STUDIES ON THE MOULTING HORMONES OF THE DESERT LOCUST *SCHISTOCERCA GREGARIA*

by

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ABSTRACT

A systematic investigation of extracts of late 5th instar nymphs of the desert locust *Schistocerca gregaria* has been carried out to detect and isolate moult-inducing substances. Moulting activity was detected by bioassay using the isolated abdomens of 4th instar nymphs as the test objects. At least three substances with moulting activity have been detected in whole locusts, and one has been isolated and identified as the known arthropod moulting hormone, 20-hydroxyecdysone. The other two active substances have been obtained as partially purified extracts. No ecdysone has been detected in any of the locust extracts examined.

A gas-liquid chromatographic method has been developed for the determination of steroid hormones of the ecdysone type in biological materials. Model steroids were used to establish the conditions for the preparation and gas-liquid chromatography of trimethylsilyl-methoxime derivatives of ecdysone and 20-hydroxy-ecdysone, and derivatives of the ecdysones were identified by mass spectrometry. The gas-liquid chromatography and the mass spectra of derivatives of the model compounds and the ecdysones are discussed.

The gas-liquid chromatographic method has been applied to the examination of extracts from whole nymphs and various

tissues for 20-hydroxyecdysone. This hormone was first detected and determined in purified extracts from whole locusts by gas-liquid chromatography, and has subsequently been determined in nymphs at daily intervals from day eight of the 4th instar to day three of adult development by this method. The titre was a maximum on the days of ecdysis, a minimum in the middle of the 5th instar, and fell to a low level following the emergence of the adult.

Prothoracic glands isolated from late 5th instar nymphs have been shown, by bioassay, to contain at least two moult--inducing substances which are similar to the two unidentified active substances from whole nymphs. It is suggested that the two active components from glands are the same as those found in whole locusts, and that they are produced in the prothoracic gland. No 20-hydroxyecdysone was detected in extracts from prothoracic glands, and it is considered unlikely that this hormone is produced in the gland.

Blood extracts from 5th instar locusts have been found to contain only a small proportion of the moult-inducing substances present in whole nymphs, and no 20-hydroxyecdysone could be detected. This hormone was also undetected in oenocyte bearing abdominal tissues, but as the proportion of oenocytes in the tissue was very low, this could not be used as a guide to the 20-hydroxyecdysone content of the oenocytes.

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INTRODUCTION

MOULTING AND METAMORPHOSIS

Insects belong to the class *Insecta* which is a subdivision of the Phylum *Arthropoda*.[1] This Phylum which groups together animals having the outer surface of the body hardened so that it forms an exoskeleton also includes *Crustacea* (crustaceans) and *Arachnida* (spiders). The exoskeleton in these classes of animals restricts their growth, and so the hardened outer cuticle is shed at intervals in the process of ecdysis or moulting. The arthropod grows rapidly while the new cuticle is soft and elastic, but as soon as this is hardened, usually only a matter of a few hours, the growth is again restricted until the next moult.

The majority of living insects belong to the sub-class Pterygota, or winged insects, and these are divided into two groups, holometabolous and hemimetabolous, which are characterised by their mode of development. The holometabolous insect hatches from the egg as a larva, which after several larval moults undergoes an abrupt metamorphosis to give a pupa. Ultimately an adult emerges from the pupa and this often does not remotely

resemble the original larva. In contrast hemimetabolous insects undergo a gradual and continuous transformation. They hatch from the egg as a nymph which closely resembles the form of a minature adult except for the absence of wings. After several nymph to nymph moults the final moult produces an adult.

DISCOVERY OF THE MOULTING HORMONE

Little was known about the actual forces which initiate and control moulting and metamorphosis in insects until 1922 when Kopeć[2] suggested the action of a hormone. He discovered that if full-grown caterpillars of *Lymantria dispar* were deprived of their brains (supra-oesophageal ganglia) 10 days after the last moult they pupated, but if the brain was removed after only seven days they failed to pupate. Similar results were observed by ligating different parts of the body, the anterior part duly pupated, but the posterior part remained unchanged unless it belonged to a caterpillar which would have pupated in a few days anyway. If the nerve cord was cut through just behind the head, pupation and further development were unaffected. Kopeć concluded that the brain produced a hormone which induces pupation. Hachlow[3] in 1931 concluded from results of his experiments with the pupae of various butterflies that some

centre of an undetermined nature situated in the thorax was controlling growth and metamorphosis. Similar conclusions were reached by Bodenstein and other researchers, and the position of the problem as it was in 1933 was reviewed by Bodenstein.[4]

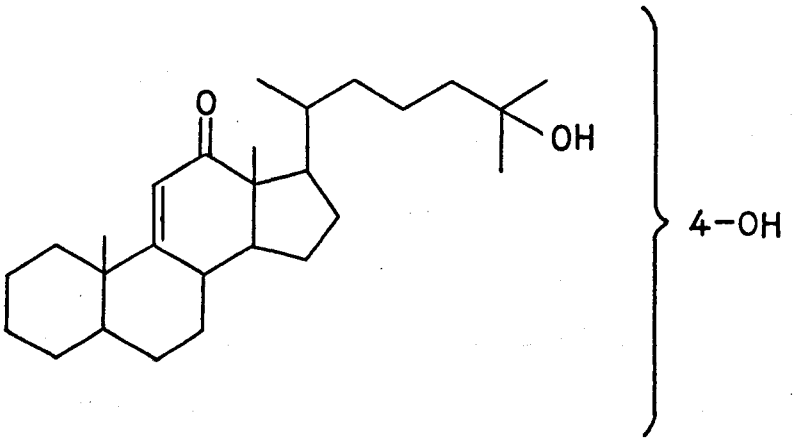
The operation of a hormonal mechanism of control in metamorphosis was proved conclusively by Wigglesworth in 1934[5,6] in his experiments on the tropical bug *Rhodnius prolixus*, and by Fraenkel in 1935[7] from his investigation into pupation in the blowfly *Calliphora erythrocephala*. They confirmed Kopeć's results and also showed that a substance transported in the blood could induce moulting and pupation.

ISOLATION OF MOULTING HORMONE

The experiments of Fraenkel on *Calliphora erythrocephala* larvae were continued by Becker and Plagge,[8,9] and they prepared for the first time extracts which were effective in producing pupation in the posterior fragments of ligated larvae. By 1939 they had developed the Calliphora test and this was improved by Karlson and Hanser[10] (1952) to give a quantitative bioassay.

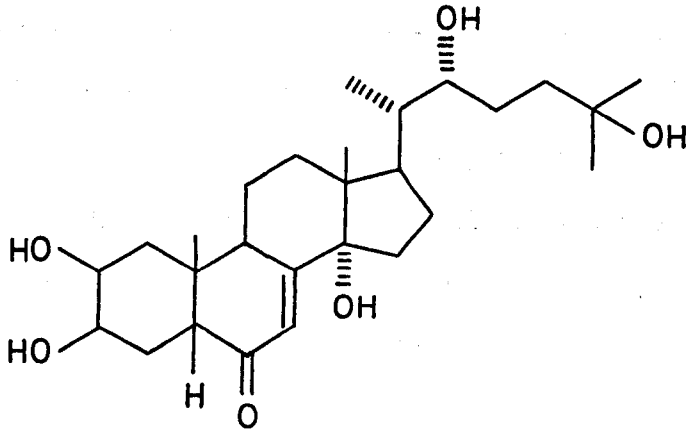
With a suitable bioassay developed, Butenandt and Karlson began the chemical work in 1943. The initial methods for concentrating the active factor were developed using pupae of the blowfly *Calliphora erythrocephala*, but later, in about 1947 pupae of the silkworm *Bombyx mori* which were available in large quantities from the silk industry, were used. In 1954 Butenandt and Karlson announced the isolation of 25 mg. of pure crystalline hormone from 500 Kg. of fresh silkworm pupae.[11] In a second communication[12] in 1956 Karlson named this hormone α-ecdysone (*2a*, see page 5) as it had become clear from further fractionation of the silkworm extract that a second, more polar substance, also active in the calliphora test was present. This he isolated as 2.5 mg. of crude crystals and named β-ecdysone (*3a*, see page 12).

