ecdysteroids present in the barnacle extracts were deduced by comparison of their retention times with those of standards. The amount of each ecdysteroid present was calculated by measuring the area of the peak and determining the concentration from a calibration curve prepared using the appropriate standard.

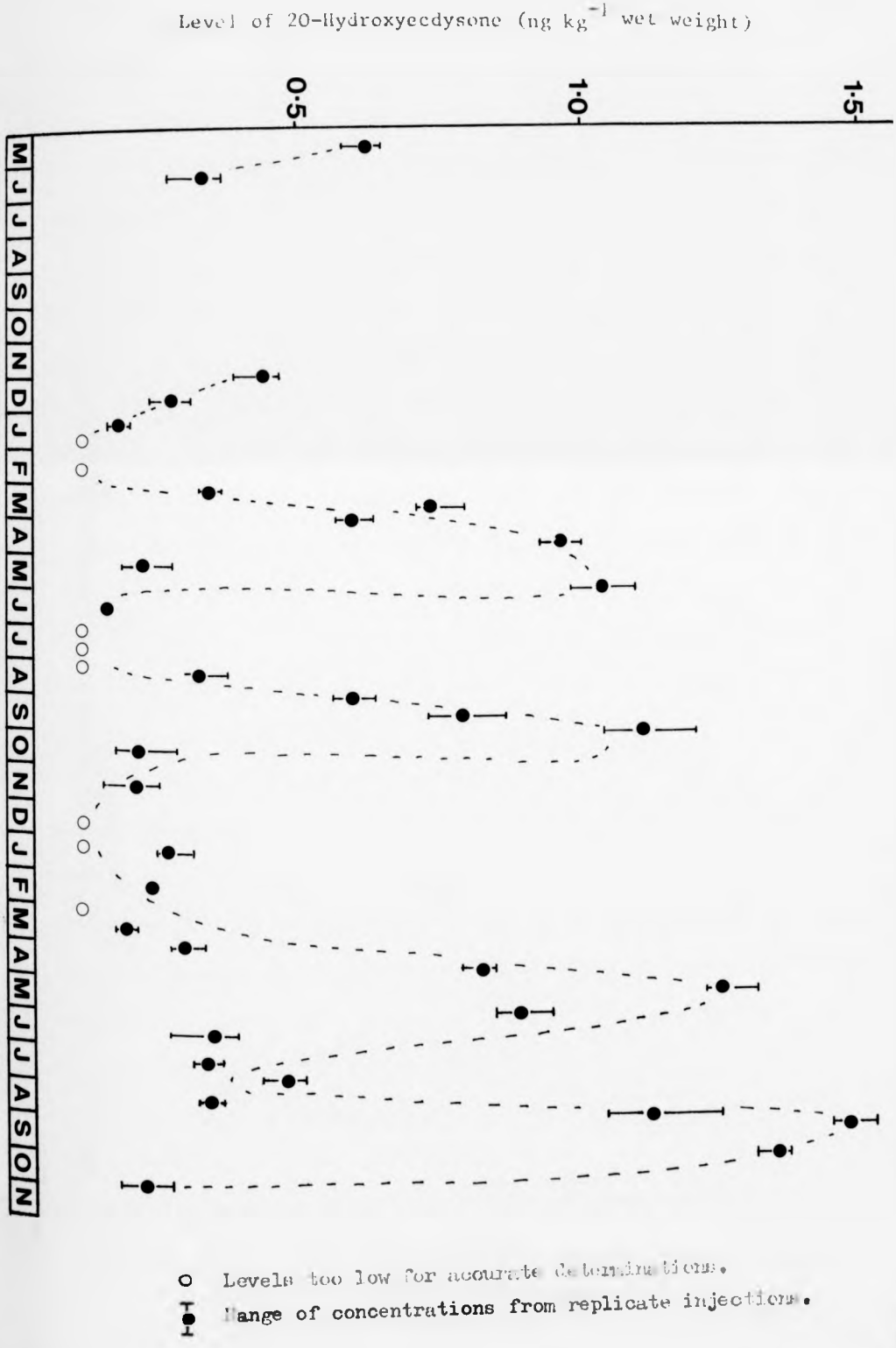
Table 11. The Seasonal Variation in the Levels of 20-Hydroxyecdysone in the Barnacle, B. balanoides.

Date		Concentration of 20-Hydroxyecdysone ($ng\ kg^{-1}$ wet weight)		Surface Temperature of the Sea (^{o}C)
		Range	Mean	
1979	14.5	580 - 632	622	10.5
	12.6	261 - 357	303	11.9
	30.11	381 - 457	435	10.2
	18.12	237 - 300	274	7.6
1980	9.1	151 - 182	170	5.8
	28.1	-	<100	5.6
	19.2	-	<100	6.8
	11.3	321 - 359	343	7.2
	26.3	767 - 826	799	6.8
	9.4	633 - 669	649	8.2
	25.4	1001 -1049	1025	9.8
	12.5	189 - 279	220	12.3
	31.5	979 -1097	1041	11.8
	19.6	149 - 162	155	13.8
	7.7	-	<100	14.6
	23.7	-	<100	14.2
	6.8	-	<100	16.2
	19.8	287 - 364	326	15.2
	3.9	549 - 629	591	17.0
	18.9	734 - 859	795	15.2
	1.10	1040 -1201	1109	12.4
	23.10	161 - 275	214	11.4

Table 11 continued.

	25.11	137 - 241	204	9.4
	21.12	-	<100	7.1
1981	10.1	-	<100	7.1
	26.1	240 - 302	262	6.8
	16.2	213 - 233	223	6.2
	5.3	-	<100	5.1
	20.3	154 - 184	180	7.3
	6.4	263 - 323	291	7.6
	30.4	882 - 946	922	8.3
	19.5	1336 -1412	1355	11.2
	9.6	853 - 936	890	12.1
	25.6	259 - 382	336	13.9
	21.7	304 - 348	327	14.9
	2.8	531 - 598	567	16.5
	17.8	310 - 349	327	16.4
	5.9	1045 -1258	1119	16.8
	24.9	1453 -1533	1487	14.8
	10.10	1316 -1372	1353	12.6
	30.10	173 - 273	220	10.7

Figure 25. Seasonal variation in the levels of 20-Hydroxyecdysone in the Barnacle, B. balanoides.



Seasonal Variation in the Levels of 20-hydroxyecdysone in the Barnacle, Balanus balanoides

After extraction and silylation of any ecdysteroid from the barnacle sample the final purified residue extracted from a TLC plate, was dissolved in toluene and aliquots of this solution were analysed by gas chromatography. From the area of the peak corresponding to 20-hydroxyecdysone the concentration of this ecdysteroid was calculated. As several injections were made per sample the range of values and average quantity for each sample are given in Table 11 As the wet weight of each barnacle sample was also recorded it was possible to calculate the concentration of 20-hydroxyecdysone per kilogram (wet weight) of barnacles and this data is also given in Table 11 and shown graphically in Figure 25. The bar lines in Figure 25 indicate the range of concentrations found from replicate injections. The graph shows the actual values of 20-hydroxyecdysone determined for each sample.

During the second year the use of an internal standard, makisterone A, was incorporated into the extraction procedure. Throughout this period the percentage recovery of this standard was calculated and the average recovery was found to be 73% with a range generally between 65-80% but on one occasion the recovery was as high as 106%. Another sample gave a low recovery of 43% and on two occasions no makisterone A was detected in the final analysis. On these three dates a further aliquot of the appropriate methanolic sample solution, obtained after the partitions, was analysed and recoveries within the accepted range were obtained. This loss, which must have occurred at the Sephadex column, or thin layer

chromatography stage indicated that the method was not infallible and errors may be present in the results for the first year when no internal standard was employed.

The levels of 20-hydroxyecdysone, the only ecdysteroid present in detectable quantities, ranged from less than 100 ng kg^{-1} wet weight of barnacles (below the minimum level of accurate detection) to 1487 ng (1.49 µg) kg^{-1}. As the average recovery was 73% of the internal standard the maximum titre of ecdysteroid found over the two year period can be calculated to be 2.02 µg of 20-hydroxyecdysone per kilogram of wet weight of barnacles. This is in the same range and larger than the quantity obtained by Bebbington (1975) who obtained 1 µg kg^{-1} 20-hydroxyecdysone wet weight of barnacles. His large sample was collected over a long period predominantly in the late summer (September) and only some of the sample would have been at the period of peak titre. This, in combination with working with very large samples, and his lengthy extraction procedure during which there must have been greater losses, would probably account for his result. All in all the two studies agree remarkably well.

The lowest values, seen as clear circles on figure 25, were samples where the presence of 20-hydroxyecdysone was detectable but the peaks seen on the gas chromatogram were too small to allow for quantification. The GC-ECD can accurately detect 20-50 pg of silylated ecdysteroid per µℓ of a standard solution that has been diluted with a large volume of special grade toluene. In the case of barnacle samples, the ecdysteroids that were extracted were still contaminated with sufficient impurities to yield a relatively large solvent front and poor base-line when analysed on the GC. The problems could be resolved by diluting the solution with excess

toluene but this resulted in the inability to quantify those samples containing very low titres of hormone.

These lowest values were obtained during late December through to early March in both years and also in the summer months of July and August in 1980. From early March there is a gradual increase in the titres to a maximum in May, after which there is a strong decline during the summer months followed by a second, slightly larger peak in early October. This is followed by a further decline in the ecdysteroid titre to the low winter levels. This general pattern was seen for both years with only slight variations, and the hormone levels are consistent with the seasonal variation of the moulting frequency of this species of barnacle as determined by Crisp and Patel (1960). The two maxima occur after liberation of the incubated larvae in the spring and just before fertilisation in the autumn with a decline in both moulting and ecdysteroid titre during the winter months of reproductive anecdysis.

Ecdysone was also extracted from B. balanoides by Bebbington (1975) but at a very low concentration of 6 ng kg^{-1}. During this study it was apparent that this ecdysteroid was also extracted from our smaller samples of barnacles as a peak with a retention time corresponding to that of ecdysone was often seen on the gas chromatogram. The levels were too low to quantify and these peaks were only seen when the titres of 20-hydroxyecdysone were high. This suggests that ecdysone production follows a similar pattern to that of 20-hydroxyecdysone but is not detected as it is present at much lower concentrations.

These results are the titres of ecdysteroid extracted from a barnacle population which contained individuals at varying stages of

the moult cycle and therefore the titres must be considered merely as an average value for that population. From the introduction it is clear that a large variation in ecdysteroid concentration is found throughout the moult cycle of crustaceans with a peak titre occurring during premoult or proecdysis (stage D). The moult cycle in barnacles has been staged by Davis et al. (1973) and the criteria for the various stages has been used by Freeman and Costlow (1979) to determine the extent of premoult during the moult cycle in the barnacle, B. amphitrite. In individuals that moulted approximately once every seven days they recorded that apolysis, the onset of premoult, commenced on day 4 and ecdysis and postmoult occurred on day 7 and lasted less than a day. This shows that barnacles exhibit a diecdysic cycle with premoult occupying over 50% of the cycle. However in a generalised crustacean moult cycle (see Introduction) the maximum ecdysteroid titre occurs at a specific point in the premoult stage, at late premoult or Stage D_2-D_3, which Freeman and Costlow (1979) recorded at day 6-7 in the barnacle. From this it can be deduced that in a constantly moulting population of barnacles, such as B. balanoides during the spring to autumn period, any extracted ecdysteroid will be obtained predominantly from barnacles at late premoult. If Freeman and Costlow's study can be directly related to this species of barnacle then our titres of 20-hydroxy-ecdysone have been obtained from 1/7-2/7th of the sample population, assuming a random distribution of barnacles at different moult stages. Therefore the maximum titre of 2.02 $\mu g\ kg^{-1}$ would in fact have been extracted from approximately 150-300 g of barnacles. These barnacles were of an average weight of 1 g and therefore the 2 µg 20-hydroxy-

ecdysone was extracted from 150-300 individual barnacles giving a concentration during the peak moulting times of 6.6-13.3 ng 20-hydroxy-ecdysone per barnacle. The concentration of ecdysteroid per barnacle during the winter months could not be so readily calculated as it has been shown that during anecdysis the barnacles are predominantly at an intermoult stage (Koulish and Klepal, 1981). The levels were expected to be very low since during the moult cycle of any crustacean the lowest titres occur at postmoult and intermoult (see Spindler et al., 1980).

Before discussion of the factors that are likely to affect this seasonal pattern it is prudent to consider the actual moulting frequency of B. balanoides throughout this period.

Determination of the Seasonal Moulting Cycle in *Balanus balanoides*

Small groups of barnacles were collected regularly and kept alive under laboratory conditions to monitor their seasonal moulting frequency in order that this cycle could be compared with the seasonal variation of ecdysteroid production determined over the same period of time. The term moulting frequency as opposed to moulting rate is used, as suggested by Barnes (1962), to define the number of times an animal moults in a given time and is the reciprocal of the intermoult period. In these experiments the number of moults per hundred barnacles per day is used as a measurement of moulting frequency.

Crisp and Patel (1960) collected small groups of *B. balanoides*, 20-30 individuals fortnightly, and maintained these groups in dishes of static seawater under ambient illumination and temperature for 16 h at a time followed by 8 h out of water. By counting the number of exuviae after the period of immersion a record of the moulting cycle from October 1954 to February 1956 was obtained. From the results a pattern emerged which they described as an annual moulting rhythm in this species. During October-November the moulting frequency was 8-12 moults per hundred animals per day but by late November a sharp reduction in this frequency was found immediately after the animals were fertilised. This was followed by a period of little or no moulting, anecdysis, for about six to eight weeks and then moulting resumed but at a slower rate until a maximum frequency was attained by May. A slight decrease was then noted during the summer months between June and August, rising again towards the

breeding season in November. The tidal level, from which the barnacles were collected, and the lunar periodicity were found to have no influence on this moulting cycle. None of the animals were fed during the study as it was found that the moulting frequency of fed and starved animals did not differ for the first 10-15 days after collection from the shore.

The rhythm of the moulting frequency closely parallels the cycle of available food found in boreal waters where two planktonic blooms occur, one in spring and one in autumn, with only a sparse food supply being available during the winter months. However, Crisp and Patel concluded that the reduction in moulting frequency and the period of anecdysis, even seen in isolated and hence unfertilised individuals, was part of basic endogenous rhythm.

Barnes (1962) though, considered that this cycle was largely determined by the environment rather than by endogenous factors. The experiments by Crisp and Patel (1960) to determine the differences in moulting frequency of starved and fed barnacles were conducted using animals collected in June. At this time the metabolic reserves in the animals would be high unlike in the animals during the winter which would probably be in a debilitated condition due to the discharge of ova and semen at the time of fertilisation in November. Barnes (1962) suggested the possibility that the period of anecdysis and subsequent slow recovery of moulting frequency were in fact due to this debilitated state which leads to a reduction or temporary cessation in feeding activity. This in turn reduces the moulting frequency. The term 'reproductive anecdysis' was then proposed by Barnes (1962) to cover the period of little or no moulting.

A series of experiments were then conducted by Barnes (1962) to determine the effect of feeding on anecdysis and found that by supplying individuals with ample food the period of anecdysis and recovery lasted for ten and fifteen days respectively before normal moulting frequency resumed. From the results of feeding during anecdysis, at a level stated to be equivalent to a diatom bloom and far in excess of the supply naturally available at this time of year, it was concluded that the low level of food rather than endogenous factors determined the moulting frequency during this period. Barnes did note however that even when an adequate food supply was available that feeding was suppressed in fertilised animals carrying egg masses. Crisp and Patel (1960) had also noted a decline in feeding activity in groups of barnacles, even in individuals which would not have been fertilised due to their isolation.

