A CHEMICAL INVESTIGATION INTO THE MOULTING HORMONES OF THE BARNACLE *Balanus balanoides*

by

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ABSTRACT

In an attempt to detect and identify the moult-inducing substances in the barnacle, Balanus balanoides, one and a half metric tonnes of barnacle material was collected and extracted. Two known arthropod moulting hormones were detected in significant amounts and estimates were made of the concentrations present in the collected barnacle material using quantitative gas liquid chromatography. The major component was identified as 20-hydroxyecdysone, at a concentration of 1 $\mu g.kg^{-1}$. However, ecdysone was detected for the first time in a crustacean at a much lower concentration of 6 $ng.kg^{-1}$. The level of 20-hydroxyecdysone detected in the barnacle extract was comparable with the levels found in crustaceans by other workers. The presence of other possible ecdysones in the extracts was indicated by gas liquid chromatography.

Methods of determining the amounts of ecdysones in biological materials were investigated. The methods included the determination of acid-induced fluorescence in ecdysone-type compounds, and gas liquid chromatographic methods using both flame ionization and electron capture detectors. In order to make this investigation, a model steroid with some of the structural characteristics of the ecdysones was synthesised by a nine-stage process. This substance was used to investigate the spectrofluorimetric and, initially, the gas liquid chromatographic analysis of ecdysones. The spectrofluorimetric method was found to be unsuitable for the concentrations of ecdysones expected in the barnacle extracts. The gas liquid chromatographic method of analysis, using electron capture detection of the trimethylsilyl derivatives of ecdysones, was found to be satisfactory and could detect levels as low as 10 - 20 picograms of ecdysones in biological materials after minimal purification.

DECLARATION

The work described in this thesis was carried out by me under the supervision of Dr. E.D. Morgan, Department of Chemistry, University of Keele.

P.M. Bebbington,
April 1975.

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All my friends, for all their moral support, including Dr. Sue Nicholls for help with the diagrams.

Janet Watson, for so ably typing this thesis.

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The following Ode was composed by Sir Edward Sullivan, Bart:

YE BALLADE OF YE BARNACLE

They sought through mazy woods and ways
  For weeks and months in days agone,
Led by the lure of mystic lays
  That whispered ever, 'On, on, on!'
No floods might stay their headstrong pride,
  No earthly ghouls nor fiends of hell:
'We'll find or perish first,' they cried,
  'The Secret of ye Barnacle!'

''Twere well,' quoth one, 'if all else fail,
  We turn our steps some other where -
To the Heron-haunts of Allendale,
  And the Necromancer wonning there.'
'Agreed!' they said, 'the road lies here':-
  And lo! or ere you'ld number three,
Arose a figure, sage, austere,
  Y-clad in the robes of wizardrie.

'Heed not the panelled gems you see -
  The Drinking Queen and the Fisher Maid -
That line my lurid walls,' said he,
  'The pictured emblems of my trade:
Your quest I know - for dreams have told
  How Odd men would come to where I dwell':-
And then he read from a script unrolled
  The Secret of ye Barnacle.

(Quoted in "Barnacles in Nature and in Myth" by Edward Heron-Allen, F.R.S., and published by Oxford University Press, 1928.)

INTRODUCTION

INTRODUCTION

FOULING: ITS CONTROL AND ECONOMICS

The accumulation of barnacles, weeds and other fouling organisms on ships' bottoms has been a problem for centuries. The cost to ship owners and national economies runs into hundreds of millions of pounds per annum. In Britain alone the bill is £50 million, while in America it is $700 million. The accumulation of fouling is such a problem because it increases the skin frictional resistance on the hull, which in turn increases fuel consumption, making it difficult to maintain cruising speeds, and reducing top speeds.

If a non-toxic surface is immersed in the sea, in a short time it will become covered with a thin film of slime. The first organisms to settle are diatoms, and these are followed in time by the microscopic protozoa. It is thought that the first organisms to settle, particularly the bacteria, condition the surface for settlement of the larvae or spores of larger species of plant or animal. The major macro-fouling species include barnacles, molluscs, hydroids, algae, sea-squirts, tubeworms and polyzoans. Barnacles are the most ubiquitous and troublesome fouling species. Over 600 species are known, but only about 40 have been found in fouling communities.

Attempts have been made to control the fouling problem since early times. Visscher[1] states that, according to Atheneus (200 B.C.), Archimedes' ships had their bottoms sheathed in lead, and were also fastened with copper belts. Lead, zinc and copper

have been used over the centuries to protect wooden hulls. Many references to anti-fouling compositions exist, ranging from cow-dung to complex mixtures of sugar, metal-salts and marble.[2,3] With the advent of iron ships, copper was found unsuitable, and so attention was focused on the development of anti-corrosive, and anti-fouling paints.

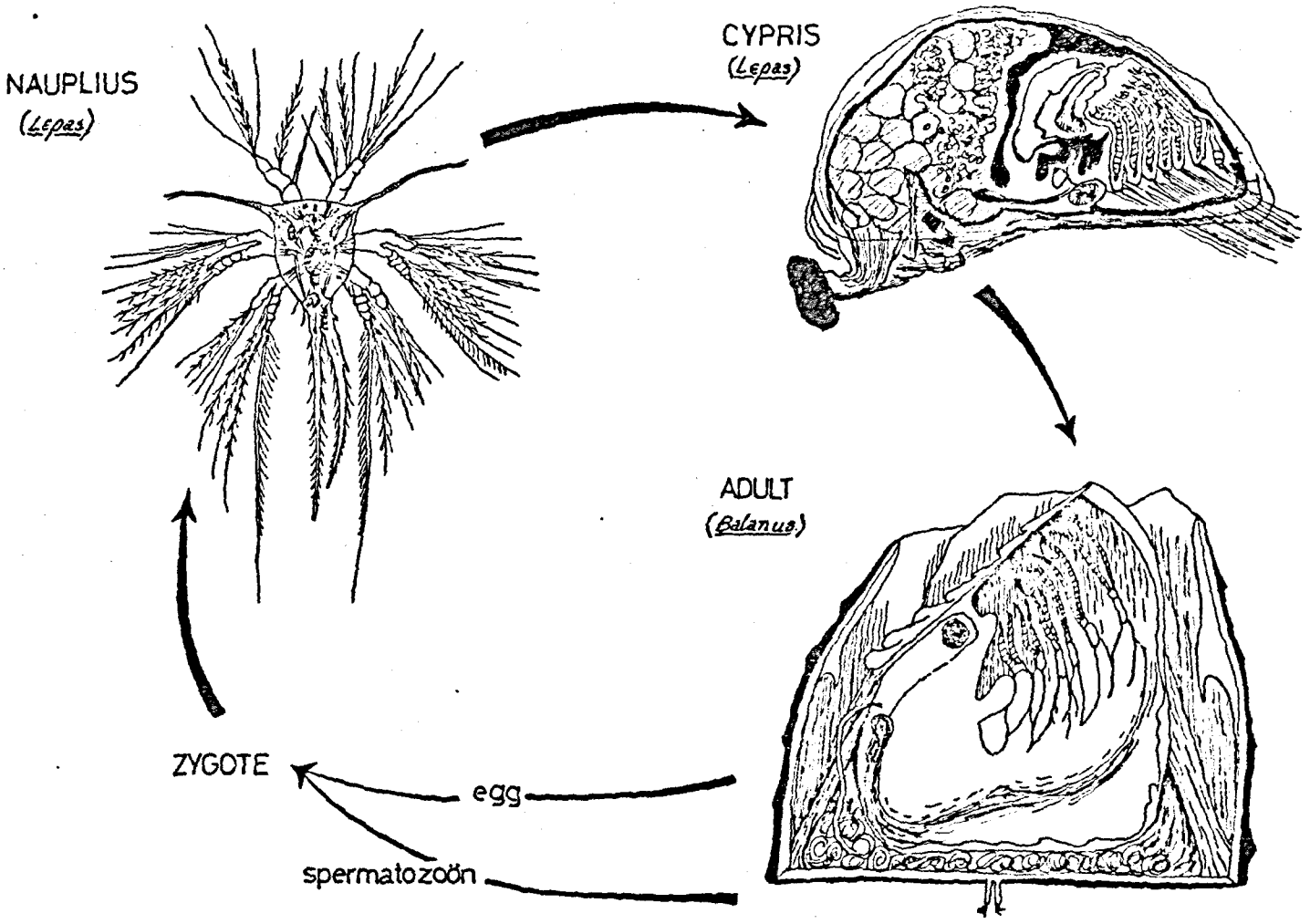
The first really exhaustive study of the fouling problem was made in the 1920's by Hentschel[4,5] in Germany and Visscher[1] in the United States. However, at the start of World War II Royal Naval vessels could count on only a few months' protection from the paints used at the time.

Recent devices for the control of fouling include systems for the release of kerosene, containing a toxic compound, alongside the keel of the ship. Ultrasonic vibrations and surface active agents have been used in attempts to control fouling but none of the latter measures have proved very effective in fouling control. For many years anti-fouling paints using cuprous oxide as their toxic ingredient have proved most satisfactory in controlling fouling. Inorganic mercury and arsenic compounds have been used but because of their toxicity to man they have not found much favour. More recently attention has been concentrated on metalo-organic compounds such as tributyltin oxide or fluoride and tri-phenyllead acetate. These compounds were at first used in place of cuprous oxide, but latterly they have been used in conjunction with it.

Considering the enormous cost of dry-docking large vessels, finding a method which will give a vessel protection for

FIGURE 1

THE LIFE CYCLE OF THE BARNACLE.



life is of prime importance. Paints available at present are capable of giving only a two-year out-of-dock period. One approach being studied in more detail is an examination of the growth and development of fouling species.

Initial studies are being made on barnacles, whose life history is well understood[6] (Fig. 1). The adults are hermaphrodite; the eggs are retained in the mantle cavity during the early stages of development and are released when they have developed into the first nauplius stage. The nauplius larva is the planktonic distribution form. The nauplii metamorphose through six progressively more complex stages to the cypris which is the benthic form, and then into the adult. The six nauplius stages vary in their adaptation to the planktonic way of life. The cyprids (Fig. 2) settle onto a substrate and wander about in search of a suitable settling place. It is thought that there are sensitive pheromone receptors in the cyprid antennae which detect pheromones put out by previously settled adults. When a suitable settling site has been selected, the cyprid attaches itself by means of cement glands in the antennae, and then rotates through 90° so that it stands on its head. It then secretes a calcarious shell (Fig. 3) and metamorphoses into the adult (Figs. 4 and 5). Barnacles, like crabs, lobsters, prawns and shrimps, are members of the class crustacea, subclass Cirripedia, which, together with the Insecta, constitute the Phylum Arthropoda.

The life cycle of the barnacle, like those of all Arthropods, is under hormonal control. There are three points at which it might be possible to interfere with the development of

Figures 2 - 5

* Figure 2. Cypris larva of the barnacle about to settle. (Micrograph approximate magnification x 180.)

* Figure 3. Newly settled larvae and young barnacles among older barnacles. (Approximate magnification x 8.)

* Figure 4. Adult specimens of Balanus perforatus and B. balanoides (foreground). (Approximate magnification x 3.)

* Figure 5. Adult barnacle under water. The valves are open and cirri extended into feeding position. (Approximate magnification x 6.)

* Taken from "Catalogue of main marine fouling organisms", Volume 1, The Barnacle, published by the Organisation for Economic Growth and Development, 1963, by courtesy of Dr. A.J. Southward, Marine Biological Association Laboratory, Plymouth.

Figure 2

Figure 3

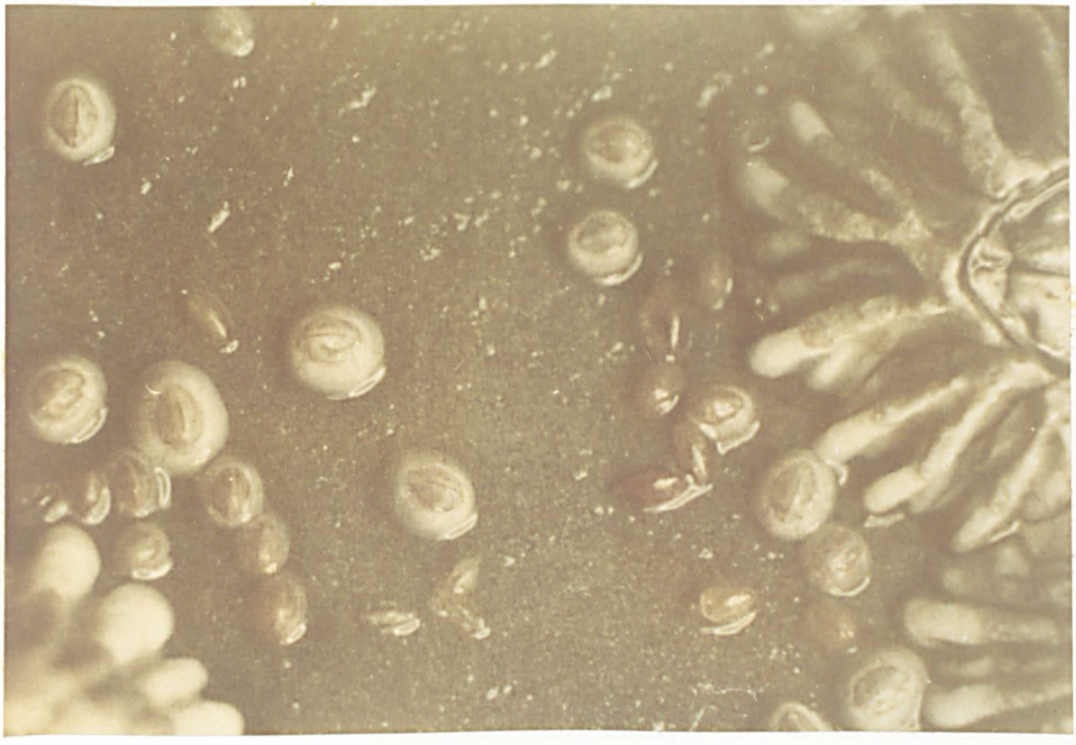


Figure 4

Figure 5

the barnacle, and effect some kind of control. These are: the moulting process; the cementation of adults to substrate; and the calcification of the outer shell. Such interference might be caused by such things as hormone mimics, which would cause mis-timing of the developmental stages. Metabolic blocking agents might be found, which would prevent proper development, or enzymes might be used to disrupt biochemical function.

At Keele we embarked on a project to extract the moulting hormones from the barnacle Balanus balanoides. This was part of a programme of research initiated by the Royal Navy, which aimed at investigating in detail barnacle physiology in the hope of finding new ways of controlling the life cycle and solving the fouling problem.

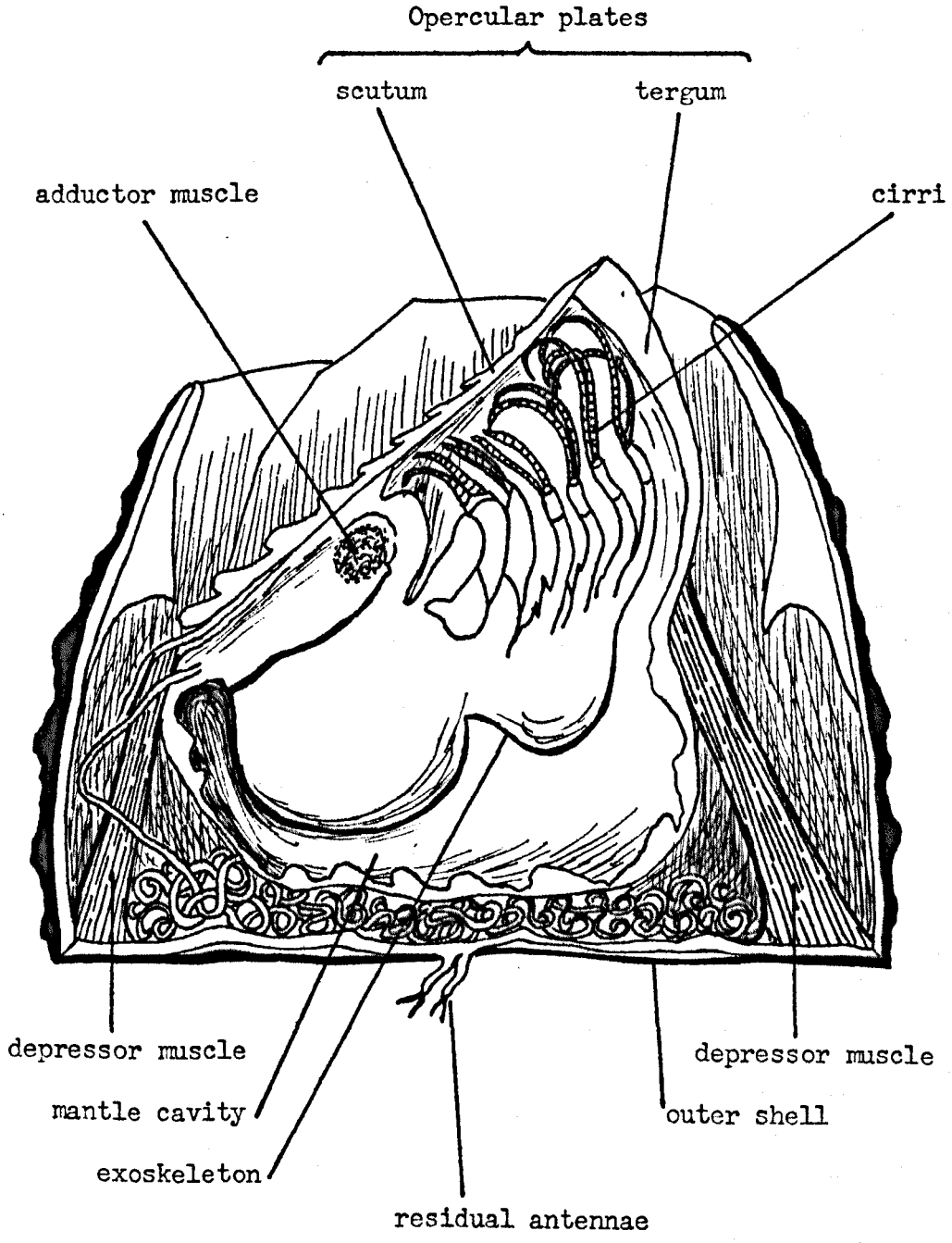
MOULTING IN ARTHROPODS

Arthropod life cycles are characterised by periods of spasmodic growth, interspersed with static periods in which no growth occurs. This condition is dictated by the nature of the hard exoskeleton (Fig. 6). The growth process can be divided into four stages: intermoult, when no growth takes place; pre-moult, when the oxygen consumption rises, glycogen is deposited in the hypodermis, and re-usable nutrients are re-absorbed from the old exoskeleton; moult, when the old cuticle, weakened by enzymic action splits and sloughs off; and postmoult, when the animal rapidly absorbs water, expanding the new exoskeleton as far as possible before it hardens, thus producing as much space for future growth as possible.

Fig. 6

Vertical Section through the Acorn Barnacle
Balanus balanoides

Animal shown withdrawn into the mantle cavity, with the thoracic feeding appendages (cirri) folded.



The regulation of these periods of seasonal activity is under neuroendocrine control, although the timing and duration of such periods vary according to geographical location, environmental conditions, age and sex of the arthropod.

FACTORS AFFECTING CRUSTACEAN MOULTING

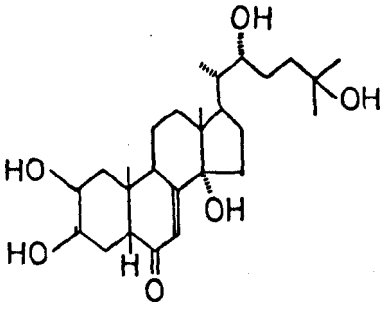
The hormonal control mechanisms of moulting appear basically similar in Crustaceae and Insecta, involving similar steroids of the ecdysone type[7,8] (Fig. 7) and a neurosecretory element. In Crustaceae (Fig. 8) a moult-inducing hormone is secreted by the Y-gland[9] in the head. A neurosecretory moult-inhibiting factor is produced in the X-organ, and stored in the sinus gland. The balance of these two components in the circulation is under the control of external stimuli such as light intensity, photo-period, temperature, salinity, food availability and living space. When the environmental conditions are right, the secretion of moult-inhibiting factors is suppressed and the animal is able to initiate a moult, under the influence of the moulting hormone produced by the Y-gland.

In insects (Fig. 8) the median region of the brain - the pars-intercebralis - is the neurosecretory region. It probably secretes several different hormones with differing effects. One of these activates a pair of glands called the prothoracic glands, which in turn secrete a substance, possibly the steroid ecdysone, which then initiates or plays a part in controlling the functional changes that lead to moulting.

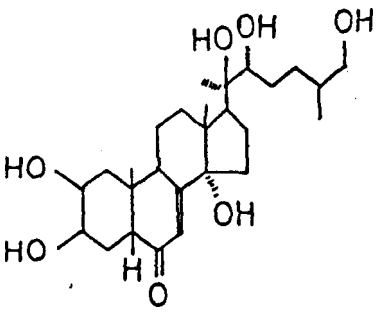
Fig. 7

Structures of Some Arthropod Ecdysones.

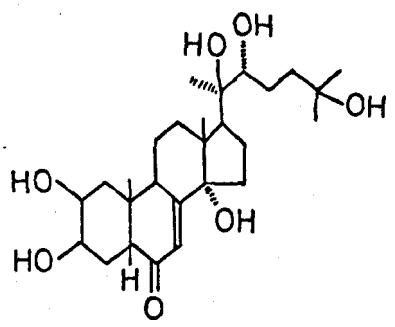
ecdysone



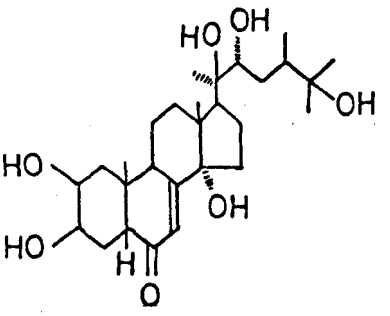
inokosterone



20-hydroxyecdysone



makisterone A



2-deoxy-20-hydroxyecdysone

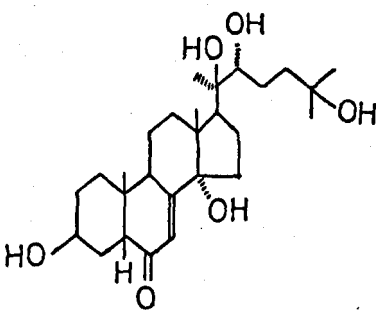
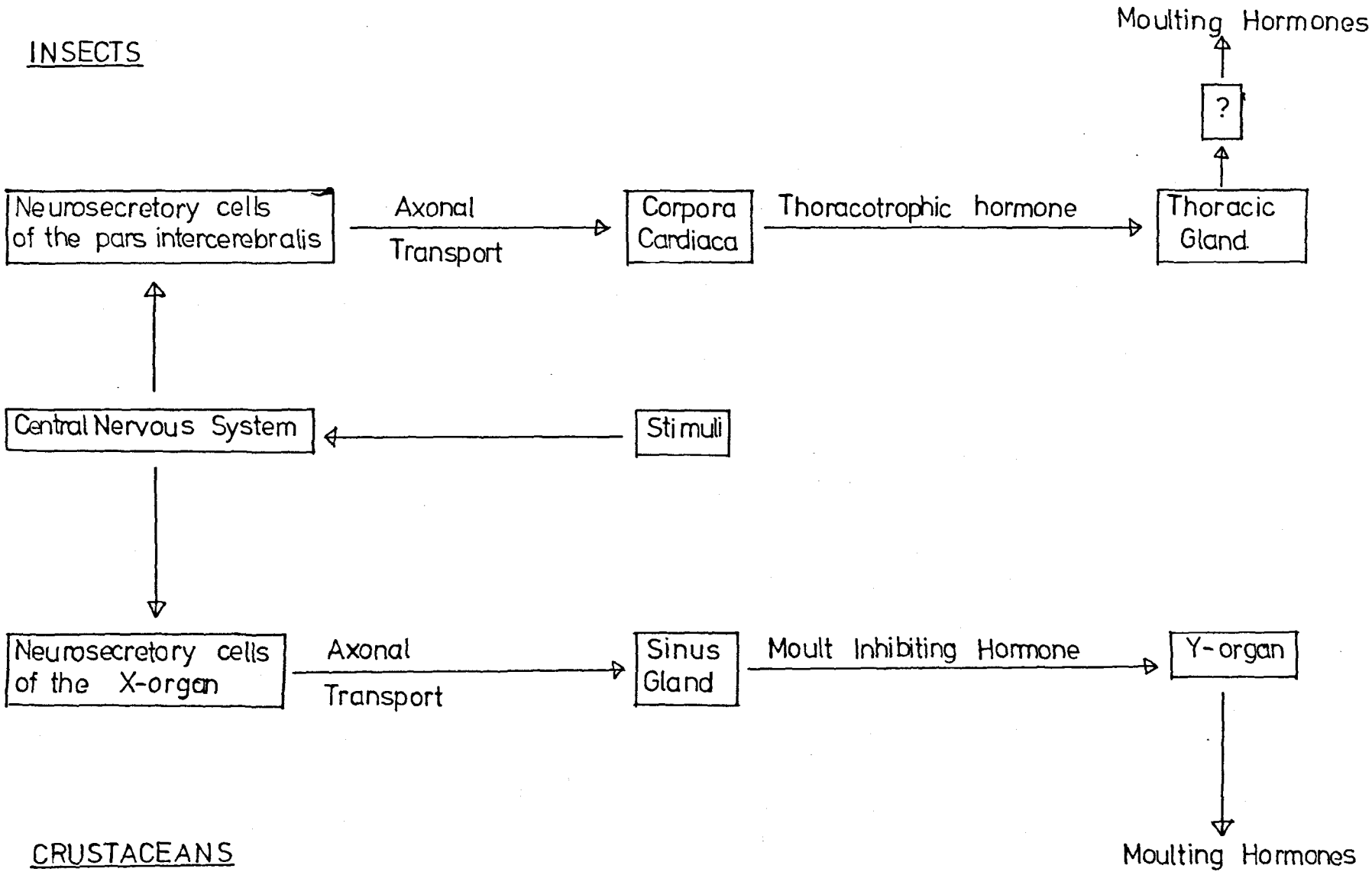


Figure 8

Moulting Hormone Secretion in Insects and Crustacea.

In both crustaceans and insects the moulting hormones are secreted by an endocrine gland whose activity is controlled by a neurosecretory hormone. In crustaceans the neurosecretory hormone inhibits the activity of the Y-organ; moulting hormone is secreted when secretion of the moult-inhibiting hormone ceases. In insects the neurosecretory hormone stimulates directly the secretion of moulting hormone.

Figure 8


INSECTS
Moulting Hormones
?
Neurosecretory cells of the pars intercerebralis
Axonal Transport
Corpora Cardiaca
Thoracotrophic hormone
Thoracic Gland
Central Nervous System
Stimuli
Neurosecretory cells of the X-organ
Axonal Transport
Sinus Gland
Moult Inhibiting Hormone
Y-organ
Moulting Hormones
CRUSTACEANS

The control mechanisms in Crustaceans and Insects are sufficiently similar for Crustacean moulting hormones to induce moulting in insects.[10] This is fortunate since it means that insects which are more convenient for laboratory culture and experiments can be used to bioassay crustacean moulting hormones.

ARTHROPOD MOULTING HORMONES

Experiments by Kopec[11] provided the first clue to the hormonal mechanism that controls growth in insects. Gabe[7] and Echalier[9] proved conclusively that moulting in crustaceans is initiated by a hormone that is produced in the Y-organ. This gland is similar to the prothoracic gland, which secretes the insect moulting hormone, ecdysone. Karlson[12] showed that an extract of the crustacean Crangon vulgaris is active in the insect Calliphora test. It was thought therefore that the crustacean moulting hormone, if not identical with the insect hormone, must be closely related to it. The concentration of hormone at an intermoult stage was found to be very small, and an attempt by Karlson and Schmialek[13] to isolate the active material from three tons of the shrimp C. vulgaris was not successful.