Characterisation of α-ecdysone (*2a*) was begun immediately, but too little β-ecdysone (*3a*) was available at that time for further studies. Accumulated chemical and spectroscopic evidence led Karlson[13] to propose the partial structure (*1*) for α-ecdysone in 1963.



(*1*)

After a further extraction[13] the chemical and X-ray crystallographic investigations were continued from 1963 to 1965 when a series of papers were published,[14-18] which culminated in the absolute configuration of α-ecdysone (*2a*) unambiguously determined as (22R)-2β,3β,14α,22,25-pentahydroxy--5β-cholest-7-en-6-one (*2a,b*) by the X-ray analysis of Huber and Hoppe.[19]



(*2a, b*)

THE CHOICE OF THE DESERT LOCUST FOR INVESTIGATION

By 1966 investigation of the moulting hormones of several other insects and crustaceans had been undertaken. Stamm[20] in 1958 had reported the isolation of α-ecdysone (*2a*), and β-ecdysone (*3a*) from adults of the Moroccan locust *Dociostaurus maroccanus* apparently confirming Karlson's result,[12] but by 1963 Burdette and Bullock[21] had detected three more active substances in silkworms. Also in 1963 Carlisle and Ellis[22] had tentatively suggested the presence of a new hormone, which they named λ-ecdysone, in the desert locust *Schistocerca gregaria* Forskål but their methods of detection were not reported. Carlisle[23] in 1965 demonstrated reciprocal activity in extracts from desert locusts and several crustaceans, and his results indicated that the groups tested probably contained similar but not identical ecdysones. Experiments by Kaplanis *et al.*[24,25] in 1966 and 1967 with the tobacco hornworm *Manduca sexta* confirmed the presence of the hormones discovered by Karlson, but a new hormone was also detected. Hampshire and Horn in their investigations from 1966 to 1968 were unable to detect α-ecdysone (*2a*) in either the crayfish *Jasus lalandei*[26,27] or the blowfly *Calliphora stygia*,[28] but moulting hormones related to α-ecdysone (*2a*) were isolated.

A report by Hoffmeister *et al.*[29] in 1965 had indicated the presence of α-ecdysone (*2a*) in the faeces of desert locusts. Carlisle and Ellis[22,30] however reported its absence in the whole insects but no details of their isolation and detection methods, except that thin layer techniques had been used, were available to support their claim.[31] Their suggestion that α-ecdysone (*2a*) is an excretory metabolite of the true moulting hormone in the desert locust should not be dismissed.

Apart from the work of Stamm[20] on adult Moroccan locusts and the preliminary examinations of the desert locust by Carlisle and Ellis,[22,23,30] all other investigations of insects for moulting hormones had been on the pupal stage of holometabolous insects. In all cases, except that of the desert locust, the bioassay used involved the rather specialised moult from larva to pupa in *Calliphora*[10] or other Diptera.[24,32,33]

It was therefore considered important to investigate thoroughly the moulting hormones present in the immature stages of a hemimetabolous insect, and if possible to use a bioassay involving the same species. Desert locusts were readily available from the breeding stock at the Anti-Locust Research Centre, and the preliminary work of Carlisle and Ellis had

shown them to be a suitable insect for such an investigation. By 1968 Dr. P.E. Ellis at the Anti-Locust Research Centre had developed a suitable bioassay using the isolated abdomens of 4th instar nymphs of the desert locust (see page 193) as the test insects, and this enabled the work described later in this thesis to be started in October 1968.

RECENT SOURCES OF ECDYSONES

Following the complete characterisation of α-ecdysone (*2a*) in 1965, a great expansion in the field of moulting hormones took place, and by 1966 α-ecdysone (*2a*) was synthesised and moulting hormones had been isolated not only from insects but also from crustaceans and plants. A brief outline of some of the relevant aspects of this expansion, which still continues, and some of the earlier work on which it was based is given below.

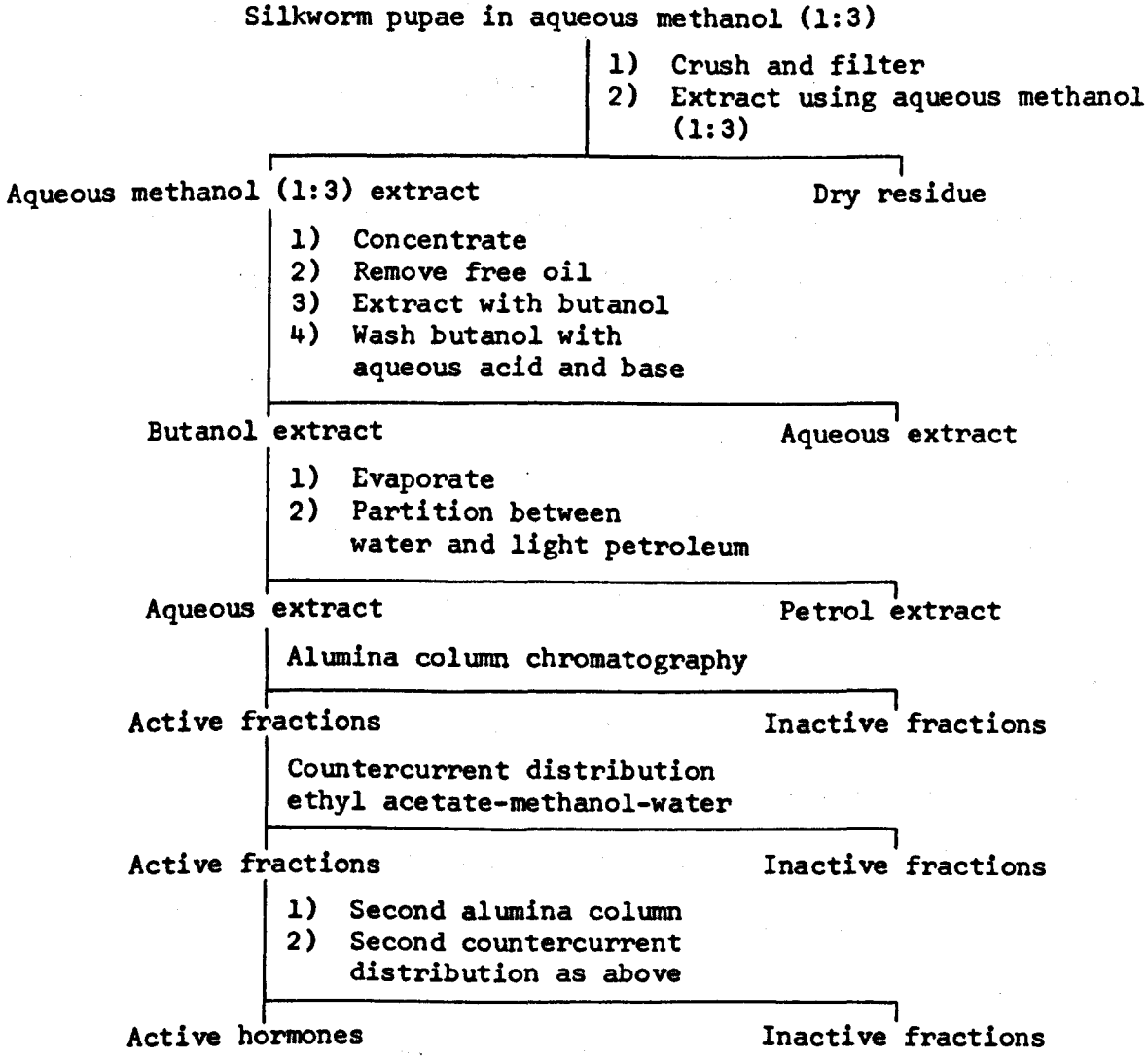
ISOLATION AND STRUCTURES OF ARTHROPOD ECDYSONES