The seasonal changes in feeding rate in B. balanoides were then determined by Ritz and Crisp (1970) who found that although ample food, artemia larvae, was made available a sharp decline in feeding was noted in October-November at the onset of the breeding season. During late November-December feeding practically ceased although food was always present. This was considered an adaption of the metabolic rate to the diminished food supplies naturally available during the winter months. An increase in feeding was found to be synchronised with the release of the first stage nauplii in the spring (a process which is synchronised with the spring diatom bloom, see Barnes, 1957) and the available food was used for growth and reserves for gonad production. The growth rate declined in summer (after June) when the sea and air temperatures

were above the optimum for the animal and when food is less readily available in the natural environment. An increase in food uptake occurred in November presumably for winter reserves and then the decline again appeared at the onset of the breeding cycle.

From these various studies it is indicative that both an endogenous rhythm and environmental conditions affect the moulting frequency during the period of reproductive anecdysis and the subsequent recovery period. The time of 6-8 weeks for the reproductive anecdysis period obtained by Crisp and Patel (1960) has been confirmed by Tighe-Ford (1974) who determined that the interval between 50% of a laboratory maintained barnacle population becoming fertilised and 50% having shed their first exuviae was 7-8 weeks. This is likely therefore to mimic the situation found naturally as the conditions used by Tighe-Ford included a continuously running sea water system maintained at ambient temperature.

For these reasons we adopted the technique used by Crisp and Patel (1960), with slight modification, in an attempt to repeat their results to obtain a seasonal moulting cycle. In our experiment we increased the number of individual barnacles collected every fortnight to between 100-200 to make the results more significant. Barnacles of approximately the same size were obtained and the settlements were cleared of any barnacle species other than B. balanoides, algae and other organisms. Isolated individuals, that is barnacles 2 cm or more from others, were also removed particularly during the winter months as these specimens were unlikely to be fertilised. The animals were then kept under ambient temperature and light conditions, as used by Crisp and Patel (1960) and Tighe-Ford (1974), for 16 h immersion and 8 h exposure. The animals were submerged in

the evening and removed from the dish of seawater first thing in the morning when any casts were collected and counted and, during the spring, the water was analysed for the presence of any stage I nauplii indicating release of newly hatched larvae.

This was the method also advocated by Crisp and Patel (1960) and Tighe-Ford (1974) but Barnes (1962) maintained his groups under total immersion. Crisp and Patel (1960) found that a period of exposure was beneficial to the barnacles, a result of which was confirmed in our laboratory where the death rate of barnacles collected from the inter tidal region rose dramatically after total immersion in an aquarium for more than six days. The dishes of seawater were also constantly agitated by aeration, unlike the static system used by Crisp and Patel (1960), as Tighe-Ford (1970) had shown that the growth rate of young barnacles was greater in flowing sea water than in static conditions. The animals were not fed but the sea water which was collected at the same time as the barnacles was only coarsely filtered and microscopic plankton may well have been present. The collected sea water was kept in a 5 gallon plastic container and continuously agitated by aeration. The sea water in which the barnacles were maintained was replaced daily from this reservoir. On several occasions when the barnacles were kept for slightly longer than a fortnight the sea water supply was supplemented with artificial sea salts prepared, as suggested by the makers, to give a salt concentration of 33 parts per thousand.

The barnacles were all collected from mean sea level below the Menai Bridge, North Wales, with the exception of groups of barnacles obtained during July to September 1980. During this period this study was conducted at Portsmouth and the moulting frequency values at this time were obtained from groups of barnacles

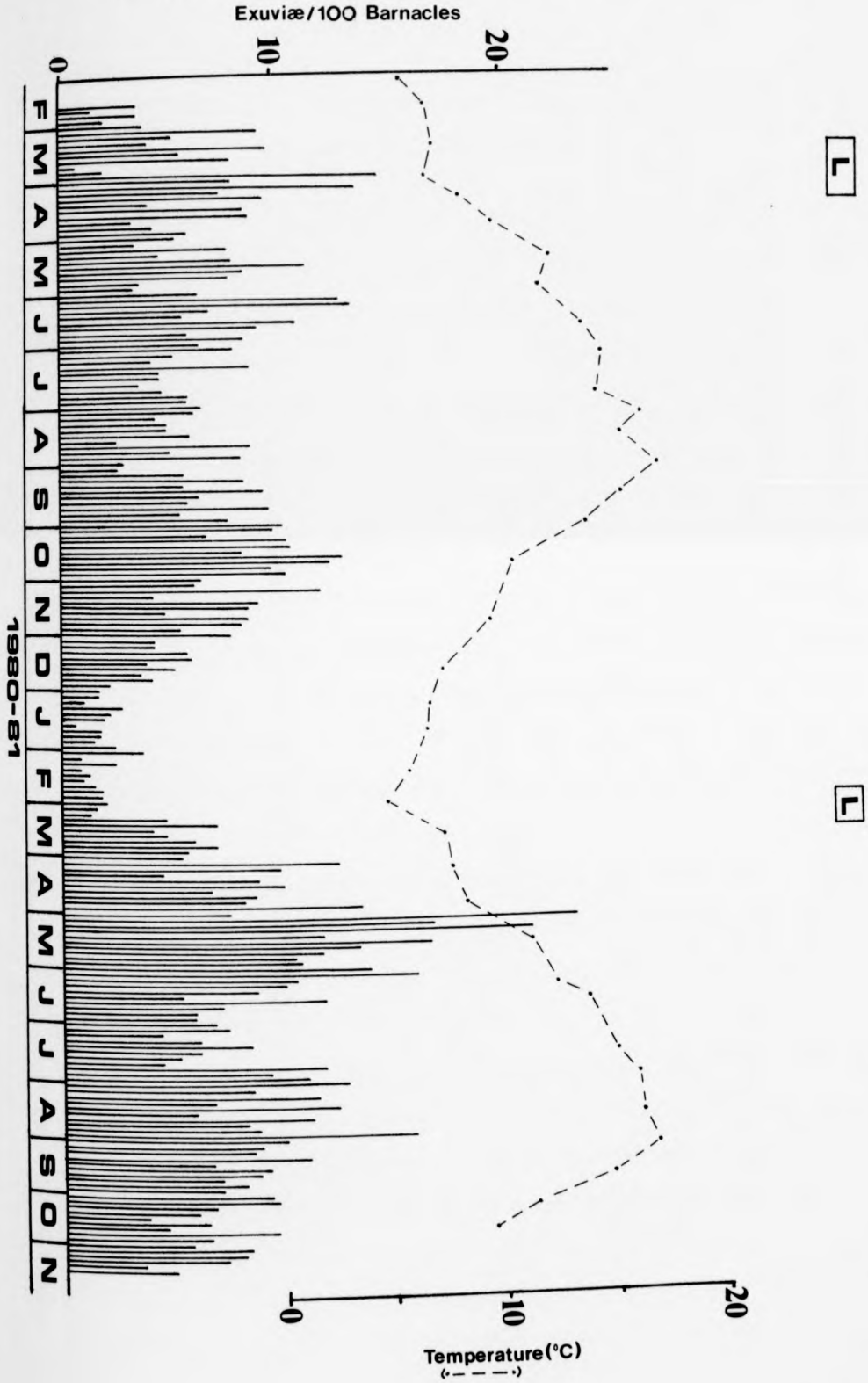
collected from Langstone Harbour, Eastney, Portsmouth. This is a more southerly and much more sheltered region than Menai Bridge and subsequently the breeding cycle may be varied. Tighe-Ford (1974) however, gives the first date of fertilisation of barnacles from this area as early November and the progress of fertilisation was similar to that found by Crisp (1959) for barnacles at Bangor.

The results of the moulting frequency (number of moults per hundred barnacles per day) obtained from February 1980 to November 1981 are shown diagramatically in figure 26. Also included on the diagram are the temperatures of the surface sea waters taken at Menai Bridge at each collection. These values are compared with the mean temperatures of sea water in which the live barnacles were maintained in the laboratory. These laboratory temperatures were taken daily, in the morning just before the immersed barnacles were removed and the exuviae counted, and the mean value for each two-three week period, before the next collection, was calculated. From the diagram it is apparent that the laboratory sea water temperature did not fluctuate from the actual sea water temperature by more than 2-3^{o}C. However, the aquarium room temperature tended to be above the sea water temperature in winter and early spring and below in summer and autumn.

The results obtained compare favourably with those found by Crisp and Patel (1960). A similar pattern is apparent with a period of reduced moulting after the November fertilisation until the following spring when the moulting rate slowly climbs to a peak in late April-early May. This is followed by a slight decline in the summer months of June and July before the rate again increases to a second maximum in late October just prior to the breeding season. The pattern is clearly recognisable but not so well defined as that obtained by Crisp and Patel (1960).

Figure 26. Seasonal Variation in the Moultingfrequency of the Barnacle, B. balanoides.

L = period of liberation of first stage nauplii.



Liberation of the First Stage Nauplii Larvae and Settlement of the Cyprid Stage Larvae

The periods of liberation during which newly hatched nauplii larvae were found in dishes containing the barnacles in 1980 and 1981 are also shown in figure 26. In 1980 the first liberation in the laboratory occurred on the 11th March and larvae were found almost daily until the 8th April but a final group of newly hatched larvae were also found two weeks later on the 23rd April. In 1981 the first recorded liberation occurred on the 5th March and further releases were noted regularly up to the 23rd March. These results parallel those reported by Crisp and Patel (1960) who found liberation to occur in the laboratory between mid March-mid April and mid March-the end of April for the years 1954 and 1955 respectively.

This data can also be correlated with the time of the first settlement of cyprid larvae. After release of the newly hatched larvae a succession of ecdyses occur and the larvae pass through six naupliar stages before the metamorphosis into the cypris or settlement larva. At the collection site at Menai Bridge the first settlements were noted on the 14th May 1979, the 25th April 1980 and the 30th April 1981. As the shore was only visited fortnightly for the collection of barnacles the actual date of the first settlement could have been any of the fourteen days prior to these dates. From these records we can back date to determine relatively accurately the time of the liberation of the 1st stage nauplii from the majority of the barnacle population at this site.

Pyefinch (1948) studied a population of barnacles at Millport in the Firth of Clyde and reported that the period when adults

contained larvae ready to hatch coincided with the appearance of both the 1st and 2nd stage naupliar larvae found in plankton hauls. He suggested that the majority of the population undergo liberation over a short period and recorded that most newly hatched larvae were found in plankton hauls on the 7th March in 1944, the 14th March 1945 and the 5th-14th March 1947. Analysis for cyprids in the plankton nets showed that although some appeared as early as the 20th March in 1947 the majority occurred between the 18th and the 27th April. Pyefinch (1948) found that cyprids took 5 days to settle in laboratory conditions but proposed that the period of settlement in natural conditions is much shorter as the appearance of cyprids in large numbers in the plankton was almost coincident with settlement on the shore. During 1947 the settlement was most heavy at the beginning of April and Pyefinch (1948) concluded that a period of approximately one month passed from liberation of Stage I nauplii to the settlement of the cyprid larvae.

Barnes (1956) also monitored the progress of the larvae of B. balanoides in the Firth of Clyde and found that the Stage I nauplii, which rapidly ecdyse to Stage II, appeared in early March about the time of the phytoplankton outburst. After Stage II the progression of the nauplii to each of the next stages was found to take 3-4 days so cyprids occur 3-4 weeks after the Stage I release. The work of Pyefinch (1948) was confirmed by Barnes and Barnes (1958) who found that the period between liberation and appearance of cyprids was 30 days irrespective of temperature and whether the estimate was made by the time between peak periods or first appearances was used.

From all these past studies it is clear that the development of settling cyprids from Stage I nauplii occurs within thirty days.

If we use this figure to back date the first or major naupliar liberation from our first settlement sightings we obtain the following dates for liberation; 1st-4th April 1979, 13th-25th March 1980 and the 18th-30th March 1981. These calculated figures correlate well with the dates found by Crisp and Patel (1960) and from our studies (see earlier) for barnacles kept under laboratory conditions.

At other stations the date of the first settlement may be regular, as reported by Moore (1935) for Port Erin where settlement occurred on the 26th April in 1933 and the 25th April in 1934, or irregular as recorded by Pyefinch (1948) for Millport. Pyefinch also cites Runnström as having noted in Liverpool Bay the first settlement in 1925 occurred on the 6th April but the maximum numbers settling occurred on the 15th of that month. At Menai Bridge it is apparent that some variation occurs annually and as the Stage I nauplii are released over a period of at least two weeks it is likely that settlement of the cyprids that develop from these nauplii will take place over a comparable period of time. Nevertheless the data obtained from the laboratory barnacles concerning the period of liberation compares favourably with the calculated date of liberation from studies of the first settlement in natural populations.

A Comparison of the Moulting Frequency of Barnacles from Different Tidal Levels.

At the site from which the barnacles were collected there is a wide tidal range and therefore a wide balanid zone. To ensure that as little variation as possible occurred between samples the barnacles were always collected from the rock face at the same tidal level. This consideration was also made in collecting groups

of live barnacles for studying the moulting frequency. However at the outset of this study groups of B. balanoides were collected from both the mean inter tidal zone and just below the mean high water mark and their moulting frequencies compared. This would confirm whether of not the tidal level actually affected the moulting frequency and possibly the ecdysteroid production.

Barnacles were collected fornightly from the end of March until the end of August and maintained in the laboratory as described earlier. The results of this study are shown diagrammatically in figure 27 and are tabulated in table 12.

From the diagrams no clear pattern of moulting frequency is visible. In the intertidal specimens, if the initial high moulting frequency in late March is disregarded, there is a gradual increase to a peak in May followed by a slight decline in moulting frequency. This is comparable with the pattern noted by Crisp and Patel (1960) and by us for 1981 (see earlier). In the barnacles collected from nearer the mean high water mark no such pattern is visible. However the period of liberation of Stage I nauplii did not differ in the two groups as larvae were released up to the 3rd April in the high water group and up to the 8th April in the intertidal specimens (a subsequent single hatch did in fact occur on the 23rd April in the latter group). If the number of moults per hundred barnacles per day is considered over the four month period (see table 15) the moulting frequency in the intertidal group is also 60% higher than that for the high water group.

The differences in moulting frequency due to tidal level has been analysed previously by Crisp and Patel (1960) who collected barnacles from low, mean and high water tidal levels and recorded the moulting frequency over ten days. This study was conducted

in January, February and September and they concluded from their results that tidal level had little influence upon the moulting frequency. Southward (1955a, b) studied the effects of tidal level on cirral beating rate in B. balanoides collected from the sites around Anglesey including the Menai Straits. He recorded that the beating rate in speciments from mean low water areas was generally lower than in barnacles from higher tidal levels, and at an exposed site in the Menai Straits the mean number of beats per ten seconds of individuals from mean high water showed a 100% increase over the rate in barnacles from the lower shore. In another experiment Southward noted freshly collected specimens from a high water tidal level beat faster than individuals collected from lower on the shore on the first day but the rates were identical on subsequent days.

As cirral activity plays a part in both feeding and respiration it may possibly reflect the general metabolic level of the barnacle. Barnacles settled near the high water area are immersed for much shorter period than those at the intertidal range or lower, particularly at the Menai Bridge where the tidal range is extensive, and have therefore a shorter period for feeding and respiration. This may result in some variation in metabolic rate and moulting frequency at different tidal levels.

The results obtained from our study confirmed that there is variation in moulting and hence possibly moulting hormone levels with tidal levels. Therefore, our practice of collecting barnacles for hormone extraction only from the same tidal region was prudent.

Table 12. A comparison of the moulting frequency of barnacles collected from the intertidal and high water zones.