In Australia, D.H.S. Horn et al.[14] undertook the isolation of crustacean moulting hormone from the crayfish Jasus lalandei, using the procedure of Karlson. They found that their extracts had a much lower activity (about 1%) in the Calliphora bioassays than did comparable extracts of silkworm pupae.[15] Horn's attempts to develop an efficient isolation procedure for crustacean moulting hormones were initially frustrated by the difficulty of measuring

the low activity of crude extracts in the Calliphora bioassay. It was considered that a crustacean might prove to be more sensitive to the crustacean moulting hormone than an insect, and Horn explored the possibility of using a crustacean bioassay. He found that if either crude or highly purified extracts of moulting hormone were injected into the freshwater crayfish Cherax destructor[16], the red chromatophores in the cuticle and fantail contracted markedly. The purified moulting hormone extracts contained, besides the moulting hormone, a hormone regulating the size of the red chromatophores of the cuticle. The two hormones had very similar properties in the counter-current distributions and column chromatograms used. It was found that the red chromatophores would respond over a very wide range of hormone concentrations, and the effect could be used as a very sensitive and quantitative bioassay for the red-concentrating hormone. Because the moulting hormone had similar properties to the red-concentrating hormone, it was possible to use the bioassay to locate fractions that also contained the crustacean moulting hormone but that were insufficiently active to give a response in the Calliphora test.

From the extraction of one ton of crayfish waste, Horn was able to extract 2 mg. of 20-hydroxyecdysone and, using the same procedure, the same hormone together with callinecdysones A and B were extracted from the crab Callinectes sapidus.[17] It was originally thought that in J. lalandei, 20-hydroxyecdysone was accompanied by smaller amounts of ecdysone. However, after extracting three tons of crayfish waste, 200µg. of the less polar moulting hormone 2-deoxy-20-hydroxyecdysone was isolated.

The X-ray crystallographic elucidation of the structure of ecdysone was carried out by Huber and Hoppe[18] in 1965 (Fig. 7). By the use of chemical and physical methods, the structure of more than twenty ecdysones has been solved since 1965. The great majority of these, known as phytoecdysones, have been isolated from a wide variety of higher plants.

All Arthropod ecdysones with the exception of 2-deoxy-20-hydroxyecdysone have a tetracyclic nucleus with hydroxyl groups in positions 2β , 3β and 14α, an unsaturated Δ^7-6-ketone system, and a cis-fusion between rings A and B. Almost all the phytoecdysones possess the same nucleus, the diversification in ecdysone structure being contained in the oxidation state of the cholestane side chain. The 5α-isomers of Arthropod ecdysones are not naturally occurring. This is surprising, since isomerisation of the A/B-cis ring junction to the A/B-trans is chemically easy, and could be used as a simple inactivation pathway.

Little is known about the absolute configuration in the oxidized cholestane side chain in ecdysones, although the nuclear stereochemistry of many ecdysones has been determined by comparison with ecdysone or synthetic compounds.

SYNTHESIS OF ARTHROPOD MOULTING HORMONES

Shortly after the structure of ecdysone was announced, two separate syntheses were carried out and simultaneously reported.

One was by the Syntex Group[19,20] and the other was a joint effort by Schering A.G. and Hofmann-La Roche.[21] The syntheses were

particularly interesting because of the extreme scarcity of the natural hormone, its possible use in insect control, and because it was the first invertebrate moulting hormone to be isolated.

In planning the synthesis of ecdysone certain factors had to be taken into consideration. These were: 1) the instability of the A/B ring junction; 2) the polyfunctionality of the molecule demanding the protection of functions introduced at early stages; 3) the lack of suitable cholestane starting materials; 4) the stereochemistry which required stereoselective operation at C-2, C-3 and C-14 in particular; and 5) the choice of timing the addition of the side chain to a developing nucleus.

Both groups used similar methods for obtaining a correctly substituted nucleus. The Syntex Group realised that the A/B-cis fusion could be obtained via an enol of the 6-keto group if there was a bulky substituent at C-2. But it was important that the latter equilibrium occurred at the correct time and not prematurely, resulting in inefficiency. It was for this reason that a 5α-hydroxyl group was used as a stereochemical holding group, capable of being selectively removed at a later stage by chromous chloride reduction of the acetate.

Bisnorcholanic acid ester was used as starting material because a robust derivative was needed, capable of surviving the sometimes severe conditions used in constructing the nucleus, and which would be available for later alkylation when side chain synthesis was performed. This was carried out directly by using an α-sulphinyl carbanion reagent, and indirectly by prior transformation of the C-24 carbomethoxy to a formyl group for Grignard alkylation.

The C-14 hydroxyl group was produced by selenium dioxide oxidation after a Δ^7-6-ketone system had been produced.

Other methods employing the epoxidation of dienol acetate, and photochemical oxygenation of unconjugated $\Delta^{8(14)}$-6-ketone system are available. In the synthesis of ecdysone by the Teikoku group[22], a lactone precursor of the dihydroxylated ecdysone side chain was employed. Several sequences led to 22-isoecdysone as a major by-product, which is hormonally inactive. 20-Hydroxyecdysone was synthesised from a 20-hydroxy-22,23-bisnorcholanic-24-aldehyde derivative, and also from a 20-keto pregnane precursor. Horn has reviewed ecdysone syntheses.[22]

PHYTOECDYSONES

The synthesis of ecdysones was difficult and the yields were so small that it appeared that only very small amounts would ever be available for research. However, Professors Nakanishi and Takemoto in Japan discovered[23] that some plants contained appreciable quantities of ecdysone-like sterols, including 20-hydroxyecdysone. Other laboratories around the world soon confirmed the Japanese findings. Butenandt and Karlson managed to extract 25 mg. of ecdysone from one ton of the silkworm Bombyx mori. This quantity of 20-hydroxyecdysone could be recovered from 25Kg. of dried leaves or roots of the Yew tree[24] Taxus baccata or from about 2·5Kg. of dried Polypodium vulgare rhizomes.[25]

BIOSYNTHESIS OF ARTHROPOD MOULTING HORMONES

Ecdysones are commonly known as the prothoracic gland

hormones, but their place or places of production in invertebrates is not known with certainty. The ecdysial glands (crustacean Y-organs or insect prothoracic glands) play some part, but just how much they are involved in ecdysone biosynthesis is not known. It is possible that the ecdysial glands control moulting by performing perhaps one or two very important key transformations in the conversion of simple sterols into the ecdysones. Their biosynthesis could be studied in more detail if their site of *in vivo* synthesis were determined.

At present, information about moulting hormone biosynthesis is confined to a small number of studies. Cholesterol was suggested as a precursor of ecdysone by Karlson and Hoffmeister[26] in mature *Calliphora* larvae. This seems reasonable, since cholesterol is an essential dietary requirement in most insects. Biosynthetic studies usually use radio labelled compounds which are injected into the insect, although problems arise due to excessive dilution of the isotope. These problems can be overcome by using a more advanced labelled precursor.

In Crustacea the last step in the biosynthesis of 20-hydroxyecdysone has been found to be the C-20 hydroxylation of ecdysone.[27] In this work, highly active tritium labelled ecdysone was used which had been synthesised by the alkylation of a bisnorcholenal derivative.

The conversion of ecdysone into 20-hydroxyecdysone has been demonstrated in moulting shrimps *Crangon nigricauda*, premoult fiddler crabs, *Uca pugilator*, and fifth instar larvae of the blow-

fly Calliphora vicina, by the isolation of 20-hydroxyecdysone and the microchemical cleavage of the vicinal 20,22-glycol system to give as one product 2β , 3β , 14α -trihydroxy-5β -preg-7-en-6,-20-dione which distinguishes ecdysone from 20-hydroxyecdysone.

Hydroxylation in vivo has been observed, and this suggests that deoxyanalogues of the moulting hormone might owe their biological activity to the fact that they can easily be hydroxylated by Arthropod enzyme systems.

Thomson et al. have shown that ^{3}H labelled 25-deoxyecdysone can be hydroxylated in vivo to give inokosterone, 20-hydroxyecdysone, and ponasterone A.[28] However, the latter is not naturally present in insects, suggesting that 25-deoxyecdysone is probably not a normal precursor of 20-hydroxyecdysone.

Recently in vitro cultures of prothoracic glands have shown that ecdysone is produced in the glands.[29] This is contrary to previous reports that ecdysones could not be detected in isolated glands.[30]

METABOLISM OF ARTHROPOD MOULTING HORMONES

At present very little is known about the structure of Arthropod moulting hormones. However, Kaplanis et al. have isolated 20,26-dihydroxyecdysone[31] which could be a metabolite. It is difficult to decide which compounds are metabolites, which are precursors, and which are the active hormones. It has been suggested that each event in an Arthropod's metamorphosis is controlled by distinct hormones.[32] Horn's group have shown that side chain

scission of 20-hydroxyecdysone occurs in Calliphora stygia to give 4-hydroxy-4-methylpentanoic acid.[33]

Ecdysones are rapidly inactivated in vivo and there is evidence for an inactivation system in the fat body of Calliphora[34], opening up possibilities for the chemical study of metabolites. Many aspects of ecdysone biosynthesis and metabolism remain to be elucidated.

DETECTION OF ARTHROPOD MOULTING HORMONES

Evidence has been found that ecdysone acts directly on the genetic template, inducing enzyme synthesis in responsive tissue.[35] In Calliphora it has been shown that ecdysone is responsible for inducing the conversion of tyrosine to N-acetyldopamine, which is the precursor of the quinones involved in the hardening and tanning of the cuticular tissues. In some insects tanning is accompanied by a distinct darkening of the puparium, and this effect has been used as a moulting hormone bioassay, using the larvae of the blowfly Calliphora erythrocephela,[36] and the housefly Musca domestica.[37]

Lack of an adequately sensitive assay system has prevented progress in moulting hormone research, where determination of small titres of moulting hormone is desirable. Bioassays, while being better than nothing, are less than adequate due to their lack of sensitivity.

A number of laboratories have investigated gas liquid chromatographic methods (G.L.C.)[38] for quantifying ecdysones, whilst

others have used high pressure liquid chromatography (H.P.L C.)[39], acid induced fluorescence[40], and radioimmune assay techniques[41]. While all these procedures are promising, the problems inherent in purifying biological samples present certain limitations to them all.

DISCUSSION

DISCUSSION

EXTRACTION AND ISOLATION OF MOULTING HORMONES FROM ARTHROPODS

In Arthropods the concentration of ecdysones is exceedingly small, as indicated in Table 1. Hence, in order to isolate enough pure material for structural studies, very large quantities of starting material are required, and very lengthy isolation procedures have to be undertaken.

The first isolation procedures were worked out by Butenandt and Karlson[15]. The method (shown in Table 2) is relatively simple and highly efficient for the extraction of ecdysone, enabling Karlson et al.[42] to isolate 250 mg. of ecdysone from one ton of dried silkworm pupae (Bombyx mori). However, the method of Karlson and Butenandt was not designed and is not as suitable for the extraction of 20-hydroxyecdysone, for the following reasons: Firstly, the partition coefficients (K) of ecdysone and 20-hydroxyecdysone (Fig. 9) are quite different in the solvent system butanol-water (1:1) (See Table 3). The partition coefficient of ecdysone is >10, while that of 20-hydroxyecdysone is only 5·3. This means that large losses could occur in the stage in which the butanol extract is washed with acid and base. Secondly, it has been found that 20-hydroxyecdysone is not always efficiently eluted from alumina, whereas ecdysone is more easily eluted.

In those examples studied, it has been found that insects contain far more ecdysone-type material than crustaceans. Therefore, whilst the losses incurred using Karlson's procedure can be tolerated when extracting insect material, a more efficient and milder method is required for the extraction of crustacean material.

Table 1

Quantities of Ecdysones in Various Arthropods at Different Stages in their Life Cycles

Species	Stage	Weight extracted (kg)	Calliphora Units/g.	Ecdysone	Weight (mg)	Ecdysone Conc. (mg/kg)	Ref. No.
INSECTA							
Bombyx mori (Silkworm moth)	Pupa	500	4·5	1*	25	0·050	15
Calliphora stygia (Blowfly)	Pupa	1·8	3·0	2*	0·15	0·083	22
Schistocerca gregaria (Desert Locust)	1-3 instar (faeces)	2·0	550	1	0·40	0·200	22
	5 instar nymph	-	-	2	-	12-240 ng/nymph	43,86
Manducta sexta (Tobacco hornworm)	pre-pupa			1	-	0·400	37
	pupa			2	-	0·500	37
				3*	-	0·075	48

*1. - Ecdysone
2. - 20-hydroxyecdysone
3. - 20,26-dihydroxyecdysone

Table 1 (cont.)

Species	Stage	Weight extracted (kg)	Calliphora Units/g.	Ecdysone	Weight (mg)	Ecdysone Conc. (mg/kg)	Ref. No.
CRUSTACEA							
Crangon vulgaris (Shrimp)	Intermoult	3000	0·015	N.I.*	-	-	13
Jasus lalandei (Crayfish)	Intermoult	1000	0·080	2*	2·0	0·002	14
Callinectes sapidus (Shore crab)	Pre-moult "green"	25	-	3*	-	0·005	17
	Pre-moult "peeler"	25	-	3,2	-	0·020	17
	Pre-moult "soft shell"	25	-	3,4*	-	0·280	17
Homarus americanus (Lobster)		5	-	2	-	0·006	51
Balanus balanoides (Acorn barnacle)		1500	-	2	1·2	0·001	

*N.I. - Not identified

1. - Ecdysone
2. - 20-hydroxyecdysone
3. - Inokosterone
4. - Makisterone A.

Table 2

FLOW DIAGRAM OF THE ISOLATION OF ECDYSONE FROM SILKWORM PUPAE

(KARLSON et al, 1963).
(Ref. 42)

Dried Silkworm pupae (1000 kg.)

↓ Extracted 3x with 75% methanol (9800 l. total) and concentrated in vacuum.

Aqueous extract (600 l.)

→ Inactive oil (45 l.) decanted after standing (12 hrs.)

↓

Defatted extract

↓ Extracted 4x with n-butanol (120 l.) and aqueous layer discarded.

Butanol extract (450 l.)

↓ Extract washed successively with ice water (60 l.), 1% sulphuric acid (60 l.), 3x with 10% aqueous sodium carbonate (60 l.), 1% acetic acid (60 l.), and ice water (100 l.). Washed butanol extract concentrated.

Active extract (4.1 kg.)

↓ Dissolved in water (30 l.) and extracted 3x with petroleum ether (6 l.). Aqueous layer retained and concentrated.

Yellow syrup (232 g., activity 50 Calliphora units (C.U.)/mg.)

↓ Chromatography on alumina(3 kg.).

Active fractions (35 g., activity 500 C.U./mg.)

↓ Countercurrent distribution in EtOAc:MeOH;H_2O (2:1:2).

Active fractions (14.6 g, activity 2500 C.U./mg.)

↓ Chromatography on alumina (500 g.)

Active fractions (2.9 g.).

↓ Countercurrent distribution as before.

Crystalline ecdysone (250 mg., activity 100,000 C.U./mg.).

D.H.S. Horn et al.[14] in Australia developed a suitable extraction (shown in Table 4) for crustacean material. Horn was careful to protect the moulting hormone from decomposition during the extraction. He used 82% aqueous ethanol containing 0·1% acetic acid to keep the extract at a neutral pH. The crayfish waste was ground up at a low temperature. The aqueous concentrate, after removal of ethanol, was extracted with a mixture of hexane and propanol (1:3 by volume), with the addition of ammonium sulphate to produce two phases. Horn used n-propanol in preference to n-butanol because the former could be removed at a lower temperature, hence avoiding possible thermal degradation. The use of alumina chromatography was avoided, with silicic acid, hydrophobic celite, and sephadex columns being used in preference. Horn took care to remove acidic impurities at a suitable point by the incorporation of potassium bicarbonate in the first tube of a countercurrent extraction.

EXTRACTION OF BARNACLES

We took similar precautions to protect our hormone, in order to obtain the maximum possible yield from our starting material (see Table 5). The barnacles were collected as rapidly as possible and killed quickly by disintegration in methanol, which denatures protein and halts enzymic activity, thus preventing loss of hormone by autolysis. Methanol is also a good solvent for most classes of biochemical compounds. Collections could be made only at low tide, at a point accessible to a laboratory where the barnacle material could be disintegrated. Collection was made at various sites in Langstone Harbour, near Portsmouth, Selsey, and Colwyn Bay in North Wales.

Table 3

Partition Coefficients (K) of Ecdysones in Various Solvent Systems (Ref. 22)

Solvent system		Ecdysone (1)	20-hydroxyecdysone (2)
Cyclohexane:butanol:water	(6:4:10)	1·27	0·16
		1·28	0·13
Cyclohexane:butanol:water	(5:5:10)	3·54	0·52
Ethyl acetate:water	(1:1)	0·32	0·06
Butanol:water	(1:1)	10[a]	5·3
Pentanol:water	(1:1)	-	3·6
Ethyl formate:butanol:water	(9:1:10)	0·75	0·2
Chloroform:methanol:water	(2:1:1)	2·0	5·0
		0·01[b]	0·4[c]
Chloroform:ethanol:water	(1:1:1)	-	0·7

a Approximate

b Dideoxyecdysone

c Ponasterone A

At the beginning of the investigation into barnacle moulting hormones, the only guide available as to the amount of hormone likely to be present in barnacles was previously published work on the mass extraction of insects and crustaceans. Most work had been done using insects, but the small amount of work done with crustaceans indicated (see Table 1) that in general the amount of hormone present in the latter was much smaller than in insects.

In order to obtain some idea of the amount of moulting hormone present, a preliminary extraction of 150 Kg. of barnacle material was undertaken, following a procedure developed by Woodbridge[43] in this laboratory for the extraction of moulting hormones from the Desert Locust (see Table 6). Samples from each stage of the extraction were bioassayed[43,45] but activity was not detected, and the fractions assumed as active were those found active in the locust work by Woodbridge. During the course of the extraction, a G.L.C. method was developed in the laboratory for the analysis of ecdysones. Using this method, a sample from the water layer of the ethyl acetate-water countercurrent extraction was analysed. This sample should have contained activity, but results indicated that the amount of 20-hydroxyecdysone present in 150 Kg. of barnacle material was less than 20 μg. At the same time, work by Tighe-Ford et al.[45] on the injection of 20-hydroxyecdysone into the barnacle B. balanoides indicated that the endogenous maximum titre of 20-hydroxyecdysone should be about 0·01 μg. per animal. Most of the barnacles were collected in the summer months. At this time of year barnacles moult about once every ten days, and therefore 10% of the sample should have the maximum titre. If the above

Table 4

FLOW DIAGRAM OF THE ISOLATION OF 20-HYDROXYECDYSONE FROM CRAYFISH (Horn et al., 1968) (Ref. 14)

Frozen crayfish waste (1000 kg.)

↓ Extracted with aqueous ethanol (3860 l.) and concentrated in vacuum.

Aqueous extract (360 l., 35 kg. dissolved solids.)

↓ Extracted with hexane ; 2-propanol (200 l. 1:3).

→ Inactive petroleum extract (110 l., 7 kg. lipids.)

→ 2-propanol : water (1:1) backwash (55 l.)

Defatted aqueous extract (515 l.)

↓ Ammonium sulphate (100 kg.) added and extracted twice with hexane:propanol (1:3, total of 360 l.). Combined hexane: 2-propanol layers concentrated in vacuum.

Aqueous extract (55 l.)

↓ Extracted with hexane (23 l.) and 2-propanol (27 l.)

→ Inactive lipid extract (27 l.)

↓ Ammonium sulphate (11 kg.) added and extracted twice with hexane (14 l.) and 2-propanol (45 l.), upper layer retained and concentrated.

↓

Table 4 (cont.)

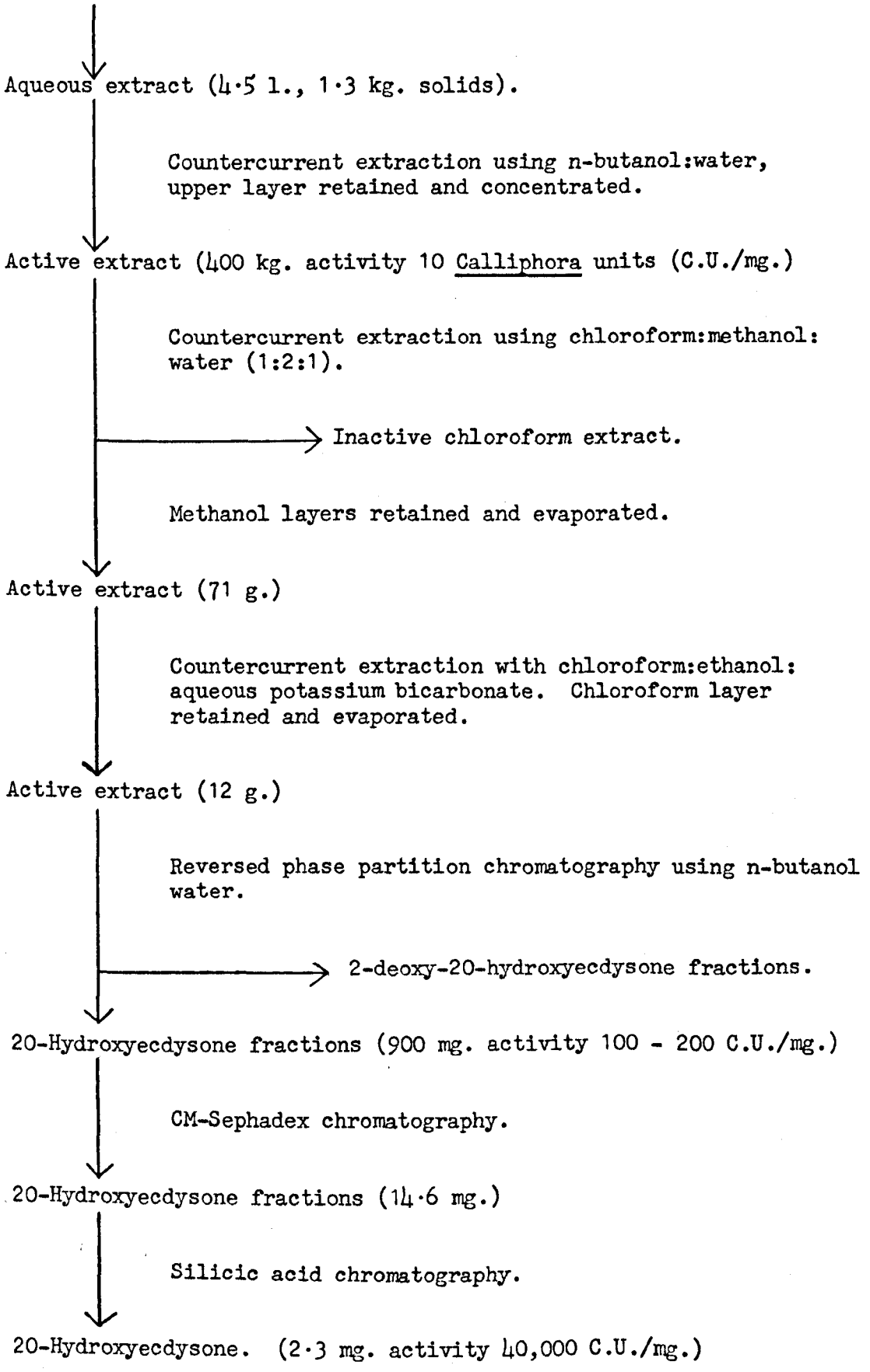

Aqueous extract (4·5 l., 1·3 kg. solids).
Countercurrent extraction using n-butanol:water, upper layer retained and concentrated.
Active extract (400 kg. activity 10 Calliphora units (C.U./mg.)
Countercurrent extraction using chloroform:methanol: water (1:2:1).
Inactive chloroform extract.
Methanol layers retained and evaporated.
Active extract (71 g.)
Countercurrent extraction with chloroform:ethanol: aqueous potassium bicarbonate. Chloroform layer retained and evaporated.
Active extract (12 g.)
Reversed phase partition chromatography using n-butanol water.
2-deoxy-20-hydroxyecdysone fractions.
20-Hydroxyecdysone fractions (900 mg. activity 100 - 200 C.U./mg.)
CM-Sephadex chromatography.
20-Hydroxyecdysone fractions (14·6 mg.)
Silicic acid chromatography.
20-Hydroxyecdysone. (2·3 mg. activity 40,000 C.U./mg.)

Table 5

FLOW DIAGRAM OF THE ATTEMPTED ISOLATION OF MOULTING HORMONES FROM BALANUS BALANOIDES

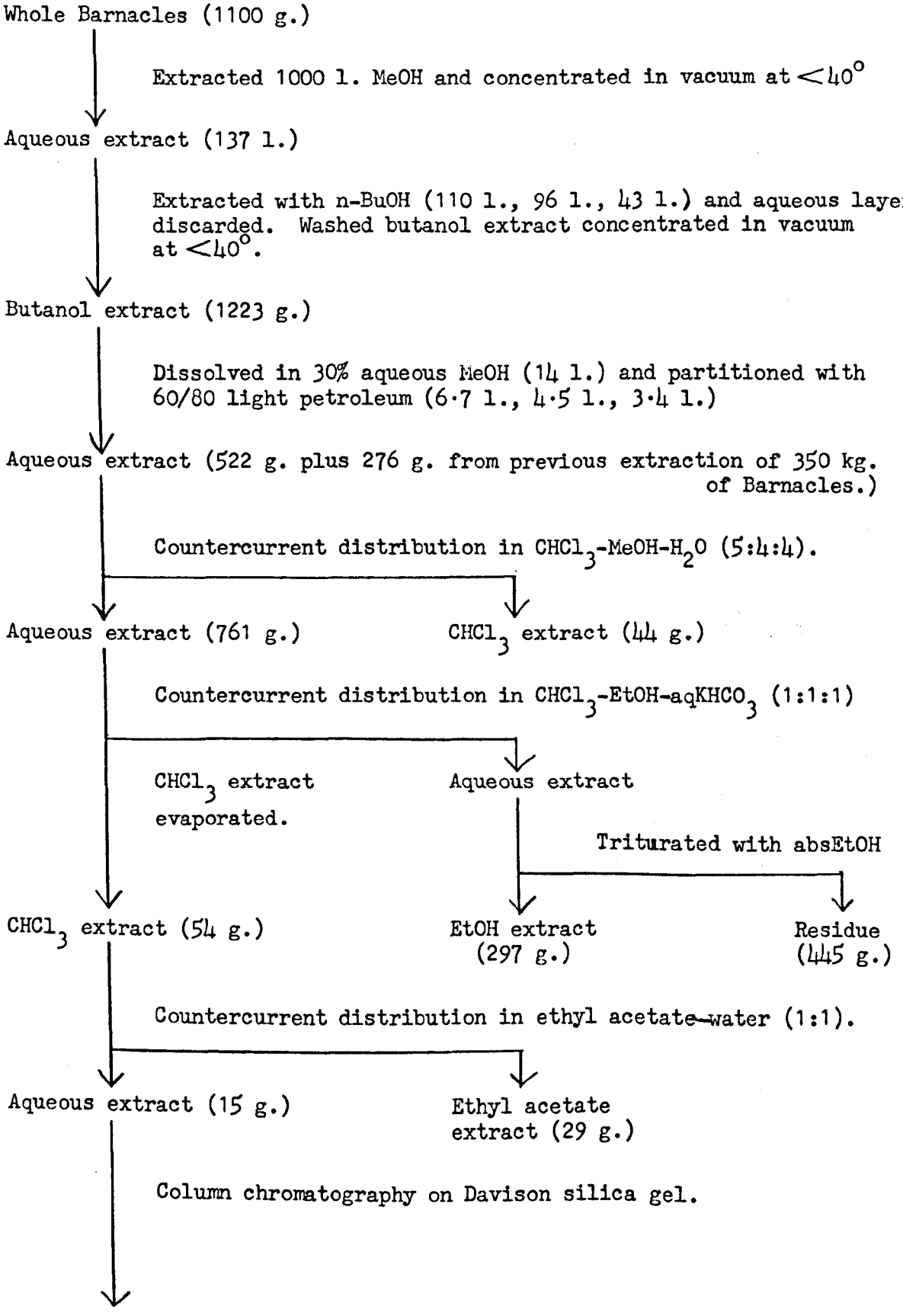


Table 5 (cont.)