The initial methods[11] used by Butenandt and Karlson (1954) in their extraction of the moulting hormone were modified, and by 1963 Karlson was able to isolate the greatly improved yield of 250 mg. of α-ecdysone (*2a*) from 1000 Kg. of silkworm

pupae.[13] This modified procedure which provided the basis for many subsequent extractions of insect material is outlined in Figure 1.

FIGURE 1

Karlson's Modified Extraction Procedure (1963)



The only other complete extraction of an insect to be reported before 1966 was the previously mentioned investigation of Stamm[20] in 1958. She reported the isolation of 11 mg. of α-ecdysone (*2a*) and 13 mg. of β-ecdysone (*3a*) from 10 Kg. of adults of the Moroccan locust *Dociostaurus maroccanus* using an extraction procedure which was essentially the same as Karlson's modified procedure of 1963 outlined in Figure 1. However despite the apparently richer source of the hormones, four consecutive countercurrent distributions were required using a system of cyclohexane-butanol-water before the pure hormones were isolated. The choice of adult locusts for the extraction was unusual as it was known that the prothoracic glands (the organ suggested in a review by Karlson[12] as the site of production of ecdysones in insects) degenerate after the imaginal moult.[34] Karlson[12] had also reported that silkworm adults yielded active extracts, but that these contained far less hormone than the pupal extracts, and possibly only hormone residues.

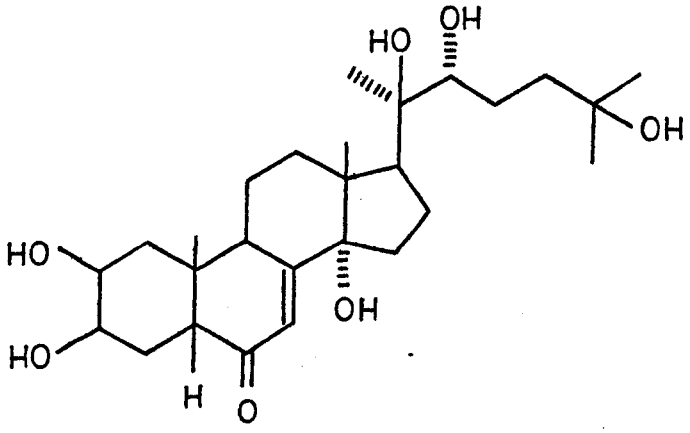
In 1966 the investigations of several German workers on silkworm extracts were published. Hocks and Wiechert[35] described the isolation of 48 mg. of an active substance, from 2.8 tons of silkworm pupae, which was slightly more polar than α-ecdysone (*2a*), and this they tentatively identified as

20-hydroxyecdysone (*3b*). Hoffmeister[36] described the extraction of 9 mg. of a new insect moulting hormone, from two tons of silkworm pupae, which was also more polar in chromatography than α-ecdysone (*2a*), and was two to three times more active in the calliphora test. This he called ecdysterone (*3c*). Later in 1966 after a study of the chemistry of this compound Hoffmeister tentatively identified it as 20-hydroxyecdysone (*3b*).[37]

Early in 1966 the experiments of a group of Australians with crustaceans and insects were announced. Hampshire and Horn[26] reported the isolation of a moulting hormone from the intermoult stage of the sea-water crayfish *Jasus lalandei* (2 mg. from one ton of waste[27]) which they named crustecdysone (*3d*, crustacean ecdysis). They assigned the structure as 20-hydroxyecdysone (*3b*) but their material showed less than a third of the activity of α-ecdysone (*2a*) in the calliphora test. Later in the same year Horn and Middleton reported[27] the isolation of crustecdysone (*3d*) as the major moulting hormone of the Saturniid oak silk moth *Antherea pernyi*. Fractionation of 31 Kg. of pupae gave 200 μg. of hormone, and this and the crayfish hormone were found to be similar if not identical to the β-ecdysone (*3a*) isolated by Karlson.[12]

These results were finally correlated in a joint publication[38] by the German and Australian groups in 1967 in

which they compared 20-hydroxyecdysone (*3b*), ecdysterone (*3c*) and crustecdysone (*3d*) isolated from the various arthropod sources. They concluded that ecdysterone (*3c*) and crustecdysone (*3d*) were identical and the same as 20-hydroxyecdysone (*3b*). This was confirmed by Hoffmeister *et al.* after a complete characterisation of their ecdysterone (*3c*).[39] In 1967 Hocks and Schulz showed that Karlson's β-ecdysone (*3a*) was also 20-hydroxyecdysone (*3b*) and they gave its absolute configuration as (22R)-2β,3β,14α,20,22,25-hexahydroxy-5β-cholest-7-en-6-one (*3a-d*). This has recently been confirmed by Dammeier and Hoppe in an unambiguous structural determination by X-ray crystallography.[40]