Date Collected	Casts per day per hundred barnacles	
	Intertidal	High Water
26-3-80	10.1	4.3
10-4-80	4.8	7.4
28-4-80	5.4	1.5
12-5-80	7.5	4.5
30-5-80	10.3	6.6
20-6-80	8.4	10.0
8-7-80	5.8	7.3
Mean no. casts/day/100 barnacles	7.4	4.5

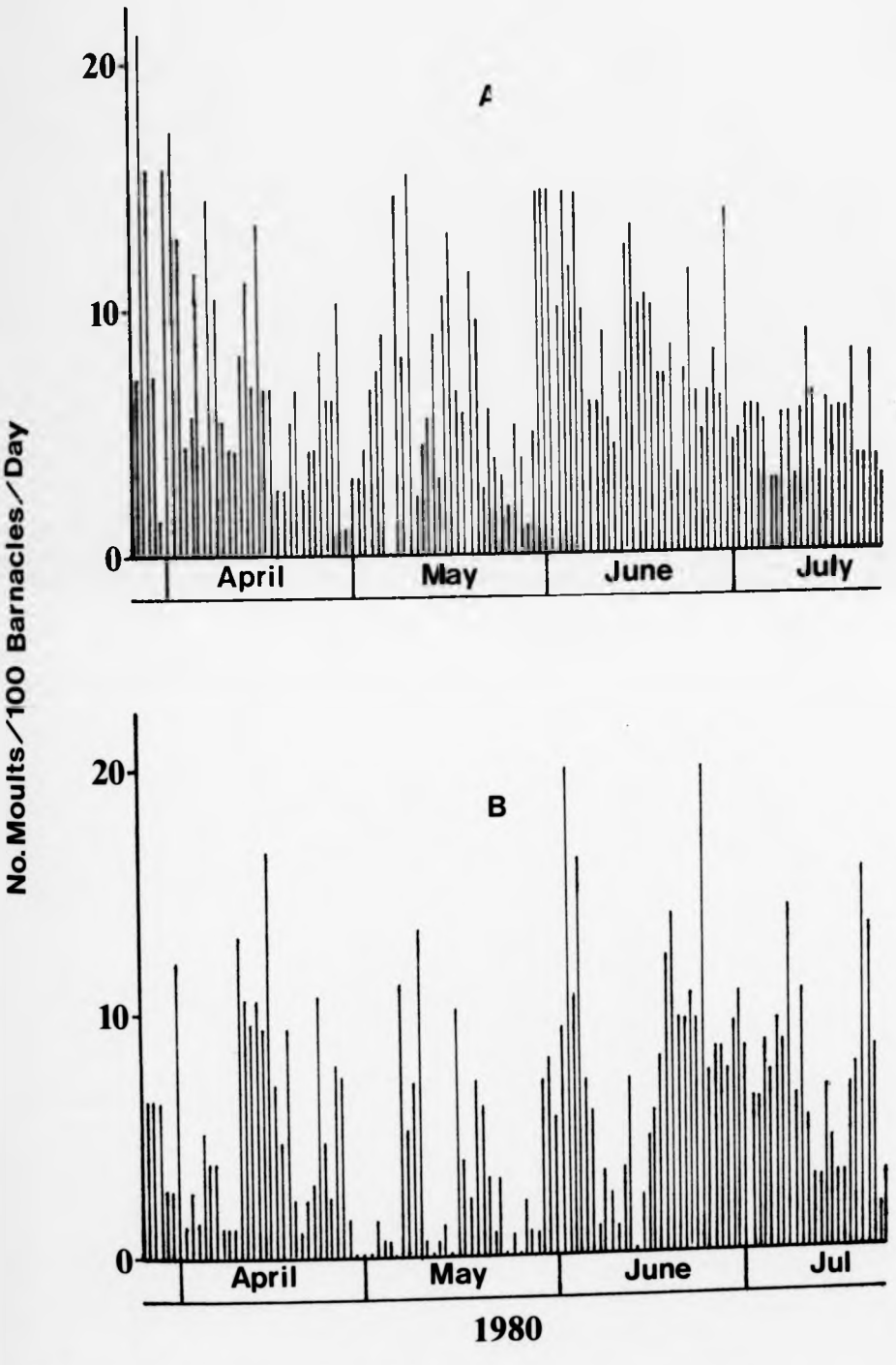


Figure 27. A Comparison of the Moulting Frequency of Barnacles from Intertidal (A) and High Water (B) Zones

Factors Affecting the Moulting Frequency and Ecdysteroid Production in the Barnacle, Balanus balanoides

The seasonal pattern of ecdysteroid production in a population of barnacles can be seen to parallel closely the pattern of moulting frequency within that population. Factors that may affect this pattern include tidal position, lunar periodicity, feeding, temperature, light and reproduction. Crisp and Patel (1960) have concluded that tidal position and lunar periodicity have no effect upon the moulting frequency. Our results indicate, however, that the tidal position may have some control over the moulting activity but from the moulting frequency results we concur with the earlier study that lunar periodicity is of little consequence. The prime factors under consideration are therefore ambient temperature, the availability of food and hence feeding of the barnacles and the relationship between moulting and the various stages involved in the breeding cycle of this species of barnacle.

(i) Nutrition and Ambient Temperature

Tighe-Ford (1977) suggested that the dietary requirements of barnacles would not be dissimilar to those of other crustaceans and that ecdysteroids are probably converted from sterols via desmosterol and cholesterol. This is similar to the pathway proposed for the synthesis of ecdysteroids in insects (see Rees *et al.*, 1980). In decapod crustaceans, cholesterol has been shown to be the major sterol, with desmosterol also present as a minor constituent, and both these compounds are present in barnacles (Tighe-Ford, 1977 and Ballantine *et al.*, 1980). Cholesterol is also the principal sterol of zoo-

and phyto-plankton, the primary food source for barnalces, and sea-water contains a number of sterols that could be involved in the biosynthesis of moulting hormones. However, sterol is unlikely to be limiting and this may reflect the general nutritive nature of the barnacle.

During the winter months, when little food is available, feeding activity in B. balanoides is suppressed (Crisp and Patel, 1960; Barnes, 1962 and Ritz and Crisp, 1970) and both moulting and the ecdysteroid titre at this time are at a low level. Nutritional reserves built up during the autumn are used during reproduction in November and to maintain developing embryos during the winter. The body weight of barnacles has been shown to decrease following fertilisation due to the transfer of sperm and the atrophy of testes (Barnes et al., 1963). Barnes (1962) concluded that some 50% of the body weight of the barnacle is lost during emission of semen and the weight of eggs released from the ovary into the mantle space is up to twice the weight of the body after the loss of semen. It is unlikely that during this debilitated state any reserve material would be utilised for the synthesis of any ecdysteroid as moulting or growth at this time would further deplete the already drained reserves of the organism. This debilitated state may in fact decrease ecdysteroid production directly, or may induce the release of a factor to prevent its production. Glandular activity would then remain at a level too low to actively effect a moult in the animal. In other crustaceans where a study of the ecdysteroid content has been made throughout the moult cycle, ecdysteroids are always detectable even during the intermoult (C) stages (Spindler et al., 1980). Koulish and Klepal (1981) have confirmed that during

the period of reproductive anecdysis, B. balanoides, remain in an extended intermoult cycle, corresponding to Stage D_o. Therefore, as found, a population of barnacles would be expected to have a low level of ecdysteroid at this time.

After the period of anecdysis the moulting frequency can be seen to increase as the levels of ecdysteroids slowly rise in a population of B. balanoides. Although the barnacles initially lose body weight during March to May (White and Walker, 1981) this is the result of larval release. This release is coincident with the increase in available food at the spring phytoplankton bloom (Barnes, 1957 and Crisp and Patel, 1960) and Crisp (1958) stated that the resumption in moulting in spring seemed to depend partly on this increase in food availability and uptake. Experiments by Crisp and Patel (1960) indicate that after anecdysis moulting resumed more rapidly in fed specimens and these authors suggested that the amount of reserved food available is important in determining the moulting acitivity.

During the summer months the available food decreases slightly and it is likely that barnacles then utilise any food reserves that have been built up during the spring phytoplankton bloom. However the mean temperature, both air and sea, increases during this period resulting in an increase in metabolic rate in the barnacles. This metabolic rate would cause rapid utilisation of any reserves and result in a reduction in moulting activity so that the moulting frequency will actually appear to decline with increase in temperature. Crisp and Patel (1960) showed that moulting of fed barnacles increased linearly with temperatures from 3-20 ^{o}C whereas in starved barnacles

any increase in temperature above 12 ^{o}C resulted in a decrease in moulting frequency. The decline in moulting frequency during the summer was seen in our laboratory experiments and was coupled with a very marked decrease in ecdysteroid level. Barnes and Stone (1974) experimented with starved animals during May-July and concluded that temperature had little effect on moulting and found no appreciable variation between a small temperature range of 10-15 ^{o}C. From this result they hypothesised that temperature independence may argue well for a hormonal control over the moult cycle, as opposed to a purely metabolic or enzyme-adaptive control. In the absence of food the production of the hormone would be low and the concentration reduced to such a level that moulting would only continue at a reduced rate. There would therefore be no response, such as a further increase in moulting, to a temperature increase unless this was accompanied with an increase in the amount of food available. Ritz and Crisp (1970) recorded a slight decrease in feeding rate in B. balanoides during the summer months and White and Walker (1981) noted a plateau in ammonia excretion during this period between peak excretion rates in the spring and autumn.

Although, our results confirm that there is a decrease in moulting frequency during the summer, the levels of ecdysteroid at this time were much lower than anticipated. The barnacles' moulting frequency during the summer was intermediate between the low winter, and the high spring and autumn rates, yet the ecdysteroid levels were comparable to the titres found during anecdysis. It is possible that the barnacles are more sensitive to moulting hormone than previously expected. This is confirmed by the positive results obtained

after injection of animals with ecdysteroids at concentrations as low as 1 ng per individual (see later). The intermediate moulting frequency can then be considered as the result of a conflict between an increase in moulting due to temperature rises coupled with a decreased response due to the low titres of ecdysteroid resulting from decreasing reserves. This is somewhat confirmed when the temperature data for the two summers of 1980 and 1981 is studied. In June, July and August 1980 when the ecdysteroid titres were exceptionally low the temperature at Valley, Anglesey, near the Menai Bridge, had a mean maximum of 17.5 ^{o}C and a mean minimum of 12.5 ^{o}C, values that were comparable with previous years. However in June and July 1981 when the ecdysteroid level can be seen to be higher, the mean maximum temperature was 15.1 ^{o}C and 16.9 ^{o}C and the mean minimum 10.2 ^{o}C and 12.2 ^{o}C respectively. These lower temperatures may have prevented the rapid utilisation of any reserves in the summer 1981 compared to summer 1980 and allowed for a higher production of ecdysteroid than normal. Due to the conflict of temperature and ecdysteroid levels affecting the moulting frequency no marked difference in moulting activity is apparent even though these variations in titre occurred.

The second peak in moulting frequency in autumn is accompanied by a second peak in ecdysteroid titre and both are coincident with the autumn plankton bloom. Similarly a second peak in nitrogen excretion, as ammonia, is seen around mid-November (White and Walker, 1981).

(ii) Reproduction

B. balanoides has adapted to the boreo-arctic conditions by requiring a period of food storage before gonad development, a period of 4-6 weeks with less than 12 hours light per day and a temperature below the critical value of 10 ^{o}C before breeding will commence (Barnes, 1963). Immediately after fertilisation in mid-November there is a decline in both moulting frequency and ecdysteroid level. In other crustaceans there is a cessation of moulting after reproduction as the eggs, which are generally attached to the outside of the body, would be lost with any exuviae. However in cirripedes, although the eggs are initially attached to the body, at oviposition the eggs are transferred along paired oviducts into elastic sacs secreted by the oviducal glands and the egg masses are then contained in two ovisacs which lie free in the mantle cavity (Walker and White, 1982). Crisp (1958) stated that there is no need for a reduction in moulting in barnacles to prevent egg loss. In fact moulting commences after anecdysis, while the animals are still gravid, but the exuviae are cast with a torn off mantle lining due to the pressure of the egg masses. The mantle lining is shed later when the hatch moult occurs (Crisp and Patel, 1960). These authors recorded that even unfertilised individuals show a reduction in moulting after the time of normal fertilisation and it was proposed that the decline was in fact due to a basic physiological rhythm. Crisp and Patel (1969) considered that the breeding in November is the result of an innate set of factors set in motion possibly as early as May and unaffected by external variables throughout the year. This internal rhythm becomes entrained at one or more points with the prevailing seasonal variables. Unlike

other barnacles species that either breed continuously, or at least throughout the summer months, B. balanoides has only one brood per year. Crisp and Patel (1969) suggested that breeding is inhibited after naupliar release in spring and that there is a delay in gonad maturation, until the autumn, which is under hormonal control. Recent studies on the effects of 20-hydroxyecdysone on barnacle oocytes in vitro have indicated that this hormone may have a gonad-inhibiting function (Fyhn et al., 1977). It may therefore be the high titres of ecdysteroid produced after the naupliar release that prevent the barnacles from progressing into a second breeding cycle. At this time there aresufficient metabolites to supply the gonads but maturation does not occur until a period between September and November (Crisp and Patel, 1969). However it is during this period that the second peak of ecdysteroid production is seen and the maturation of the gonads may be initiated earlier during the summer when 20-hydroxy-ecdysone is found at a much lower concentration. Crisp and Patel (1969) have also intimated that other processes that occur after oviposition which are an aid to winter survival may also be under hormonal control. During this period the levels of ecdysteroids are at their lowest and it may be the absence of this hormone or the presence of another factor that controls these processes.

(iii) Moult-Inhibiting Substances

Tighe-Ford (1977) deduced from earlier studies that development and growth in barnacles is regulated by hormonal systems similar to those in other arthropods. Davis and Costlow (1974) and Cheung (1974) confirmed the presence of a substance that acted as a moult-inhibiting

hormone (MIH) in barnacles and Cheung also suggested the presence of a moult accelerating factor (MAF). The work of Tighe-Ford (1974) demonstrated the positive effects that 20-hydroxyecdysone had on increasing and synchronising moult activity in barnacles. Cheung and Nigrelli (1974) have induced moulting in explants, penes and appendages, of barnacles with 20-hydroxyecdysone. Further effects have been attributed to the presence of ecdysteroid by Freeman (1977 and 1979) and Freeman and Costlow (1976 and 1979). Studies of the action of 20-hydroxyecdysone on explants of barnacle mantle tissue both alone and in the presence of a MIH have shown that the ecdysteroid stimulates protein synthesis, enhances apolysis and cuticle formation and controls chitinolysis during the early part of proecdysis. From these results the authors concluded that during the moult cycle of a barnacle it is the reduction in the levels of MIH rather than 20-hydroxy-ecdysone that trigger the events leading to proecdysis. Freeman and Costlow (1979) also showed that while maintaining MIH at a fixed level the inhibitory effect could be overcome by elevated levels of 20-hydroxyecdysone and concluded that the transition from intermoult to premoult, as measured by apolysis, is the result of variation in the relative concentrations of 20-hydroxyecdysone and MIH. As the tissue approaches premoult there is also a heightened response to ecdysteroid which is considered to occur by the reduction of MIH (although it is possible that the response is the result of an increase in moulting hormone, or indeed both effects).

A very helpful study now would be to characterise the MIH and to monitor the variation in its titre, and the titre of ecdysteroid, throughout the actual moult cycle. The seasonal variation of MIH titres in a barnacle population could also be determined and a reciprocal pattern to that of ecdysteroid may be seen.

Conjugated Ecdysteroids in Balanus balanoides

During the extraction of free ecdysteroids from barnacles any conjugated hormone would have been extracted into the water phase of the second solvent partition system. This phase was retained and subjected to enzymatic hydrolysis to release any ecdysteroids that may be present as polar conjugates. The free form could then be silylated and detected by GC-ECD. Throughout the two year period, from November 1979 to November 1981, the results of these analyses were predominantly negative. Positive results were obtained during the months of December, January and February and the retention time of peaks detected by the GC-ECD were identical to those for 20-hydroxyecdysone. The levels present were very low and the peaks detected in December, 1979 and 1980, and January and February 1981 were too small to quantify accurately the amount present. However the samples were much purer than the free ecdysteroids isolated from the barnacle sample and the concentration of conjugated 20-hydroxyecdysone during these periods could be calculated to be less than 40 ng kg^{-1} wet weight of barnacle. In the months of January and February 1980 the amount of conjugate present was at such a level to allow an accurate quantification to be made. The January sample contained 126 ng 20-hydroxyecdysone conjugate kg^{-1} wet weight of barnacles and the February sample, 174 ng conjugate kg^{-1} wet weight of barnacles. At no other time during the study was there any indication that conjugates of 20-hydroxyecdysone, or any other ecdysteroid, were present.