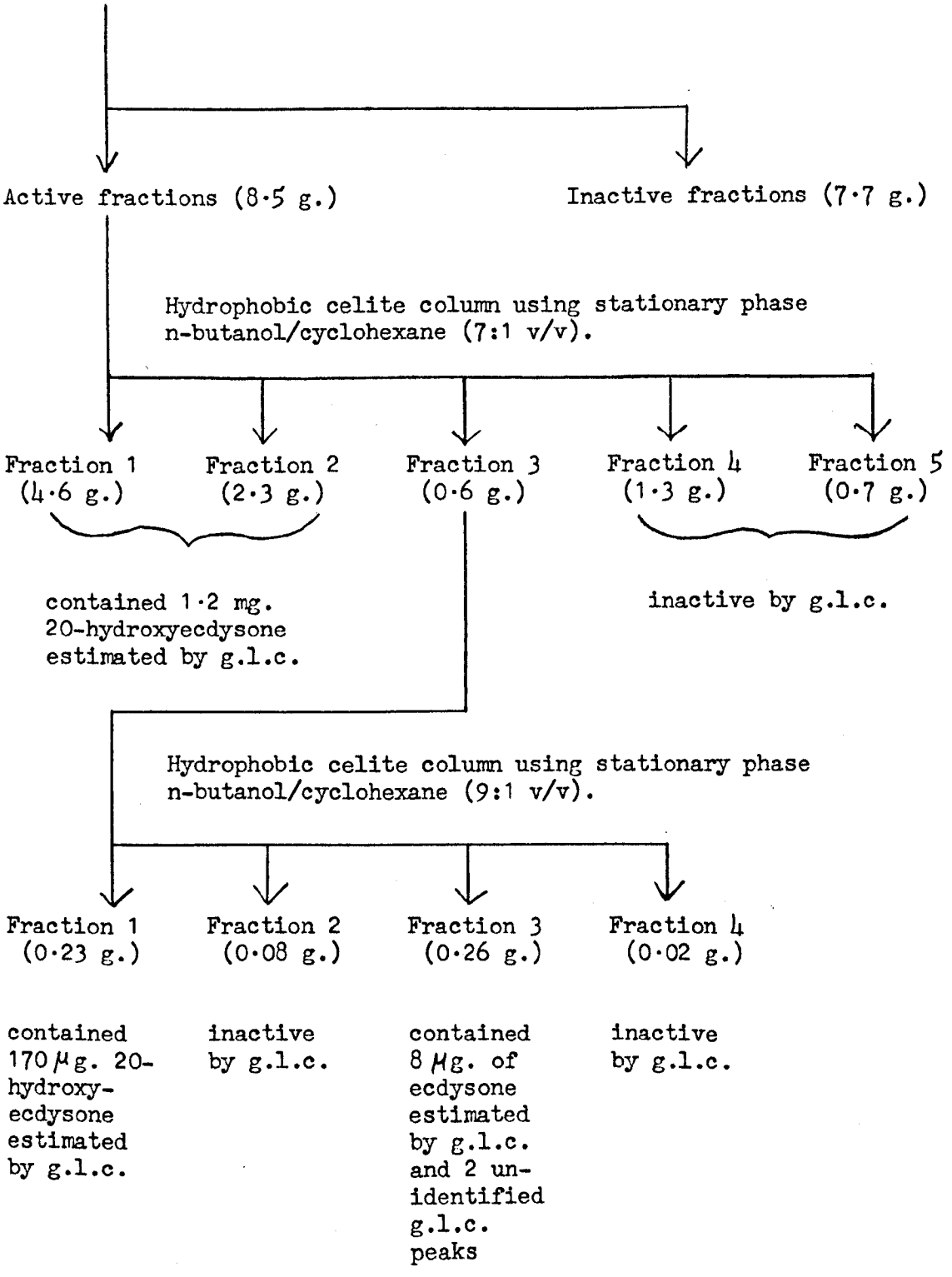

Active fractions (8·5 g.)
Inactive fractions (7·7 g.)
Hydrophobic celite column using stationary phase n-butanol/cyclohexane (7:1 v/v).
Fraction 1 (4·6 g.)
Fraction 2 (2·3 g.)
Fraction 3 (0·6 g.)
Fraction 4 (1·3 g.)
Fraction 5 (0·7 g.)
contained 1·2 mg. 20-hydroxyecdysone estimated by g.l.c.
inactive by g.l.c.
Hydrophobic celite column using stationary phase n-butanol/cyclohexane (9:1 v/v).
Fraction 1 (0·23 g.)
Fraction 2 (0·08 g.)
Fraction 3 (0·26 g.)
Fraction 4 (0·02 g.)
contained 170 µg. 20-hydroxy-ecdysone estimated by g.l.c.
inactive by g.l.c.
contained 8 µg. of ecdysone estimated by g.l.c. and 2 un-identified g.l.c. peaks
inactive by g.l.c.

Table 6

EXTRACTION OF MOULTING HORMONES FROM THE DESERT LOCUST, SCHISTOCERCA GREGARIA (Woodbridge, 1971) Ref. 43

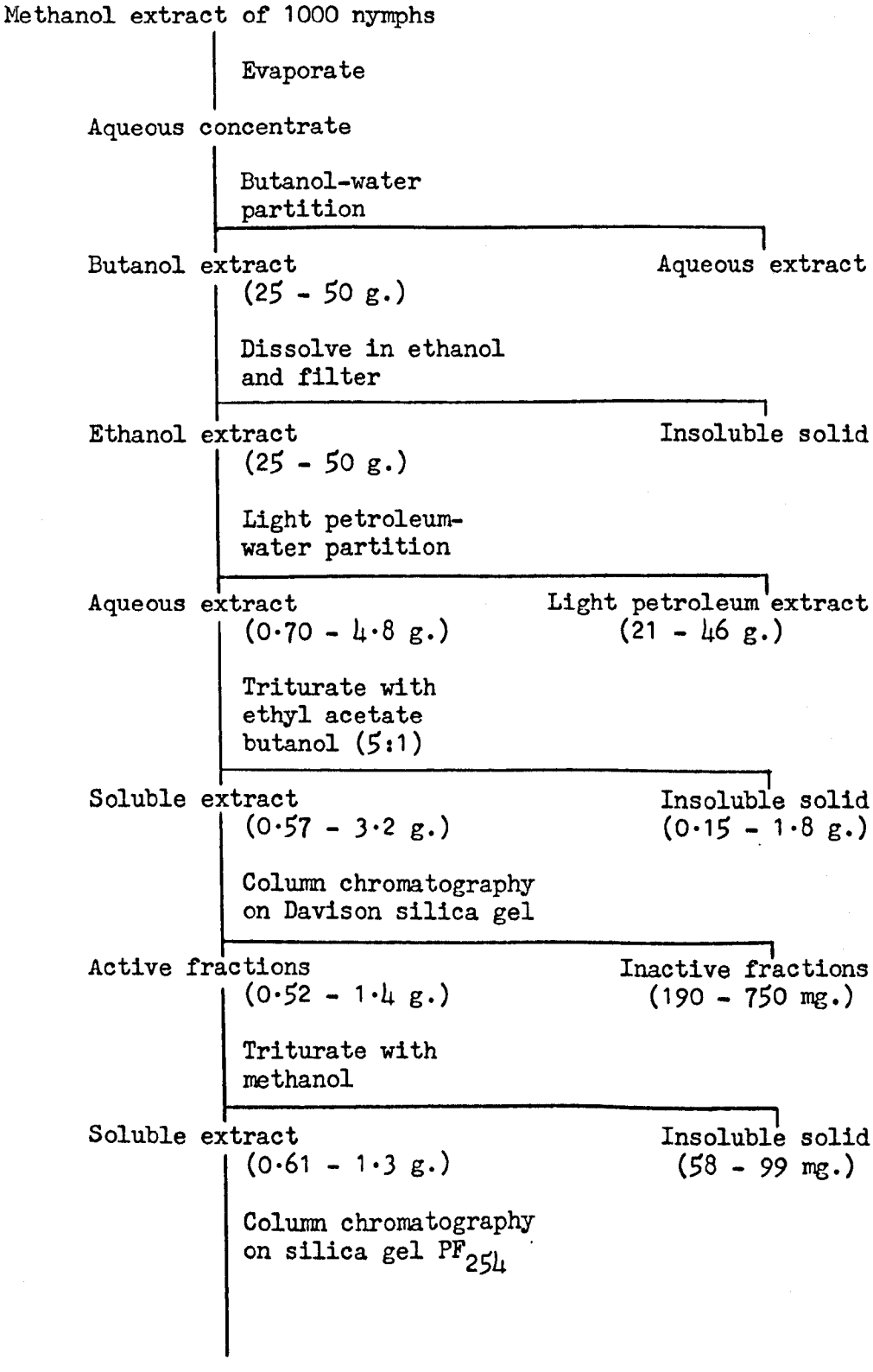
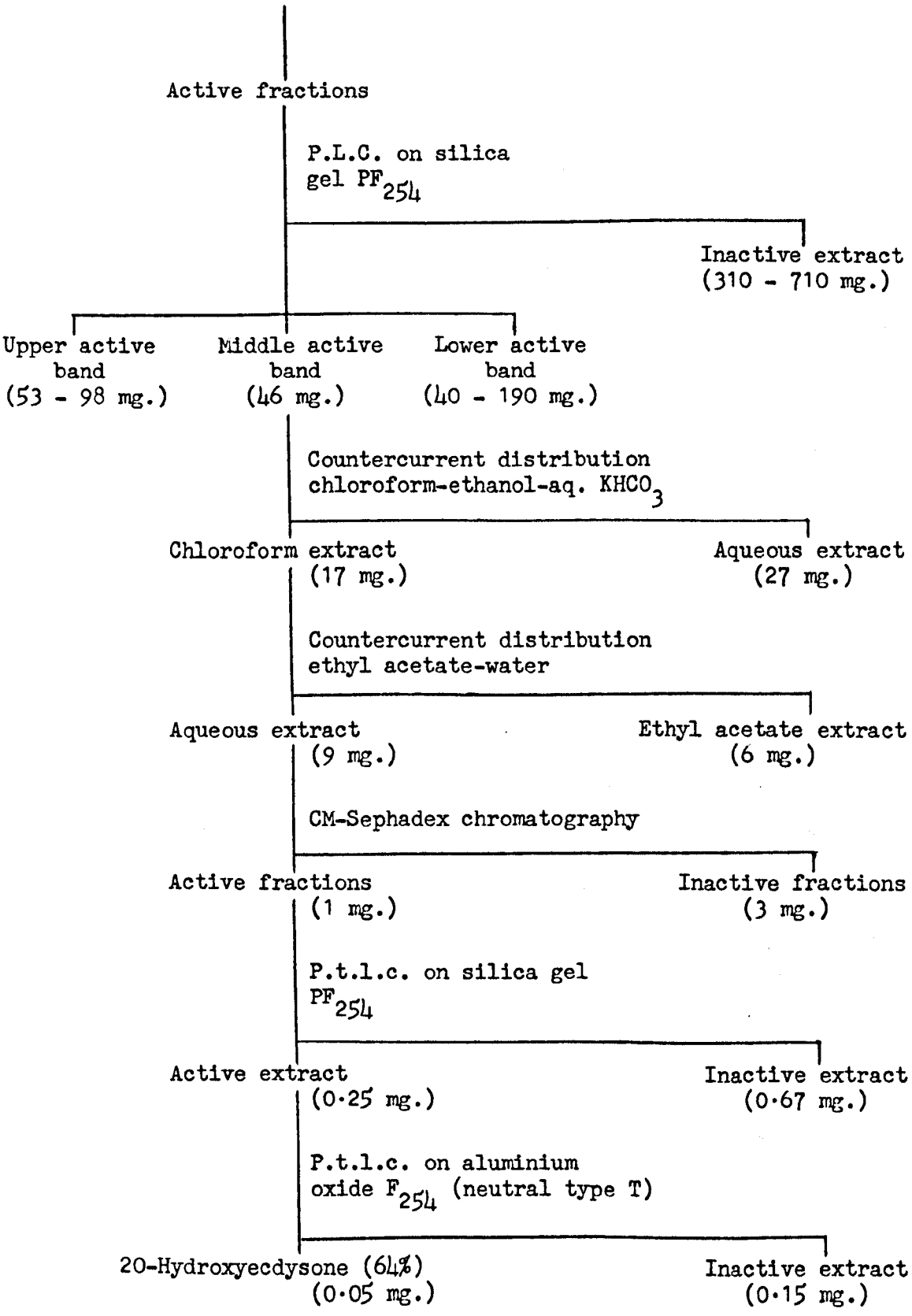


Table 6 (cont.)


Active fractions
P.L.C. on silica gel PF_{254}
Inactive extract (310 - 710 mg.)
Upper active band (53 - 98 mg.)
Middle active band (46 mg.)
Lower active band (40 - 190 mg.)
Countercurrent distribution chloroform-ethanol-aq. $KHCO_3$
Chloroform extract (17 mg.)
Aqueous extract (27 mg.)
Countercurrent distribution ethyl acetate-water
Aqueous extract (9 mg.)
Ethyl acetate extract (6 mg.)
CM-Sephadex chromatography
Active fractions (1 mg.)
Inactive fractions (3 mg.)
P.t.l.c. on silica gel PF_{254}
Active extract (0·25 mg.)
Inactive extract (0·67 mg.)
P.t.l.c. on aluminium oxide F_{254} (neutral type T)
20-Hydroxyecdysone (64%) (0·05 mg.)
Inactive extract (0·15 mg.)

conditions prevailed in 150 Kg. of barnacles, then the maximum amount of 20-hydroxyecdysone to be expected would be 15 μg, which is below the detection limit of the G.L.C. method then used. In the light of the above, somewhere in the region of 1500 Kg. of barnacle material would have to be extracted in order to obtain reasonable quantities of material for structural studies.

It was decided to attempt a large scale extraction of a further 1100 Kg. of barnacle material. The scale-up of the extraction procedure produced a number of problems concerned with the handling of large quantities of semi-solids and liquids.

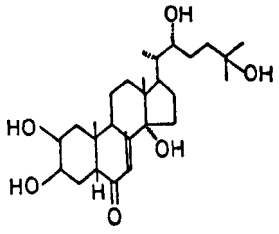
The hard calcarious shells proved difficult to grind, and after trials using a small laboratory homogenizer, a satisfactory system using a 2 H.P. double-acting homogenizer with stainless steel blades was developed. Grinding produced a semi-solid mass which had to be separated into solid and solution phases. This proved to be the most difficult stage to carry out quickly and cleanly. Filtration on a battery of 24 cm. diameter Büchner funnels was efficient but very slow, because the filter papers quickly became clogged by fine solid shell debris. Gravity filtration in muslin bags was found to be quick, but left appreciable quantities of liquid in the debris. This could have been removed by repeated washing; however, it would have required much larger quantities of methanol, which later would have had to be distilled off.

As a compromise procedure the barnacle material was ground in plastic bins, the solid allowed to settle, and the liquid decanted off. The remaining solid residue was re-ground in methanol and

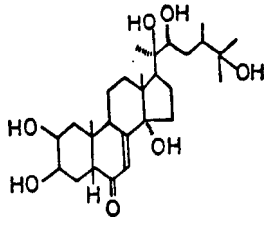
Fig. 9

Structures of Ecdysones.

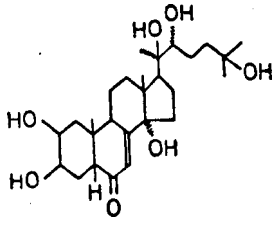
ecdysone



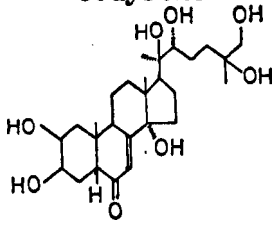
makisterone A



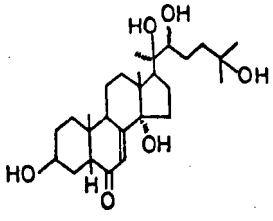
20-hydroxy-ecdysone



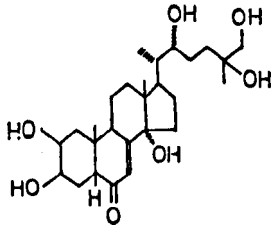
20,26-dihydroxy-ecdysone



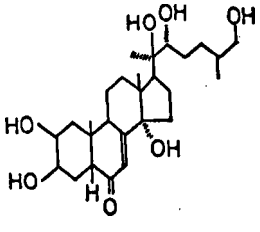
2-deoxy-20-hydroxyecdysone



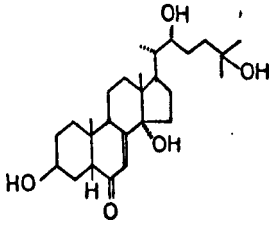
26-hydroxy-ecdysone



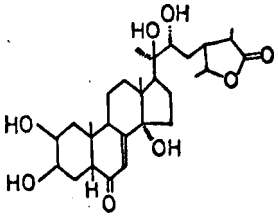
inokosterone



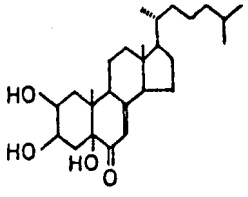
2-deoxyecdysone



cyasterone



2β,3β,5α-trihydroxy-cholest-7-ene-6-one



allowed to settle again. After the second decantation, the remaining gritty residue was filtered, using either a Büchner funnel or a muslin bag. This again was slow until a 6" diameter hydraulic press was acquired. Although the press held only about one litre of liquid, it speeded up considerably and increased the efficiency of the process, producing an essentially dry cake which was discarded.

The aqueous methanolic filtrate, approximately 1000 litres, had to be reduced to about 1/10 of its original volume. The evaporation was carried out under an atmosphere of nitrogen at reduced pressure to try and stop atmospheric oxidation, and at less than 40°C in order to prevent thermal degradation of the hormone material. The distillation was carried out in three 20-litre distillation units and a rising film evaporator. The total distillation rate was about 30 litres per hour.

The 1100 Kg. of barnacles collected produced 137 litres of aqueous concentrate after evaporation of the methanol. The latter had to be partitioned with n-butanol (110 litres). In order to perform this partition on such a large scale, a semi-automatic apparatus had to be designed, shown in Fig. 10. Each phase to be partitioned was pumped by a peristaltic pump to the mixing chamber at a metered rate, where the phases were vigorously stirred. After stirring, the mixed phases were allowed to run into a 35-gallon settling tank. The process was made semi-continuous by adjusting the feed into the mixing chamber so that it was equal to the rate of run-off of mixed phases into the settling tank. The residence time of the phases in the mixing chamber was such as to allow the same degree of mixing of phases as could be obtained by performing the partition in a

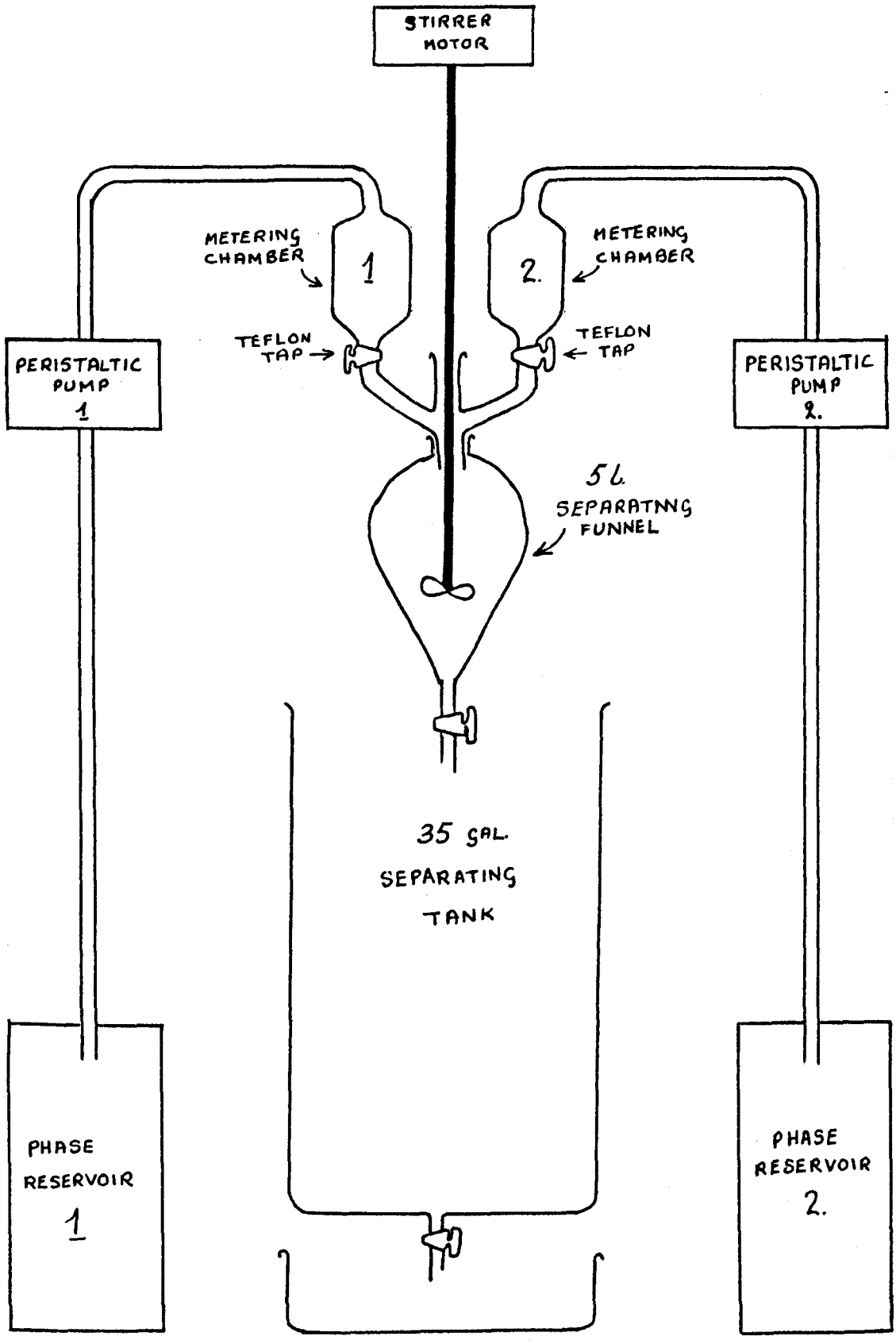
Figure 10

Semi-automatic Liquid/Liquid Partitioning Apparatus

The semi-automatic liquid/liquid partitioning apparatus was developed to enable large volumes of two immiscible phases to be partitioned. It enabled the phases to be thoroughly mixed in required proportions, with a degree of mixing similar to that obtained by manual agitation of small batches. The mixed phases were continuously run into a 35-gallon capacity tank where they were allowed to separate. The flow rate through the system was variable. In normal operation this was approximately 30 litres per hour through each pump. This allowed 35 gallons ($\sim$160 l.) of the combined phases to be partitioned in 5 - 7 hours.

Fig. 10

Semi-automatic Liquid/Liquid Partitioning Apparatus.



separatory funnel. After phase separation the aqueous layer was partitioned twice more with n-butanol (96 litres, 43 litres) in the same apparatus. The separation of phases was reasonably rapid and little trouble was encountered with emulsions. The combined n-butanol layers were washed with a small quantity of water (7·5 litres) which was then backwashed with n-butanol (25 litres). The combined n-butanol extracts were evaporated to give an 'active' residue (1·22 Kg.).

The residue from the n-butanol phase was partitioned between light petroleum (b.p. 60° - 80°) (6·7, 4·5 and 3·4 litres) in the apparatus described above, after having been dissolved in a mixture of methanol and water (3:7) (14 litres). It was found necessary to use aqueous methanol instead of pure water so as to avoid the formation of severe emulsions. This partition was designed to remove neutral lipids. The 'active' aqueous phase was evaporated to leave a residue (522 g.), to which was added 276 g. of a similar residue from a previous extraction.

The latter combined residues were subjected to a counter-current extraction using the system methanol-chloroform-water. The procedure used was that of Horn[14]. In one part of this paper, Horn reports that he used chloroform-methanol-water in the proportions 4:5:4, and in another part he records the proportions 1:2:1. In our extraction the former proportions were used, but as a check a small quantity of the residue from the aqueous phase was repartitioned using the latter proportions of methanol-chloroform-water, resulting in a further extraction of $<4\%$ of the partitioned material into the

chloroform phase. This partition, which was designed to remove polar lipids, was not very successful in reducing the bulk of the material. Only 44 g. of material was extracted into the chloroform phase, leaving 761 g. in the aqueous phase. Material from both phases was bioassayed, using the locust abdomen bioassay[43]. The chloroform phase residue was inactive, but the aqueous phase residue was too toxic and killed the locusts. Attempts were made to de-toxify the material by absorbing the toxic substances into silica gel but this was not successful. Difficulties with the bioassay on barnacles at this stage required that purification should be continued, on the presumption that activity would be found in the same phases as was found by Horn et al.

The aqueous phase residue was subjected to a further counter-current extraction, using the system ethanol-chloroform-aqueous potassium bicarbonate (1:1:1 by volume) in five tubes. Potassium bicarbonate was added to remove any acidic impurities which could degrade the hormone. Woodbridge[43] had used this procedure in the isolation of 20-hydroxyecdysone from the locust Schistocerca gregaria, and had used the double-withdrawal method. Horn in his paper says that he had used the latter, but detailed examination of the description of the method he actually used indicates that he actually followed up the fundamental process by a method known as completing the square[46]. Calculations show that, assuming the partition constant K of 20-hydroxyecdysone in the system chloroform-ethanol-water (1:1:1 by volume) has a value of 0·7, a significantly better separation is not achieved by using the double-withdrawal technique in preference to completing the square. This is particularly important when it is considered that the double-withdrawal method uses much more solvent and entails many more mani-

pulations. In the present extraction the method of completing the square was used after the fundamental procedure. The active material, according to Horn, should be in the chloroform phase. The chloroform phase was evaporated to dryness, leaving a residue (54 g.) which was subjected to a countercurrent extraction in the system ethyl acetate-water (1:1) in four tubes. In this system ecdysones are found in the aqueous phase which was evaporated to give a residue (14·9 g.).

Chromatography has been used to advantage for the separation of arthropod moulting hormones. Preparative thin-layer chromatography has been widely used for the separation of ecdysones in synthetic and isolation work. However, it has been found less suitable for the isolation of very small amounts of ecdysone, because large losses are often incurred. Column chromatography is more satisfactory for isolation work because the absorbent is more easily washed free of impurities and manipulation losses are less. In general, separations as good as those achieved by thin-layer chromatography can be obtained if a high enough ratio of absorbent to material being chromatographed is used.

Adsorption chromatography is the method of choice for the separation and isolation of unsaponifiable components of lipophilic substances, such as lipids and steroids. As a rule, adsorption methods are first used as a rough fractionation of the mixture. In crude extracts especially, the mutual competition of substances for active sites on the adsorbents may be an advantage. Care must be taken when exposing labile substances such as the ecdysones to alumina. Even moderately active aluminas tend to dehydrate tertiary alcohol groups forming olefins. This is one reason the otherwise highly efficient adsorption chromatographic process has been supplanted by partition

procedures. An important advantage of adsorption chromatography over partition chromatography is that a higher loading can be employed with the former.