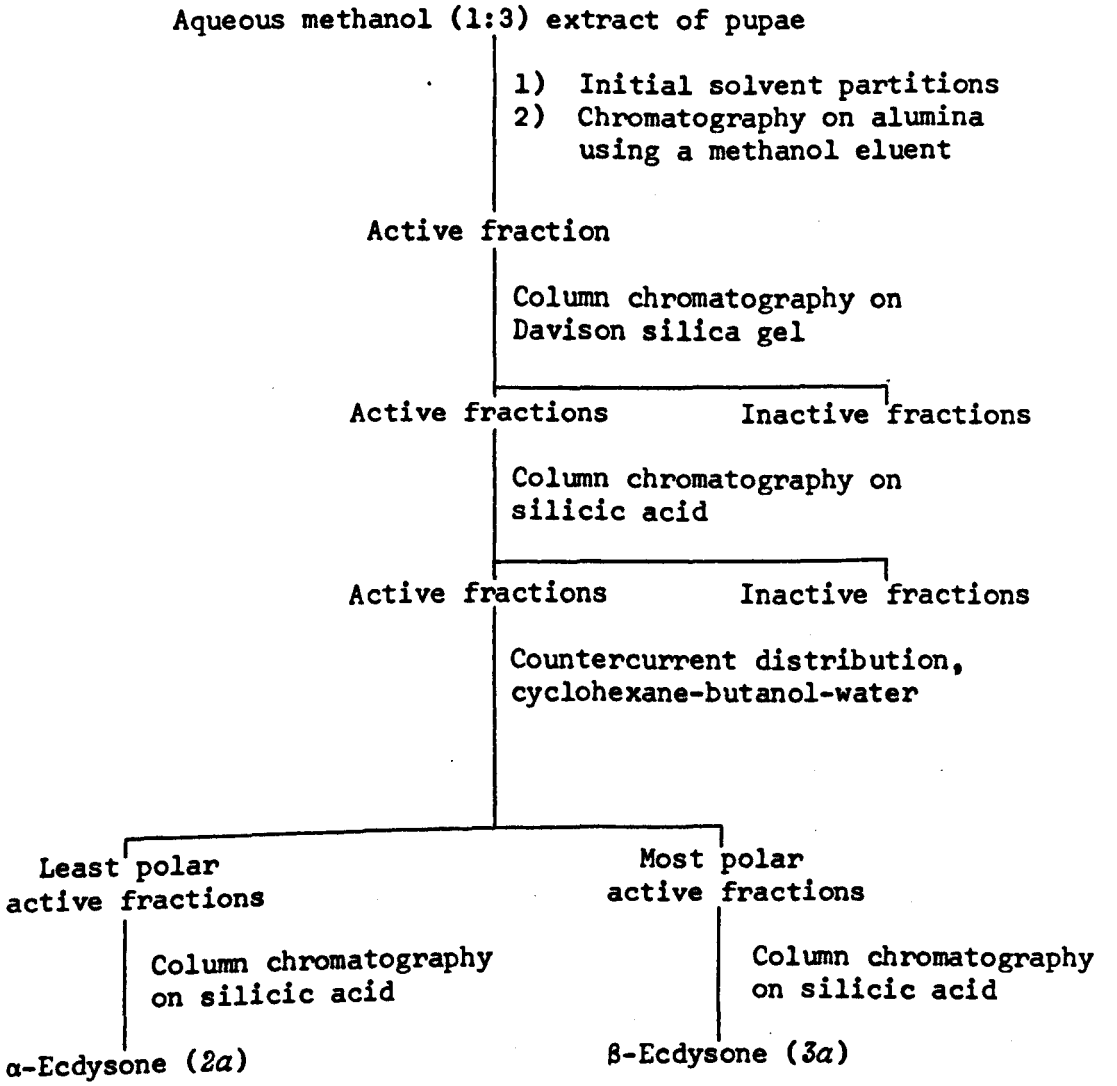


(*3a - d*)

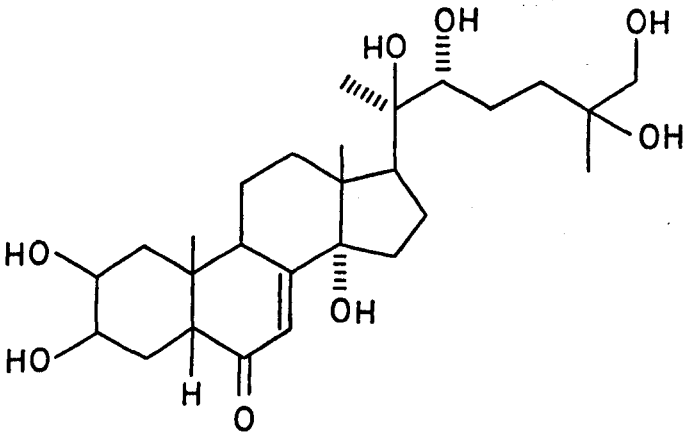
The last investigation of an arthropod for moulting hormones to be described in 1966 was carried out on pupae of the tobacco hornworm *Manduca sexta* by Kaplanis *et al.*[24] They isolated two substances active in the calliphora test from pupae selected by bioassay at their maximum hormone titre. Characterisation of these compounds showed that one was α-ecdysone (*2a*) and the other β-ecdysone (*3a*). The extraction of 12.7 Kg. of pupae yielded 4.4 mg. of α-ecdysone (*2a*) and 3.7 mg. of β-ecdysone (*3a*) and is noteworthy because the latter stages of the isolation differed from those of Karlson's procedure (see Figure 1, page 9). These stages are outlined in Figure 2.

FIGURE 2

Extraction Procedure for Tobacco Hornworm Pupae (1966)



This group reported[25] the isolation of a third moulting hormone in 1967 more polar in countercurrent distribution than crustecdysone (*3d*) and its structure was shown to be 20,26-dihydroxyecdysone (*4*)



(*4*)

At about this time (1966) the name α-ecdysone (*2a*) was modified to ecdysone (*2b*) which is now generally accepted, but the several names for 20-hydroxyecdysone (*3b*) except for β-ecdysone (*3a*) have continued in use*. A brief review of the

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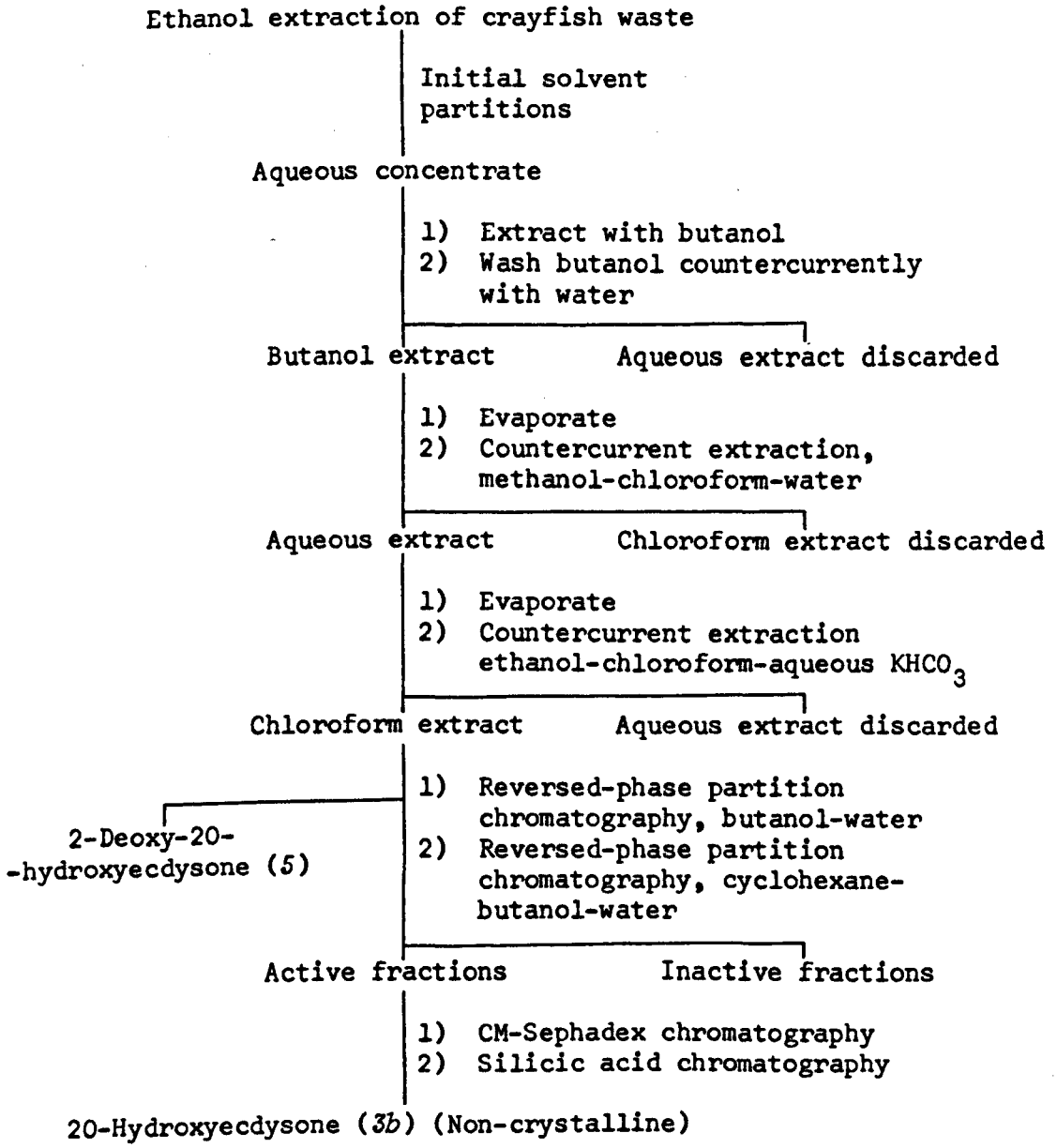
*Hampshire and Horn, in their communication[26] accepted for publication in December 1965, assigned for the first time the 20-hydroxyecdysone structure to a crustecean moulting hormone which they named crustecdysone (*3d*). The use of a nomenclature based on ecdysone (*2b*, ecdysis, moulting) as the parent compound with related compounds named as derivatives of ecdysone (*2b*, e.g. crustecdysone *3d* then becomes 20-hydroxyecdysone *3b*) would seem to be preferable. Such a nomenclature will be used in all subsequent parts of this thesis.