The appearance of the conjugated 20-hydroxyecdysone is coincidental with the period of the lowest titre of the free ecdysteroid. The time when the conjugate was detected at the highest

level, January and February is the period in the season that the free ecdysteroid is below the level of accurate detection, i.e. at less than 100 ng kg^{-1} wet weight of barnacles. So during this period the predominant source of ecdysteroid is a conjugated form of 20-hydroxy-ecdysone. The winter months are also the period of low moulting frequency when the barnacles undergo 'reproductive anecdysis'. Ultrastructural studies of the epidermis and cuticle during this anecdysis has shown that the barnacles are in a state of extended intermoult (Koulish and Klepal, 1981), the stage in the moulting cycle of other crustacea when the moulting hormone titre is at its lowest. The conjugated ecdysteroid may therefore be an inactivation product produced to maintain the circulating levels of moulting hormones at such a low concentration that the induction of moulting is prevented. However, there is a second period of reduced moulting when the titres of free ecdysteroid are also very low during July and August and no conjugates were detected at this time in either years.

In insects' eggs the quantities of ecdysteroids are vast, much greater than in the larvae, and almost entirely conjugated. It was therefore expected that we would find substantially higher titres of conjugated ecdysteroids in the barnacles during the winter months after fertilisation. Although McCarthy and Skinner (1979a and b) have recorded the presence of conjugates of three ecdysteroids in intermoult stage crabs, conjugates that have been found in other crustacea have been located in developing ovaries or embryos. Spindler (1980, private communication) found traces of conjugates in newly hatched larvae of brine shrimp and McCarthy and Skinner discovered conjugates of 20-hydroxyecdysone in embryos of the blue crab. Lachaise et al., (1981) recorded that conjugates of ecdysteroids

were present in developing ovaries of the shore crab but at very low levels in comparison with the titres of the corresponding free ecdysteroids. In insects 95% of ecdysteroids found in developing ovaries and newly laid eggs may be in the conjugated form. From these results we tentatively suggest that the conjugated 20-hydroxy-ecdysone evolved from the developing embryos rather than the adult barnacles. To prove this hypothesis a further project would need to be undertaken to extract developing embryos from barnacles throughout the breeding season and monitor the presence, and any changes, of free and conjugated ecdysteroids. The embryos develop in two sacs within the mantle and can be readily removed from the barnacle by carefully removing the adult from its point of attachment and rupturing the basal membrane. The two sacs can then be teased out. This study, and an effective breeding programme that would allow for the collection of a very large number of barnacle larvae at each development stage for ecdysteroid analysis, are the logical progressive steps in the further study of moulting hormones in barnacles.

Effects of Injected Material upon the Moulting of Adult Barnacles

In recent years there has been a growth in the understanding of the role of ecdysteroids in the moulting of crustaceans. The effects of ecdysone and 20-hydroxyecdysone, applied exogenously as a solution of known concentration and endogenously by injecting the same, have been shown to induce accelerated proecdysis and ecdysis in decapods, isopods and amphipods (Krishnakumaran and Schneiderman, 1970, and Vernet, 1976). However the doses employed were often of a very high concentration far in excess of the levels now known to occur naturally (see Introduction).

Larvae of the barnacle Elminius modestus have been exposed to exogenous solutions of juvenile hormone analogues (Tighe-Ford, 1977) and solutions of 20-hydroxyecdysone have been used to induce moulting in isolated penes of the barnacle B. eburneus by Cheung and Nigrelli (1974). Several experiments have been conducted to monitor the effect of ecdysteroids on explants of barnacle tissue. Freeman and Costlow (1976 and 1977) found that apolysis and protein synthesis were stimulated in epidermal tissue from B. amphitrite when cultured with 20-hydroxyecdysone, but very high levels of 0.5-2.0 $\mu g\ ml^{-1}$ of ecdysteroid were employed. This technique was also employed to show the antagonistic action of 20-hydroxyecdysone and a moult inhibiting hormone (MIH) from a shrimp eyestalk extract. Ovarioles of the same barnacle species have been incubated with 20-hydroxyecdysone by Fyhn et al. (1977) and results suggested that this hormone may well be gonad inhibiting as well as moult stimulating. The effects of both ecdysone and 20-hydroxyecdysone on inner mantle tissue explants of B. amphitrite were studied by Freeman and Costlow (1979) who found both ecdysteroids

equally effective when using concentrations of 10-20 μg ml^{-1}. Freeman (1980) using similar tissue, reported that 20-hydroxyecdysone and MIH both act directly upon the epidermis to control chitinolysis during early proecdysis.

Although a variety of authors have used injection techniques to study ecdysteroid effects on crustaceans (see Vernet, 1976) only two authors have employed this method in the study of moulting in barnacles. Tighe-Ford (1968) devised a micro-injection technique and injected extracts of barnacle central nervous system (CNS) into B. balanoides. The technique was then employed to monitor the effect of injecting barnacles with 20-hydroxyecdysone (Tighe-Ford and Vaile, 1972a and b; Tighe-Ford, 1974), barnacle extracts containing an unknown concentration of ecdysteroid, and azadirachtin, an insect anti-feedant, (Tighe-Ford, 1974). The injection of 20-hydroxy-ecdysone increased and synchronized the moulting activity in barnacles suggesting that the effect was hormonal. Single injections induced a rapid response but a series of doses showed an initial reduction in moulting followed by marked activity. Levels of between 20-200 ng per individual were injected, one experiment monitored the effect by a low dose of only 5 ng per barnacle, and responses when the animals were moulting at a low (late winter) and high (late autumn) frequency were noted. This technique, however, necessitated the drilling of a hole through the shell of the barnacle close to the base of the lateral plate (see Experimental) and injecting the solution into the haemocoel between the basal mantle tissues. In controls that were injected with sterile sea water, or distilled water, an increase in moulting over uninjected controls was noted and determined to be a response to wounding. Calcification around the site of the injection

within the mantle tissue was also seen. This effect was less apparent in barnacles that were subjected to single rather than multiple injections.

Davis and Costlow (1974), proposed a second technique for injecting barnacles by forcing open the opercular valves with forceps and injecting a solution into the epithelium of the adductor scutorum using a mounted glass needle. By staining, it was shown that injected material circulated throughout the body within an hour. The technique was employed to study the effects of injecting specimens with CNS extracts from barnacles and eyestalk extracts from crabs. This technique, unlike that of Tighe-Ford's, was less injurous to the organism and therefore did not affect the duration of the intermoult cycle.

As part of this study a short period of experimentation was conducted at the Exposure Trials Station Laboratory, Eastney. During this period further experiments on the effects of injecting material into barnacles were conducted. Although the disadvantages of Tighe--Ford's micro-injection scheme were known this technique was chosen due to availability of equipment and the size of barnacles available. Davis and Costlow's method used barnacles with a diameter of 12-17 mm, much larger than the species of B. balanoides obtained at Eastney which had a mean diameter of 9.5 mm.

Solutions of 20-hydroxyecdysone, ecdysone and 20-hydroxyecdysone of a known concentration extracted from barnacles were injected into barnacles during the summer months when the moulting activity was known to be at an intermediate frequency (see earlier discussion). Groups of barnacles were injected, in a single dose, with 20-hydroxy-ecdysone at 1, 2, 5 or 10 ng per barnacle. From the results of

Bebbington (1975) and this study (see earlier) the concentration of ecdysteroid injected by Tighe-Ford (1974) into barnacles is now known to be in excess of the levels naturally occurring. For this reason the effects of lower levels were studied which more closely mimicked the levels calculated to occur naturally. This set of injections was also considered as an attempt to determine the efficiency of this technique as a bioassay and the effects of both standard 20-hydroxyecdysone and 20-hydroxyecdysone, at similar concentrations, extracted from barnacles at varying times of the year were compared.

Effects of 20-hydroxyecdysone

Barnacles used in this experiment were collected in early July and acclimatised for two weeks to a period of 16 h submersion (overnight) in running seawater at ambient temperature and light and 8 h exposure. During this period the mean daily moulting frequency was found to be 9.3 moults per day per 100 barnacles, with a range of 7.7-13.3.

For the determination of the effects of pure 20-hydroxyecdysone and crude preparations from barnacle extracts, ten groups of thirty individual barnacles per group were prepared. One group was kept as uninjected controls and another as controls injected with sterile sea water. Four groups were then injected with pure 20-hydroxyecdysone dissolved in sterilised sea water. The three samples of ecdysteroid extracted from barnacles were not readily soluble in the sterile sea water and to each a small volume of acetone was initially added to aid dissolution. The solution was then made up to volume with the sea water. For this reason a second group of injected controls were prepared and dosed with sterile sea water containing acetone in the appropriate proportion. Each of these ten groups were then placed

in one of the chambers of the flowing sea water system and submerged immediately after injection.

To ensure that the moulting frequency was not affected by any other factor arising from the design of the system the groups of barnacles were not maintained in the same chambers constantly. After the daily recording of number of moults the barnacles were removed from the sea water system, but carefully kept in their groups, while the tanks were cleaned of faeces and any other debris. When the groups were replaced at night they were moved round one chamber, i.e. on day 1 group 1 were in the first compartment, on day 2 the second and so on and group 10 moved from the last to the first compartment. Thus the groups were continually cycled throughout the apparatus.

The experiment ran for 28 days and the results, shown as number of moults per day per hundred barnacles, are given in tables 13 and 14. To facilitate analysis the results are divided into cycles or periods in which the number of exuviae are 90% or more the number of live barnacles in that group. This, however, assumes that no barnacle moulted more than once per cycle. Any deaths were also recorded. Only six injected individuals died, 3.3%, three injected controls and three barnalces that had been injected with ecdysteroids extracted from barnacles.

Moulting increased in the injected controls and those groups injected with standard 20-hydroxyecdysone. In the groups injected with ecdysteroids from barnacles at >0.4, 1.6 and 6.4 ng per barnacle (samples, 1, 2 and 3) no marked increase in moulting activity was apparent but there was an indication that the injected material inhibited this action (see tables 13 and 14).

Table 13.

The action of 20-hydroxyecdysone upon the moulting activity of barnacles expressed as the number of exuviae per day per 100 animals using groups of 30 animals.

Day	Controls		20-hydroxyecdysone (ng/barnacle)			
	Untreated	Sea-water	1	2	5	10
1	3.3	3.3	0	6.7	3.3	10.0
2	3.3	10.0	3.3	3.3	0	3.3
3	3.3	10.3	6.7	0	3.3	6.7
4	13.3	10.3	13.3	20.0	20.0	13.3
5	46.7	10.3	16.7	16.7	53.3	80.0 113
6	23.3	20.7	53.3	40.0	20.0 100	0
7	3.3	44.8 110	10.0 103	13.3 100	3.3	3.3
8	0	13.8	6.7	13.3	0	10.0
9	3.3 100	3.5	3.3	10.0	13.3	0
10	30.0	6.9	3.3	10.0	13.3	20.0
11	20.0	10.3	23.3	13.3	10.0	36.7
12	23.3	10.3	16.7	10.0	20.0	10.0
13	0	13.8	10.0	10.0	26.7	10.0
14	10.0	13.8	10.0	13.3	6.7	10.0 100
15	23.3 107	17.2	6.7	13.3	6.7 100	6.7
16	23.3	10.3 100	6.7	3.3 97	0	3.3
17	3.3	3.5	16.7 104	20.0	6.7	3.3
18	6.7	6.9	6.7	13.3	3.3	6.7
19	3.3	10.3	13.3	10.0	10.0	10.0
20	16.7	6.9	6.7	13.3	13.3	10.0

D

/ continued

Table 13 - continued

21	16.7		6.9	3.3	3.3		10.0	10.0
22	6.7		13.8	6.7	3.3		6.7	3.3
23	3.3		17.2	0	0		3.3	3.3
24	6.7		6.9	3.3	3.3		3.3	6.7
25	3.3		10.3	0	13.3		10.0	10.0
26	0		3.5	13.3	6.7		3.3	3.3
27	3.3		0	0	13.3		0	10.0
28	3.3	96	0	13.3	6.7	106	16.7	16.7 104
Total	303.3		296	273	303		287	317

I = a cycle or period in which the number of exuviae cast is 90% + the number of barnacles alive in the group.

D = death

Table 14.

The action of crude 20-hydroxyecdysone extracted from barnacles upon the moulting activity of barnacles expressed as the number of exuviae per day per 100 barnacles using groups of 30 animals.

Day	Controls		Sample		
	Untreated	Sea-water	1	2	3
1	3.3	10.0	3.3	0	13.3
2	3.3	13.3	3.3	3.3	3.4
3	3.3	6.7	0	3.3	3.4
4	13.3	6.7	0	3.3	6.9
5	46.7	13.3	28.6	30.0	38.0
6	23.3	23.3	32.1	30.0	27.6
7	3.3	20.0	14.3	13.3	6.9
8	0	16.7	3.6	13.3	3.4
9	3.3	10.0	10.7	6.7	10.3
10	30.0	0	10.7	3.3	0
11	20.0	0	3.6	10.0	6.9
12	23.3	0	25.0	13.3	13.8
13	0	27.6	10.7	13.3	17.2
14	10.0	6.9	10.7	16.7	10.3
15	23.3	3.6	3.6	13.3	13.8
16	23.3	10.7	17.8	16.7	6.9
17	3.3	7.1	7.1	13.3	6.9
18	6.7	7.1	0	0	10.3
19	3.3	21.4	7.1	3.3	6.9
20	16.7	10.7	10.7	6.7	10.3
	100	110	96	103	103
	107	D	D	100	D
		D	107		
		105			

/ continued

Table 14 - continued

21	16.7			17.8		7.1	10.0		20.7
22	6.7			14.2		17.8	10.0		10.3
23	3.3			3.6		17.8	23.3		13.7
24	6.7			0		10.7	20.0		0
25	3.3			0		3.6	0		17.2
26	0			0		7.1	13.3		3.4
27	3.3			3.6		3.6	0		6.9
28	3.3	I	96	3.6		7.1	6.9	D 94	0
Total	303			258		278	297		289

I = a period in which the number of exuviae is 90%, or more, of the number of animals.

D = death

The increased moulting activity shown by the standard solution is shown graphically in figures 28 and 29. Figure 28 shows the cumulative response over the four weeks and it is clear that the accelerated activity is short-lived and only affects one cycle. All the injected groups then reverted to a normal second cycle where the moulting frequency is on average 10 moults per 100 barnacles per day.