After the solvent partitions, the bulk of barnacle material had been reduced sufficiently to use column chromatography. It was decided to try to achieve further purification and concentration of active material by chromatography on silica gel. The residue from the aqueous phase of the ethyl-acetate-water countercurrent extraction (14·9 g.) was chromatographed on a column of 'Davison' silica gel deactivated with water. The column was eluted with benzene containing increasing amounts of methanol. This column had been used to advantage by Woodbridge[43] in the purification of locust extracts. At this stage a new and much more sensitive gas chromatographic method became available for the detection of ecdysones down to the picogram level[44]. This involved the formation of trimethylsilyl-ethers (T.M.S.) of ecdysones and the use of an electron capture detector (e.c.d.) on the gas liquid chromatograph (g.l.c.). The fractions from the column referred to were pure enough to be analysed for ecdysones by g.l.c. (The method is described later.) The analysis showed that all of the ecdysone type material was contained in fraction 3 and the amount was estimated at about 20 μg. (See Table 7.)

Having achieved a rough separation of ecdysone type compounds from chemically dissimilar compounds, a technique was sought which would further separate the components of the ecdysone fraction obtained from the silica gel column. Reverse-phase chromatography can achieve more efficient separations than liquid-liquid extraction or adsorption

Table 7

Column Chromatography of Barnacle Extract from the Aqueous Phase of the Ethyl Acetate-Water Countercurrent on Deactivated Silica Gel.

Fraction	Eluent	Wt. fraction (g.)	Volume (ml.)
1	10% n-butanol in benzene	0·43	4700
2	5% methanol in benzene	3·18	3500
3	25% methanol in benzene	8·48	6500
4	50% methanol in benzene	1·85	4075
5	100% methanol	1·17	4075
6	15% water in methanol	1·03	2020

Details of Column

1) I.D. = 45 mm.

2) Packing: silica gel (450 g.), grade 950, mesh 60 - 200, deactivated with 10% water.

Table 8

Column Chromatography of Fraction 3 from the Silica Gel Column on Hydrophobic Celite.

Fraction	Eluent	Wt. fraction (g.)	Volume (ml.)
1	Water	4·6	1400
2	10% methanol in water	2·3	1800
3	20% methanol in water	0·6	1000
4	30% methanol in water	1·3	2000
5	40% methanol in water	0·7	2800

Details of Column

1) I.D. = 45 mm.

2) Packing: hydrophobic celite (290 g.) containing a mixture of n-butanol-cyclohexane (7:1 v/v, 177 ml.) as stationary phase.

   All eluting solvents were saturated with stationary phase.

chromatography. Karlson and Butenandt[15] used the system n-butanol-cyclohexane-water (7:3:10 by volume) in a countercurrent distribution extraction when attempting to enrich silkworm extracts during the isolation of ecdysone. Horn et al.[14] attempted to use this system for the extraction of 20-hydroxyecdysone from J. lalandei but experienced troublesome emulsions. He therefore investigated reverse-phase partition chromatography with hydrophobic celite[47] and n-butanol-cyclohexane as the stationary phase, and water containing increasing amounts of methanol, and saturated with stationary phase, as the moving phase. This system had proved very useful for the enrichment of crayfish extracts.

The fraction, obtained from chromatography of barnacle extracts on silica gel deactivated with water, and shown by gas chromatography to contain ecdysones, was re-chromatographed on hydrophobic celite with a mixture of n-butanol-cyclohexane (7:1) as the stationary phase. It was then eluted with water containing increasing amounts of methanol, and saturated with stationary phase, a similar system to that used by Horn[14]. (See Table 8.) The column fractions were again analysed by g.l.c. and 20-hydroxyecdysone was shown to be present in fractions 1 and 2 (Fig. 11), the total amount being calculated as 1·2 mg. Both the hexakis(trimethylsilyl) ether and the heptafluorobutyrate derivative of this material was shown to have a retention time identical with those of the corresponding derivatives of authentic 20-hydroxyecdysone on a 1·5% column of OV-101 (Fig. 12).

Fraction 3 from the column showed several peaks in the ecdysone region on the gas chromatograph (Fig. 13). This fraction was re-chromatographed on hydrophobic celite with a slightly more

Table 9

Column Chromatography of Fraction 3 from the First Hydrophobic Celite Column on Hydrophobic Celite.

Fraction	Eluent	Wt. fraction (g.)	Volume (ml.)
1	Water	0·233	220
2	10% methanol in water	0·080	150
3	20% methanol in water	0·263	275
4	30% methanol in water	0·020	340

Details of Column

1) I.D. = 20 mm.

2) Packing: hydrophobic celite (32 g.) containing a mixture of n-butanol-cyclohexane (9:1 v/v, 20 ml.) as stationary phase.

All eluting solvents were saturated with stationary phase.

polar stationary phase (butanol-cyclohexane 9:1). The column was eluted with water saturated with stationary phase containing increasing amounts of methanol (Table 9). About 20 mg. of each fraction obtained was treated with 100 µl of trimethylsilylimidazole (T.M.S.I.) and, after t.l.c. clean-up, was analysed by gas chromatography.

Fraction 1 was found to contain a further 170 µg. of 20-hydroxyecdysone. From these results it was possible to estimate that the total amount of 20-hydroxyecdysone present in the extract obtained from an initial wet weight of 1500 Kg. of barnacles was approximately 1·4 mg.

Fractions 2 and 4 obtained from the above-mentioned column did not contain any peaks in the retention time-range expected for T.M.S.I. derivatives of ecdysones[44]. The gas chromatogram of fraction 3 (Fig. 14) from the same column contained the same group of peaks observed in the gas chromatogram of fraction 3 from the first reverse-phase column (Fig. 13), although the peak which, on the basis of its retention time, could be assigned to 20-hydroxyecdysone, was absent. This 20-hydroxyecdysone had been eluted in fraction 1 above. In fraction 3 from the second reverse phase column, peaks A and B (Fig. 14) were found to have the same retention time as those produced by the pentakis-TMS ether of ecdysone. These two peaks, A and B, were identified as the pentakis-TMS ether derivative of ecdysone and an epimer produced during reaction. Co-injection of an authentic pentakis-TMS ether derivative of ecdysone, with derivatised fraction 3, increased the height of peaks A and B without introducing any new peaks. The fact that the material present in peaks A and B had the same retention time as ecdysone under the g.l.c. conditions used suggests

that it was itself an ecdysone with a retention time shorter than that of a hexakis TMS derivative of 20-hydroxyecdysone. Out of the large number of ecdysones which have been isolated, only a limited number have been identified in arthropods.

The possible identities of the ecdysone present are therefore also limited in number. The first possibility is inokosterone (callinecdysone A) (Fig. 9). This is a structural isomer of 20-hydroxyecdysone and has been isolated from at least one crustacean (Callinectes sapidus)[17]. However it was found that the TMS ether of this compound had a longer retention time than that of 20-hydroxyecdysone and a much longer retention time than those of the compound responsible for peaks A and B (Fig. 14). Thus peaks A and B are not produced by the TMS ether of inokosterone.

Another possibility which can be eliminated is makisterone A (callinecdysone B) (Fig. 9) which has also been isolated from C. sapidus[17]. This compound has a similar structure to 20-hydroxyecdysone, but has an extra methyl group at C-24. The increased molecular weight could be expected to increase significantly the retention time of a TMS ether derivative over that of a similar derivative of 20-hydroxyecdysone.

A further possibility is 2-deoxy-20-hydroxyecdysone which has also been isolated from a crustacean, the marine crayfish Jasus lalandei[14]. This compound (Fig. 9) has the same number of hydroxyl groups as ecdysone but differs in the position of one of them. Although the totally silylated derivate of each compound would have the same molecular weight, the spacial arrangement of the TMS ether groups would be different, and hence the overall shape of the molecule would also be different. Thus it would be expected that the retention times of their

Figure 11

G.L.C. Analysis of Fractions 1 and 2
from the First Hydrophobic Celite Column.

Fraction 3 from the 'Davison' silica gel column was chromatographed on hydrophobic celite (290 g.) containing a mixture of n-butanol-cyclohexane (7:1 v/v)(177 ml.) and eluted with water containing increasing amounts of methanol saturated with stationary phase (Table 8). After derivatisation, samples from fractions 1 and 2 with TMSI the hexakis TMS ethers produced were gas chromatographed on a 3 ft. x $^1/_8$" helical column of 2% OV 101 on CQ, using nitrogen as carrier gas (flow rate 45 ml min^{-1}, column temp. 272°). The method of detection was electron capture (^{63}Ni).

The peak at longer retention time **is possibly due to the hexakis TMS ether of 20-hydroxyecdysone, whilst the peak at shorter time is possibly due to the C-9 epimer, in which the C-9 proton is 'β' instead of 'α' to the plane of the steroidal ring system.**

Fig. 11

G.L.C. Analysis of Fractions 1 and 2
from the First Hydrophobic Celite Column*

(* See Table 8)

Figure 12

The Hexakis TMS Ether and Heptafluoro-butyrate Exchanged Derivative of Authentic and Barnacle-derived 20-hydroxyecdysone.

The 20-hydroxyecdysone obtained from barnacles was compared with authentic pure 20-hydroxyecdysone by first of all forming their hexakis TMS ethers and then exchanging some of the TMS residues with heptafluorobutyryl derivatives.

The g.l.c. retention times of both derivatives were compared on a 6 ft. x $\frac{1}{8}$" helical column of 1·5% OV 101 on CQ, using nitrogen as carrier gas (flow rate 45 ml min^{-1}, column temp. 272°). The method of detection was electron capture (^{63}Ni).

Fig. 12

The Hexakis T.M.S. Ether and Heptafluorobutyrate Exchanged Derivative of Authentic and Barnacle-derived 20-Hydroxyecdysone.

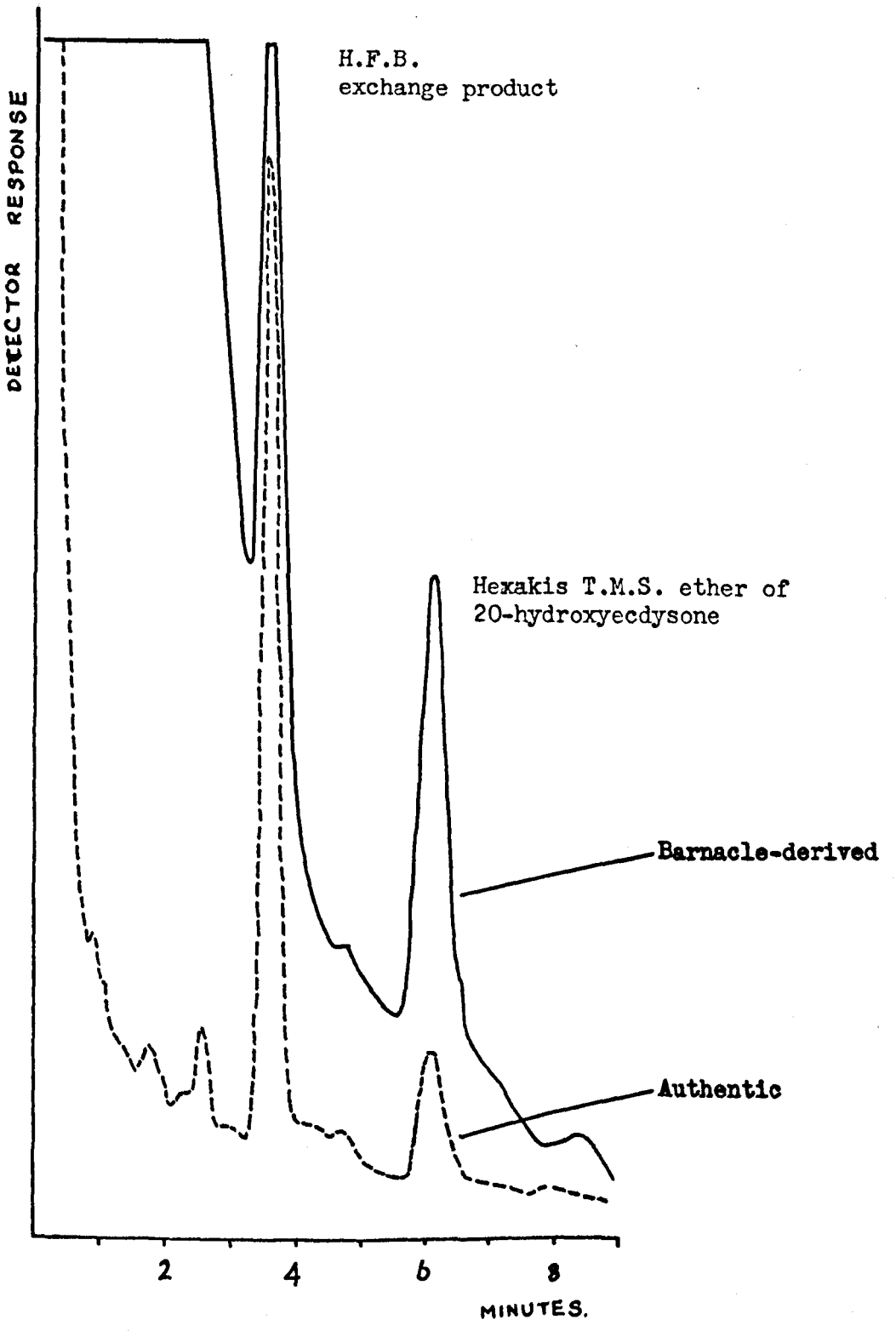


Figure 13

G.L.C. Analysis of Fraction 3 from the First Hydrophobic Celite Column.

Fraction 3 from the 'Davison' silica gel column was chromatographed on hydrophobic celite. (For details, see Legend to Fig. 11.)

Peak C was identified as being due to the hexakis TMS ether derivative of 20-hydroxyecdysone by comparison of retention time. (See Figs. 11 and 16.)

Fig. 13

G.L.C. Analysis of Fraction 3 from the First Hydrophobic Celite Column*.

(* See Table 8.)

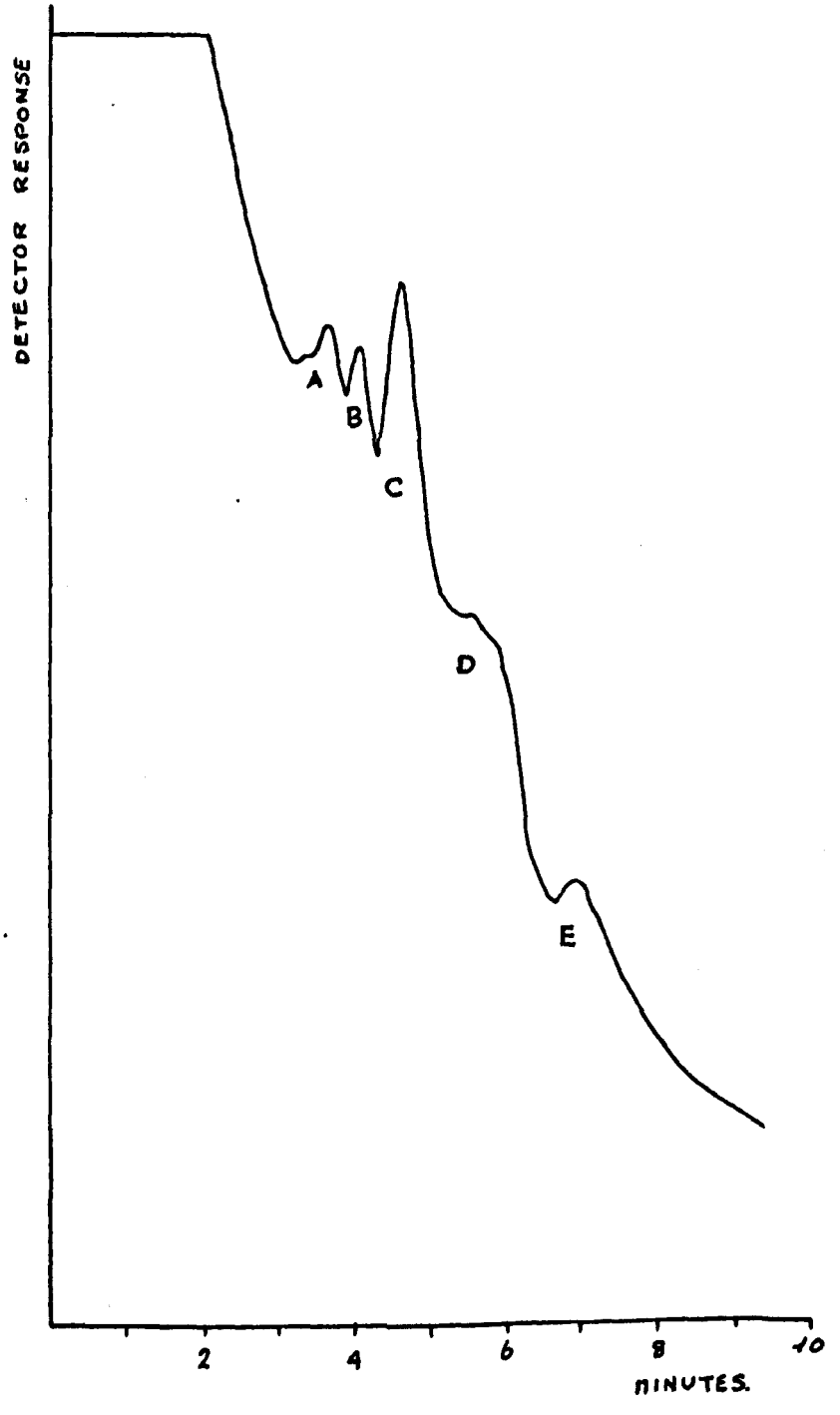


Figure 14

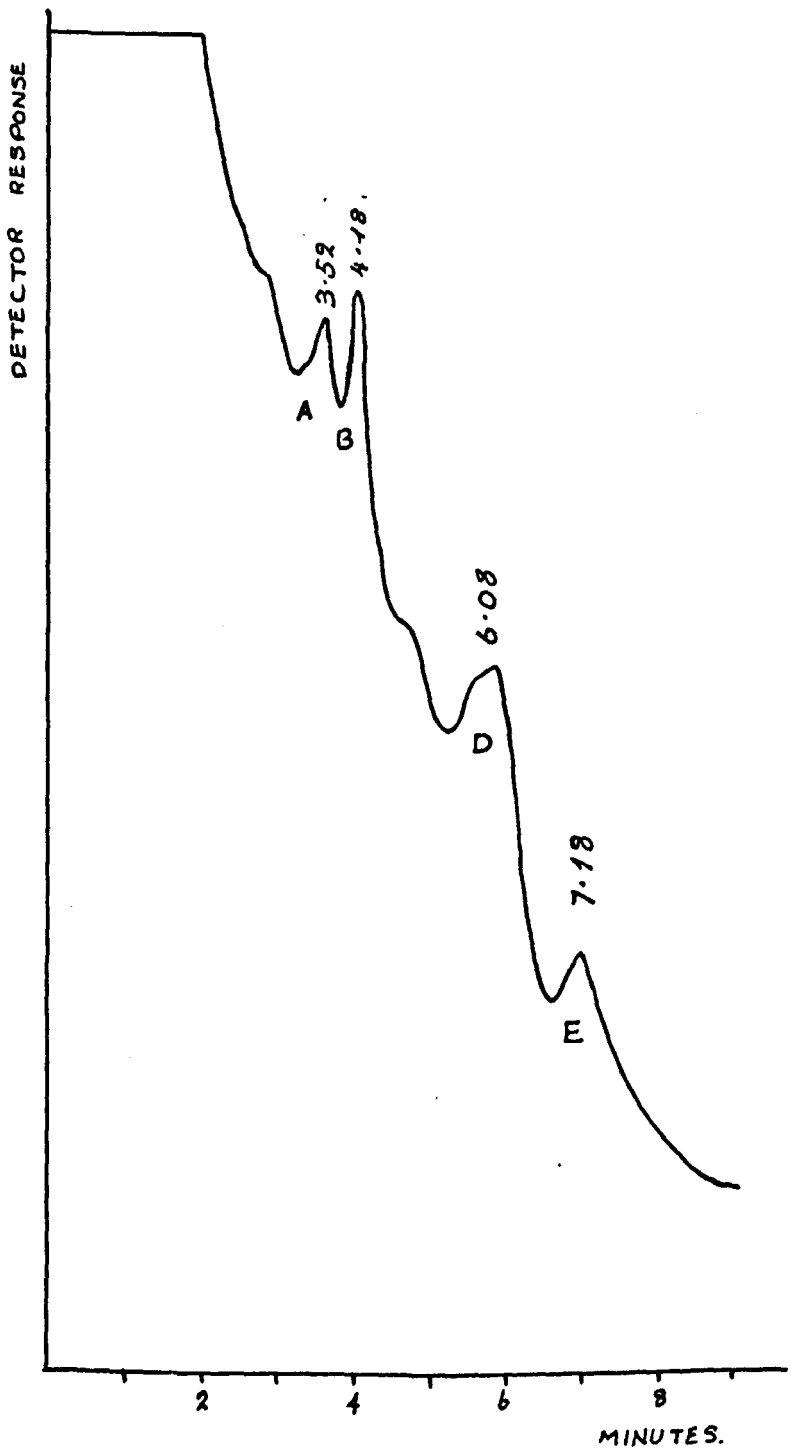
G.L.C. Analysis of Fraction 3 from the Second Hydrophobic Celite Column.

Fraction 3 from the first hydrophobic celite column was re-chromatographed on hydrophobic celite (32 g.) containing a mixture of n-butanol-cyclohexane (9:1 v/v)(20 ml.) and eluted with water containing increasing amounts of methanol saturated with stationary phase (Table 9). After derivatisation of Fraction 3 using TMSI, the TMS ether derivatives formed were chromatographed as described in the Legend to Fig. 11. It was found, by comparison, that the retention times of peaks A and B, and those of the pentakis TMS ether of authentic ecdysone were identical (Figure 15).

Fig. 14

G.L.C. Analysis of Fraction 3 from the Second Hydrophobic Celite Column*.

(* See Table 9)



Figures 15 - 17

G.L.C. Traces of TMS Ether Derivatives of Pure Ecdysone, 20-hydroxyecdysone and Inokosterone Standards.

Authentic samples of ecdysone, 20-hydroxyecdysone and inokosterone were reacted with TMSI to form the TMS ether derivatives. These were gas chromatographed under the conditions described in Fig. 11, and used as standards for identification of peaks in g.l.c. traces of barnacle extracts treated with TMSI.

Fig. 15

G.L.C. Trace of Pentakis T.M.S.
Ether Derivative of Pure Ecdysone.

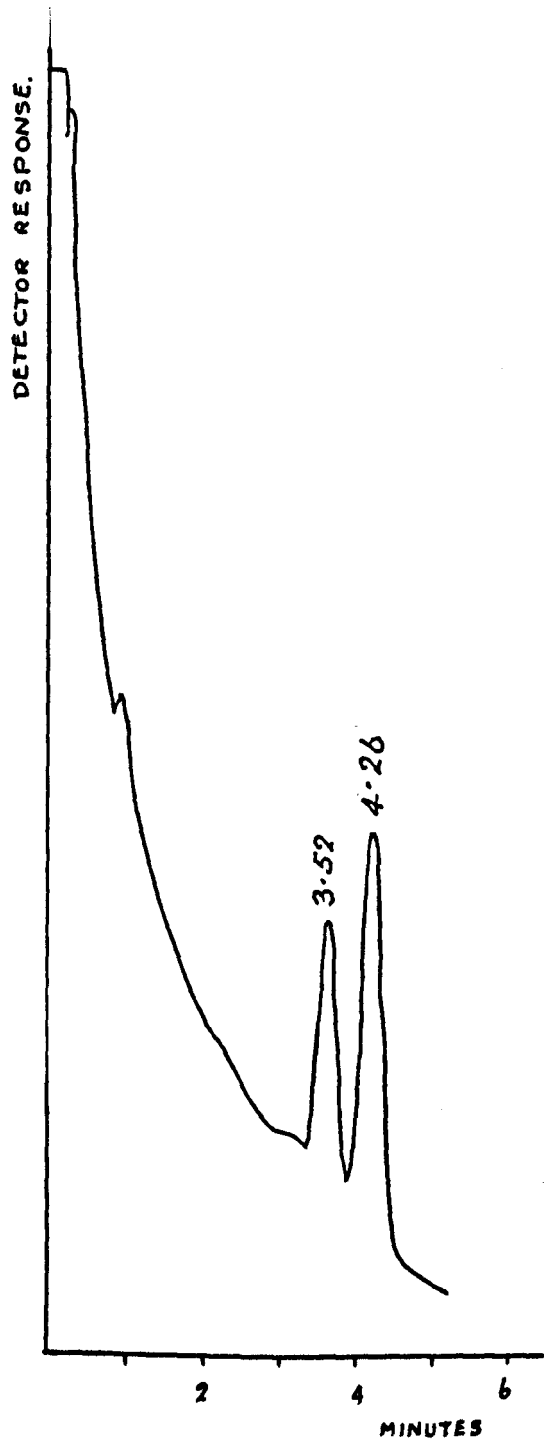


Fig. 16

G.L.C. Trace of the Hexakis T.M.S. Ether Derivative of Pure 20-Hydroxyecdysone.