relative activities in the calliphora test of the steroids with moulting activity isolated from arthropods up to 1967 has been published.[41]

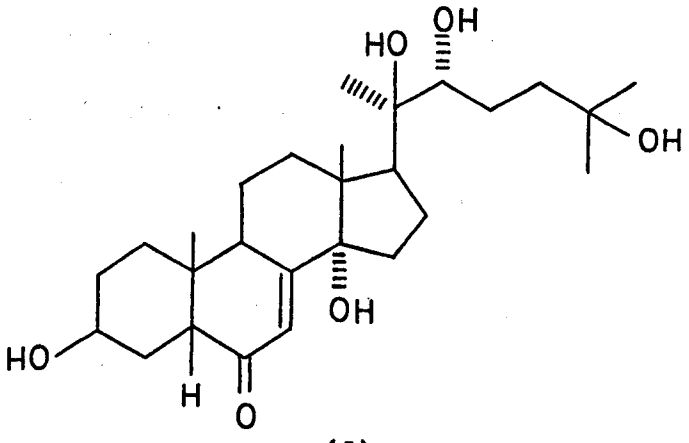
The Australian group of Horn *et al.* finally published[42] in 1968 the procedure used in their extraction of 20-hydroxyecdysone (*3b*) from crayfish waste reported[26] in 1966. They had found during their initial investigations in 1962 that crude extracts of crayfish waste had less than one hundredth of the moulting activity of comparable extracts from silkworm pupae. Their procedure was much longer than any extraction method previously described which reflected the difficulty of the isolation, however only the latter part differed greatly from Karlson's procedure of 1963 (see Figure 1, page 9). In the initial stages of the extraction ethanol was substituted for methanol, propan-2-ol for butanol, and pure hexane was used instead of light petroleum. The column chromatography on alumina was avoided because of the possible decomposition of the small amount of hormone. Two bioassays were used in the development of the method, the calliphora test, and a novel but indirect assay using the freshwater crayfish *Cherax destructor*. The main sequence of the extraction procedure is briefly outlined in Figure 3.

FIGURE 3

Extraction Procedure for Crayfish Waste (1968)



The extraction of crayfish thoraxes as shown above had yielded not only 20-hydroxyecdysone (*3b*) but also a small amount of a less polar moulting hormone.[42] By 1968 fractionation of three tons of crayfish waste had yielded 200 µg. of this material, and this was used for a structural determination which showed it to be 2-deoxy-20-hydroxyecdysone (*5*).[43] No ecdysone (*2b*) could be detected.

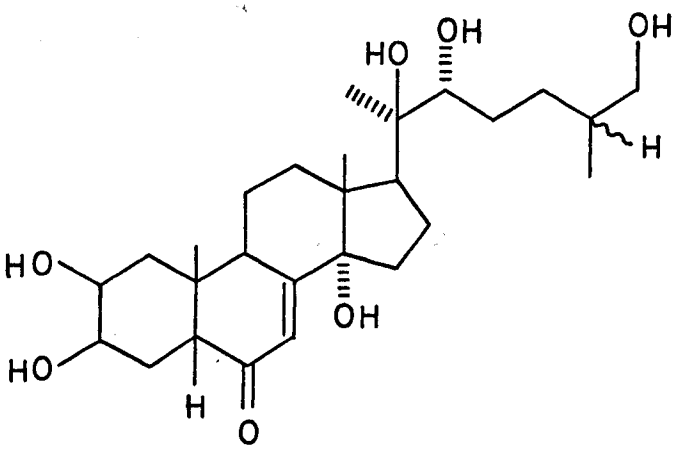


(*5*)

Three more arthropods have been investigated for moulting hormones since 1968 by the Australian group. In 1969 Galbraith and Horn[28] reported the extraction of the brown blowfly *Calliphora stygia* and the blue blowfly *Calliphora vicina* at different stages of puparium formation using the procedure outlined in Figure 3. The only moulting

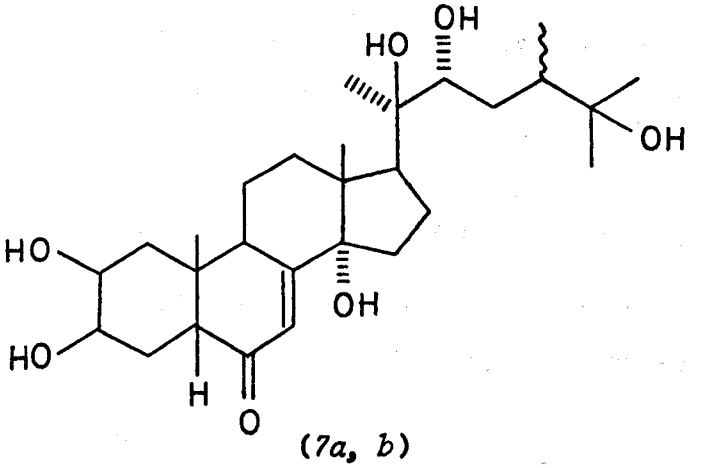
hormone which they detected in significant amounts was 20-hydroxyecdysone (*3b*), ecdysone (*2b*) if present occurs in much smaller quantities. This result may be contrasted with the reports by Karlson in 1956[12,44] which stated that pupae of the blue blowfly *Calliphora erythrocephala* contain both ecdysone (*2b*) and 20-hydroxyecdysone (*3b*). Ecdysone (*2b*) was considered to be the principle moulting hormone present and evidence was presented by Karlson and Hoffmeister[45] in 1963 for its biosynthesis in *Calliphora erythrocephala* from cholesterol.

In February 1969 the isolation of three moulting hormones from the female marine crab *Callinectes sapidus* at three different stages of development (see page 144) was reported.[46] 20-Hydroxyecdysone (*3b*) was present in quantities ranging from 4 to 280 µg. per Kg. of crabs, and two new arthropod moulting hormones were isolated and named callinecdysone A (*6a*) and callinecdysone B (*7a*). Callinecdysone A (*6a*) was present in quantities ranging from 5 to 20 µg. per Kg. of crabs. Its structure was reported as either one of the two stereo-isomers of 25-deoxy-20,26-dihydroxyecdysone (*6a,b*) or a mixture of them both after comparison of its spectral properties with those of inokosterone (*6b*) an ecdysone isolated from a plant.[47]



(*6a, b*)

Callinecdysone B (*7a*) was isolated only from the stage just after moulting at a level of 24 µg. per Kg. of crabs. The spectra of this hormone and a plant counterpart makisterone A (*7b*)[48] were identical and the structure was reported as being one of the two possible C-24 isomers of 24-methyl-20-hydroxyecdysone (*7a,b*).