Figure 29 shows the response over the first fourteen days and emphasises the rapid reaction to the 10 ng dose. Here the moulting activity became synchronized and 80% of the barnacles in this group moulted on the fifth day after injection. Some synchronization was also apparent during the second moulting cycle in this group when a total of 56.7% moulted on days 11 and 12. A marked but less rapid reaction was seen with the 5 ng injected group where 53.3% moulted simultaneously five days after being injected. This was the lowest concentration that Tighe-Ford (1974) had used in his study and he found that although some response was obtained the activity was closely similar to that of sea water injected controls. This may have resulted from his study being conducted with barnacles already undergoing a relatively high moulting frequency in the autumn. Barnacles used in our study were moulting at an intermediate rate and therefore may have been more 'susceptible' to the injected material at these low levels. We also obtained some reaction to lower doses of 1 and 2 ng per barnacle. Although the cycle in these groups was closely similar to that of the injected controls it can be seen that a synchronized response occurred six days after injection when 40% and 53.3% respectively moulted in the two groups. In the injected controls the response arose seven days after wounding when 44.8%

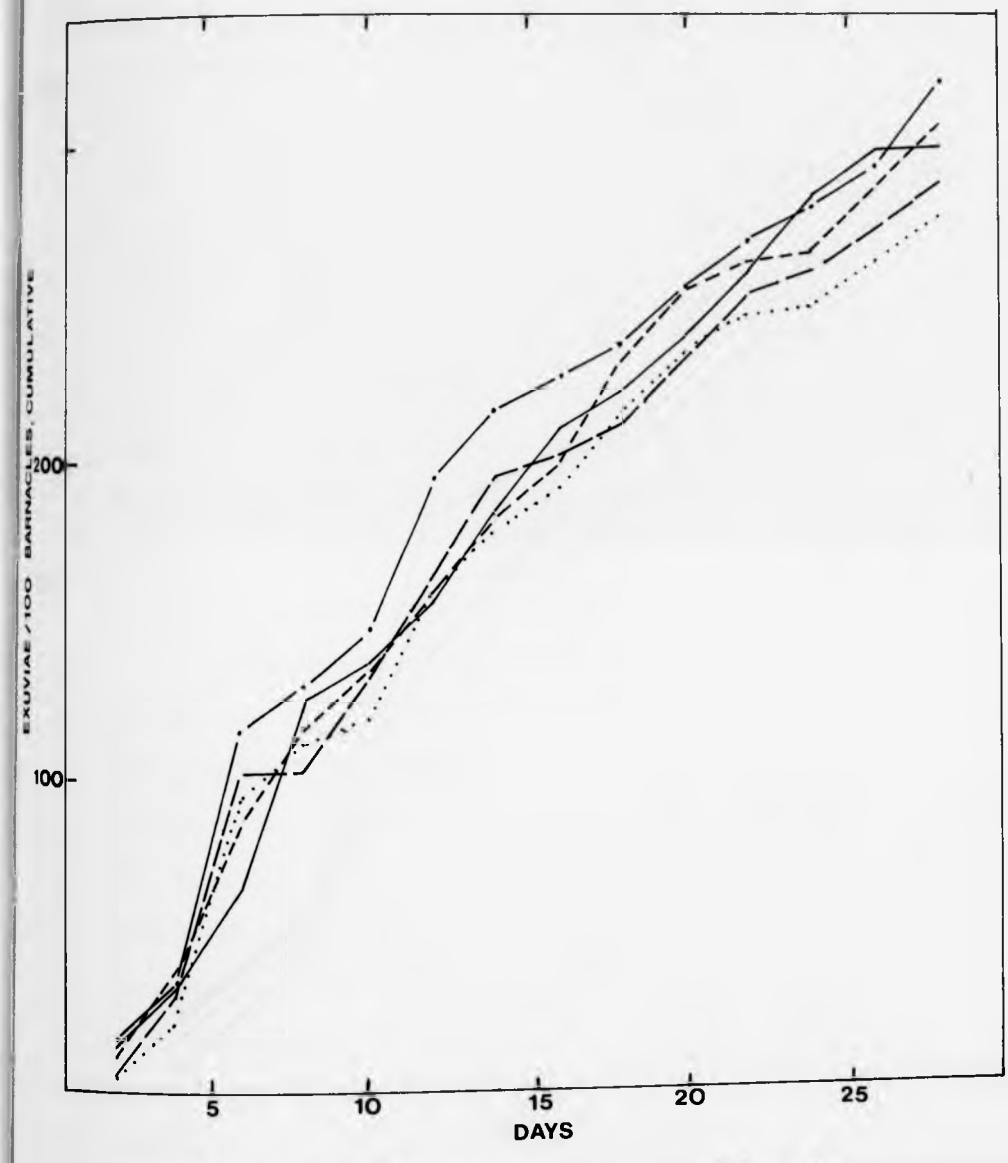


Figure 28. Moulting response of barnacles injected with 20-hydroxyecdysone.

Controls, sea-water injected (——);
1 ng ecdysteroid injected (....);
2 ng ecdysteroid injected (----);
5 ng ecdysteroid injected (—— ——);
10 ng ecdysteroid injected (· —— ·)

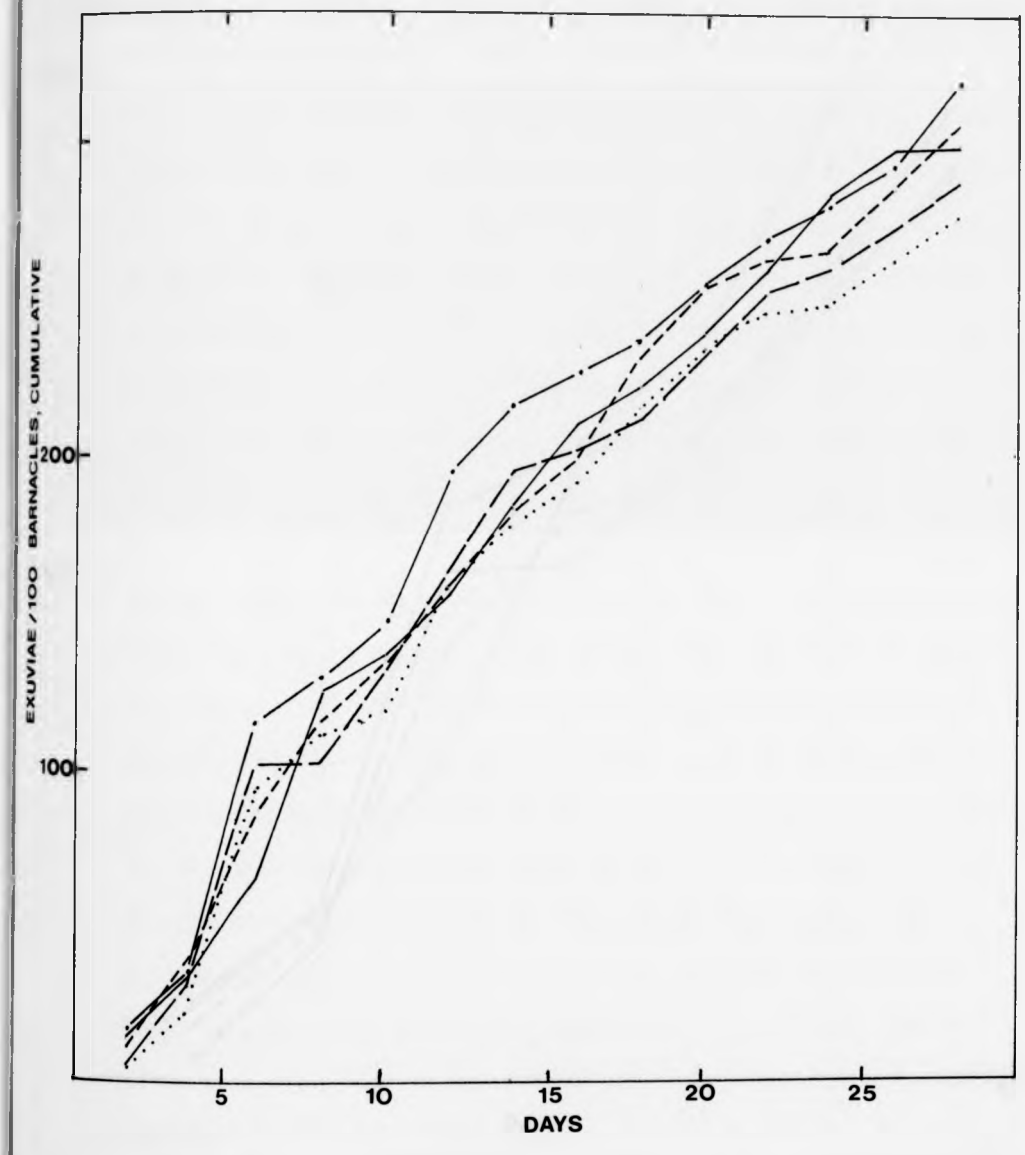


Figure 28. Moulting response of barnacles injected with 20-hydroxyecdysone.

Controls, sea-water injected (——);
1 ng ecdysteroid injected (····);
2 ng ecdysteroid injected (----);
5 ng ecdysteroid injected (—— ——);
10 ng ecdysteroid injected (· —— ·)

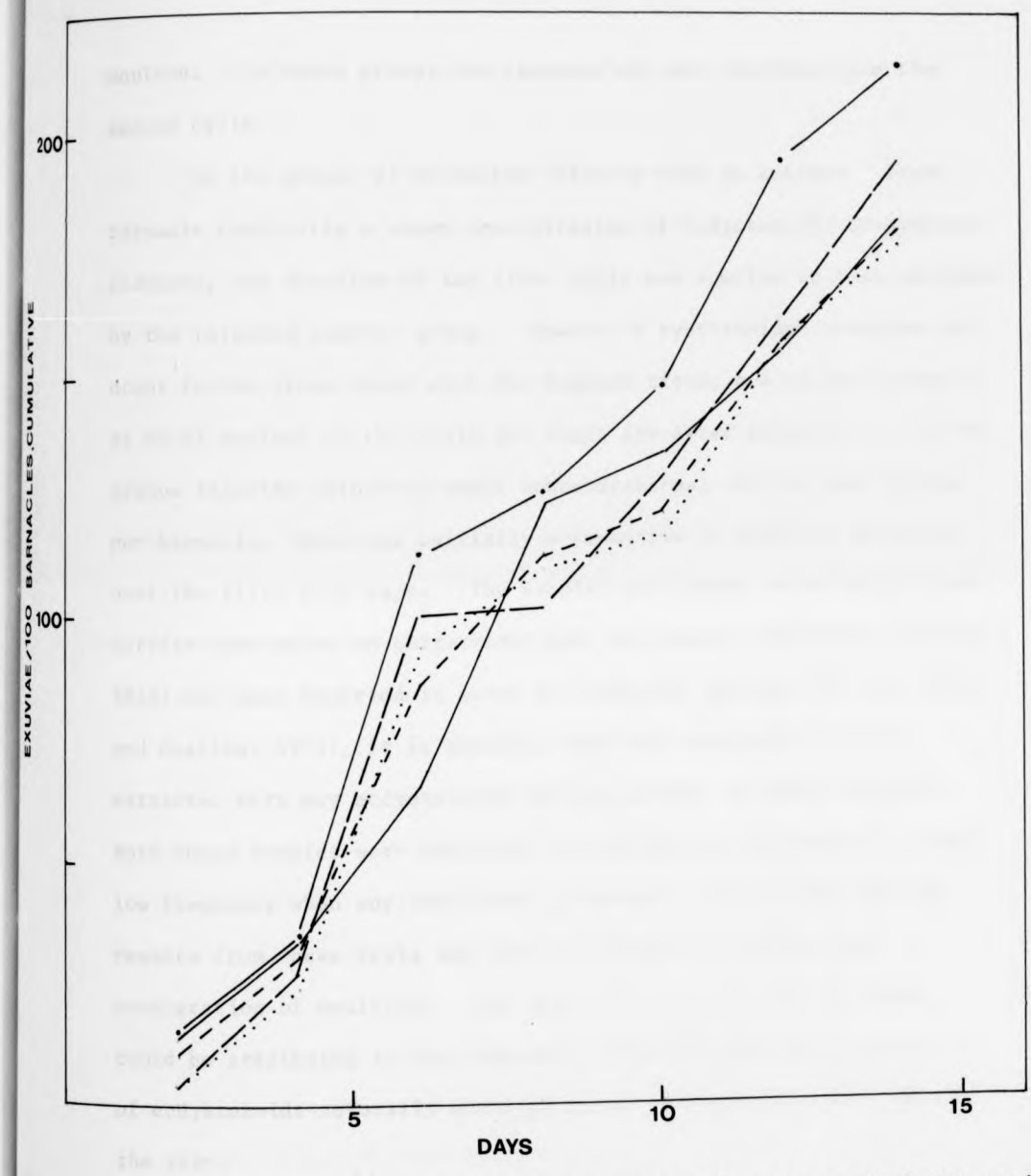


Figure 29. Moulting response of barnacles injected with 20-hydroxyecdysone.

Controls, sea water injected (——);
1 ng ecdysteroid injected (····);
2 ng ecdysteroid injected (----);
5 ng ecdysteroid injected (—— ——);
10 ng ecdysteroid injected (· —— ·)

moulted. In these groups the response did not continue into the second cycle.

In the groups of barnacles injected with an extract from barnacle containing a known concentration of ecdysteroid, 20-hydroxy-ecdysone, the duration of the first cycle was similar to that obtained by the injected control group. However a synchronized response did occur in the group dosed with the highest titre, 6.4 ng per barnacle, as 65.6% moulted on the fifth and sixth day after injection. In the groups injected with very small concentrations, >0.4 ng and 1.6 ng per barnacle, there was initially a reduction in moulting activity over the first four days. The samples were known to be impure (see earlier discussion on extraction) and, as a moult inhibiting substance (MIS) has been reported to occur in barnacles (Cheung, 1974 and Davis and Costlow, 1974), it is possible that this substance had been extracted with any ecdysteroids and was present in these samples. Both these samples were extracted from barnacles moulting at a very low frequency when any inhibitory substance would be high and the results from these tests may indicate inhibition rather than acceleration of moulting. The moulting activity that was seen could be attributed to the response to wounding and the presence of ecdysteroids naturally occuring in the barnacle at this time of the year.

From these studies it is apparent that the use of this technique as a bioassay is limited primarily due to the seasonal variation in moulting frequency in this barnacle species. The response to similar doses of 20-hydroxyecdysone varies according to the time of year when the barnacles are collected. Tighe-Ford (1974) recorded variation when using specimens moulting at a low and high rate and this study

now records the effect when using barnacles at an intermediate state. The technique also suffers from the wounding response which masks any effect from injections of materials at low concentrations. It was also difficult to ensure that the material was satisfactorily injected into the haemocoel of the barnacle as it would be very easy to penetrate the mantle walls and inject the sample into the mantle space from where it would be ejected when the animal opened its opercular valves on submersion. Tighe-Ford used barnacles specially grown on perspex sheets to enable him to visualise the injection and prevent this occurrence, unlike on this occasion when barnacles settled on rocks were utilised. Undoubtedly Davis and Costlow's (1974) method is superior and if older and larger specimens of B. balanoides are obtained this technique would be preferable.

A further disadvantage of using this method is the fact that the individual barnacles are at varying stages in their moulting cycle. Davis and Costlow (1974) have shown that the effect of injecting a barnacle with CNS extracts varies depending on the moulting stage of the animal.

Effects of Ecdysone

Barnacles were collected in early August and prepared as before. Six groups of thirty barnacles per group were arranged, one group maintained as injected controls and another as uninjected controls. Individuals in the other four groups were injected with ecdysone at either 1, 2, 5 or 10 ng per barnacle. They were immediately placed in the flowing sea water system, cycled daily as before, and the number of moults per group per day noted. The results of the 28 day

experiment are shown in table 15, displayed in the same manner as in the previous experiment.

In this experiment the mortality rate of barnacles was very high and occurred in both the uninjected control group as well as in injected groups. Two deaths occurred in both the controls in the first seven days and a single death was recorded in each of the three lowest dosed groups within the same period. A very high mortality rate occurred in the group whose individuals were injected with the highest concentration of ecdysone, 10 ng per barnacle, where four barnacles died within six days and a further two deaths were recorded after twelve days yielding a 20% mortality rate.

From table 15, it is apparent that the injected material did not synchronise or increase the moulting frequency of the barnacles, neither was there any apparent inhibition of this activity. The moulting activity of the groups injected with ecdysone is closely similar to that of the injected control group. It was expected that some, or all, of the injected material would be converted to the major moulting hormone 20-hydroxyecdysone, and that a response would be seen similar to that which occurred with the barnacles that were injected directly with 20-hydroxyecdysone. The effect did not occur.

Ecdysone has been found to be present in crustacea (see Introduction) and has been isolated from the barnacle B. balanoides but at a much lower concentration than the major ecdysteroid, 20-hydroxyecdysone. Ecdysone has been injected into a variety of crustacea (see Vernet, 1976) and has been shown to have a positive effect upon moulting in decapods, amphipods and isopods. This is

Table 15.

The action of ecdysone upon the moulting activity of barnacles expressed as the number of exuviae per day per 100 barnacles using groups of 30 animals.