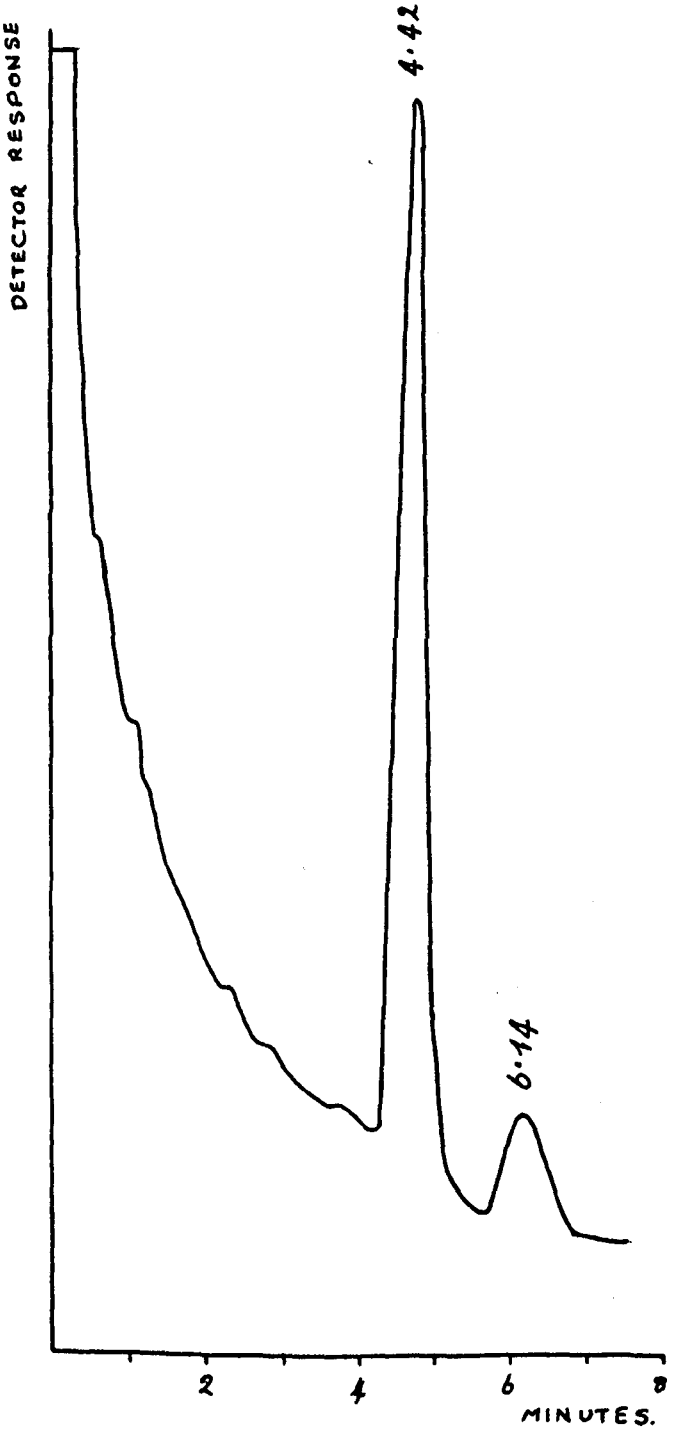
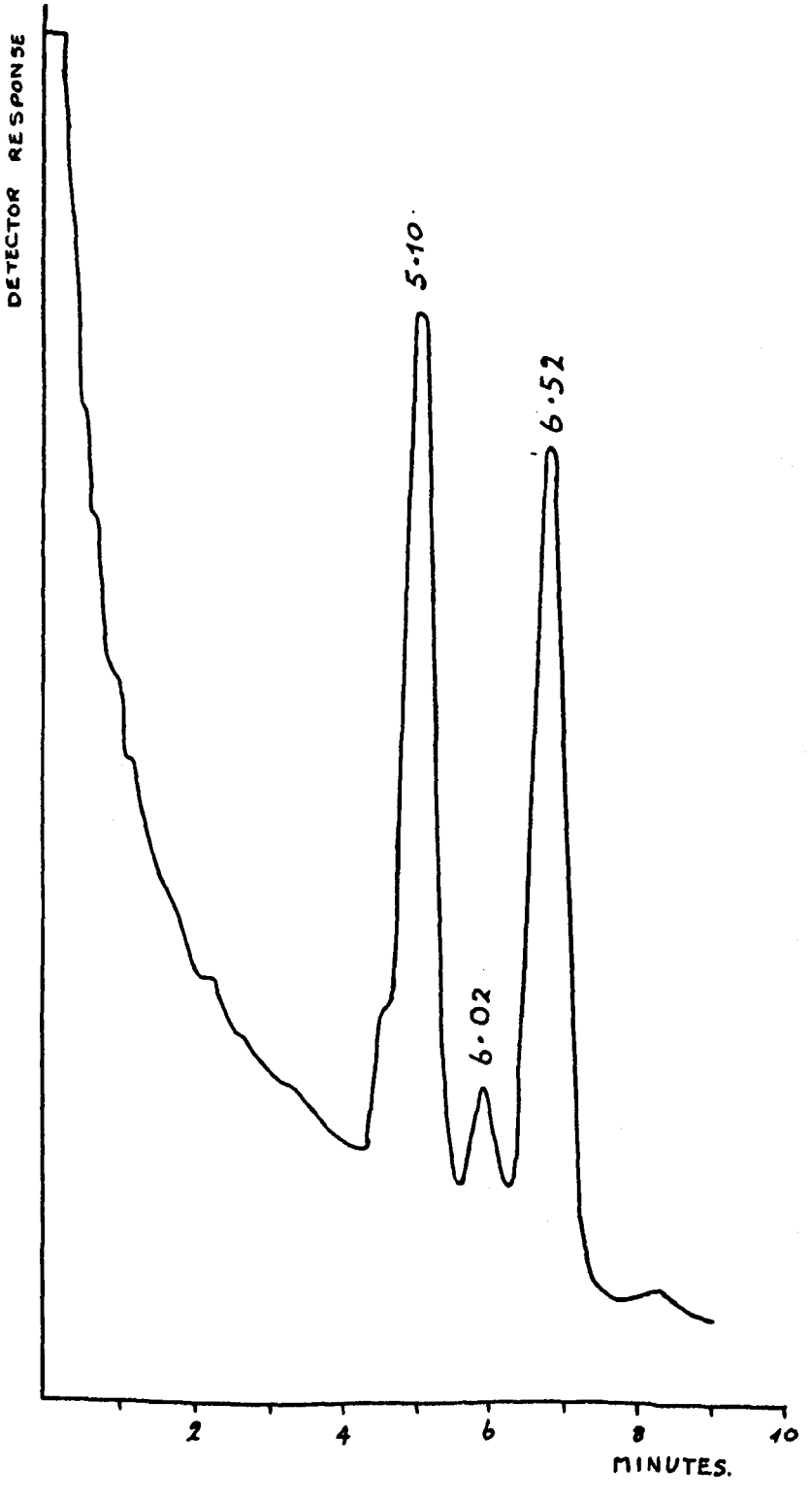


Fig. 17

G.L.C. Trace of the Hexakis T.M.S.
Ether Derivative of Pure Inokosterone.



derivatives would also be significantly different. If peaks A and B are due to TMS derivatives of 2-deoxy-20-hydroxyecdysone, it is unlikely that co-injection of the pentakis-TMS derivative of ecdysone with derivatised barnacle material would produce peaks of exactly coincident retention times. Hence in the absence of an authentic sample of 2-deoxy-20-hydroxyecdysone to test this hypothesis, it is reasonable to suppose that peaks A and B are not due to derivatives of this compound.

A fourth possibility is 20,26-dihydroxyecdysone (Fig. 9), which has so far been isolated only from an insect, the Tobacco Hornworm, Manducta sexta[48]. This compound also has a similar structure to 20-hydroxyecdysone but has an extra hydroxyl group at C-26. The molecular weight being greater than that of 20-hydroxyecdysone, the retention time of the silylated derivative would be expected to be longer than that of 20-hydroxyecdysone.

A fifth possibility is provided by 26-hydroxyecdysone which was also isolated from M. Sexta[49]. Since this compound has a hydroxyl group at C-26 (Fig. 9), it has the same number of hydroxyl groups as 20-hydroxyecdysone, and hence the molecular weight of its TMS-ether derivative would be the same as that of the corresponding derivative of 20-hydroxyecdysone. It would therefore be expected to have a longer retention time than ecdysone and peaks A and B.

A sixth possibility is ecdysone itself, which has been shown by co-injection of its pentakis-TMS-ether derivative, with a derivatised barnacle extract, to have an identical retention time as peaks A and B. Ecdysone has been isolated from insects[15], but so far not from

crustaceans. However it might be present as a precursor of other ecdysones; it has been shown that ecdysone is a precursor of 20-hydroxyecdysone in insects[29].

A final possibility is presented by the ecdysone recently found by Bollenbacher[50] in the crustacean Pachygrapsus crassipes after in vitro culture of their Y-organs. This compound appears to have 3 to 4 hydroxyl groups in undefined positions. The TMS ether of this compound would therefore be expected to have a retention time less than that of ecdysone and thus of peaks A and B.

Of the possibilities considered above, the pentakis-TMS derivative of ecdysone is most likely responsible for peaks A and B.

Other peaks were observed on the gas chromatograph (Fig. 14) of derivatised fraction 3 of the second reverse phase column. These peaks had longer retention times than 20-hydroxyecdysone, and could not be positively identified. They represent either electron capturing compounds of quite different structure from the ecdysones, or more polar ecdysones. It is possible that they are metabolites of 20-hydroxyecdysone.

Our extraction procedure has shown that 20-hydroxyecdysone is the major moulting hormone present in the barnacle B. balanoides. Ecdysone itself was also present at low but significant levels. The presence of other moulting hormones cannot be excluded, as other substances with ecdysone-like retention times were observed on the g.l.c. trace, but these could not be identified. It was possible to quantify the total amount of 20-hydroxyecdysone present in the extract from 1400 Kg. of barnacle material. This was 1·4 mg. The amount of

ecdysone present was also estimated. This was 8 μg. These figures were obtained by quantitative g.l.c.

This situation is comparable with the results of investigations in other species. Horn et al.[14] found 20-hydroxyecdysone to be the major moulting hormone present in J. lalandei, whilst 2-deoxy-20-hydroxy-ecdysone was also present but at much lower levels. Faux et al.[17] showed that in C. sapidus at the postmoult stage 20-hydroxyecdysone was the most important moulting hormone, while inokosterone and makisterone A were present in small amounts. The level of 20-hydroxyecdysone found in B. balanoides was of similar magnitude to those found in other species. The concentration of 20-hydroxyecdysone in B. balanoides was 1 $\mu g.kg^{-1}$.* Horn found 2 $\mu g.kg^{-1}$ in J. lal ndei, and Faux found 280 $\mu g.kg^{-1}$ in post-moult C. sapidus. Gagosian et al.[51] found 6 $\mu g.kg^{-1}$ in postmoult Homarus americanus. (See Table 1.)

As well as 20-hydroxyecdysone, which was shown to be responsible for peak C, other small peaks were found on the g.l.c. trace (Fig. 14). Peaks A and B have been identified as ecdysone. Peaks D and E probably represent ecdysones with a similar physiological function to inokosterone and makisterone A found in C. sapidus, although neither of these peaks was due to inokosterone. In C. sapidus makisterone A and inokosterone occur at relatively high levels at particular stages during the life cycle and peak titres were connected with particular moult stages (Table 10). It is probable that hormones of similar function occur in B. balanoides, also in conjunction with the various stages of ecdysis. Such hormones could be

* Hormone concentrations quoted as $\mu g.kg^{-1}$ of collected weight of barnacles.

Table 10

Moulting Hormones from Callinectes sapidus at Three Stages during Ecdysis.*

(Weights in $\mu g.kg^{-1}$ extracted.)

	Stages of Ecdysis		
	"green"	"Peeler"	"soft-shell"
Inokosterone	5	20	-
20-hydroxyecdysone	-	4	280
Makisterone A	-	-	24

* from Faux et al.[17]

expected to be detectable in extracts derived from a mixed population of B. balanoides, since only a proportion of the individuals present in that population would be in a particular ecdysal stage at the time of sampling. Peaks D and E might also represent metabolites of 20-hydroxyecdysone possibly lacking physiological significance.

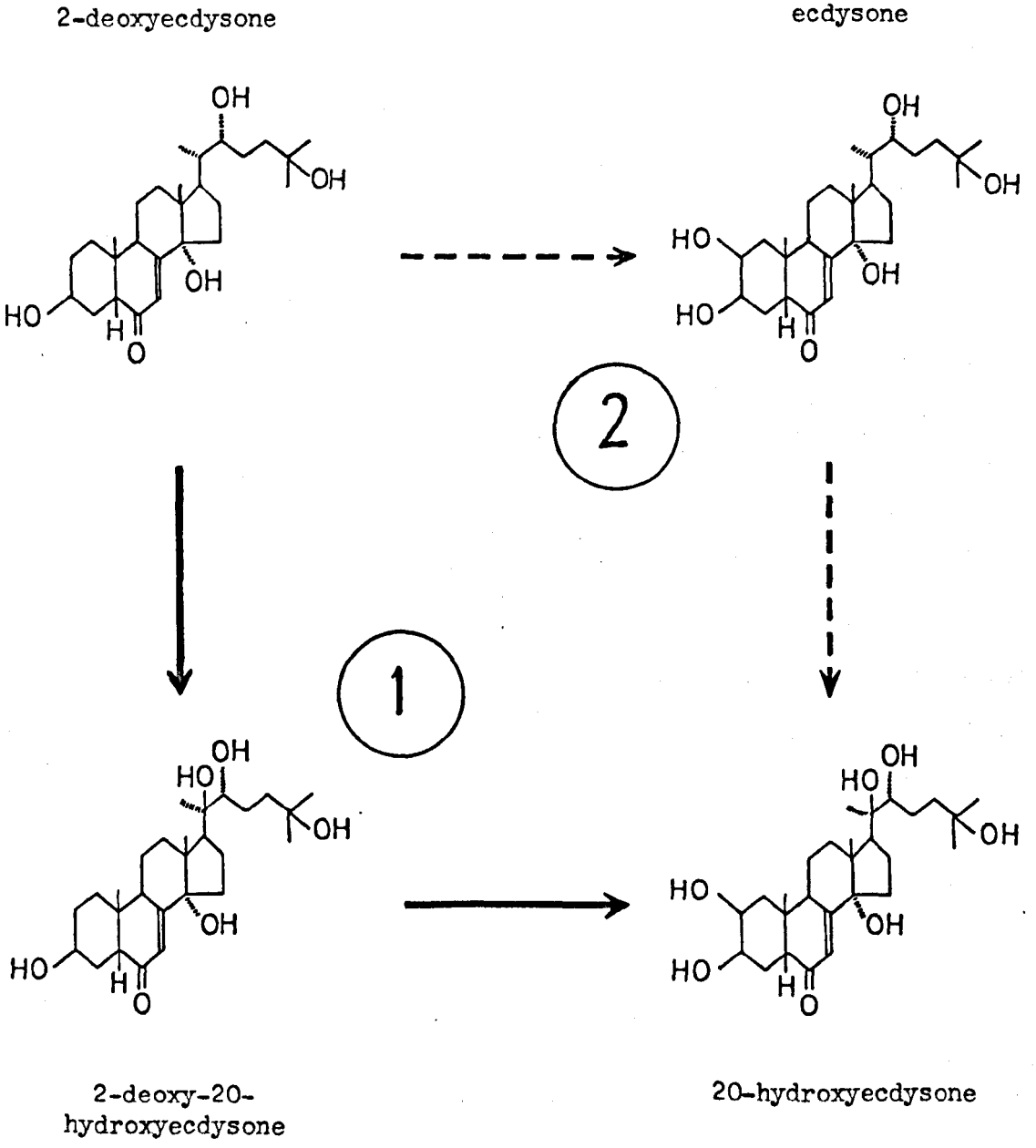
Ecdysone was found in the extract of B. balanoides at a very low concentration of 6 $ng.kg^{-1}$. Horn found comparably low levels of 2-deoxy-20-hydroxyecdysone in J. lalandei: 70$ng.kg^{-1}$. Because the two substances occur at such low but similar concentrations, it is reasonable to suppose that they perform a similar function, and that they may represent prohormones.

Ecdysone has been shown to be produced by insect prothoracic glands in vitro[29] and it is known that in insects ecdysone is rapidly metabolized to 20-hydroxyecdysone[52]. This conversion has also been shown to take place readily in the shrimp Crangon nigricauda and the crab Uca pugilator during premoult and moulting periods[52]. 2-Deoxy-ecdysone (Fig. 9) and 2-deoxy-20-hydroxyecdysone are both active in the Calliphora test, suggesting that they are both precursors of 20-hydroxyecdysone[53,54].

Two pathways have been postulated for the biosynthesis of 20-hydroxyecdysone in arthropods[55]. The first one involves conversion of 2-deoxyecdysone to 20-hydroxyecdysone via 2-deoxy-20-hydroxyecdysone. The second pathway involves conversion of 2-deoxyecdysone via ecdysone. (Fig. 18) The first pathway has been found in J. lalandei, and has been postulated as the main pathway in other arthropods[55]. The second pathway probably occurs in C. nigricauda and U. pugilator as well as

Fig. 18

Possible Immediate Precursors
of Arthropod Ecdysones.



C. stygia[52,54]. In B. balanoides the identification of ecdysone would suggest that pathway 2 is the more important.

The discovery of ecdysone in extracts from B. balanoides has further importance in that so far it has been isolated only from insect material. The level occurring in the B. balanoides extract suggests that, rather than its being entirely absent from other crustacean extracts, it occurred at levels undetectable by the methods used. Only the increased sensitivity of the g.l.c. method[44] has enabled the detection of its presence in B. balanoides.

2-Deoxyecdysone has not as yet been found in arthropods. However it is interesting at this point to note Bollenbacher's[50] recent results. He has shown that the main secretory product from cultured Y-organs of Pachygrapsus crassipes was an ecdysone having three to four hydroxyl functions. This was found together with 2-deoxy-20-hydroxy-ecdysone. It is interesting to speculate on the identity of this as yet partially unidentified ecdysone.

For the purposes of comparison with the data available from other arthropods, hormone concentrations were expressed as $\mu g.kg^{-1}$ wet weight of barnacle material extracted. Because in the case of barnacles this includes the hard calcareous shell, which represents a large proportion of the total weight, these results are more informative when calculated on the basis of extractable tissue present, exclusive of the shell. As is shown in Table 11, up to 59% w/w of the weight of barnacle material collected was calcareous shell. The concentrations of hormones extracted from the barnacle calculated on the basis of extractable tissue are compared with the concentrations calculated on the basis

Table 11

Composition of Barnacle Body

	Weight of fraction	% weight collected
Total weight barnacle material	1432 g.	100%
Residue remaining after methanol extraction (mainly calcareous shell)	837 g.	59%
Methanol extractable material	35 g.	2·5%

Thus, in 1400 kg. barnacle material collected, methanol would extract approximately 35 kg. material (2·5%).

Table 12

Comparison of Concentrations of 20-Hydroxyecdysone Based on Weight of Material Collected and Weight of Methanol Extractable Material.

	Total Material Collected	Total Material Collected Minus Shell
Weight	1400 kg.	35 kg.
Concentration of 20-hydroxyecdysone	1·0 μg kg^{-1}	40 μg kg^{-1}
Concentration of ecdysone	6·0 ng kg^{-1}	230 ng kg^{-1}

of wet weight collected in Table 12. Most of the barnacles were collected in the summer months. At this time about 10% of the barnacles collected are moulting, and about 10% of these have the maximum titre of moult-inducing hormone. Injections of 20-hydroxyecdysone into B. balanoides[45] indicated that the endogenous maximum titre should be about 0·01 µg per animal. If these conditions prevailed in the 1400 kg. sample of barnacles, then the maximum amount of 20-hydroxyecdysone to be expected would be 140 µg (assuming one barnacle weighs about 1g.). If the calculation of the maximum endogenous titre of 20-hydroxyecdysone in B. balanoides was based on an accurate assumption, then it would have been expected that the total amount of 20-hydroxyecdysone extracted would have been less than the predicted amount of 140 µg. The fact that 1400 µg. were extracted would suggest that the basic assumption was erroneous, and it is now well established that 20-hydroxyecdysone acts in a multiplicity of ways as a general growth hormone and not just as a moult-inducer[56,57,58].

Considering also that samples taken contain individuals at all stages of moulting, that the value of 1·4 mg. represents an average, and that it is known from recent work that a very sharp peak of hormone is produced at one stage in the moult cycle of insects, followed by its rapid disappearance, then the actual peak of hormone titre may be extremely high for a short time in barnacles. Such a very brief, high pulse of activity would be very interesting to study in detail.

BIOASSAYS OF EXTRACTS

In order to monitor the concentration of moult-inducing substances as the extraction progressed, bioassays were carried out at each stage, using either the barnacle[45,59] or locust[43,60] bioassay. At the beginning of the investigation the exact identity of the barnacle moulting hormone(s) was not known. However, there was no reason to believe that the nature of the barnacle moulting hormone(s) would differ from those found in other arthropods. It was extremely unlikely, therefore, that moulting hormone activity would have been lost during an extraction procedure designed to isolate ecdysones, and thus some activity was expected in the bioassay.

Unfortunately at no time during the extraction was any significant activity detected in either the barnacle or locust assay. There are several possible explanations for this.

The most obvious possibility, particularly during the early stages of the extraction procedure, was the presence of material toxic to the bioassay animals in the extracts injected. The possibility appeared to be substantiated by the high mortality rate among the bioassay animals. The toxicity of extracts could have been due to residual traces of solvents; all solvents, however, were removed by pumping bioassay samples under high vacuum for several hours. The toxicity might also have been due to the concentration of some toxic substances derived from the animal during the extraction procedure. However, had this been the case, it might have been expected that the mortality rate would have gradually increased or decreased as the extraction proceded from stage to stage. But there appeared to be no

significant trend and the mortality rate remained almost constant.

Another possibility was that the bioassay material was not sufficiently soluble in aqueous medium to allow all of it to be dissolved for injection. To overcome this difficulty, an emulsifying agent, Triton-X100, was included in some of the bioassay samples and in their corresponding controls. The number of deaths in the Triton controls and in the animals injected with bioassay samples containing Triton were comparable. There was no significant increase in moulting hormone activity. However, the Triton itself appeared to be somewhat toxic, and thus the results of the addition of an emulsifying agent were somewhat inconclusive.

Various workers have shown that exogenous ecdysones are rapidly inactivated by insects and crustaceans[61,62,63,64]. This inactivation has been shown in insects to proceed via the formation of conjugates, usually glucosides, sulphates, esters, and glucuronides[65,66]. The possibility of conjugates being present in barnacle extracts was investigated by enzymic hydrolysis of the bioassay samples. Neither β-glucosidase nor esterase treatment increased moulting hormone activity on subsequent re-bioassay of the sample. There is the possibility that the major conjugates present were not α-glucosides or esters, but were either glucuronides or sulphates. Rather than using specific enzymes to identify the particular conjugates involved, it would perhaps have been more satisfactory to have used a less specific mixture of enzymes with a general hydrolytic activity.

A further possibility which might account for the lack of significant activity in the bioassays might have been a species

difference between hormone and bioassay material. Shortly after the start of the investigation, the barnacle bioassay became impracticable, and the locust abdomen assay had to be used. Carlisle[67] has shown that each species is more sensitive to homologous hormone than to one derived from another species. Perhaps the locust assay was not sufficiently sensitive to the levels of moulting hormone present in barnacle extracts.

A final possibility was that the levels of moulting hormone were just too low to be detectable by conventional bioassay methods. Subsequently, using a gas liquid chromatographic assay for ecdysones[44], it was determined that the total barnacle extract contained 1400 μg of 20-hydroxyecdysone. It was calculated that this represented 0·001 μg per barnacle. Tighe-Ford suggests, on the basis of injection of pure 20-hydroxyecdysone into barnacles, that the endogenous titre was about 0·01 μg per animal. Most bioassays were carried out at an arbitrary dose of 10 barnacle equivalents per test abdomen; which means that the dose applied to each locust was 0·01 μg which might have been expected to produce a positive result.

The fact that a positive result was not obtained could be explained on the basis that Tighe-Ford's calculations were arrived at from the injection of pure 20-hydroxyecdysone, whereas bioassay material possibly included related substances having an inhibitory action.

SYNTHESIS OF ECDYSONES

Ecdysone and its derivatives are not readily available, and early on in the present work an ecdysone-type compound was required for model studies for gas chromatography, liquid chromatography and fluorescent analysis.

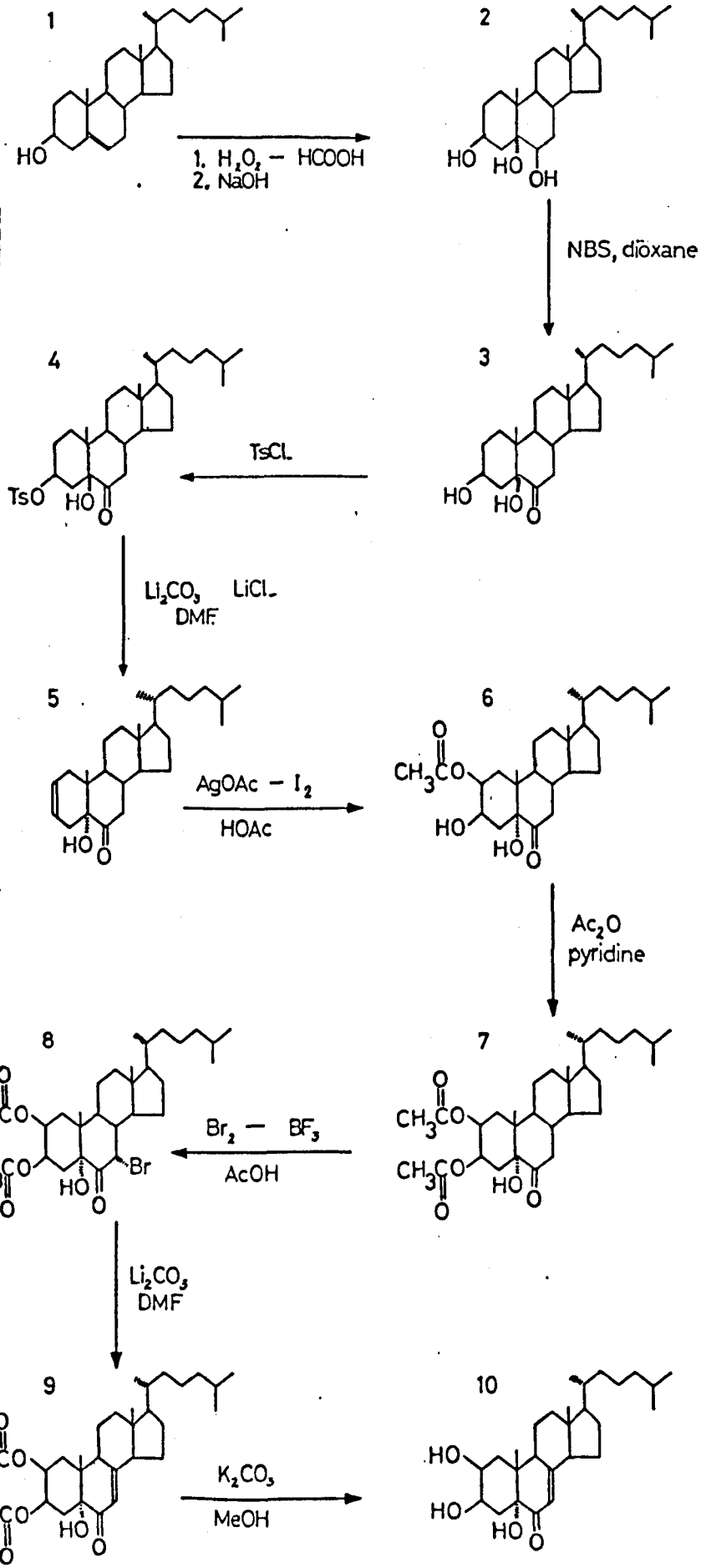
The synthesis of a complete ecdysone molecule would be difficult because of the number of steps involved and the poor overall yield. Therefore the synthesis of a simpler molecule containing some of the structural features of the ecdysone molecule was thought desirable for use as a model compound in analytical studies. The compound chosen was 2β ,3β ,5α -trihydroxycholest-7-ene-6-one (Fig. 19, structure 10.).

The ecdysone molecule (Fig. 20, structure 1) contains five principal structural elements which distinguish this group of natural products from other sterols. These are: 1) the vicinal 2β ,3β -diol group in the A ring, 2) the 5β -hydrogen atom at the A/B ring junction, 3) the α,β unsaturated 7-ene-6-one grouping in ring B, 4) the 14α-hydroxyl group at the C/D ring junction, and 5) the 17β cholestane side chain containing eight carbon atoms.

The various structural features are introduced by methods dependent on the structure of the starting material for the synthesis. As an example, the 2β ,3β -diol group can be formed either by the cis-hydroxylation of compounds having a double bond between C-2 and C-3, or by bromination of 3-oxo-5α -steroids followed by reaction of the bromo compound formed with silver acetate to introduce a 2β -acetoxy-group. The diol group is obtained by reduction of the 3-oxo group after hydrolysis of the 2β-acetoxy group.

FIGURE 19

SYNTHESIS OF 2β,3β,5α-TRIHYDROXYCHOLEST-7-ENE-6-ONE FROM CHOLESTEROL.



Auto-oxidation of the 3-oxo-5α-steroid produces the 2,3-diketone which can be reduced to the diol by sodium borohydride[68]. The Δ^7-bond is usually produced via bromination of the 6-oxo-compound at C-7 and dehydrobromination. It can also be introduced directly by oxidation of $\Delta^{5,7}$-steroids[69,70]. The 14α-hydroxyl function can be introduced in several ways, the most common being by allylic oxidation of the 7-ene-6-one system with selenium dioxide[71]. Other methods involve the formation and oxidation by peracids of the enol acetate of 7-ene-6-ones[72], microbiological hydroxylation[73], and photochemical oxidation of $\Delta^{8(14)}$-6-oxosteroids[74].