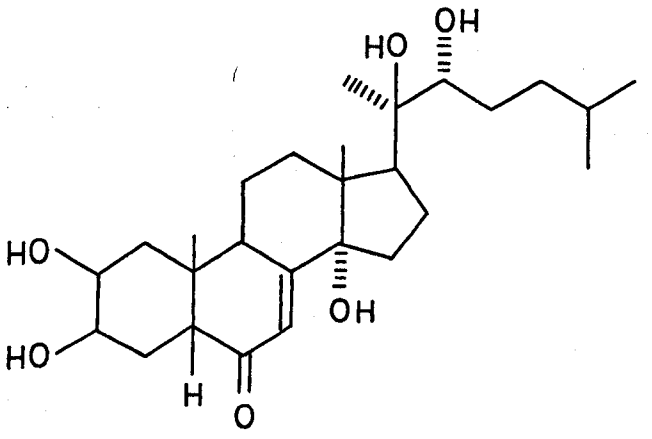
(*7a, b*)

From the investigations so far carried out insects appear to be a better source of moulting hormones than crustaceans unless the latter are selected at times of maximum hormone titre.[46] Indeed an attempt by Karlson and Schmialek in 1959 to isolate the active material from three tons of shrimps *Crangon vulgaris* was unsuccessful.[49] No further arthropod ecdysones have as yet been isolated and characterised. At the present time crustecdysone forms a common link as it is the only moulting hormone to be isolated from all the arthropods so far investigated, and the only one detected by Horn *et al.* in two of the insects.[27,28]

PHYTOECDYSONES

The discovery that plants were also sources of steroids active in promoting moulting in arthropods was announced in 1966 by Nakanishi *et al.*[50] in Japan. They isolated four substances active in the calliphora test[51] from leaves of the evergreen tree *Podocarpus nakaii*, which they collected in Taiwan. These they named ponasterones, and 4.8 Kg. of dried leaves yielded 2 g. of ponasterone A (*8*), 50 mg. of ponasterone B, 500 mg. of ponasterone C and 20 mg. of ponasterone D. The planar structure of ponasterone A (*8*) corresponded to 25-deoxy-20-hydroxyecdysone

(*8*), but the name ponasterone was retained as the configuration of the side chain hydroxyl groups were then still unknown. The stereochemical identity of ponasterone A with 25-deoxy--20-hydroxyecdysone (*8*) has subsequently been established.[52,53]

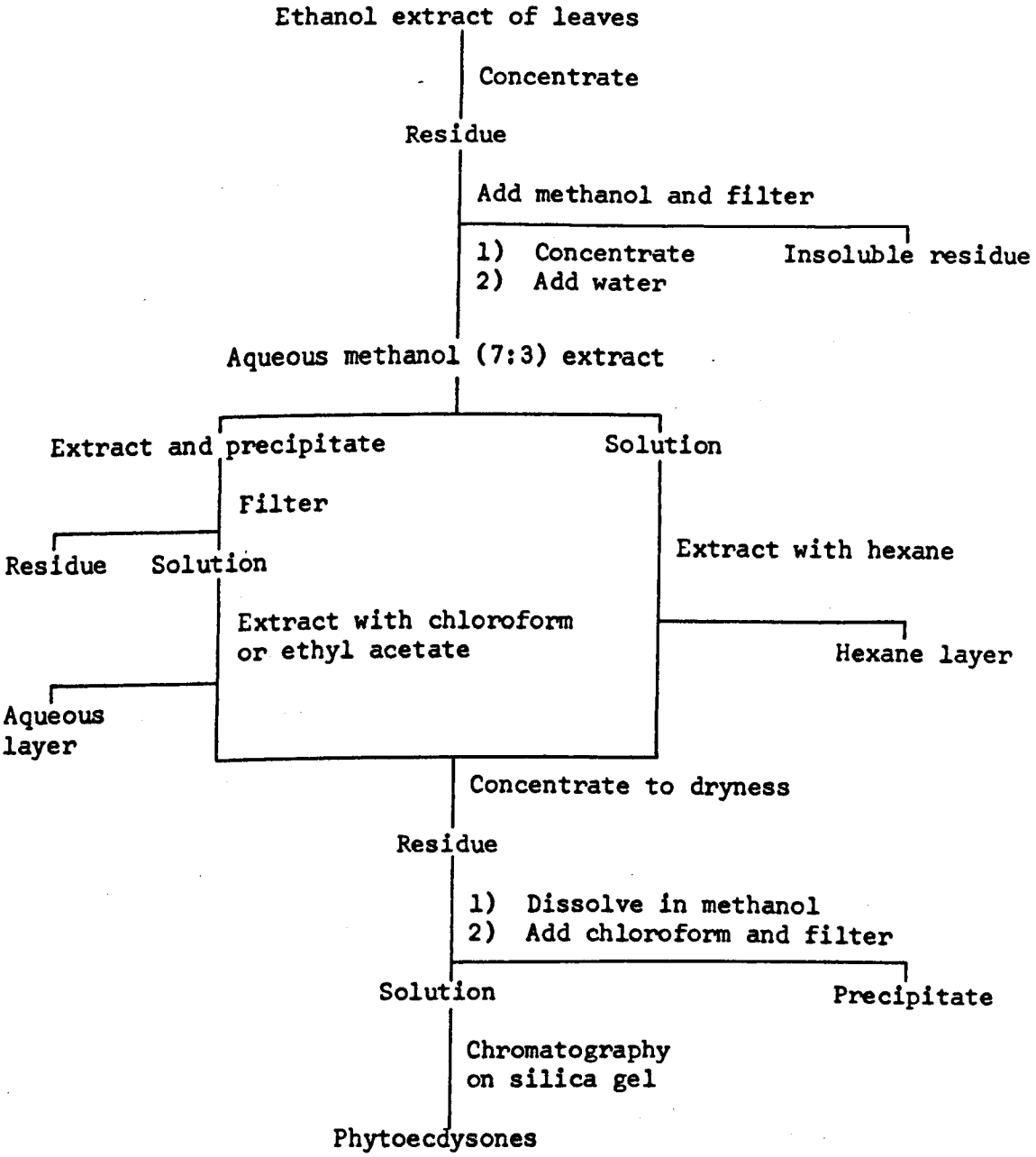


(*8*)

By 1967 the Japanese group had developed a general extraction procedure for the isolation of phytoecdysones from plant leaves[54] and this is outlined in Figure 4.

FIGURE 4

Extraction Procedure for Plant Leaves (1967)



Since the initial discovery of phytoecdysones in plants in 1966, groups of workers from several different countries have entered the field and large numbers of extractions have been reported.[55] Many of these were performed using similar extraction schemes to the one outlined in Figure 4 (see page 23), but there have been a number of variations. Kaplanis and Thompson (1967) used their tobacco hornworm procedure (see Figure 2, page 14) to isolate ecdysone (*2b*) and 20-hydroxyecdysone (*3b*) from the dry pinnae of the bracken fern *Pteridium aquilinum*.[56] Jizba, Herout and Šorm have used polyamide powder for column chromatography in the latter stages of their isolation of phytoecdysones from *Polypodium vulgare* rhizomes.[57] These they called polypodines, and polypodine A which represents approximately 1% (dry weight) of the rhizomes is identical with 20-hydroxyecdysone (*3b*).[58] It is interesting to note that these authors record that Volmar and Reebe in 1933 in an earlier investigation[59] of *Polypodium vulgare* reported a substance called polypodine which had the same characteristics as polypodine A, but they concluded from their tests that it was a glycoside.