Day	Controls: Untreated	Controls: Sea-water	Ecdysone (ng/barnacle): 1	2	5	10
1	0	0	3.3	6.7	0	0
2	6.9	0	6.9	3.3	6.7	10.7
3	3.4	16.7	20.7	20.0	3.3	25.0
4	17.2	10.0	3.5	13.3	30.0	21.4
5	10.3	16.7	10.3	16.7	20.0	17.8
6	21.4	33.3	25.0	26.7	23.3	0
7	14.2	3.3	14.3	10.0	3.4	11.5
8	25.9	25.0	17.8	13.8	3.4	15.4
9	7.4	25.0	10.7	20.7	6.9	11.5
10	3.7	14.3	10.7	13.8	17.2	7.7
11	11.1	7.1	21.4	0	10.3	15.4
12	7.4	14.3	14.3	10.3	20.7	11.5
13	3.7	3.6	17.8	27.6	6.9	12.5
14	7.4	10.7	0	10.3	10.3	29.2
15	14.8	7.1	10.7	6.9	13.8	4.2
16	14.8	10.7	7.1	10.3	6.9	12.5
17	7.4	10.7	7.1	10.3	6.9	8.3
18	11.1	10.7	14.3	3.4	10.3	8.3
19	0	3.6	14.3	6.9	6.9	12.5
20	7.4	7.1	7.1	6.9	10.3	0

D = 99; 2D = 105; D = 102; D = 97; D = 103; 2D = 100; D = 104; 2D = 102; 104

/ continued

Table 15 - continued

21	3.7 I 100	7.1	3.6	0	3.4	12.5
22	7.4	7.1	3.6	6.9	3.4	12.5
23	11.1	7.1	10.7	6.9	10.3	12.5
24	14.8	10.7	14.3	13.8	10.3	8.3
25	14.8	7.1	10.7	20.7	10.3	8.3
26	7.4	7.1	10.7	6.9	13.8	4.2
27	11.1	14.3	7.1 I 96	13.8 I 107	10.3	8.3 I 96
28	11.1	14.3 I 97	14.3	6.9	6.9	20.8
Total	277	305	312	314	287	323

I = a period when the number of exuviae is 90%, or more, of the number of animals.

D = death

the first attempt to determine its effect in cirripedes. King and Siddall (1969) demonstrated that 23,24-^{3}H-ecdysone injected into the shrimp, Crangon nigricauda and the fiddler crab, Uca pugilator was converted, up to 75%, to 20-hydroxyecdysone. A similar result was found in the shore crab, P. crassipes by Chang et al., (1976), and in the green crab, C. maenas by Lachaise et al., (1976). The rate of conversion was found to be dependent upon the stage of development of the organism. Lachaise et al., (1976) demonstrated that although the injected ecdysone was converted to 20-hydroxyecdysone, and a very small concentration of six other metabolites, only labelled ecdysone was excreted. Kuppert et al., (1978) injected ^{3}H-ecdysone into crayfish, Orconectes limosus, and found that after one hour 60-70% of the total radioactivity was excreted as the unchanged ecdysteroid. However the remaining ecdysone was converted to labelled 20-hydroxy-ecdysone but little of this was detected in excretions. McCarthy and Skinner (1979a) followed the metabolism of ^{3}H-ecdysone injected into the land crab, Gecarcinus lateralis and also found that the labelled ecdysteroid was predominantly converted to 20-hydroxyecdysone very rapidly. Further metabolism yielded quantities of 20,26-dihydroxy-ecdysone and polar conjugates of all three free ecdysteroids were found. The polar conjugates were presumed inactivation products and were predominantly excreted in the urine and faeces along with labelled free ecdysteroids.

From all these studies it is apparent that there is a rapid conversion of ecdysone to 20-hydroxyecdysone coupled with an initial excretion of labelled ecdysone. Injected ecdysone is also converted to a series of minor metabolites of which the conjugates appear to be inactivation products which are excreted primarily in the urine.

From the results of injection of ecdysone into barnacles there appears little evidence to support the conversion of ecdysone in this organism. However this may be the result of the low dosage and using a population of individuals at varying stages of the moult cycle. If in cirripedes, as in other crustacea, a high percentage of the initial concentration of ecdysone is rapidly excreted then the amount of 20-hydroxyecdysone converted from injected material may be of too low a concentration to show any positive effects. However, in the previous series of experiments it was apparent that an injection of as little as 1 ng of 20-hydroxyecdysone showed some effect. It may therefore be necessary to perform this experiment with barnacles at a fixed stage in the moulting cycle. Lachaise et al., (1976) recorded that the conversion rate of ecdysone to 20-hydroxyecdysone was dependent upon the stage of development and found the most rapid conversion occurring when the crab under test was a stage D_o-D_1, early premoult. Similarly King and Siddall (1969) noted a variation of 50% in conversion rate in U. pugilator at premoult and intermoult stages. Maisslat and Legrand (1970) found that ecdysone injected into the isopod Ligia oceanica was most effective when the organism was at stage C and least effective when at the end of stage D or at stages A and B.

The concentrations of ecdysone injected into the barnacles were of a similar amount to the concentration of 20-hydroxyecdysone used in the previous experiment to allow for a direct comparison between the activities of these two ecdysteroids. However from the reports of similar experiments in other crustacea it is now considered that these levels were of too low a concentration to show any significant

effect over and above the wounding response. The low concentrations naturally present suggest that this ecdysteroid is merely a very short lived intermediate metabolite that, as in other crustacea, is rapidly converted to the major moulting hormone, 20-hydroxyecdysone. To determine this more accurately these experiments would have to be repeated, preferably using larger specimens and the injection technique devised by Davis and Costlow (1974), using larger amounts of ecdysone. Davis et al., (1973) and Koulish and Klepal (1981) have shown that it is possible to determine the moult stage of individual barnacles but to obtain groups of individuals at similar stages would entail extensive experimental procedures.

EXPERIMENTAL METHODS

Solvents and their Purification

Methanol and acetone used for the preparation of solutions of ecdysteroids and for the Sephadex column chromatography were obtained from Fisons (Distol Pesticide grade).

Toluene (1 ℓ) for GC-ECD was purified by shaking with cool concentrated sulphuric acid (2 x 100 ml), then distilled water (1 ℓ), and finally 5% sodium bicarbonate solution (1 ℓ) to remove all traces of acid. The toluene was then dried overnight with either anhydrous magnesium sulphate or calcium sulphate, distilled from phosphorus pentoxide and stored over molecular sieves 4A. Its purity was checked by evaporating a 10 ml portion to 200-500 μℓ with a stream of nitrogen and injecting 1-2 μℓ of the concentrated solution onto the gas chromatograph fitted with a ^{63}Ni ECD. The breadth of the solvent peak indicated its purity and should be no more than 1 min.

Pyridine for silylation reactions was dried overnight with calcium hydride before distillation and storage over molecular sieves.

Diethyl ether (1 ℓ) was dried overnight with granular calcium chloride and then distilled from sodium wire and stored over molecular sieves.

Pure ethanol was obtained using the method of Perrin (1966). Magnesium turnings (5g) and iodine (0.5g) were placed in a 2 ℓ flask with absolute ethanol (100 ml) and warmed until the reaction abated (solution turned from brown to white). Then further ethanol (1 ℓ) was added and the whole refluxed for 1h before fractional distillation, the ethanol being collected at 77-79°C.

All other solvents were laboratory grade and were dried in the conventional way with sodium or molecular sieves where required.

Water and acetone used for washing glassware was distilled, as was the water used for partition, and thin layer, chromatography.

Cleaning of Glassware and Reacti-vials

Glassware was cleaned by soaking overnight in caustic detergent followed by a thorough washing with tap water, distilled acetone and finally distilled water. The 1 cm^3 Reacti-vials (Magnus Scientific, Sandbach) were first soaked overnight in chromic acid (3g chromium trioxide, 5 ml of water made up to 100 ml with concentrated sulphuric acid), then in saturated sodium bicarbonate and finally extensively rinsed with distilled acetone and water. They were then dried in an oven at 130-140^{o}C. Sample bottles used for storage of solutions of standard ecdysteroids and barnacle samples were similarly cleansed.

Preparation of Trimethylsilylimidazole

The preparation followed that described by Barkoffer and Rittor (1965) and adapted by Wilson (1979). Imidazole (13.6g) plus hexamethyldisilazane (24.2g) and two drops of concentrated sulphuric acid were placed in a three-necked 100 ml flask and refluxed under nitrogen for 2h. Fractional distillation of the mixture under vacuum gave trimethylsilylimidazole as a colourless mobile liquid which was collected with a Perkin triangle with an average yield of 85%. The compound was tested for purity by preparing the TMS derivatives of 20-hydroxyecdysone, and also by its NMR spectrum. The TMSI was stored in 1 ml glass vials sealed under nitrogen and kept at 4^{o}C until required.

Preparation of Trimethylsilyl Ethers of Pure Ecdysteroids

A sample of ecdysteroid (0.2-1.0 mg) was weighed on a micro-balance and dissolved in methanol or acetone (Fisons Distol Pesticide grade), to give a concentration of 500 μg ml^{-1}. An aliquot of the solution (50 μℓ, i.e. 25 μg ecdysteroid) was evaporated to dryness in a 1 cm^3 Reactivial (Pierce and Warriner, Chester) with a stream of warm nitrogen. The ecdysteroid was dissolved in a mixture of purified pyridine and TMSI in the ratio of 2:1 (generally 65 μℓ and 35 μℓ respectively). The vial was sealed with a screw cap and then left either for 30 min at room temperature (20°C), 5h at 120°C or 60h at 140°C depending upon the trimethylsilyl ether required. The solution was allowed to cool and an aliquot (10 μℓ) removed and diluted with ECD grade toluene to give a final concentration of 1-10 ng $\mu\ell^{-1}$ of the derivative. A sample of this solution (1 μℓ) was injected onto the GC column and the retention time of the derivative noted. The time required for complete conversion to a single derivative was originally found by monitoring the course of the reaction at regular timed intervals (see Discussion). The remaining solution in the Reactivial was either stored in a refrigerator, where the TMS ether was stable for several weeks, or purified directly by thin layer chromatography (see later).

Preparation of Thin Layer Chromatography Plates

Silica gel (500g of Kieselgel 60 PF_{254}, Merck) was washed with methanol (3 x 1 ℓ) followed by diethyl ether (3 x 1 ℓ) with gentle warming and stirring on a stirrer hot plate, the solvent being decanted after each washing. After the final wash the silica was dried and sieved through a number 100 sieve (Endecotts Test Sieves Limited) and

stored in a sealed container. The washed silica (25g) was slurried with distilled water (25 ml) and spread onto glass plates, either four 20 x 20 cm or eight 10 x 20 cm, to a thickness of 0.6 mm. The glass plates were thoroughly cleaned before use by soaking in a caustic detergent solution followed by several washes with distilled water and a final rinse in distilled acetone. The slurried plates were then left to air dry at room temperature for 24h, activated before use by heating in an oven at 105°C for 1h, then cooled and stored in a dry box over granular calcium chloride.

Thin Layer Chromatography of Pure Ecdysteroids and their TMS ethers

For TLC analysis an aliquot of a solution of ecdysteroid in either methanol or acetone was taken containing at least 10 µg and placed as a spot onto the origin of a normal phase 10 x 20 cm 0.6 mm TLC plate. Alongside the ecdysteroid a spot of ecdysone and 20-hydroxy-ecdysone were also placed and the plate was then developed for 15 min in either dichloromethane:methanol (85:15) or chloroform:95% ethanol (85:15). After development the plate was dried with the aid of a hair dryer and the ecdysteroids were visualised under UV light at 254 nm and R_f values calculated.

For TLC analysis of TMS ethers of pure ecdysteroids the volume of the reaction mixture after silylation was initially reduced by evaporating some of the pyridine with a stream of nitrogen. A portion of the concentrated solution, containing at least 10 µg, was then placed as a spot onto the origin of a normal phase 10 x 20 cm 0.6 mm TLC plate alongside the tetrakis-TMS ether of ecdysone, which was prepared by silylating ecdysone for 5h at 120°C and here used as a standard. The plate was then developed in toluene:ethyl acetate (7:3) for 15 cm,

removed and dried with the aid of a hair drier. The derivatised ecdysteroids were then visualised under UV light at 254 nm. To confirm that the spot seen under UV illumination was the derivatised ecdysteroid the appropriate R_f zone was scraped from the plate into a 5 ml centrifuge tube and shaken with diethyl ether (3 ml). After centrifuging, the diethyl ether was gradually pipetted into a Reacti-vial and evaporated to dryness with a stream of nitrogen, the final residue being taken up in ECD-grade toluene to give a concentration of 1-10 ng $\mu\ell^{-1}$ of ecdysteroid derivative. A portion of this solution (1 $\mu\ell$) was injected onto the GC column, using its retention time to confirm that the spot on the TLC plate was the silylated ecdysteroid.

To determine the percentage recovery of TMS ethers of ecdysteroids after TLC or if the amount of derivatised ecdysteroid was below the level of detectability by UV fluorescence (ie. < 10 μg), then the pyridine solution after silylation was reduced in volume while still warm with a stream of nitrogen and the remainder applied as a band to the origin of a 20 x 20 cm TLC plate. This was developed and dried as before and the silica from R_f 0.5-0.9 removed and packed into a glass column (25 x 1.0 cm), the lower end of which was constricted and plugged with glass wool. The ecdysteroid derivative was eluted from the silica with dried, distilled diethyl ether (15 ml) into a centrifuge tube. The ether was evaporated to a small volume with a stream of nitrogen and gentle warming with a hair drier, ensuring that water did not condense inside the tube. This reduced volume, plus two ether washings (2 x 250 $\mu\ell$), was transferred to a 1 cm^3 Reacti-vial and reduced to dryness. The residue was taken up in a known volume of purified toluene and diluted suitably for analysis by gas chromatography.

Preparation of Reverse Phase Thin Layer Chromatography Plates

Commercially available reverse-phase thin layer chromatography (RP-TLC) plates (OPTI-UP RPTLC plates, Fluka AG, Switzerland) were used in the analysis of pure ecdysteroids. Reverse phase TLC plates were also prepared from the normal phase 10 x 20 cm 0.6 mm TLC plates and from 10 x 10 cm high performance TLC plates (Whatman HP-K UV_{254} HPTLC, Uniscience Ltd., Cambridge, UK) according to Stahl (1969). This preparation involved coating the normal phase TLC plates with a non-polar liquid phase. This was simply achieved by 'chromatographing' a plate in dichloromethane containing the appropriate percentage of "Nujol" grade of liquid paraffin ($2\frac{1}{2}$, 5, $7\frac{1}{2}$ or 10% vol/vol). Primarily a $7\frac{1}{2}$% loading was used. The plate was then dried in a fume cupboard and stored over granular calcium chloride. Immediately before use the plate was equilibrated by suspension over the proposed running solvent for 1h in a sealed chromatography tank. For determination of percentage recoveries of low levels of ecdysteroids it was found necessary to pre--wash the Whatman HPTLC plates before preparing reverse phase plates. This was achieved by running the plate in dichloromethane and then removing a strip of silica corresponding to a band of R_f 0.9-1.0. The plate was then dried and prepared as a reverse phase plate as before.

Reverse Phase Thin Layer Chromatography of Pure Ecdysteroids

The prepared plates were equilibrated for 1h in the solvent vapour and then solutions of ecdysteroids (10 µl of 500 µg ml^{-1} in methanol) were placed as spots at the origin. The plates were then developed in a tank of methanol:water (60:40), removed and allowed to dry in a fume cupboard before visualising under UV light at 254 nm.