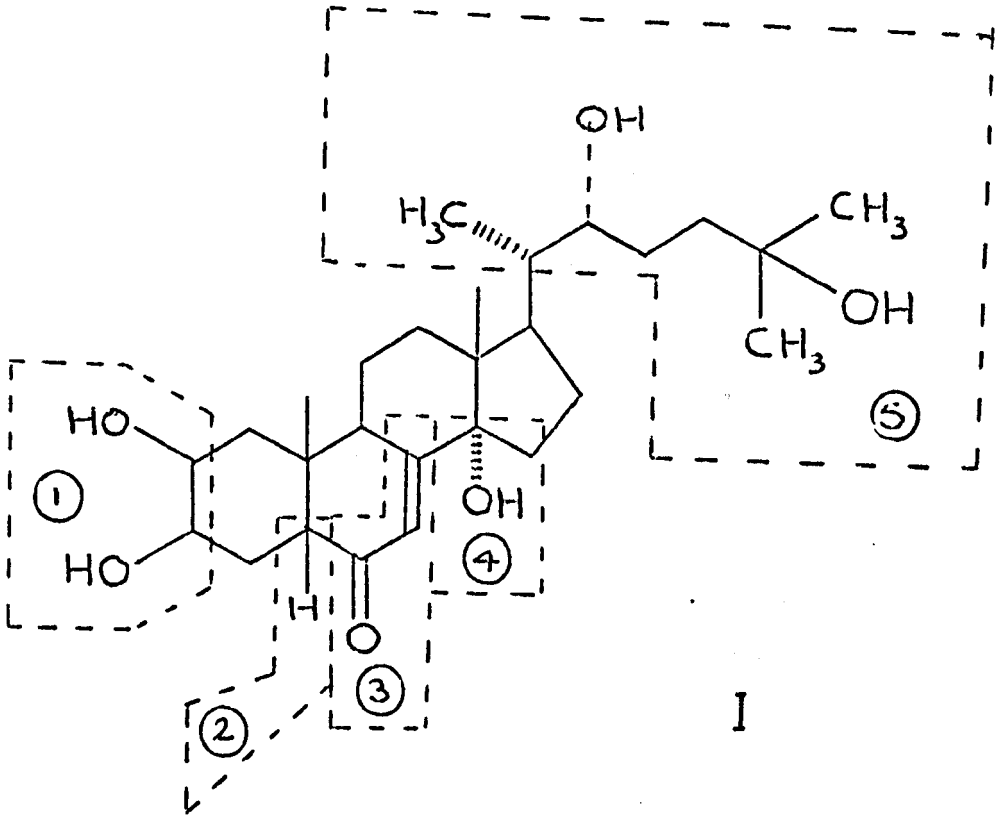
The required side chain is normally introduced by the reaction of steroids of the pregnane type, having ketonic or aldehydic groups at C-20, with the lithium or magnesium salt of the corresponding acetylene alcohols containing all the required substituents.

If the ecdysone molecules synthesised are to have moulting hormone activity, then there must be a cis-junction of the A and B rings, i.e. the proton at C-5 must be β. In 6-oxosteroids the 5α-isomer is usually more stable than the 5β-isomer[75]. However, in 2β,3β-dihydroxy-6-oxosteroids like the ecdysones, the meta-diaxial interaction of the 2β-hydroxyl group and the methyl group at C-19 in the 5α-isomer (Fig. 20, II) lowers its stability with respect to the 5β-isomer (Fig. 20, III) where such interactions are absent. In an equilibrium mixture therefore, the 5β-isomer tends to be predominant. This phenomenon is used to advantage in the synthesis of ecdysones from 5α-steroids.

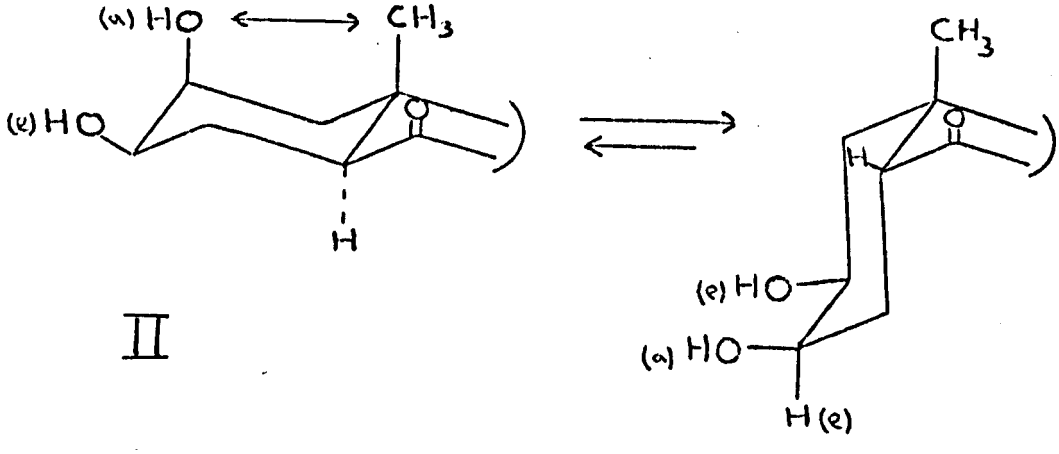
There are a number of syntheses of ecdysone which differ quite considerably both in the types of reactions used to introduce

Fig. 20

The Structural Elements of the Ecdysone Molecule



I



II

III

the various functional groups, and in the order in which the groups are introduced.

The scheme used to synthesise 2β,3β,5α-trihydroxy-cholest-7-ene-6-one (Fig. 19) was based on the synthesis of ecdysone by Siddall et al.[76,77]. Cholesterol (structure 1) was chosen as starting material because of its availability and cheapness. It was initially oxidized with performic acid to 3β,5α,6β-trihydroxy-cholestane[78] (structure 2), and further oxidized at C-6 by N-bromo-succinimide to produce 3β,5α-dihydroxycholestan-6-one (structure 3). Both reactions gave a high yield of products. By t.l.c. analysis the 3β,5α-dihydroxycholestan-6-one shown in structure 3 appeared to be contaminated with a small amount of 3β,5α,6β-trihydroxycholestane shown in structure 2. However, the tosylate (structure 4) obtained from the reaction of structure 3 with p-toluenesulphonyl chloride gave only one spot on t.l.c. after recrystallization from ethyl acetate and the correct elemental analysis and spectra for structure 4.

Detosylation of structure 4, with lithium carbonate and lithium chloride in boiling dimethylformamide, went very smoothly to give 5α-hydroxycholest-2-ene (structure 5). The reaction of structure 5 with moist silver acetate and iodine in glacial acetic acid, to give 2β-acetoxy-3β,5α-dihydroxycholestan-6-one proved difficult, giving a poor yield of product (structure 6). Meakin et al.[79] had reported a synthesis of 2β,3β-dihydroxycholestan-6-one from cholest-2-ene. They stressed the importance of the acetic acid being freshly distilled and the silver acetate freshly prepared; and recommended that the reaction be carried out under an atmosphere of nitrogen. Meakin et al. had shown by titration that the electrophilic iodine generated by

silver acetate and iodine in moist acetic acid fell appreciably in air, even at 20°, but that this loss was avoided by using a nitrogen atmosphere. Using the conditions described by Meakin *et al.*[79], 5α-hydroxycholest-2-en-6-one (structure 5) was successfully converted with iodine and silver acetate in moist acetic acid into 2β-acetoxy-3β,5α-dihydroxycholestan-6-one (structure 6) in good yield.

The C-3 hydroxyl group of structure 6 was easily acetylated, using acetic anhydride in pyridine to give the diacetate (structure 7). The diacetate was brominated at C-7 using bromine in acetic acid with boron trifluoride as catalyst to give 2β,3β-diacetoxy-7α-bromo-5α-hydroxycholestan-6-one (structure 8). The configuration of the bromine atom at C-7 was checked by determination of the coupling constant for the protons at C-8 and C-7 (Fig. 21).

The fact that the bromine atom in structure 8 was 7α- instead of 7β- enabled structure 8 to be readily dehydrobrominated, using lithium carbonate in boiling D.M.F. to give 2β,3β-diacetoxy-5α-hydroxycholest-7-ene-6-one (structure 9). The hydrolysis of structure 9 initially proved difficult, the ultraviolet extinction coefficient of the hydrolysis product being much lower than was to be expected for the pure compound. The hydrolysis had previously been carried out using a 2% solution of potassium carbonate in 90% aqueous methanol, but van Bever *et al.*[80] had shown that 2β,3β-diacetoxy-14α-hydroxy-5β-androst-7-ene-6,17-dione could be hydrolysed using a 0·033% solution of potassium carbonate in methanol. The hydrolysis of structure 9 was carried out successfully, employing the latter mild conditions. The reaction was followed by t.l.c. which showed that the hydrolysis was complete after 15 minutes at 50°. It is possible that the stronger

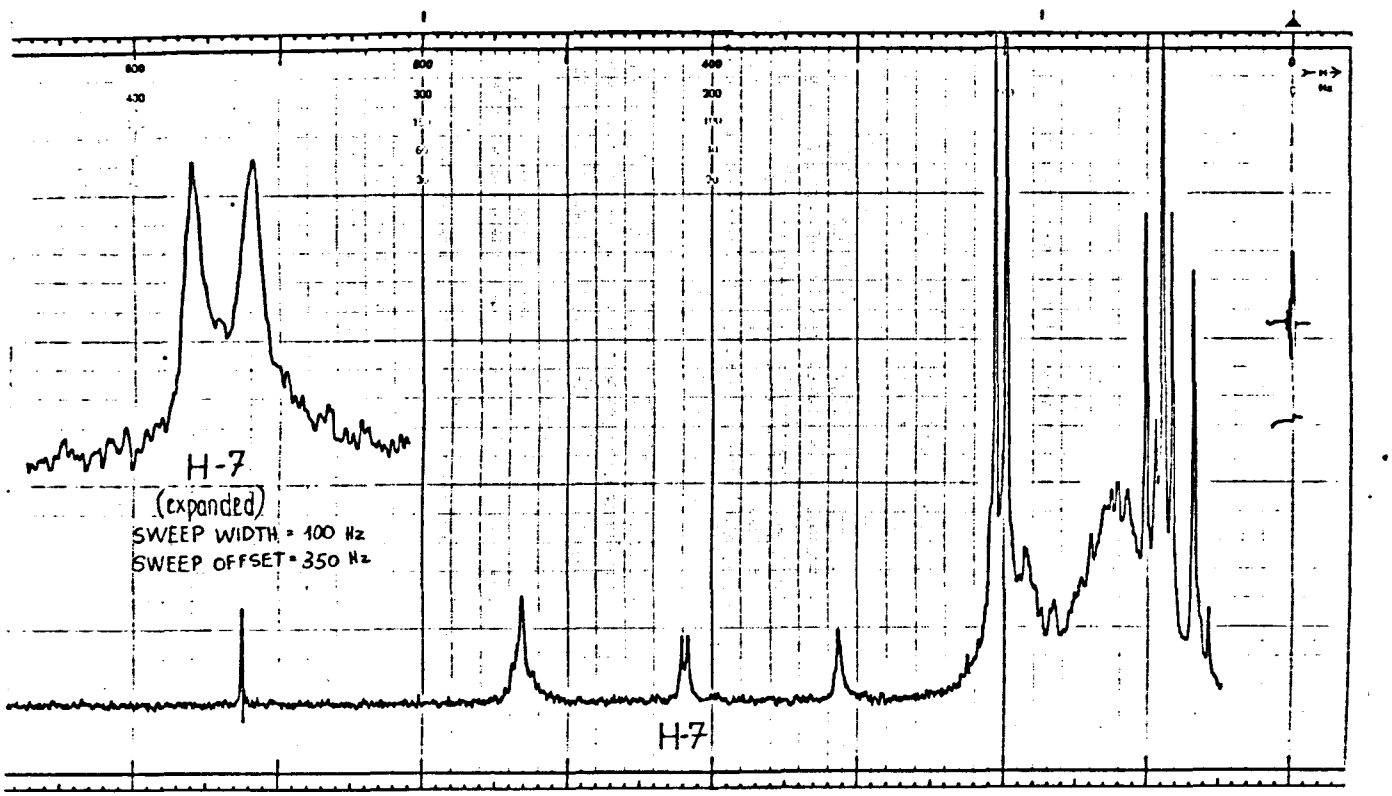
Figure 21

NMR Spectrum of 2β,3β-diacetoxy-7α-bromo-5α-hydroxycholestan-6-one.

The spectrum was obtained on a Varian HA 100,100 MHz instrument, the sample being dissolved in deuterochloroform. Expansion of the peak due to the proton at H-7 showed that the coupling constant between protons at H-7 and H-8 was 4·2 Hz. Thus the bromine atom at C-7 was 'α' in relation to the plane of the ring.

Fig. 21

NMR Spectrum of 2β,3β-diacetoxy-7α-bromo-5α-hydroxycholestan-6-one (See Fig. 19, structure 8).

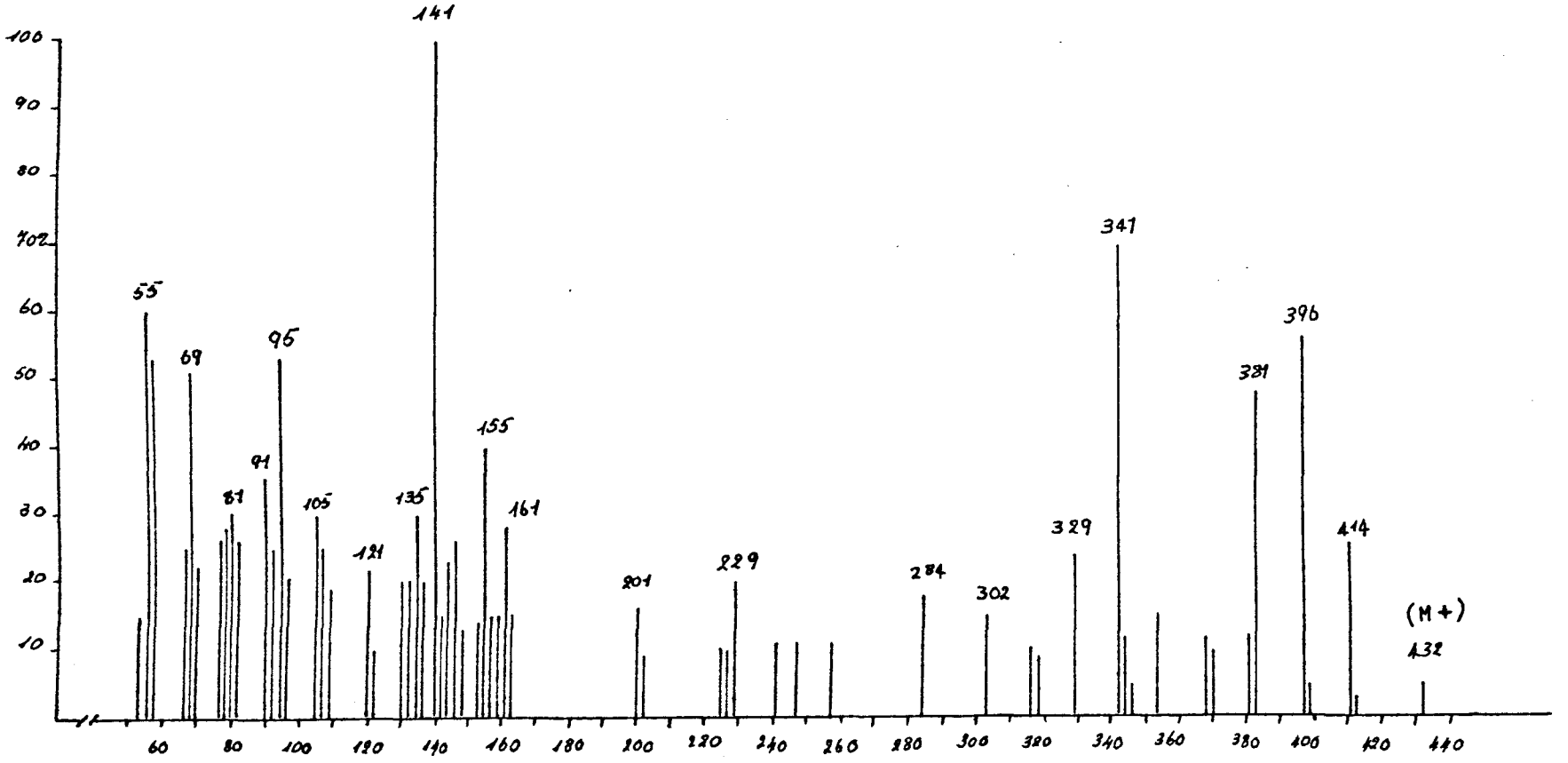


hydrolysis conditions used previously not only hydrolysed the diacetate but also caused some isomerization of the Δ^7-bond out of conjugation with the carbonyl group. Every effort was made in the isolation of the hydrolysis product, 2β,3β,5α-trihydroxycholest-7-ene-6-one (structure 10) to use the mildest possible conditions so as to avoid the possibility of isomerization.

The final product (structure 10) was obtained as colourless compound, soluble in pyridine but practically insoluble in most other solvents. It was crystallized from methanol in a microcrystalline form. The insolubility of structure 10 is probably due to the nearness to each other of the two hydroxyl groups and the carbonyl group which could make solvation very difficult. The g.l.c. and fluorescent analysis of compound (structure 10) are described on pages 65 - 67.

Fig. 22

Mass Spectrum of 2β,3β,5α-Trihydroxycholest-7-ene-6-one.



DETERMINATION OF ECDYSONES BY SPECTROFLUORIMETRY

Work on the isolation of arthropod moulting hormones from animals has in the past been relatively slow because of the need to rely almost entirely on bioassays to give an indication of the hormonal activity of the various extracts obtained. Bioassays tend to be lengthy, slow, expensive, unspecific and not always sensitive enough to detect the very low concentration of moulting hormone in extracts. Chemical methods for ecdysterol analysis would be more desirable.

One promising method appeared to be acid-induced fluorescence[81]. Many sterols are known to fluoresce in concentrated sulphuric acid, and this fact has been used for quantitative analysis at very low concentrations. A preliminary report stated that ecdysones fluoresce in sulphuric acid[82]. A principle requirement for the estimation of 20-hydroxyecdysone in arthropod extracts would be sensitivity to submicrogram quantities of the sterols, comparable to that of the already available insect bioassays.

Absorption of radiant energy, subsequently re-emitted at a higher wave-length, causes the fluorescent emission[83]. The sensitivity of fluorimeters has now been so developed as to be limited not by the minimum intensity of light which they are capable of detecting, but by the magnitude of the blank value for any particular determination. A number of factors can affect this blank value: 1) The presence of trace amounts of fluorescent impurities in the solutions being measured, 2) Instrumental defects, 3) The blank value may be increased by the use of an inefficient filter system in filter fluorimetry, and 4) Fluorescence from secondary filters may be caused by excited light from the

solution or cuvette.

All the factors affecting the blank value can be minimized by using efficient mono-chromators for isolating the exciting and fluorescent light, instruments with well designed cell compartments, and cuvettes made from material with minimum fluorescence.

However, given that all the largely practical difficulties contributing to the blank value can be overcome, there still remains one problem which is not easily solved. This is Raman emission from the pure solvent. Raman emission occurs at longer wavelengths than the exciting light and can cause serious interference when weakly fluorescent solutions are being measured.

All solvents containing hydroxyl groups show a Raman band in the region of 3300 cm^{-1}, and ethanol has an inflection at about 3000 cm^{-1}, due to the presence of both C-H and O-H bonds in this substance.

The intensity of fluorescence is proportional to concentration, and a limiting intensity due to virtually complete absorption of the excited light can be expected. Nevertheless, at a certain concentration the intensity usually goes through a flat maximum and then decreases in relation to any further increase in concentration. The same value of fluorescence intensity could therefore correspond to two different values of concentration, a phenomenon which is due to 'self-quenching'.

Gilgan and Zuick[40] have briefly described the use of sulphuric acid-induced fluorescence of 20-hydroxyecdysone. They obtained a linear relationship between fluorescence and concentration in the range 0·005 µg. to 10 µg. but, because of high blank values they found the

Figure 23

Concentration of 2β,3β,5α-trihydroxycholest-7-ene-6-one versus Fluorescence Intensity.

'A' and 'B' show plots of the acid-induced fluorescence of 2β,3β,5α-trihydroxycholest-7-ene-6-one after one hour at room temperature in a solution of absolute ethanol-concentrated sulphuric acid (1:1 v/v) against sterol concentration. Plot 'B' shows this graph expanded in the concentration range 0 - 2·0 $\mu g\ ml^{-1}$.

procedure limited to amounts in excess of 0·05 μg. This sensitivity is comparable with the barnacle bioassay which is sensitive to at least 0·02 μg. of crystalline 20-hydroxyecdysone per animal, and with that of the Calliphora assay which is sensitive to 0·01 μg. of ecdysone per animal. The suitability of this method of analysis of trace amounts of ecdysones in animal tissues was not however investigated by these workers.

Because of the scarcity of 20-hydroxyecdysone, the synthetic compound 2β,3β,5α-trihydroxycholest-7-ene-6-one (Fig. 20, structure 10) was used in our own investigations into the possibility of using a fluorescent analysis for ecdysones. The compound in a solution of ethanol-concentrated sulphuric acid (1:1) produced a fluorescent emission at 435 nm when excited by radiation of 400 nm.

Fig. 23 shows a plot of concentration of synthetic sterol against fluorescent intensity. The shape of curve A can be explained in terms of 'self-quenching'. One problem is that two values of concentration can have the same fluorescent intensity. However, graph B reveals that there is a linear relationship between fluorescent intensity and concentration of sterol in the region 0·1 μg. - 2·0 μg. Unfortunately the gradient of the line in this region was not very steep. It would have been more satisfactory had the increase in fluorescence been greater in proportion to the increases in concentration.

The emission spectrum of the ethanol-sulphuric acid solution of the synthetic sterol is shown in Fig. 24. Some interference was found from a Rayleigh scatter peak, and a Raman peak, which was present at 457 nm. These peaks overlapped the fluorescence peak to a certain

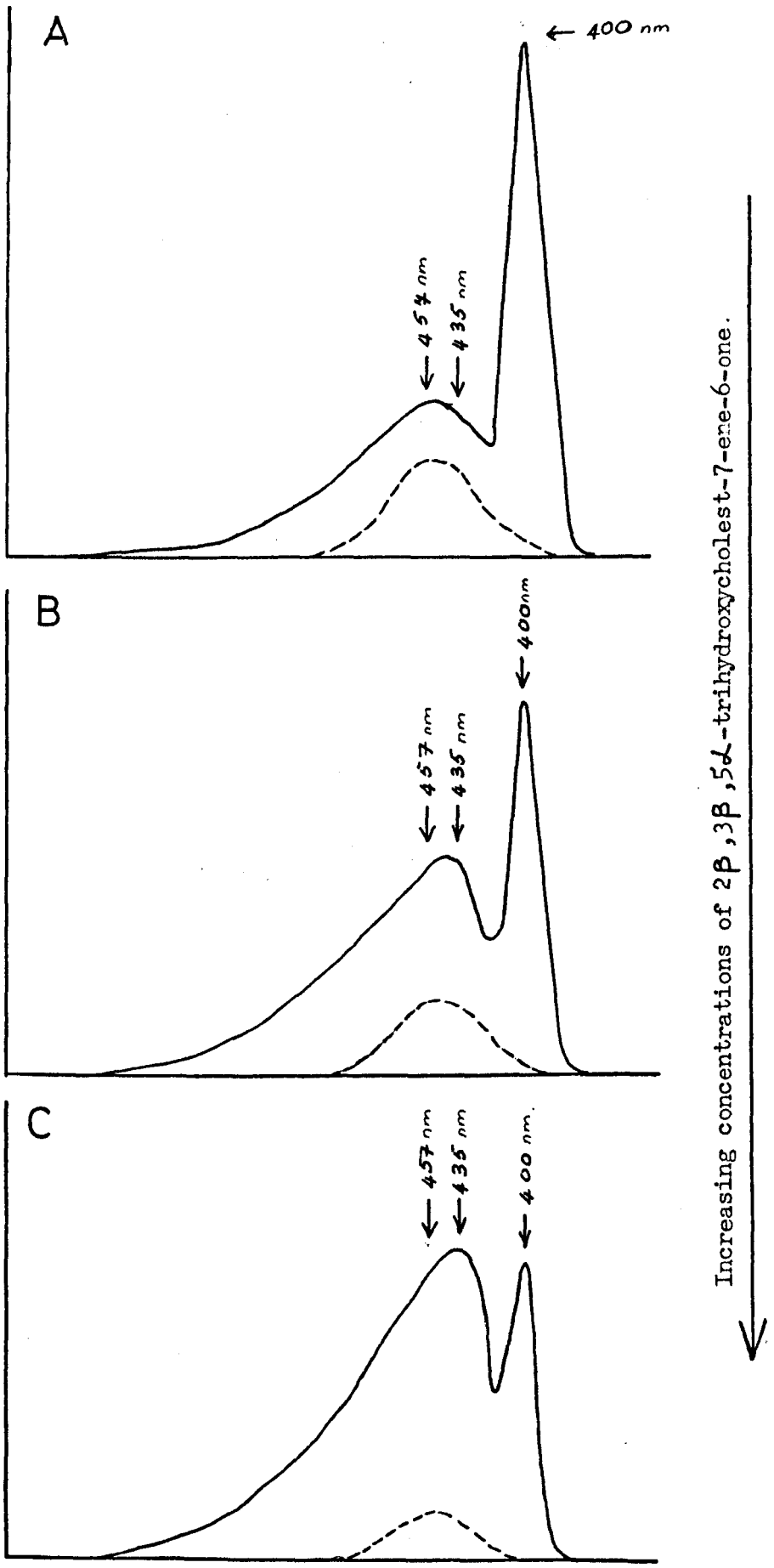
Figure 24

Representative Emission Spectra of 2β,3β,5α-trihydroxy-cholest-7-ene-6-one in Ethanol-Sulphuric Acid.

Curves A, B and C show representative fluorescent emission spectra of 2β,3β,5α-trihydroxycholest-7-ene-6-one in absolute ethanol-concentrated sulphuric acid (1:1 v/v). The peak at 400 nm was due to Rayleigh scatter. The peak at 437 nm was due to Raman emission which tended to obscure the fluorescence peak at 435 nm at lower sterol concentrations. The broken line represents the differing contributions of the Raman peak to the total emission.

Fig. 24

Representative emission spectra of 2β, 3β, 5α-trihydroxycholest-7-ene-6-one in ethanol-sulphuric acid (1:1 v/v).



extent. In the concentration range below 0·2 $\mu g.ml^{-1}$, the intensity of the Raman peak amounted to at least 50% of the total peak height. In the lower concentration ranges the contribution of the Raman peak to the fluorescence peak became a limiting factor to the sensitivity. At concentration ranges of synthetic sterol below 0·1 $\mu g. ml^{-1}$, the intensity of the Raman peak in proportion to that of the fluorescence emission was such that the latter could not be accurately measured.

In order to ascertain the usefulness of the fluorescent method for estimating ecdysone concentrations in barnacle extracts, the synthetic sterol was mixed with barnacle material. Here further problems were encountered as a result of interference from fluorophores in the barnacle material. The intensity of the interference was such that it was impossible to detect the sterol in the mixed samples.

Although the fluorometric assay provides a useful means of estimating ecdysone concentrations, it would seem to be of only limited application. It is most accurate and useful when the concentration of interfering fluorophores is negligible in comparison with the concentration of ecdysone in the sample. Therefore, if the fluorometric assay is to be used, it must incorporate an extremely efficient clean-up procedure.