Horn announced almost simultaneously with Nakanishi's initial discovery of phytoecdysones the presence of a

phytoecdysone in the wood of the Australian brown pine *Podocarpus elatus*.[60] This was later identified as 20-hydroxecdysone (*3b*), and was isolated in considerable quantities (0.05%) from the bark.[61] The extraction method combined some aspects of the crayfish procedure (see Figure 3, page 17) and some aspects of Nakanishi's procedure (see Figure 4, page 23) for leaf extractions. Column chromatography on alumina has been used extensively by Takemoto *et al.* in the latter stages of their plant extractions.[62,63]

Recently Hori (1969) reported[64] the separation of crude plant extracts with moulting hormone activity using an automatic column-chromatographic method employing amberlite XAD-2 resin. The eluent was monitored at three ultraviolet wavelengths and a separation of phytoecdysones was achieved. This technique was suggested for the exploratory investigation of ultraviolet-absorbing natural products and should have important applications in the detection of ecdysones and their metabolites.[65]

Research into new sources of phytoecdysones has been extensive and the extracts from many groups of plants have been investigated for moulting activity. The scale of the investigation can be appreciated from the statement by Imai and Nakanishi (1969) that additional sources[66] of phytoecdysones

have been discovered by the screening of 1056 species selected from 186 families of Japanese plants.[67] Activity is found in ferns and higher plants[68] and gymnosperm extracts show marked activity.[69] Even mulberry leaves contain phytoecdysones (0.001%).[70] Extracts of fungi and sea-weeds however, show no activity.[68]

Up to the present time about 50 named phytoecdysones have been isolated and all the plant counterparts of the arthropod moulting hormones so far extracted have been found. The rapid pace of discovery however caused confusion in the nomenclature of the compounds, and all the named phytoecdysones are represented by only 34 different structures. The phytoecdysones isolated up to 1970 have been reviewed.[55,71,72]

SYNTHESIS OF ECDYSONES

The synthesis of ecdysones and their analogues has been undertaken in several laboratories and two fundamentally different approaches have been used. The first, which was the basis of the earliest syntheses, began with comparatively simple starting steroids, and the synthesis of ecdysone (*2b*) using this approach was announced almost simultaneously in 1966 by two independent groups, Siddall *et al.* at Syntex,[73,74] and

Furlenmeier *et al.* at Schering AG-Hofmann-La Roche.[75] A bisnorcholenic acid derivative was the starting material used in each case. These multi-stage syntheses which gave poor overall yields of ecdysone (*2b*) have been reviewed by Berkoff[55] and Furlenmeier *et al.*[76]

Several subsequent syntheses[77-82] of ecdysones by different groups have used this same approach. The problem has been attacked in the majority of cases by the construction of the steroid nucleus followed by the side chain, but Mori *et al.* developed the inverse procedure.[83,84] Most of the syntheses have many stages in common while exhibiting one or more new ones. Ergosterol[85] and stigmasterol[86] have both been used as starting materials.

The second and more recent synthetic approach has been to modify the phytoecdysones available in gram quantities. This technique has been used by Horn *et al.*[87-90] and Hikino *et al.*[91] to prepare, in a few stages, ecdysone analogues varying in their degree and pattern of hydroxylation.

STRUCTURE AND ACTIVITY

With the large number of ecdysone analogues now available from natural and synthetic sources, investigations of structure-activity relationships are being undertaken.[92]

A clear pattern has not as yet emerged but several functional groups on the steroid skeleton are associated with high activity in moulting.[93] It is notable however that the synthetic chemists have not as yet succeeded in improving greatly on the activity of the natural ecdysones, many synthetic analogues being less active by factors ranging between ten and one hundred times.[88,89,92,94] Indeed certain synthetic steroids exhibiting only some of the features of the ecdysone molecule have inhibitory effects in insects.[92,95,96] The potential use of moulting hormones and their analogues as pesticides has been reviewed.[97-99]

The moulting hormone activity of some synthetic ecdysone analogues may reflect *in vivo* biosynthetic transformations, and Kaplanis *et al.* suggested this explanation for the low level of activity in a 6-hydroxyecdysone analogue.[92] The current information on metabolic pathways of steroids in insects has recently been discussed by Thompson.[100]

RECENT TRENDS

The sudden availability of ecdysones from 1966 onwards has allowed research into many aspects of the field to be vigorously pursued. Research into the mechanism of hormone action has been undertaken, and the effects of ecdysones on

the process of sclerotisation and the many aspects of insect metabolism have been investigated. These have been briefly reviewed.[55,101-103] The titres of moulting hormones in insects (see page 144) and the mechanisms of *in vivo* deactivation[104] and excretion[65] of moulting hormones have also been investigated. Research into the centres of moulting hormone production (see page 176) has been continued. Human tissues have been assayed for moulting activity[105] but none has yet been found, and the effects of ecdysones on protein synthesis and tumour inhibition in vertebrates are being investigated.[55,106]

DISCUSSION

The object of this work was to undertake a systematic investigation of nymphs of the desert locust *Schistocerca gregaria* for substances exhibiting moulting activity, with the ultimate aim of isolating and identifying any active components detected. Fifth instar nymphs, selected because they were the largest juvenile form of the desert locust, were available from the Anti-Locust Research Centre, London, and were used in these studies.

Throughout the work locust extracts were tested for moulting activity by Dr. P.E. Ellis at the Anti-Locust Research Centre. The bioassay used is described in the Experimental section (see page 193) and was developed for these investigations by Dr. Ellis, so that desert locusts themselves could be used as the test objects to determine the activity of extracts.

ISOLATION STUDIES

Fifth instar locusts were collected at the Anti-Locust Research Centre from the general stock to provide the material

for the large scale extractions of whole locusts. The nymphs were collected in the 8th to 11th day of the instar (see page 195) as preliminary investigations had shown that this corresponded to the period of maximum hormone titre.[31] As the nymphs became available they were homogenised with methanol to a fine pink--brown paste in a mechanical blender at a concentration of approximately 150 locusts per litre. This was stored in five gallon drums at room temperature. Methanol was chosen as Karlson had found it the best solvent for insect material,[12] and it can simultaneously act as a preservative when material is collected over extended periods.

The facilities at the Anti-Locust Research Centre only permitted storage of the homogenate at room temperature although lower temperatures would have been preferable.[24,28] Throughout the work undertaken at Keele care was taken to prevent thermal, biological or oxidative degradation of moulting substances in extracts. All the homogenate on arrival from the Anti-Locust Research Centre was put into cold storage (-5 to 0°) to await extraction, and extracts from all stages in the isolation were stored under an atmosphere of nitrogen at temperatures below 5°. Solutions were evaporated either *in vacuo* or under an atmosphere of nitrogen at temperatures below 40°.[42]

At the outset little was known about the nature of the

moulting hormones in the desert locust, except that Carlisle and Ellis[30] had indicated the presence of 20-hydroxyecdysone and a new moulting hormone which they called λ-ecdysone, but no details were available to support their claims.[31] As a complete investigation was being undertaken the possibility that several substances exhibiting moulting activity might be present, as detected by Burdette and Bullock in silkworm extracts,[21] was not overlooked. The further possibility was anticipated that these might appear in different fractions at some stage in an extraction from that expected for the then known insect moulting hormones, ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone. A preliminary extraction was therefore carried out beginning with Karlson's procedure of 1963 (outlined in Figure 1, page 9).