Ecdysone was used as a typical ecdysteroid for determination of recoveries from RP-TLC plates. Each plate was spotted at the origin with a series of concentrations ranging from 2 µg (which could be visualised under UV light) to 10 ng (which could not). After development in methanol:water (60:40), the plate was dried and viewed under UV light and the R_f value of ecdysone found from the concentrated spot. An area of silica from 1 cm above to 1 cm below this value was scraped into a 5 ml centrifuge tube and shaken with methanol (3 ml), the mixture centrifuged and the solution decanted. The silica was washed once more with methanol (1 ml), the mixture centrifuged and the solution decanted and combined with the first washing. The methanol solution was then gradually pipetted into a 1 cm^3 Reacti-vial, evaporated to dryness with a stream of warm nitrogen and thoroughly dried under vacuum in a drying pistol at 50°C for 1h. The recovered ecdysone was then converted to its TMS ether by reaction in a mixture of purified pyridine (15 µℓ) and TMSI (10 µℓ) for 30 min at room temperature. The solution was then diluted with ECD grade toluene to give a final concentration between 200 pg $µℓ^{-1}$ - 1 ng $µℓ^{-1}$, and 1 µℓ of this solution was injected onto the GC column. The resultant peak area was used to determine the percentage recovery by comparison with a calibration curve for ecdysone.

Microcrystalline Column Chromatography

Microcrystalline cellulose (7g, E. Merck, Darmstadt) was placed in a glass column (18 cm x 10 mm i.d.) with a drawn out end plugged with glass wool. The support was then washed by eluting with benzene saturated for 24h with an equal volume of 50% isopropanol in water. The barnacle sample, with added ecdysone (10 µg), was reduced to a small volume (500 µℓ) and introduced onto the surface of the column

and eluted with the benzene solution. Fractions (5 ml) were collected up to 50 ml and each was evaporated to dryness into a 1 cm^3 Reacti-vial, with the aid of a stream of nitrogen and a hair drier. The residue was then dried thoroughly in a drying pistol under vacuum and at 50°C for 1h. The dried residue was then reacted with pyridine (65 µℓ) and TMSI (35 µℓ) for 5h at 120°C. The solution, after cooling, was then diluted with toluene to give a solution of approximately 1-2 ng $µℓ^{-1}$ of ecdysone and 1 µℓ was injected onto the gas chromatograph column. The active fraction that contained the ecdysone was then found and the percentage recovery calculated.

Floridin Earth Column Chromatography

Floridin Earth (50g) or Florex (BDH Chemicals Ltd., Poole, England) was prewashed with saturated sodium bicarbonate (150 ml), distilled water (3 x 150 ml) and finally dry distilled ethanol (150 ml). The support was then dried overnight in a drying cabinet containing granular calcium chloride. The washed Floridin Earth (7g) was then placed in a glass column (18 cm x 10 mm i.d.) with a drawn out end plugged with glass wool. The support was then washed by eluting with hexane (20 ml).

The sample, in methanol, was reduced to a small volume (500 µℓ) and introduced onto the top of the support and was eluted with the following solvents; hexane (10 ml), hexane:diethyl ether (10 ml each of 70:30, 50:50 and 30:70), diethyl ether (20 ml), diethyl ether:acetone (10 ml of 70:30 and 20 ml of 50:50), acetone (10 ml), acetone:ethanol (20 ml of each of 95:5, 90:10 and 80:20) and finally ethanol (20 ml). Each fraction was collected and evaporated to dryness using a stream of warmed nitrogen into a 1 cm^3 Reacti-vial and silylated with pyridine (65 µℓ) and TMSI (35 µℓ) for 5h at 120°C. The solution was then

diluted with toluene and 1 μℓ was injected onto the gas chromatograph fitted with an electron capture detector. The active fractions that contained the ecdysteroids were then found and the percentage recovery calculated.

Sephadex Gel Column Chromatography

Purification of the ecdysteroids present in the biological samples necessitated the use of two columns using components of Whatman Ion Exchange Columns (Uniscience Limited, Cambridge) with a support of Sephadex LH-20 (Pharmacia Fine Chemicals), and either acetone or methanol as eluant.

The Sephadex (25g) was swollen overnight in the appropriate solvent (100 ml). The acetone-eluted column incorporated a precision bore glass column 45 cm x 15 mm (i.d.) and was packed with the swollen Sephadex as a slurry to give a column of 30 cm in length (equivalent to 20g of dry Sephadex). The methanol-eluted column was 60 cm x 15 mm (i.d.) and was packed to give a column 47 cm in length (equivalent to 20g of dry Sephadex). Both columns were sealed with polypropylene barrel assemblies of 15 mm diameter fitted with capillary feed tubes. The tube at the base of the column was fitted with a T-piece (Phase Sep., Chester), one arm of which was connected to a capillary tube (0.75 m) leading to a solvent reservoir (250 ml), the other arm was fitted with a Luer fitting (Phase Sep.) into which a Mininert syringe valve (Phase Sep.) was located. The glass reservoir was connected to the feed capillary tubing with a ¼" O.D. glass-to-teflon union (Magnus Scientific, Sandbach).

By introduction of the sample through a 1 ml glass syringe, fitted into the Mininert syringe valve, onto the base of the column

the sample could be developed by gravity feed from the reservoir. Fractions eluted from the column were collected from the capillary feed tube projecting from the barrel assembly at the top of the column. This procedure ensured that no air bubbles were introduced into the system and that these relatively volatile solvents did not evaporate from the column and cause 'cracking' of the support. The solvent flow rate for the acetone eluted column was maintained at 1 ml min^{-1} and for the methanol eluted column, 0.33 ml min^{-1} by varying the position of the reservoir. When not in use the capillary feed tube at the top of the column could be sealed.

To determine the elution profile of the ecdysteroids found in barnacles the columns were calibrated with ecdysone, 20-hydroxyecdysone and makisterone A, by injecting a 10 µg sample in 1 ml of the appropriate solvent onto the column with a 1 ml syringe. The syringe was then washed with 500 µℓ of the respective solvent, and this washing was also injected onto the column immediately after the sample. For the acetone eluted column fractions (5 ml) were collected from the time of injection until 200 ml had been obtained. With the methanol eluted column, fractions (2 ml) were collected until a total volume of 100 ml was obtained. Each fraction was then pipetted gradually into separate 1 cm^3 Reacti-vials and evaporated to dryness with a warm stream of nitrogen gas. The Reacti-vials were then placed under vacuum in a drying pistol at 50°C for 1h. The fractions were then silylated with pyridine (65 µℓ) and TMSI (35 µℓ) for 5h at 120°C. After cooling they were suitably diluted with ECD grade toluene and 1 µℓ was injected onto the GC, the amount of ecdysteroid in each fraction being calculated by comparison of peak area for each specific ecdysteroid with a callibration curve. These results gave the elution profiles of ecdysone, 20-hydroxy-

ecdysone and makisterone A, developed on acetone and methanol eluted Sephadex LH-20 columns.

The recovery of ecdysteroids from each of the columns was found using ecdysone and 20-hydroxyecdysone at levels down to 500 ng. Either 2, 1 or 0.5 µg was injected onto the column and the active fraction collected, evaporated down on a Rotary evaporator to a small volume and then transferred to a 1 cm^3 Reacti-vial where the sample was finally evaporated to dryness. This was then dried in the pistol, silylated and gas chromatographed as before and the % recovery of the ecdysteroid from the column calculated.

Preparation of Columns for Gas Chromatography

Coiled glass columns (0.9 m or 1.5 m x 4 mm internal diameter) were thoroughly cleaned with distilled water and acetone and oven dried at 140°C. Each column was then silanised by filling with a solution of 20% dimethylchlorosilane (Hopkins and Williams, Chadwell Heath, Essex) in toluene for 2h. The solution was then pipetted out and the glass column dried with a stream of warmed nitrogen.

A support of 1.5% w/w OV101 silicone phase (Magnus Scientific, Sandbach) on Chromosorb W (Magnus Scientific) was prepared. The Chromosorb W (30g) was shaken with OV101 (0.45g) in toluene (50 ml), and the solvent removed on a rotary evaporator using a Kolbe flask (250 ml). The support was then dried by warming on a fluidised bed, the finings being removed by a flow of nitrogen up through the support.

Before packing each glass column was plugged with silanised glass wool at the end connected to the detector. The glass column was then packed with the prepared support with the aid of a vibrator and a vacuum line connected to the end plugged with glass wool. The column

was packed to within 7.5 cm of the end and firmly plugged with glass wool. Before connecting to the detector the columns were conditioned by heating to 300°C for 24h with a nitrogen carrier gas flow of 50-60 ml min^{-1}.

In repeated use, resolutions deteriorated and the retention times gradually decreased. The life of a column was prolonged by regularly replacing the first few centimetres of packing (approximately every 300 injections), and by injecting 10 µl samples of "Silyl-8" (Pierce and Warriner, Chester) onto the column at 250°C with the detector disconnected.

Gas Chromatography with an Electron Capture Detector

Gas chromatography (GC) was performed on a Pye Series 104 gas chromatograph fitted with a ^{63}Ni electron capture detector (ECD) connected to a Pye electron capture amplifier.

Regular use of the ECD leads to contamination. The central electrode was removed and cleaned with metal polish and the barrel was cleaned by placing in a 1 ℓ beaker, containing hexane, in an ultrasonic bath for 1-2h. Alternatively the detector could be cleaned without disassembly by purging it at 400°C with hydrogen gas (McCown, 1978). Both methods proved adequate for slight contamination, but otherwise clearing the detector must depend upon the design and maker's instructions. Mild contamination could sometimes be removed by increasing the detector temperature to 350°C and the purge gas to 50-60 ml min^{-1} overnight.

Oxygen-free nitrogen was used as a carrier gas and was dried and further purified by passing over molecular sieves and through an 'oxy--trap' (Alltech Associates). The carrier gas was maintained at a flow rate of 50-60 ml min^{-1} with the column at 285°C and the detector at 300°C. When the column was not in use the carrier gas was switched

off and purge of 15 ml^{-1} min of nitrogen, dried and purified as before, was maintained through the detector which was kept at its working temperature of 300°C.

Injections of 1-2 µℓ of a sample in ECD grade toluene were made directly "on-column" with a 5 µℓ syringe (Scientific Glass Engineering, London) fitted with an 11 cm needle which reached onto the top of the column packing.

Preparation of Calibration Graphs using Pure Ecdysteroids

A known quantity of ecdysteroid (10 µg) in purified pyridine (65 µℓ) and TMSI (35 µℓ) was converted to its TMS ether as described previously. After formation of the desired derivative a quantity of the reaction mixture (10-20 µℓ) was diluted in toluene and then serially diluted to give a range of concentrations of ecdysteroid from 200 pg $\mu\ell^{-1}$ - 5 ng $\mu\ell^{-1}$. 1 µℓ samples were then injected onto the GC (in triplicate) and from the peak areas obtained a calibration curve was prepared. Calibration graphs for ecdysone, 20-hydroxyecdysone and makisterone A were prepared.

Due to contamination and slight variations in the sensitivity of the ECD these calibrations were repeated regularly particularly before and after determinations of biological samples.

Analysis of Two New Solvent Partition Systems

Ecdysone, 20-hydroxyecdysone and makisterone A were analysed in two new solvent partition systems to determine their partition co-efficients.

In separate tests each ecdysteroid (100 µℓ of a methanolic solution of 250 µg ml^{-1} i.e. 2½ µg) was added to aqueous (80%) methanol (200 ml) and partitioned with petroleum ether (b.p. 40-60) : isopropanol (200 ml

of 99:1). After decanting the lower aqueous phase, the upper ether phase was backwashed twice (2 x 100 ml) with aqueous (80%) methanol. The four phases, the original aqueous and ether layers plus the two aqueous backwashes, were then analysed to determine the percentage recovery of the ecdysteroid in each. Each solution was evaporated to dryness on a rotary evaporator at 50°C and under vacuum to a small volume. This volume was then gradually transferred to a 1 cm^3 Reacti--vial and evaporated to dryness with a stream of nitrogen and a hair dryer. Complete dryness was ensured by placing the Reacti-vial into a drying pistol under vacuum and at 50°C for 1h. The residue was then silylated with pyridine (65 µl) and TMSI (35 µl) for 5h at 120°C. The solution, when cool, was diluted with ECD grade toluene and 1 µl was injected onto the gas chromatography column. The area of the peak corresponding to the derivatised ecdysteroid was then measured and the amount of ecdysteroid recovered was calculated from the appropriate calibration curve.

The results of these tests indicated that none of any of the three ecdysteroids, was lost in the ether phase. Over 85% of each ecdysteroid was recovered from the first aqueous methanol partition and the remainder in the first aqueous methanol backwash. The inclusion of a second backwash was retained to ensure complete recovery of any ecdysteroid.

A second solvent partition system was analysed in the same manner. The ecdysteroid was added to isopropanol:water (51 ml of 15:36) which was then partitioned with hexane (20 ml). After recovering the lower aqueous phase, the hexane layer was backwashed twice with aqueous isopropanol (25 ml of 15:36). Analysis of all four phases as before showed that all the ecdysteroids were recovered in the first aqueous

phase (mean recovery of 103.9%). Nevertheless the two washes were retained to ensure complete recovery of any ecdysteroid for biological material.

Collection of Barnacles

Regular collections of barnacles (∿ 500g), approximately once per fortnight, were made at low tide from a barnacle population at mean sea level on the north-facing side of one of the supports of the Menai Bridge on the Anglesey side of the Menai Straits. The barnacles were carefully scraped from the stonework, with a 2" paint scraper, into a 1 ℓ beaker after ensuring that the area was free from other organisms such as whelks, mussels, marine worms, algae, newly settled barnacles or barnacles of other species. The sample was then transferred into a pre-weighed jar containing methanol (500 ml) which was sealed and transported to the laboratory. While at the collection site the surface sea temperature was taken and live specimens (100-200) were obtained by collecting barnacle-encrusted loose rocks. A 5 ℓ container was used to collect sea water for use in studying the moulting rate of the live specimens.

The Use of Makisterone A as an Internal Standard in the Analysis of Ecdysteroids in Barnacles

A sample of Makisterone A (100 µℓ of 10 ng $\mu\ell^{-1}$ in methanol i.e. 1 µg) was added to the barnacle sample before grinding. The percentage recovery of the Makisterone A was used to determine the efficiency of the sample purification and to ensure accurate quantification of the concentration of 20-hydroxyecdysone in the barnacle sample.

Extraction and Determination of Ecdysteroids from Biological Material

Methanol (500 ml) was added to the collected barnacles and the whole sample was ground using a one quarter horse power grinder (Parvalux, Unishears Mixers Ltd., Stourbridge). The mixture was vacuum filtered through an 11 cm diameter scintered glass funnel (Scinta Glass 2, Gallenkamp), the residue being re-ground and washed twice with methanol (2 x 500 ml) and filtered. Both the residue and the filtrates were retained, the shell fragments being dried at 120°C until at a constant weight and the combined methanolic extracts being evaporated to dryness on a rotary evaporator, under vacuum, at 50°C to give a viscous dark green oil.

The residue from the methanolic filtrates was partitioned between light petroleum (b.p. 40-60) : isopropanol (99:1, 200 ml) and aqueous methanol (water:methanol 4:1, 200 ml), the upper light petroleum phase being backwashed twice with aqueous methanol (2 x 100 ml) before being discarded. The combined aqueous methanol extracts were then reduced to dryness in a rotary evaporator, under vacuum, at 50°C.

This residue was then partitioned between butanol (200 ml) and water (200 ml), the lower aqueous phase being backwashed twice with butanol (2 x 100 ml). Water (50-100 ml) was added to the combined butanolic extracts and the solution reduced to dryness on the rotary evaporator, under vacuum, at 50°C by distillation as an azeotropic mixture. The butanol residue was partitioned between hexane (20 ml) and aqueous isopropanol (water:isopropanol 36:15, 102 ml), the upper hexane phase being backwashed twice with aqueous isopropanol (2 x 51 ml) before being discarded. The combined aqueous isopropanol extracts were reduced to dryness on a rotary evaporator, under vacuum, at 50°C, the final residue being taken up in methanol (20 ml of Distol Pesticide grade, Fisons).

The lower aqueous phase from the butanol-water partition was retained for the analysis of any conjugated ecdysteroids. The water was evaporated to dryness, under vacuum on a rotary evaporator at 50°C, by distillation as an azeotropic mixture with butanol. The residue was dissolved in acetate buffer 0.1M pH 5.2 (20 ml) in preparation for enzymatic analysis as described later.