The fluorescent assay is probably not specific for individual ecdysones, but is probably specific for a fluorophore produced from the tetracyclic nucleus in the ethanol-sulphuric acid mixture, possibly by dehydration and subsequent increased conjugation from the 7-ene-6-one system. It thus represents a potentially useful general ecdysone assay.

GAS CHROMATOGRAPHIC ANALYSIS OF ECDYSONES

The analysis of ecdysones by gas liquid chromatography (g.l.c.) is difficult because of the fact that ecdysones are large polar molecules which are heat-sensitive and non-volatile. In order to analyse ecdysones by g.l.c. it is necessary to form suitable volatile derivatives. The methods of derivatisation and types of derivatives formed are limited because of the sensitivity of ecdysones to heat, acids and bases. Since ecdysones are polyfunctional compounds, derivatisation often results in mixtures of derivatives being formed. The g.l.c. separations which have been carried out involve the formation of trimethylsilyl ethers, O-methoximes, and heptafluorobutyrate ester derivatives.

The ease with which ecdysones can be silylated depends upon the position and immediate environment of the hydroxyl groups on the sterol skeleton[84]. When hydroxyl groups are in unhindered positions on the sterol nucleus, they are able to be silylated with quite mild reagents such as bis(trimethylsilyl) acetamide (BSA). For example, in 3β,14α-dihydroxycholestane and 3β,14β-dihydroxycholestane, both the hydroxyl groups are readily silylated with BSA at room temperature. When hydroxyl groups are sterically hindered they are more difficult to silylate. For example, in 3α,20α-dihydroxypregnane the hydroxyl group at C-20 is difficult to silylate with BSA, but the 20-hydroxyl group in 3α,17α,20α-trihydroxypregnane is silylated more easily. The 15β-hydroxyl group in 3β,14β,15β-trihydroxycholestane is easier to silylate with BSA than that in 15β-hydroxypreg-4-ene-3,20-dione which needs trimethylchlorosilane (TMCS) as a catalyst. Surprisingly, it appears that the introduction of a hydroxyl group next to a hindered

hydroxyl group sometimes eases the silylation of the hindered hydroxyl group. In 5α-hydroxycholestane-3,6-diacetate the 5α-hydroxyl group is easily silylated with BSA at room temperature. However, the 5α-hydroxyl group in 5α-hydroxycholestan-6-one-3-acetate is difficult to silylate and silylation needs to be catalysed by TMCS, possibly because of intra-molecular hydrogen bonding in the ketone. Conjugated ketones, such as that present in 17β-hydroxyandrost-4-ene-3-one, are enolised when treated with a mixture of BSA-TMCS, forming a mixture of the mono 17β-TMS ether, and the 3-TMS-enol ether, so producing several peaks at shorter retention time than the 17β-TMS ether.

THE G.L.C. PROPERTIES OF 2β,3β,5α-TRIHYDROXYCHOLEST-7-ENE-6-ONE

It was decided to investigate the G.L.C. analysis of ecdysones, using the synthetic compound 2β,3β,5α-trihydroxycholest-7-ene-6-one (Fig. 19, structure 10.). This compound has the additional feature of a 5α-hydroxyl group, similar to the 5β-hydroxyl group found in a few phytoecdysones. Also, the 5α-hydroxyl group is next to a carbonyl group which will make its derivatisation difficult.

BSA could be used to derivatise the sterol, but this would derivatise only the 2β and 3β-hydroxyl groups, and not the 5α-hydroxyl group, because of the latter's proximity to the 6-keto function. A mixture of BSA and TMCS would have derivatised all three hydroxyl groups, but caused enolisation of the 7-ene-6-one system, producing a mixture of TMS ethers and TMS-enol ethers, thus giving rise to a number of peaks on the g.l.c. The synthetic sterol was reacted with trimethylsilylimidazole (TMSI) at 80° for 20 hours. However, on analysis of the

derivative produced by g.l.c., using F.I. detection, a number of peaks were produced which suggested that the derivative was decomposing on the column. In the light of more recent information[84], however, it is possible that the TMSI used for derivatisation could have contained a trace of TMCS which catalysed the enolization, hence producing a number of peaks on the g.l.c.

Because of the initial failure to obtain a useful g.l.c. derivative of the synthetic sterol using TMSI as described above, it was decided to protect the ketone group by methoxime formation[85]. It has been reported that the 5α-hydroxyl group in 5α-hydroxycholestan-3,6-diacetate can be derivatised using BSA alone, so it is quite possible that the 5α-hydroxyl group in the O-methoximated synthetic triol could be derivatised using BSA alone. However, since the ketone function was now protected and enolisation could not take place, it was decided to use stronger silylating conditions to ensure that the 5α-hydroxyl group would be derivatised. The derivatisation reagent used was a mixture of TMSI-BSA-TMCS (5:5:4). The mixed derivative, formed by silylation of the O-methoxime derivative of the synthetic triol overnight with the latter reagent mixture, was analysed by g.l.c., which showed only one peak; the syn and anti methoximes not being separated on the column used. The derivative was subjected to combined g.l.c. - m.s. The mass spectrum and G.L.C. trace obtained (Figs. 25 and 26) showed that the mixed derivative was in fact 2β,3β,5α-tris(trimethylsilyloxy)cholest-7-ene-6-one methoxime.

This mixed derivative produced a satisfactory derivative, but each determination took at least two days to complete which, for a potential routine procedure, is a little lengthy.

Figure 25

G.L.C. - M.S. of 2β,3β,5α-tris(trimethylsilyloxy)cholest-7-ene-6-one methoxime.

408 (M - 270); 438 (M - 180); 498 (M - 180); 528 (M - 45 - 15 - 90); 545 (M - 45 - 90); 618 (M - 45 - 15); 633 (M - 45).

Fig. 25

G.L.C. - M.S. of 2β,3β,5α-tris(trimethylsilyloxy)cholest-7-ene-6-one methoxime.

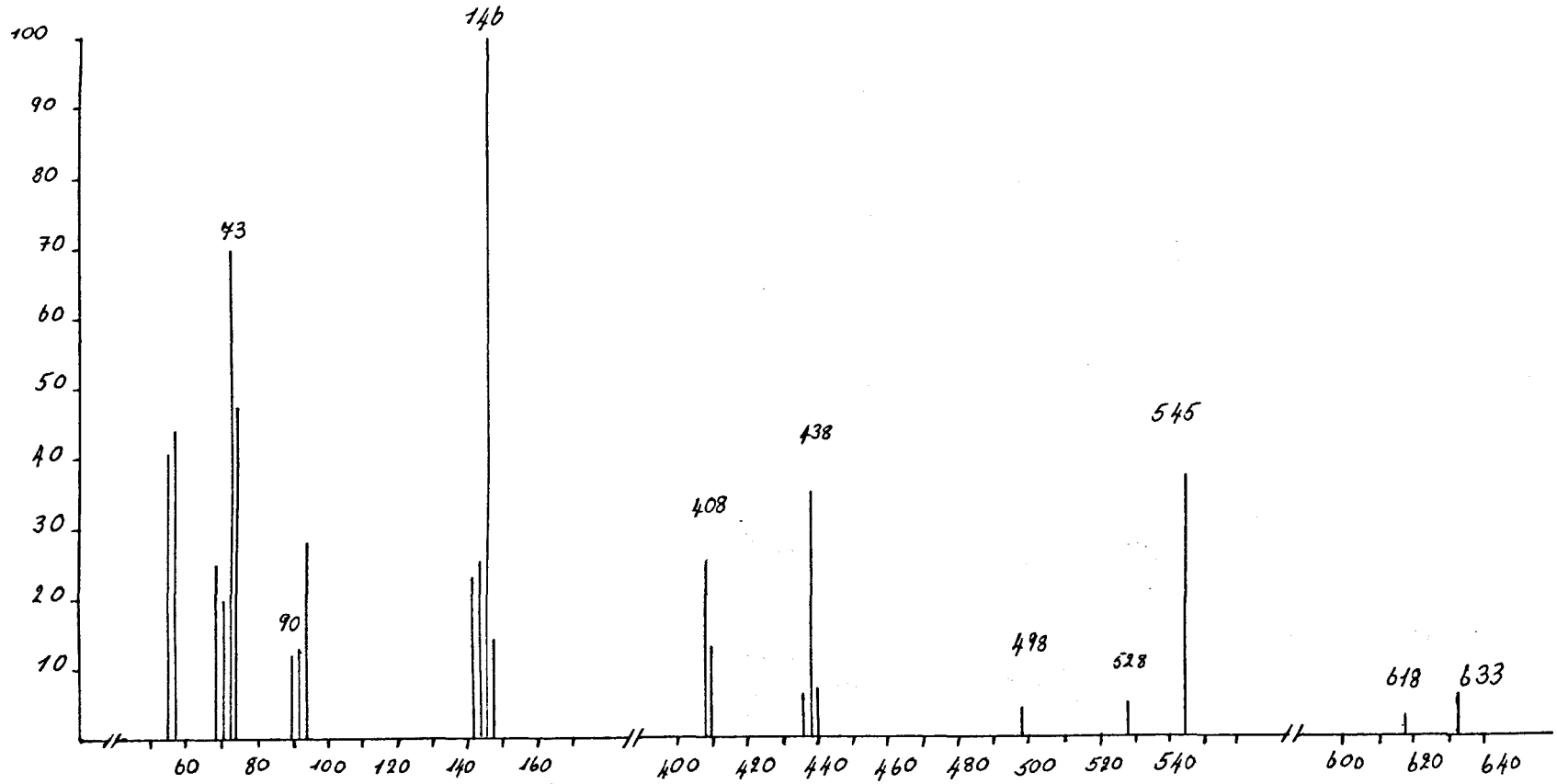


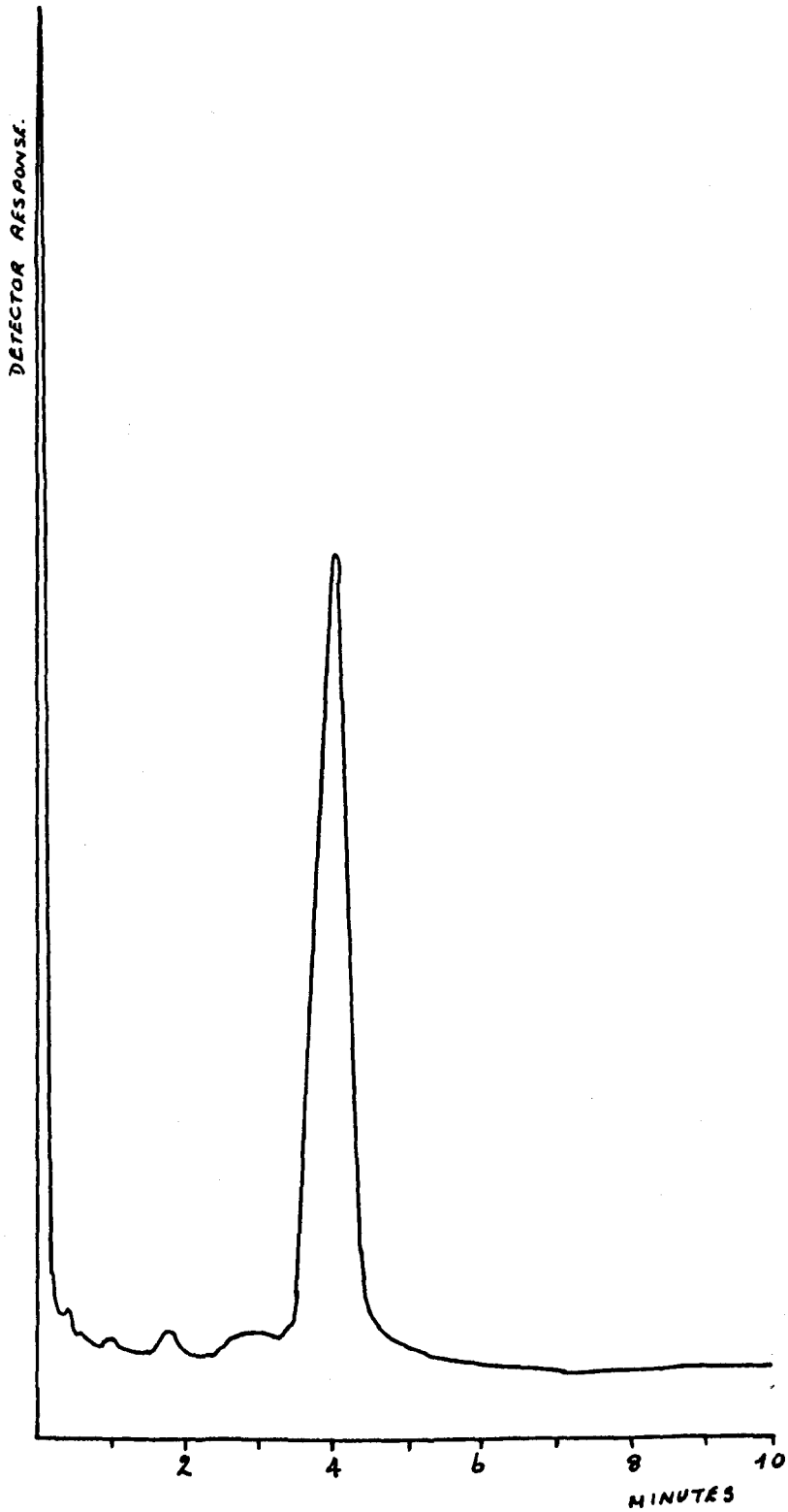
Figure 26

G.L.C. Trace of 2β,3β,5α-tris(trimethylsilyloxy)cholest-7-ene-6-one methoxime.

2β,3β,5α-trihydroxycholest-7-ene-6-one was reacted with O-methoxime hydrochloride and the methoxime formed was reacted with a mixture of TMSI-BSA-TMCS (5:5:4) to form 2β,3β,5α-tris(trimethylsilyloxy)cholest-7-ene-6-one methoxime. This was gas chromatographed, using an 18" column packed with 1% OV 101 on CQ at 230° using nitrogen as the carrier gas (60 ml min^{-1}) and F.I. detection.

Fig. 26

G.L.C. Trace of 2β,3β,5α-tris(trimethylsilyloxy)-cholest-7-ene-6-one methoxime.



GAS CHROMATOGRAPHIC ANALYSIS OF ECDYSONES USING AN ELECTRON CAPTURE DETECTOR (e.c.d.).

Although the method described above for the analysis of ecdysone type molecules was adequate, it was lengthy and only as sensitive as the limiting sensitivity of the flame ionization detector (FID) which is about 50 nanograms. Because of the extremely low concentration of ecdysones in arthropods, and hence the expected low levels prevailing in barnacle extracts, a more sensitive method for the detection of ecdysones was desirable. Such a method became available in the laboratory, using an electron capture detector[44]. This method was potentially capable of detecting picogram quantities of ecdysones.

The g.l.c. analysis of ecdysones using an e.c. detector was first reported by Ikekawa[83]. Ikekawa first silylated the molecule, and then prepared heptafluorobutyryl derivative by an exchange reaction between trimethylsilyl and heptafluorobutyryl (HFB) moieties. He reported that the HFB derivatives had excellent g.l.c. properties, and could be detected by FID and e.c. detectors. The method of Ikekawa was used to assay for 20-hydroxyecdysone by Gagosian[51] in the isolation of the moulting hormones of the lobster Homarus americanus.

Borst and O'Connor[29] have shown that the TMS derivative of 20-hydroxyecdysone can be detected at levels below 50 nanograms., without forming the heptafluorobutyrate derivative. In our own laboratory it was discovered that the TMS ethers of 20-hydroxyecdysone could be detected at low levels by an electron capture detector. If 20-hydroxyecdysone was reacted at 22°, then only the penta-TMS derivative was formed. This was because the 20-OH group was being hindered by the

bulky TMS-ether grouping at C-22. If 20-hydroxyecdysone was reacted with commercial TMSI for one hour at 100°, then two peaks were observed on the chromatogram, the peak at shorter retention time being the more intense initially and its intensity increasing with prolonged heating. If, however, 20-hydroxyecdysone was treated in the same way with TMSI prepared in the laboratory, then the peak at longer retention time was by far the more intense. From mass spectral analysis it was found that both peaks had the same molecular weight.

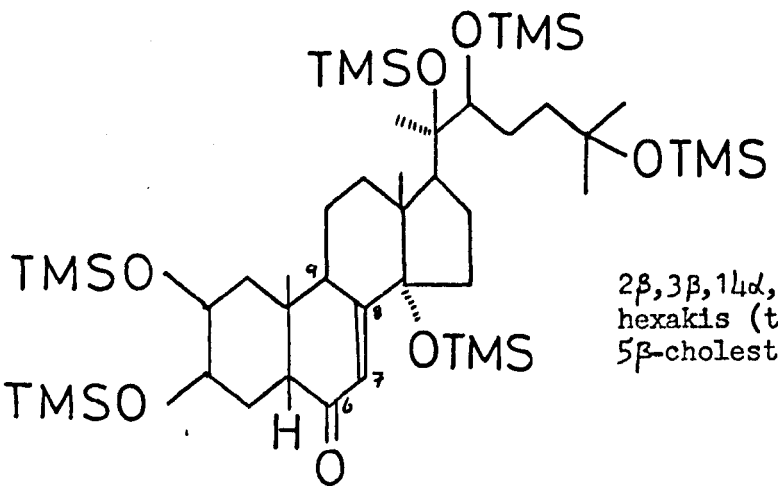
The appearance of two peaks is apparently due to the partial isomerization at some as yet undetermined point in the molecule. The retention time difference between the 5β and 5α isomers is not great enough to account satisfactorily for the observed facts. One possible explanation requires epimerization around the C-9 centre after enolization of the α,β-unsaturated linkage as shown in Fig. 27. After epimerization, the proton at C-9 would be 'β' instead of 'α' to the plane of the ring system, which would deform the ring quite substantially. This would give the molecule a very different shape and hence alter its retention time on the g.l.c.

On heating for one hour at 100° with commercial TMSI, the predominant peak was the one at shorter retention time. However, using TMSI prepared in the laboratory, the peak of longer retention time predominated. When determining ecdysones in crude biological material, two peaks of about equal intensity were obtained using TMSI prepared in the laboratory. It was concluded that the commercial TMSI contained a small amount of trimethylchlorosilane (TMCS), since addition of small amounts of TMCS could catalyse isomerization, producing the peak of shorter

Fig. 27

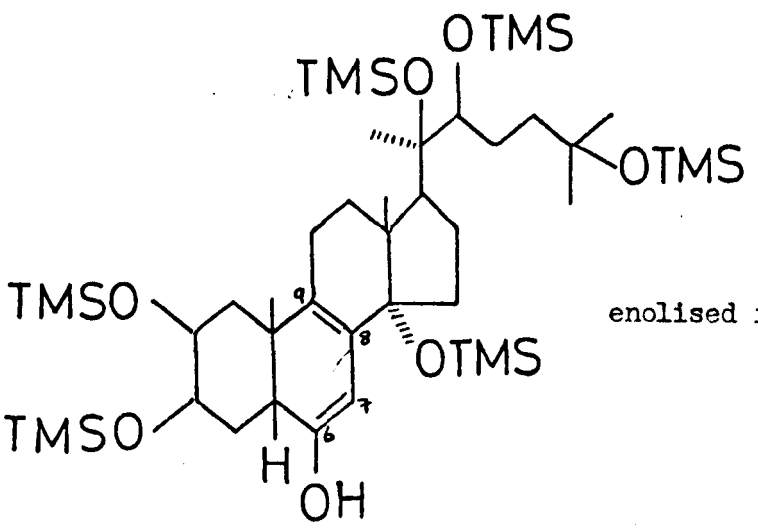
Structure of Derivatives Formed for G.L.C. Analysis.

1.



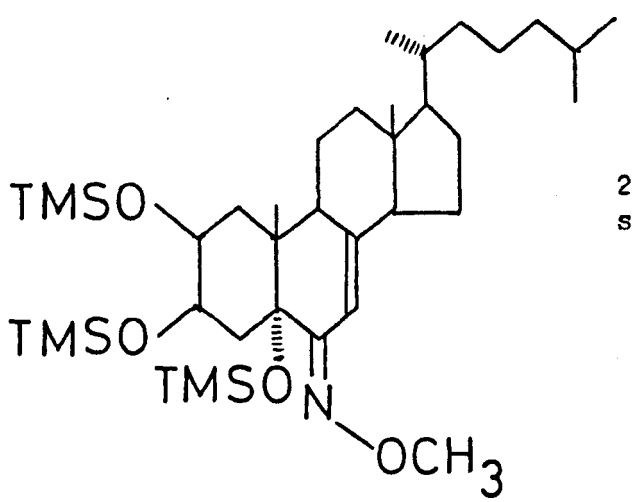
2β,3β,14α,20S,22R,25-hexakis (trimethylsilyloxy)-5β-cholest-7-ene-6-one.

2.



enolised form of (1)

3.



2β,3β,5α-tris(trimethylsilyloxy)cholest-7-ene-6-one.

retention time. Alternatively, imidazole from the TMSI could catalyse the isomerization.

The method as it is used at present is particularly reliable and trouble-free. The response of the e.c.d. is linear over the range 5 - 700 picograms for compounds such as ecdysone, 20-hydroxyecdysone, inokosterone, cyasterone, and 2β,3β,14α-trihydroxy-5β-cholest-7-ene-6-one. It is equally sensitive to TMS ether derivatives and heptafluorobutyrate derivatives. The analysis of natural products for ecdysones by g.l.c. using e.c. detection of TMS ether derivatives is more selective than e.c. detection of heptafluorobutyrate derivatives. This is because fewer unrelated compounds will form TMS derivatives which are detectable by e.c.d.

The isomerization problems have been resolved and the method of derivatization now produces one peak instead of two. The peak produced is the one of longer retention time which is due to the natural isomer. To obtain one peak the silylation is carried out in dry pyridine for six hours at 100°C.

Pyridine probably acts as the conjugate acid for the imidazole produced from reacted TMSI, and the conjugate base for TMCS. In pyridine the isomerization does not take place, because both the imidazole and TMCS are neutralized by pyridine, which is not acidic or basic enough to catalyse the isomerization.

CONCLUSION

An extraction procedure has been developed which will isolate moulting hormones from barnacles. The procedure resulted in the preparation of extracts which were sufficiently refined to allow the moulting hormones to be identified and quantified. Unfortunately, the concentration of moulting hormones was not high enough to enable them to be easily isolated in a pure state. The major moulting hormone in Balanus balanoides was found to be 20-hydroxyecdysone at a concentration of 1 $\mu g.kg^{-1}$. Ecdysone was present at a much lower concentration of 6 $ng.kg^{-1}$. The possible presence of other ecdysones was indicated by gas liquid chromatography, but these could not be identified.

The level of 20-hydroxyecdysone found in extracts of Balanus balanoides was comparable with the levels of moulting hormone found in crustaceans by other workers. Ecdysone has not previously been found in crustaceans. It was probably detected in barnacle extracts only because of the extreme sensitivity of the detection method used.

The low levels of moulting hormones found in crustaceans, compared with the levels found in insects, may be due to the differences in the availability of material suitable for mass extraction. Insect material can be obtained with all the individuals at a known stage in the life cycle, whereas it is difficult to collect crustacean material containing individuals at similar stages in their life cycle. This is an important consideration when it is known that peak hormone titres occur transiently on or before the time of ecdysis. Such transient peak titres would be swamped by the comparatively low concentration of moulting hormones present at other periods of the

moult cycle.

The problem of studying moulting hormones which occur at such low concentrations in crustacean material will be overcome only when either synchronous culture populations become readily available for study, or when an *in vitro* organ culture can be successfully performed. *In vitro* culture would have a particular advantage over mass extraction procedures in yielding a cleaner hormone preparation from smaller quantities of starting material, and avoiding the necessity of handling large quantities of calcareous shell.

A further advantage arising from *in vitro* culture of specific organs would be the possibility of studying *in vivo* hormone biosynthesis and catabolism. In the barnacle specific endocrine organs have not yet been identified. However, the nervous systems of other crustaceans have been shown to be involved in hormone production. Therefore, *in vitro* culture of barnacle nervous systems could produce important information about cirripid endocrinology.

Bioassays are not an adequate measure of moulting hormone activity in crustacean extracts because of the low concentrations of moulting hormones in crustacea, and because of the low sensitivity of the assays available. Although it is convenient to assay crustacean material on insect test material, it would be preferable to have a readily available crustacean bioassay. A further disadvantage of bioassays is the susceptibility of test material to toxic substances in extracts. For these reasons a chemical assay was sought.

Acid-induced fluorescence of ecdysone-type compounds was investigated as a possible chemical assay for ecdysones. Although

this method could produce a reliable estimate of ecdysone-type substances present, it was of use in the analysis of biological materials for ecdysone only when the material was free of interfering fluorophores. Unfortunately, this method lacked sensitivity in the concentration ranges expected in barnacle material. This method also had the disadvantage of not differentiating between different ecdysones. It would be interesting to investigate the nature of the fluorescent species produced by ecdysones in concentrated sulphuric acid.

The gas liquid chromatographic properties of the trimethylsilyl ether derivatives of ecdysones were also investigated in the hope of finding a sensitive assay method. Gas liquid chromatography, using a flame ionization detector, was found not to be sensitive enough to detect hormone levels in biological extracts, having a detection limit of 50 ng. Gas liquid chromatography of trimethylsilyl ethers of ecdysones, using electron capture detection, was shown to be a very satisfactory method for their determination, with adequate sensitivity, having a linear response to the trimethylsilyl ethers of ecdysone in the range 5 - 700 picograms. This method could be used with biological materials with minimal purification, and it was capable of differentiating between ecdysones.

The gas liquid chromatographic method of estimating ecdysone could be used in a study of how average moulting hormone titres vary in barnacles throughout the year. This would indicate the period of most sensitive growth.

Such an investigation is planned. It involves an initial experiment to determine the recovery of 20-hydroxyecdysone from small (0·3 - 0·5 kg.) samples, which would be followed by analysis of barnacle samples taken at monthly intervals from the same area.

EXPERIMENTAL

MATERIALS AND METHODS

1. Extraction.

Solvents of general purpose grade were used for preliminary extraction of barnacle material and the subsequent solvent partitions.

The barnacle material was ground using a "Unishear" 2 H.P., three phase, double-acting grinder.

Filtration was carried out using a battery of four 10-litre Büchner flasks, fitted with 24 cm. diameter polythene funnels with a removable drainage tube. A hydraulic press, 6" in diameter and with an approximate capacity of one litre, was also used for filtration. This was kindly loaned by Progress Engineering Ltd., Burslem, Stoke-on-Trent.

Large-scale distillation was carried out using three "Quickfit" distillation sets each of 20-litre capacity, as well as a "Quickfit" rising film evaporator with a Büchi steam generator. Small-scale evaporations were carried out using a Büchi rotary evaporator, incorporating a controlled temperature bath.

Large-scale solvent partitions were carried out using a specially designed semi-automatic liquid/liquid partitioning apparatus illustrated in Fig. 10. Other smaller-scale solvent partitions were carried out in a standard laboratory separatory funnel of up to 20-litre capacity.

2. Column Chromatography.

Column chromatography was carried out using glass columns

fitted with teflon Roto-flow taps.

Packing materials used were: 1) 'Davison' silica gel, grade 950, mesh 60 - 200, 2) Hydrophobic celite prepared by treating celite as described by Howard et al.[47]. Eluting solvents were re-distilled and dried before use.

3. Thin Layer Chromatography.

Thin layer chromatography was carried out on 5 x 20 cm. or 20 x 20 cm. glass plates, usually coated with a 0·25 mm. layer of PF_{254} (Merck) fluorescent silica gel. Plates used for the preparation of samples for g.l.c. were coated with a 0·6 mm. layer of PF_{254} silica gel. Plates were equilibrated overnight over a saturated solution of sodium chloride.