The methanol solution obtained after the partition chromatography was then further purified by Sephadex gel column chromatography. A portion of the methanol solution (5-10 ml), equivalent to between a quarter and a half of the sample, was reduced to a small volume (1 ml) by evaporating the solution gradually, with a stream of nitrogen and a hair dryer, into a 3 ml glass sample-tube. This concentrated solution, plus two methanol washings (2 x 250 µl) of the tube, was injected onto the base of the methanol-eluted sephadex column. The active fraction containing any ecdysteroids was eluted from the column between 45-80 ml after the injection. This volume was retained and reduced (to ∿ 1 ml) under vacuum, on a rotary evaporator at 50°C.

The reduced volume was then transferred to a centrifuge tube to which acetone (10 ml of Distol Pesticide grade, Fisons) was slowly added. After shaking, any precipitate was removed by bench centrifuging, the acetone decanted and the precipitate re-dissolved in methanol (500 µl) and re-precipitated with acetone (10 ml). The mixture was again centrifuged and the decanted acetone extracts combined and reduced to a small volume (1 ml) by rotary evaporation or with the aid of a stream of nitrogen and a hair dryer. This solution, plus two acetone washings of the vessel (2 x 250 µl), was injected onto the base of the acetone--eluted sephadex column. The column was developed as described previously and the active fraction (75-160 ml after the sample injection)

containing any ecdysteroids was retained. This volume was reduced by rotary evaporation under vacuum at 50°C, and the concentrate then transferred to a 1 cm^3 Reacti-vial and evaporated to dryness with a stream of nitrogen. The resultant viscous orange residue was dried under vacuum in a drying pistol at 50°C for 1h.

The two Sephadex columns, after collection of any ecdysteroids, were flushed out by eluting with 100 ml of the appropriate solvent. The columns were then sealed, ready for use for the next sample.

After dissolving the dried residue in purified pyridine (200 µl), TMSI (100 µl) was added, the vial sealed and placed in an oven at 120°C for 4½h to convert any ecdysteroids to their trimethylsilyl ethers. After cooling the reaction mixture was spread onto the origin of an 0.6 mm normal phase TLC plate. The reactivial was washed with ECD grade toluene (2 x 50 µl) and the washings also spread at the origin. The TLC plate was then developed in toluene:ethyl acetate (7:3) for 15 cm. After drying, the plate was viewed under short wave UV light to determine the presence of any large quantities of derivatised ecdysteroids. Generally the amounts were undetectable by this method and a R_f band of 0.55-0.9, which would contain all the TMS ether of any ecdysteroids present, was scraped from the plate. This was packed into a glass column and any derivatised ecdysteroids eluted into a centrifuge tube with diethyl ether as described previously. The ether was reduced to a small volume with a stream of nitrogen and this volume plus two ether washings (2 x 250 µl) of the centrifuge tube were transferred to a 1 cm^3 Reacti-vial and reduced to dryness. The residue was taken up in ECD grade toluene (100 µl) and samples (1 µl) were injected onto the gas chromatograph. The quantity of any ecdysteroid present was determined by comparison of the peak area of the sample against a calibration graph.

The mean value was then calculated. The percentage recovery of the internal standard, makisterone A, was also calculated from the calibration curve.

Extraction and Hydrolysis of Ecdysteroid Conjugates

The aqueous phase from the butanol:water partition was evaporated to dryness by azeotropic distillation with butanol on a rotary evaporator at 50°C. The residue was then dissolved in Walpole's acetate buffer (20 ml, 0.1M at pH 5.2) and divided into two portions (2 x 10 ml), one of which was subjected to enzymatic hydrolysis and the other was incubated under the same conditions, but with no enzyme, as a control. The samples were then incubated overnight at 37°C in a thermostatted waterbath. After incubation the samples were made up to 100 ml with distilled water and extracted with butanol (200 ml in three portions: 100, 50 and 50 ml). The butanol extractions were combined and evaporated to dryness, the residue was analysed in the normal manner by silylation of any ecdysteroids liberated by the enzymatic hydrolysis.

The conditions for the enzymatic hydrolysis were as used by Wilson (1979) with the exception that only acetate buffer was used. The buffer was prepared from acetic acid (10.5 ml of an 0.2M solution) made up to 100 ml with distilled water to give an 0.1M solution of pH 5.2. The pH of the solution was checked with a pH meter, and if necessary, adjusted with dilute sodium hydroxide solution or dilute hydrochloric acid. The quantities of enzyme used were as suggested by Wilson (1979) and were as follows: β-glucuronidase at 110 Fishman units ml^{-1}, aryl sulphatase at 800 Ray units ml^{-1} (both from Koch Light), α-D-glucosidase at 2.2 units ml^{-1} and β-D-glucosidase at 1 unit ml^{-1} (both from Sigma).

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Determination of the Moulting Frequency of Balanus balanoides

A small group of live barnacles (100-200 individuals) attached to a rock was collected from the intertidal zone at the same time as each sample for ecdysteroid determination. Any newly settled individuals or barnacle other than B. balanoides were removed and the group was placed in a dish (2 ℓ) containing natural or artificial seawater (Tropic Marin or Instant Ocean). The water was continuously aerated and changed daily. The barnacles were subjected to a period of sixteen hours submersion (overnight) and eight hours exposure (daytime) at ambient light and temperature. At the daily water change the number of casts were counted and recorded as the number of moults per hundred barnacles.

Collection and Maintenance of Barnacles for the Micro-Injection Technique

Barnacles of the species B. balanoides were collected in July from settlements on small rocks at low tide in Langstone Harbour, Eastney, Portsmouth. Any newly settled individuals or barnacles of any other species were removed. The barnacles were measured across the carino--rostral axis and only those having a length of between 0.7-1.34 cm were retained. These were arranged into groups of thirty individuals, with a mean carino-rostral length of approximately 1 cm, and acclimatized for two weeks to a period of 16h submersion (overnight) and 8h exposure (daytime) in a flowing sea water system at ambient temperature. Their daily moulting rate was recorded as the number of moults per hundred barnacles by daily counting the casts present in the tanks containing the barnacles.

The flowing sea water system for studying the moulting rate of groups of barnacles consisted of two perspex tanks, joined together by a pipe, of 65 cm x 22 cm and 12 cm in depth, each divided into five

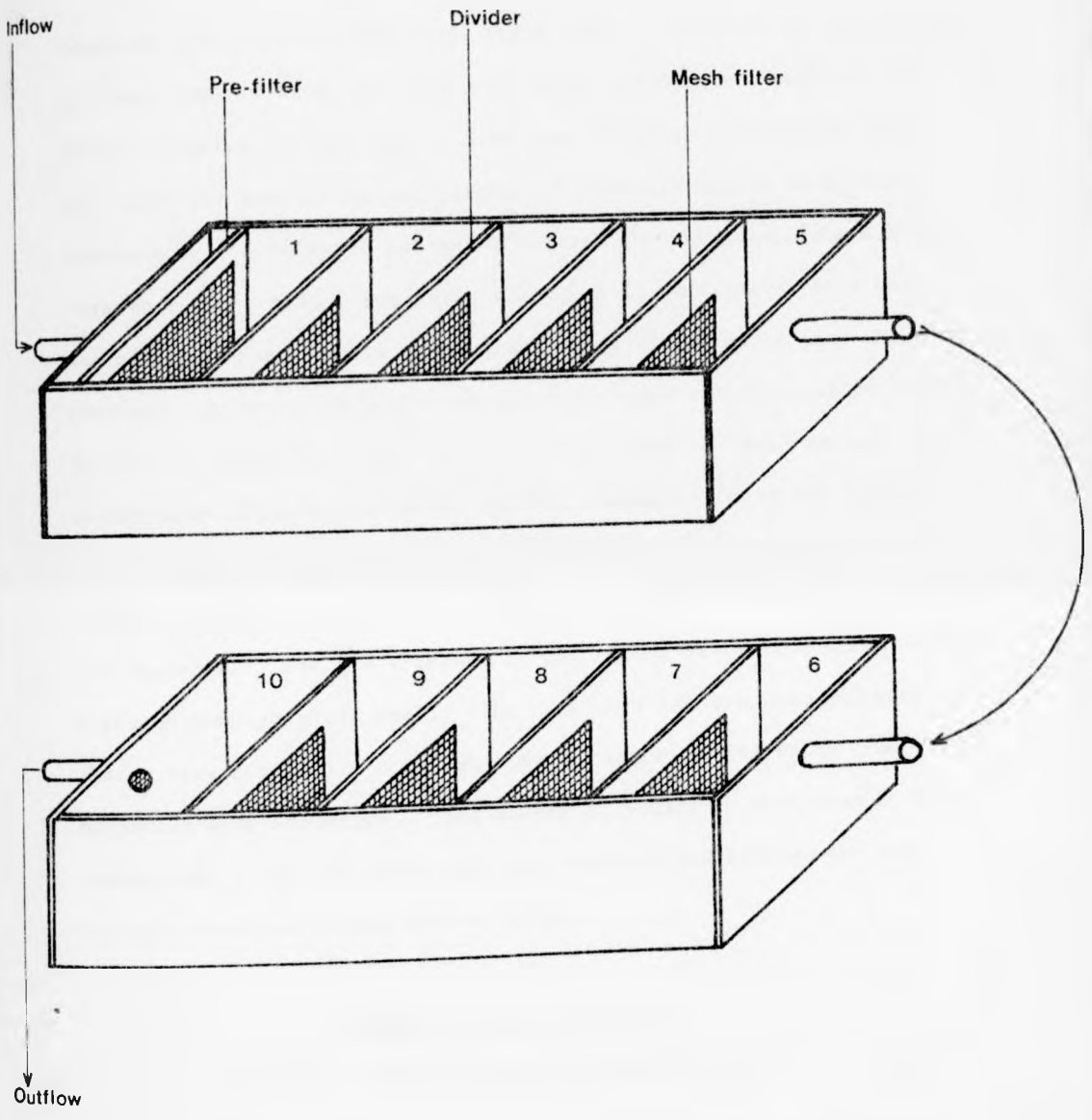

Figure 30.

The continuous flowing sea-water system (see text for details).

chambers with perspex panel (see figure 30). An area (10 cm x 6 cm) had been removed from the centre of these dividers and replaced with plastic netting of mesh size 0.5 mm thus allowing a continual flow of sea water but preventing any passage of barnacle moults to adjacent chambers. The filtered sea water entered the chambers, from a 250 ℓ reservoir via a connecting tube, through a 15 mm diameter hole which was covered in netting to prevent the entry of any casts into the first chamber. After passing through the ten chambers the sea water flowed out of the system through a pipe 15 mm in diameter, mesh-covered to retain casts from barnacles in the last chamber. Both the inflow and outflow pipes were located 10 cm from the base of the tanks to ensure that a constant depth of 10 cm of seawater was maintained in each of the ten chambers. The 250 ℓ reservoir was filled daily with fresh seawater filtered through glass wool. The reservoir tap was then adjusted so that a flow rate of 15 ℓ h^{-1} for 16h overnight during which time the barnacles were submersed. The number of moults in each chamber were counted daily and the tanks were then emptied and cleaned and the barnacles exposed during the day (8h).

Micro Injection Technique

This method was adapted from the technique described by Tighe-Ford (1968 and 1974).

After the shell plates of a barnacle had been gently scrubbed clean a small hole was drilled close to the base at the rostral end of a lateral plate (see figure 31) with a modeller's drill fitted with an 0.52 mm high speed twist drill (Dormer Drills, Sheffield). Drilling at this site ensured that no injury was caused to the body or the lateral or rostral depressor muscles controlling the operculum. A 5 cm needle

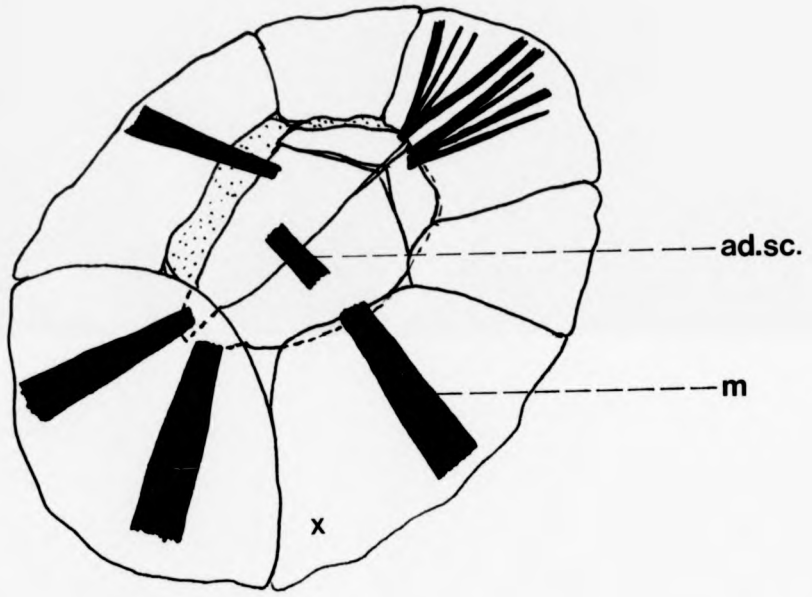

Figure 31. Diagram to Show the Position for Drilling into a Barnacle for the Microinjection Technique.

x - site for drilling

ad.sc. - adductor scutorum muscle

m. - muscle

attached to a 5 µℓ syringe (Scientific Glass Engineering, London) was then carefully inserted into the haemocoel between the basal mantle tissues, to a depth of approximately 2 mm, and 2 µℓ of fluid was injected. To prevent bacterial infection after removal of the needle a few grains of "Crystamycin" (Glaxo Laboratories Ltd., Greenford) were placed into the drill hole which was then dried and plugged with warmed "guttapercha" (Dental Temporary Stopping, Amalgamated Dental Trade Distributors Ltd.). The barnacle was then submersed in sea water.

Injection of Barnacles with Ecdysteroids and Determination of their Moulting Frequency

Ten groups of thirty acclimatised barnacles were taken for injection experiments. One group was retained as uninjected controls and one group injected with 2 µℓ each of sterilized, filtered sea water. Each individual of the remaining eight groups was injected with a solution (2 µℓ) of sterilized, filtered sea water containing either; 1, 2, 5 or 10 ng of pure ecdysone or 1, 2, 5 or 10 ng of pure 20-hydroxyecdysone (both from Simes, Milan). Each group was then placed in one of the separate ten chambers of the flowing sea water system (described earlier) and maintained at ambient temperature with a 16h submersion and an 8h exposure for 28 days. Each day the number of moults from each group were recorded and any deaths noted.

A second set of experiments was conducted, using the micro-injection technique, to study the effect of injecting barnacles with impure 20-hydroxyecdysone extracted from barnacles. Extracts of barnacles, containing ecdysteroids, collected for analysis from the Menai Bridge in December, February and May were purified to the point just prior to silylation and then divided into half. One half was analysed in the

normal manner and the level of 20-hydroxyecdysone present calculated. The second half, after drying in a 1 cm^3 Reacti-vial, was taken up in acetone (10 µℓ) and then when required was prepared for injection into barnacles by dilution with sterilised, filtered sea water (100 µℓ). These dilutions gave concentrations of 20-hydroxyecdysone at > 0.2 ng $µℓ^{-1}$ (February), 0.8 ng $µℓ^{-1}$ (December) and 3.2 ng $µℓ^{-1}$ (May).

Five groups of thirty acclimatized barnacles were taken, one being retained as uninjected controls and one as injected controls with each individual barnacle being injected with sterilised, filtered sea water (2 µℓ). The remaining three groups were then injected with a solution (2 µℓ) of the extracted 20-hydroxyecdysone at a concentration of > 0.4, 1.6 or 6.4 ng. The five groups were then placed in separate chambers of the flowing sea water system as described previously and maintained at ambient temperature with a 16h submersion and an 8h exposure. The number of moults per group were counted daily and any deaths of individual barnacles noted.

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