4. Solvents and Chemicals.

N-N'-dimethylformamide and pyridine were dried with barium oxide powder and purified by distillation. Toluene and benzene were distilled and dried over sodium wire, as this was found to be sufficient purification prior to use with the e.c. detector of the g.l.c. Acetic acid was refluxed over and distilled from chromic oxide. Acetic anhydride was distilled from phosphorus pentoxide. Concentrated sulphuric acid was distilled under reduced pressure. Absolute ethanol was refluxed over potassium hydroxide pellets and distilled.

Laboratory reagent grade cholesterol was used as starting material for the synthesis of 2β,3β,5α-trihydroxycholest-7-ene-6-one. Other reagents used in the synthesis procedure, unless otherwise

stated, were of laboratory reagent grade.

Silylating reagents, heptafluorobutyrylimidazole and heptafluorobutyric acid, were obtained from Pierce Chemical Co. Trimethylsilylimidazole was also prepared in the laboratory from trimethylchlorosilane and imidazole by Mr. C.F. Poole.

The esterase for enzymic hydrolysis was obtained from Sigma Feinbiochemica, GmbH, and the α-glucosidase, having a specificity for α-1,4 and α-1,6 glycosidic linkages, was a gift from Professor J.B. Lloyd, Biochemistry Research Unit, University of Keele.

5. Instrumentation.

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected.

Infra red (I.R.) absorption spectra were measured on a Perkin-Elmer 257 grating spectrophotometer. The spectra were determined either in solution ($CHCl_3$) or as nujol mulls.

Ultraviolet (U.V.) absorption spectra were recorded on a Unican S.P.800 spectrophotometer, using 1 cm. cells and commercial 95% ethanol as solvent.

Nuclear magnetic resonance spectra (NMR) were recorded on either a Perkin-Elmer R10 60 MHz, or a Perkin-Elmer R24 60 MHz instrument, or a Varian HA 100, 100 MHz instrument. Results are quoted as τ values, using an internal standard of trimethylsilane (10·00τ).

Mass spectra were measured with an Hitachi-Perkin-Elmer R.M.U. 6E mass spectrometer, using an electron accelerating potential

of 2·5 KV, an ionization energy of 80 e.V., and a trap current of 60 mA. The instrument is of medium resolution with a single focusing magnetic sector. The instrument is fitted with a Watson-Biemann separator for g.l.c. - M.S. work. The separator is operated at 310°C.

Gas liquid chromatography was carried out using a Pye model 84 chromatograph, fitted with a flame ionization detector and an electron capture detector. Operating conditions and details are given at a later stage in the experimental section.

Fluorescence was measured using an Hitachi-Perkin-Elmer model 204 spectrofluorimeter with a xenon lamp source.

LARGE SCALE EXTRACTION OF BARNACLES FOR MOULTING HORMONES

1. Initial Solvent Extraction.

Between September 1971 and September 1972, barnacle material (1100 Kg.) was collected from Langstone Harbour, near Portsmouth, Selsey and Colwyn Bay. This material was ground on the day of collection with methanol in the ratio 1:1 w/v, using a 2 H.P. double-acting homogenizer. The shells were filtered off, using Büchner funnels and a hydraulic press. The residue on the filters was washed with an amount of methanol equivalent to that used in the initial grinding. The methanol (1000 litres) was evaporated off under vacuum in an atmosphere of nitrogen to give an aqueous concentrate (137 litres). Care was taken to keep the boiler temperature $< 40^{\circ}C$ during the evaporation which was carried out using three 20-litre capacity pot stills and one rising film evaporator. The combined rate of evaporation, using the three stills and the rising film evaporator, was approximately 30 litres $hr.^{-1}$.

2. Butanol-Water Partition.

The aqueous concentrate left after the evaporation of methanol was partitioned with n-butanol (110 litres, 96 litres, 43 litres), using a specially designed semi-automatic apparatus shown in Fig. 10. This apparatus incorporated reservoirs for the n-butanol phase and for the aqueous concentrate. These phases were pumped separately by peristaltic pumps through 100 ml. metering vessels into a 5000 ml. mixing chamber where they were vigorously stirred. The proportion of the two phases entering the mixing chamber was set in the approximate ratio of 1:1 v/v. The rate of feed to the mixing chamber

was controlled by adjusting the flow rate through the peristaltic pumps. After thorough mixing of the phases, the mixture was run into a 35-gallon capacity separating tank, where the phases were allowed to separate. The process was continued until all of both phases had been partitioned. The combined n-butanol extracts were washed with water (7·5 litres) and the water layers from the n-butanol backwash were washed with more n-butanol (25 litres). All the n-butanol was evaporated, using the large-scale distillation apparatus, to give a residue (1·22 Kg.) Water was added at intervals during the evaporation to facilitate formation of the minimum boiling butanol-water azeotrope.

3. Light Petroleum (b.p. 60° - 80°) - 70% Aqueous Methanol Partition.

The residue from the n-butanol extract was dissolved in 70% aqueous methanol (14 litres) and, using the semi-automatic apparatus, was partitioned with light petroleum (b.p. 60° - 80°) (6·7 litres, 4·5 litres, and 3·4 litres). The aqueous layer was retained and evaporated to leave a residue (522 g.) The light petroleum phase contained chiefly neutral lipids, together with some non-polar sterols.

4. Methanol-Chloroform-Water Countercurrent Extraction.

The residue from the aqueous phase of the 60/80 light petroleum - 70% aqueous methanol partition (522 g.) was combined with a similar residue from an extraction of 300 Kg. of barnacles which, by the same procedure, gave 276 g. of residue after evaporation of the aqueous methanol phase.

The combined residues from the total of 1400 Kg. were dis-

solved in methanol (8 litres) and added to a mixture of water (8 litres) and chloroform (10 litres). After thorough mixing, the chloroform layer was removed and extracted twice with methanol (8 litres) and water (8 litres). The aqueous methanol layers obtained were extracted countercurrently with a second portion of chloroform (10 litres). The combined chloroform layers were evaporated to yield a residue (Extract A, 44 g.). The aqueous layers were concentrated to yield a residue (Extract B, 761 g.).

5. Ethanol-Chloroform-Aq.Potassium bicarbonate (1:1:1 v/v) Counter-current Extraction.

The above Extract B was transferred, with potassium bicarbonate (200 g.) to the first of five separating funnels (20 litres) and partitioned between a mixture of ethanol (6·4 litres), water (6·4 litres), and chloroform (6·4 litres). A countercurrent distribution was carried out, using the double withdrawal method as described by Horn et al.[14]. The five lower layers were combined and evaporated to dryness to yield a residue (Extract C, 54 g.). The upper layers were concentrated to dryness and then triturated with absolute ethanol (4 litres). The light brown solid which precipitated was filtered off and washed with ethanol (Extract D, 445 g.). The ethanol filtrate was evaporated to yield a dark brown syrup (Extract E, 297 g.). Extracts C, D and E were all bioassayed using the locust abdomen test, but the results were inconclusive.

6. Ethyl Acetate-Water (1:1) Partition.

The residue from the chloroform phase of the ethanol-chloro-

form-aq. potassium bicarbonate (1:1:1) countercurrent (54g.) was subjected to a countercurrent extraction in the system ethyl acetate-water (1:1).

On addition of ethyl acetate and water to the chloroform extract, a dark brown precipitate formed which was not very soluble in either ethyl acetate or water. The mixture was transferred to the first of three 5-litre separating funnels, together with two litres each of water and ethyl acetate. The thick black solid did not dissolve even with shaking. It was left in the separating funnel and later added to the ethyl acetate phases. The partition was then carried out in the fundamental way. The top and bottom phases were then double-withdrawn as described by Horn[14].

The combined aqueous phases and the combined ethyl acetate phases were evaporated in the usual way. The black solid left in the first tube was dissolved in chloroform and added to the combined ethyl acetate phases. Some emulsions were formed during the partition but these were broken by light centrifugation.

The residue from the aqueous phase was 14·9 g., and that from the ethyl acetate phase was 29·4 g. Both residues were brown gums, the ethyl acetate extract being the darker. These residues were bioassayed using the locust abdomen test and again the results were inconclusive.

7. Column Chromatography on a Column of Davison Silica Gel.

The aqueous residue from the ethyl acetate-water countercurrent (14·9 g.) was chromatographed on Davison silica gel (450 g., deactivated with 10% water, grade 950, mesh 60 - 200, column I.D.,

45 mm.).

The column was packed in sodium dried benzene, and the material to be chromatographed was applied to the top of the column in a small amount of 10% n-butanol in benzene. The column was then eluted as shown in Table 7. Each fraction from the column was screened for 20-hydroxyecdysone by gas chromatography of its hexakis TMS ether derivative, using an e.c. detection method described later.

This showed that all the 20-hydroxyecdysone present was contained in fraction 3. This fraction was therefore carried forward in the purification scheme.

8. First Reverse Phase Partition Chromatography using a Hydrophobic Celite Column.

Fraction 3 from the Davison silica gel column was re-chromatographed on hydrophobic celite prepared as described by Martin et al.[47] Hydrophobic celite (290 g.), containing a mixture of butanol-cyclohexane (7:1 v/v)(177 ml.), was packed in water saturated with the butanol-cyclohexane mixture into a 45 mm. I.D. glass column.

The material to be chromatographed was applied to the top of the column dissolved in stationary phase (30 ml.), and eluted with water saturated with stationary phase containing increasing amounts of methanol. The elution of the column is shown in Table 8. Each fraction from the column was screened for 20-hydroxyecdysone by the g.l.c. - e.c.d. method.

Fraction 1 from the above column was found to contain 755 µg.

of 20-hydroxyecdysone and fraction 2 contained 455 µg. of 20-hydroxyecdysone. The g.l.c. trace of fraction 3 indicated that this fraction possibly contained other ecdysones. This fraction was further refined. (See Figs. 11 - 13.)

9. Second Reverse Phase Partition Chromatography Using a Hydrophobic Celite Column.

Fraction 3 from the first hydrophobic celite column was re-chromatographed on hydrophobic celite (32 g.), containing a mixture of n-butanol-cyclohexane (9:1 v/v) (20 ml.) packed into 20 mm. I.D. glass column.

The material to be chromatographed was applied to the top of the column in stationary phase (4 ml.), and eluted with water saturated with stationary phase containing increasing amounts of methanol. The elution of the column is shown in Table 9. Each fraction was screened for ecdysones by the g.l.c. - e.c.d. method.

Fraction 1 from the above column was found to contain 20-hydroxyecdysone (170 µg.), ecdysone (8 µg.) and two other unidentified peaks, which could possibly be due to ecdysone-type compounds. (See Fig. 14.)

ANALYSIS OF COLUMN FRACTIONS BY GAS LIQUID CHROMATOGRAPHY FOR 20-HYDROXYECDYSONE.

Samples were removed from each column fraction obtained for analysis by g.l.c. These samples were dried and freed of any residual solvent by pumping down to a pressure of approximately 0·1 mm. Hg. for two hours. From this dried sample 20 - 30 mg. were removed and weighed into a 'Reacti-Vial'. This accurately-weighed sample was heated with trimethylsilylimidazole (TMSI) (100 μl.) at 140°C for 20 hours. The cooled mixture was then diluted with an equal volume of e.c.d. grade toluene, and subjected to preparative thin layer chromatography, on a 20 x 20 cm. plate coated with a 0·6 mm. layer of methanol-washed PF_{254} silica gel. The plate was eluted with an ethyl acetate-toluene (1:9 v/v) solvent system.

The hexakis TMS ether derivative of 20-hydroxyecdysone is known to have an R_f value in the t.l.c. solvent system used of 0·71. The area of silica gel between R_f 0·50 and R_f 0·85 was therefore scraped off and eluted in a short glass column with diethyl ether ($\sim$ 6 ml.). The ether was evaporated from the eluate, using a stream of nitrogen. The residue obtained after evaporation of the ether was dissolved in a known volume of toluene (usually 100 μl.), and gas chromatographed on a 3' x 1/8" column of 2% OV 101 on CQ, using nitrogen as a carrier gas at a flow rate of 45 ml. $min.^{-1}$. The column temperature was 272°. A ^{63}Ni electron capture detector with a pulse height of 47 - 60 V., a pulse width of 0·75 μ sec. and a pulse period of 50 μsec. was used.

All solvents used in the preparation of samples for e.c.d. -

g.l.c. analysis were shown to be pure by prior e.c.d. - g.l.c. analysis.

The response of the e.c. detector to TMS ether derivatives of 20-hydroxyecdysone was found to be linear over the range 5 - 700 picograms. It was therefore possible to quantify the amount of 20-hydroxyecdysone present in column fractions by comparison of the peak areas obtained by injection of known concentrations of the TMS ether derivative of 20-hydroxyecdysone with those obtained by injection of hexakis TMS ether derivatives of column fractions.

1. Preparation and Gas Liquid Chromatographic Analysis of the Heptafluorobutyrate Derivative of 20-Hydroxyecdysone. (Fig. 12)

Samples of column fractions which were shown to contain 20-hydroxyecdysone by the method described above were again reacted with trimethylsilylimidazole. The hexakis TMS ether derivative of 20-hydroxyecdysone which was produced was reacted with heptafluorobutyrylimidazole (20 μl.) and heptafluorobutyric acid (2 μl.) in toluene (20 μl.) This mixture was heated at 55°C for two hours. After cooling, benzene (1 ml.) was added and the solution washed with 8% w/v sodium bicarbonate solution. The benzene solution was dried over molecular sieve, made up to a known volume with dry benzene and injected into the gas liquid chromatograph as described in the previous section.

2. Gas Liquid Chromatographic Analysis of 2β,3β,5α-Trihydroxycholest-7-ene-6-one. (Fig. 26)

2α,3β,5α-Trihydroxycholest-7-ene-6-one (1·2 mg.) was mixed with methoxyamine hydrochloride (14 mg.) in pure dry pyridine and left

overnight in a stoppered tube. The mixture was extracted with ethyl acetate from 5% v/v hydrochloric acid in 10% w/v aqueous sodium chloride. The ethyl acetate extract was dried over molecular sieve, evaporated and the residue silylated with a mixture of trimethylsilyl-imidazole (10 μl.), bis(trimethylsilyl) acetamide (10 μl.) and trimethylchlorosilane (8 μl.). The mixture was left overnight in a stoppered tube, at room temperature. 1 μl. was then removed and examined by gas liquid chromatography using a 1% OV 101, 18" column at 230°C, and nitrogen as carrier gas, flow rate 60 ml.min^{-1} with a flame ionization detector.

The TMS ether-O-methoximated sterol derivative was also examined by combined g.l.c. - m.s. The direct link gas liquid chromatography - mass spectrometry (g.l.c. - m.s.) was carried out using a Pye model 64 gas chromatograph with a 1·5' x 1/8" helical glass column packed with 1% OV 101 on CQ. The carrier gas used was helium with a flow rate of 18 ml.min.$^{-1}$. The g.l.c. was linked to the R.M.U. 6E mass spectrometer via a Watson-Biemann interface operated at 310°C. (Figs. 25 and 26.)

ANALYSIS OF 2β,3β,5α-TRIHYDROXYCHOLEST-7-ENE-6-ONE, BY ACID-INDUCED FLUORESCENCE. (FIG. 24.)

A stock solution with a concentration of 0·02 mg. ml.$^{-1}$ of 2β,3β,5α-trihydroxycholest-7-ene-6-one (Fig. 19, structure 10) was prepared in absolute ethanol. This was diluted with concentrated sulphuric acid and absolute ethanol to give the range of sterol concentrations required for the preparation of a calibration curve. The ratio of ethanol to sulphuric acid was carefully maintained at 1:1 v/v. The solutions of sterol in ethanol-sulphuric acid (1:1 v/v) were left to stand for one hour at room temperature. This was found to be the optimum time for the development of maximum fluorescent emission at 435 nm. after irradiation at a wavelength of 400 nm. The fluorescent emission of various concentrations of sterol were plotted against fluorescent intensity to produce the calibration curve shown in Fig. 24.

ENZYMIC HYDROLYSIS OF EXTRACTS

Extracts from the countercurrent distribution partition in chloroform-ethanol-aq. potassium bicarbonate (1:1:1 v/v) were treated with the enzymes α-glucosidase and an esterase.

Samples of 20 - 30 mg. were weighed into 10 ml. screw-capped tubes. In order to solubilize the samples they were mixed with a 10% w/v aqueous solution of Triton-X100 (scintillation grade) (10 μl.mg.$^{-1}$) and the mixture was dissolved in acetone. The acetone was evaporated under a stream of nitrogen at room temperature. Samples were dried and freed from any residual solvent by pumping down to a pressure of 0·1 mm. Hg. for two hours before being treated with enzyme.

The glucosidase reaction was carried out using α-glucosidase with a capability of hydrolysing 0·145 μmoles of p-nitrophenyl-α-glucoside per min. per mg. enzyme at pH 5·0 and 37°C. The reaction was stopped with methanol.

Samples were also treated with an esterase capable of hydrolysing carboxylate esters. This enzyme was capable of hydrolysing 100 μmoles of ethyl butyrate to butyric acid and ethanol per min. per mg. enzyme at pH 8·0 and 25°C. The reaction was again stopped with methanol.

Samples which had been treated with the enzyme were blown

down to dryness at room temperature, and the residues extracted with methanol. Samples of the residue from the methanol extraction were bioassayed.

Samples of extracts were bioassayed both before and after treatment with enzymes. In all cases the results were inconclusive, probably because of the toxicity of the extracts to the locust abdomens.

SYNTHESIS OF 2β,3β,5α-TRIHYDROXYCHOLEST-7-ENE-6-ONE.
(FIG. 19, STRUCTURE 10).

3β,5α,6β-Trihydroxycholestane (structure 2).

Cholesterol (40 g.) and 90% formic acid (400 ml.) were heated to 70° - 80° for half an hour with stirring. On cooling to 20°, 30% hydrogen peroxide (50 ml.) was added. The mixture was stirred for two hours, after which time a solution having a blue fluorescence had been produced. The solution was left overnight. Boiling water was added with stirring, and the resulting white gum was filtered off after cooling, washed with a small amount of water, dried superficially, and dissolved in methanol (1100 ml.). Aqueous sodium hydroxide (25% w/v, 50 ml.) was added, producing a greenish yellow solution after heating on a water bath for 10 minutes. The solution was acidified with 40% aqueous sulphuric acid and diluted with water (400 ml.) The white precipitate produced was filtered off after cooling, washed with water (2000 ml.), air-dried, and vacuum-dried at 40° to give 3β,5α,6β-trihydroxycholestane (38·5 g. 89%) m.p. 230° - 233°, (lit[78] 236° - 238°), $\nu_{max.}$ 3400 and 2900 cm.$^{-1}$.

The product was extremely insoluble and the crude product was carried onto the next stage.

3β,5α-Dihydroxycholestan-6-one (structure 3).

3β,5α,6β-Trihydroxycholestane (35 g.) was dissolved in dioxan (315 ml.) and water (35 ml.). N-Bromosuccinimide (18 g.) was added with stirring, the temperature being controlled at 25°. The

solution became deep red in colour and a precipitate was formed. Stirring was continued for a further one and a half hours, when the excess N-bromosuccinimide was destroyed using aqueous sodium sulphite solution (i.e. until the red colour had disappeared). Water (900 ml.) was added, the mixture allowed to stand in ice for two hours, and it was then filtered and dried to give 3β,5α-dihydroxycholestan-6-one (32·6 g. 92%), m.p. 230° - 234° (lit[78] 231° - 233°), $\nu_{max.}$ 3400, 2900 and 1710 cm.$^{-1}$

3β-(p-toluenesulphonyloxy)-5α-hydroxycholestan-6-one.

3β,5α-Dihydroxycholestan-6-one (28·9 g.) was dissolved in pyridine (103 ml.), p-toluene sulphonyl chloride (34·4 g.) was added with stirring, and the temperature was controlled at about 30° with an ice bath. The mixture was allowed to stand overnight, and was then poured onto melting ice (800 ml.). The white precipitate obtained was filtered off, washed with water (1000 ml.), dried in air, and then vacuum-dried at 40°. The crude product was recrystallized from ethyl acetate to give 3β-(p-toluenesulphonyloxy)-5α-hydroxycholestan-6-one (22·1 g. 57%), m.p. 141° - 143°, (from ethyl acetate) $\nu_{max.}$ 3400, 2900, 1710 and 1600 cm.$^{-1}$, NMR ($CDCl_3$) 2·5(q, aromatic protons), 7·6 (S, aromatic - CH_3 protons). (Found: C, 71·1; H, 9·2. $C_{34}H_{48}O_5S$ requires: C, 71·3; H, 9·2%).

5α-Hydroxycholestan-2-ene-6-one.

3β-(p-toluenesulphonyloxy)-5α-hydroxycholestan-6-one (40 g.) was dissolved in freshly distilled dimethyl formamide (230 ml.), and

then lithium bromide (18·6 g.) and lithium chloride (18·6 g.) were added. The mixture was refluxed for one hour, filtered hot, cooled, and then poured into ice water (800 ml.). The precipitate was filtered off, washed with water, air-dried, and then vacuum-dried at 40° to give 5α-hydroxycholestan-2-ene-6-one (29 g.), m.p. 143° - 145° (from methanol), $\nu_{max.}$ 3400, 2900, 1710 and 1670 cm.$^{-1}$, NMR ($CDCl_3$) τ 4·35 (m, Δ^2- en protons). (Found: C, 80·5; H, 10·9. $C_{27}H_{44}O_2$ requires: C, 81·0; H, 11·0%). The crude material was carried onto the next stage without purification.

2β-Acetoxy-3β,5α-dihydroxycholestan-6-one.

Glacial acetic acid (20 ml.) was neutralized with 35% ammonia using phenolphthallin as indicator, and added to a cold solution of silver nitrate (25 g.) in distilled water (50 ml.). The precipitated silver acetate was washed rapidly with distilled water and acetone, and stored over phosphorus pentoxide in a darkened evacuated desiccator. Iodine (20 g.) was added in portions over a 30 minute period to a stirred mixture of 5α-hydroxycholest-2-ene-6-one (21·6 g.) in glacial acetic acid (1000 ml., previously refluxed over and fractionated from chromic oxide) and silver acetate (27 g., freshly prepared), under nitrogen at room temperature. After about one hour, when all the iodine had been consumed, water (10 ml.) was added and stirring was continued under nitrogen overnight. Ether (1500 ml.) was added, the mixture was filtered, and then the filtrate was washed with water. The residue obtained by evaporation of the dried ether extract was triturated with light petroleum (b.p. 60° - 80°) to give 2β-acetoxy-3β,5α-dihydroxycholestan-6-one (10·7 g., 47%), m.p. 161° - 163° (from toluene), $\nu_{max.}$ 3400, 2900 and 1720 cm.$^{-1}$, NMR ($CDCl_3$) τ 4·9 (m, H - 2),

5·9(m, H - 3), 8·0(S, CH_3COO-), (Found: C, 73·0; H, 9·9. $C_{29}H_{48}O_5$ requires: C, 73·1; H, 10·1%).

2β,3β-Diacetoxy-5α-hydroxycholestan-6-one.

2β-Acetoxy-3β,5α-dihydroxycholestan-6-one (10·0 g.) was dissolved in dry pyridine (50 ml.) and acetic anhydride (46·5 ml., previously distilled from phosphorus pentoxide) was added with stirring. The mixture was allowed to stand overnight, after which time water (300 ml.) was added with cooling. The solution was extracted with ether (3 x 400 ml.), and then the ether extract was washed with normal sulphuric acid (3 x 250 ml.), 10% aqueous sodium bicarbonate solution (3 x 250 ml.), and water (3 x 250 ml.). The ether extract was dried over anhydrous magnesium sulphate and the ether evaporated to give 2β,3β-Diacetoxy-5α-hydroxycholestan-6-one (9·9 g., 91%), m.p. 184° - 186° (from light petroleum [b.p. 60° - 80°]) $\nu_{max.}$ 3450, 2900 and 1720 $cm.^{-1}$., NMR ($CDCl_3$) τ 4·7(m, H - 2), 6·3(S, 5α - OH), 7·9, 8·0 (S, 2 x CH_3COO-), (Found: C, 71·6; H, 9·8. $C_{31}H_{50}O_6$ requires: C, 71·8; H, 9·7%).

2β,3β-Diacetoxy-7α-bromo-5α-hydroxycholestan-6-one.

2β,3β-Diacetoxy-5α-hydroxycholestan-6-one (4·6 g.) was dissolved at 60° in glacial acetic acid (100 ml.) and treated with a solution of bromine (1·3 g.) and boron trifluoride etherate (1·5 ml.) in acetic acid (50 ml.). The solution was kept at 60° for half an hour, allowed to stand for two hours, poured into water (200 ml.) and then the precipitate was filtered off, air-dried and vacuum-dried at

40° to give 2β,3β-diacetoxy-7α-bromo-5α-hydroxycholestan-6-one (3·3 g., 62%), m.p. 183° - 186° (from methanol/ether, phase change 125° - 130°), $\nu_{max.}$ 3450, 2900 and 1720 cm.$^{-1}$., NMR ($CDCl_3$, 100 M. Hz.)τ 4·68 (m, H - 2 and H - 3), 5·81(d, J 4·2 Hz. 7β - H), 6·87(S, 5α - OH), 7·94, 8·20(S, 2 x CH_3COO-), (Found: C, 60·8; H, 8·3. $C_{31}H_{49}O_6Br$ requires: C, 62·3; H, 8·3%).

2β,3β-Diacetoxy-5α-Hydroxycholest-7-ene-6-one.

2β,3β-Diacetoxy-7α-bromo-5α-hydroxycholestan-6-one (3 g.) was refluxed with lithium carbonate (3 g.) in freshly distilled dimethyl formamide (75 ml.) for one hour. The mixture was filtered hot, cooled and poured into ice water (200 ml.). The precipitate was filtered, air-dried and then vacuum-dried at 40° to give 2β,3β-diacetoxy-5α-hydroxycholest-7-ene-6-one (1·8 g., 70%), m.p. 184° - 186° (from ether/light petroleum [b.p. 60° - 80°]), $\nu_{max.}$ 3450, 2900, 1735, and 1670 cm.$^{-1}$., $\lambda_{max.}$ (EtOH) 250 nm (ϵ = 11340), NMR ($CDCl_3$) τ 4·3 (d, H - 7), 4·7(m, H - 2 and H - 3), 7·95, 8·05(S, 2 x CH_3COO-), (Found: C, 72·2; H, 9·4. $C_{31}H_{48}O_6$ requires: C, 72·1; H, 9·4%).

2β,3β,5α-Trihydroxycholest-7-ene-6-one.

2β,3β-Diacetoxy-5α-hydroxycholest-7-ene-6-one (0·45 g.) in methanol (40 ml.) was heated to 30° to achieve solution and allowed to cool to 22°. Potassium carbonate (15·3 mg.), methanol (6·5 ml.) and water (5 ml.) were added in solution over a period of one hour. On addition, the mixture became pale yellow in colour, and as the addition proceeded there was some precipitation. The mixture was heated at 50°

(when the precipitate redissolved) until, after about 10 minutes, t.l.c. showed that all of the starting material had reacted, producing two spots at lower R_f. After a further 15 - 20 minutes, t.l.c. showed little change and the reaction mixture was cooled in ice, and ice water (30 ml.) was added to ensure complete precipitation. The precipitate was filtered off with difficulty, air-dried and then vacuum-dried over phosphorus pentoxide to give 2β,3β,5α-trihydroxycholest-7-ene-6-one (0·12 g., 32%), m.p. 230° - 233° (from methanol), $\nu_{max.}$ 3350, 2900, 1680 and 1610 $cm.^{-1}$., $\lambda_{max.}$ (EtOH) 250 nm (ϵ = 11250), NMR (d_6-DMSO) τ 4·6 (d, H - 7), 4·2(m, H - 2), 5·8(m, H - 3), (Found: C, 75·0; H, 9·9. $C_{27}H_{44}O_4$ requires: C, 75·0; H, 10·2%).

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