The homogenate prepared from approximately 350 nymphs gave after filtration and evaporation an aqueous concentrate (I) which unlike the corresponding extract from silkworm pupae contained only a small amount of free oil. This aqueous concentrate was partitioned between butanol and water, and the butanol phase was partitioned successively with cold aqueous acid and base solutions followed by washing to neutrality with water. The butanol extract gave an oil (II) which corresponded to one quarter

of the material in the initial extract* and this showed positive moulting activity.

An attempt to partition the oil (II) between light petroleum and water according to Karlson's procedure failed, as an emulsion was formed. The oil was therefore dissolved in a small volume of absolute ethanol and the solution was filtered to remove insoluble material. The ethanol was evaporated and the oil remaining was then successfully partitioned between water and light petroleum. The extract (III) isolated from the aqueous phase showed positive moulting activity as had been the case in Karlson's extraction of silkworm pupae.[13] The overall purification factor at this stage was however only 740 times compared with 4300 times in the case of the silkworm pupae which contained a much larger proportion of lipids than do desert locusts.

The aqueous extract (III) and a small amount of insoluble material (IV) which had also originated from the light petroleum-water partition were combined and the combined material was chromatographed on a column of alumina as described

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*When purification factors are quoted in the discussion they were calculated from the weight of active extract derived from a certain weight of nymphs, assuming no loss of moulting activity during the course of the extraction.

The mean weight of a desert locust nymph in the 8th to 11th day of the 5th instar as determined from six groups of ten nymphs each is 1.5 ± 0.1 g.

in the Experimental section. The column was eluted as shown in Table 2 (see page 199). Nine fractions were collected and these were divided into four groups A to D to reduce the number of bioassays required. This grouping was based partly on Karlson's results[13] and partly on the weight distribution observed for the fractions.

At this stage in the extraction these groups were further combined with corresponding material originating from the extraction of 1050 nymphs. The groups A and B, first eluted, were inactive, but groups C and D showed positive moulting activity. Karlson had found the bulk of his moulting activity in the silkworm extract corresponding to group C, and it was from this material that he isolated ecdysone and 20-hydroxyecdysone, but he did not report on the activity of his other alumina column fractions.

The subsequent stages in Karlson's procedure involved two countercurrent distributions, but these were avoided because a large number of fractions for bioassay would have been produced. Instead it was decided to select chromatographic techniques producing only a few extracts for bioassay, so that the relative levels of activity in extracts could initially be determined using more concentrated fractions.

Kaplanis *et al.* in their extraction procedure for the

isolation of moulting hormones from tobacco hornworm pupae (outlined in Figure 2, page 14), had successfully used columns of silica gel and silicic acid for the fractionation of their extracts which were subsequently found to contain both ecdysone and 20-hydroxyecdysone. Their results using the house fly bioassay indicated no loss of ecdysones on either absorbant, and in their preliminary work using silica gel to fractionate ^{14}C-ecdysones they noted no decomposition of active material.[24]

On the basis of these results the active extract (V) obtained by pooling the active fractions from the alumina column stage was chromatographed on a column of Davison silica gel according to the procedure of Kaplanis *et al.*[24] The column was eluted as shown in Table 3 (see page 201) and a small portion of material from each of the six fractions collected was used for a quantitative bioassay. The dose per abdomen was arbitarily chosen as 20 µl. of a 5% solution prepared by dissolving the bioassay sample in water. Only the material in fractions two and three showed moulting activity. All the active material isolated from the tobacco hornworm pupae was detected in fraction three and this fraction contained the bulk of the active material from the locust extract.

Karlson had reported that when a hormone rich starting material such as silkworm pupae was extracted, ecdysone could be

crystallised from the most active alumina column fractions,[12] and Carlisle and Ellis had reported the use of thin layer techniques in their claim to have detected ecdysones in the desert locust.[30] Thin layer chromatography (t.l.c.) of the silica gel column fractions (see page 202) however showed that all the fractions were complex mixtures, and the identification of any components as ecdysones was not possible.

The active material from the silica gel column (fractions two and three) was therefore combined and chromatographed on a column of silicic acid, and Bio-sil A was used as the absorbant as the Unisil used by Kaplanis *et al.*[24] was not available. The column was eluted as shown in Table 4 (see page 203) and material from each of the four fractions collected was subjected to bioassay. The dose per abdomen for each extract was selected on the basis of the number of nymphs from which that extract was derived (see page 194), as it was considered that this would give a better indication of the relative levels of activity in extracts when comparing one stage in the extraction with another. Each fraction was assayed at one nymph equivalent and one fifth of a nymph equivalent per abdomen, and at these dose levels all four fractions eluted from the column showed moulting activity.

The results showed that the column had given a poor

separation of components, and the bulk of the weight and activity had been eluted in the first fraction followed by a decrease in weight and activity for subsequent fractions. The ecdysones isolated by Kaplanis *et al.* from the tobacco hornworm pupae were eluted from the silicic acid column exclusively in fraction two. T.l.c. of the four fractions showed that they were all still complex mixtures.

This preliminary investigation had shown that substances exhibiting moulting activity could be isolated from desert locust nymphs and partially purified by the extraction procedure so far described. Examination of the extracts from the latter stages in this isolation had shown that they were still complex mixtures of components, and that considerable purification would be required to effect isolation of the active substances. As the quantity of active extract remaining after the silicic acid column stage was only about 500 mg., it was decided to undertake a large scale extraction based on a modification of the above procedure, to provide a larger quantity of material for further investigation.

The modified extraction was begun with the homogenate prepared from 2271 nymphs. The methanol extract obtained after filtration was evaporated to the aqueous concentrate (VI) which was partitioned between butanol and water. All the active

material was extracted into the butanol phase which was evaporated to give the oil (VII). The preliminary procedure was modified at this stage and the butanol partitions with acid and base were avoided as it had been reported that hormones of the ecdysone type were labile under such conditions.[107] It was anticipated that an active substance could be partially or completely deactivated by this treatment and that its loss of activity might have gone unnoticed.

The active extract(VII) from the butanol phase was treated directly with absolute ethanol, and the soluble portion, after evaporation of the ethanol, was partitioned between light petroleum and water. The phases were evaporated and the extracts from both of the above stages were tested for moulting activity (see Tables 5 and 6, pages 206 and 207). All the active material was detected in the portion of the extract soluble in ethanol, and this active material was extracted solely into the aqueous phase of the partition to give the active extract VIII.

The extraction of a further 2300 nymphs according to the modified procedure described above yielded a similar active extract (VIIIa) from the aqueous phase of the light petroleum--water partition. This was chromatographed on a column of alumina as described in the preliminary extraction procedure, but a modified method of absorbing the material onto the alumina

which is described in the Experimental section (see page 208) was used. The insoluble material (IX) which could not be dissolved for chromatography was shown to be inactive, and the column was eluted with a wider range of solvents than used in the preliminary procedure (see Table 7, page 209).

The materials eluted were combined into six groups, A to F, as shown in Table 7, and as a spread of moulting activity had been expected each group was assayed at a dose equivalent to two nymphs per abdomen. The high level of moulting activity expected in the ethyl acetate-methanol fraction was observed, but the material eluted with methanol and aqueous methanol also showed relatively high moulting activity. The results in Table 7 indicated a much wider spread of activity into fractions eluted with polar eluents than found by Karlson in his extraction of silkworm pupae, and indicated that an active substance more polar than 20-hydroxyecdysone might be present in the extract.

The extract from the combined fractions two to four, group B, was the only other material to show moulting activity, but as this was at a low level it probably represented the first traces of active material, the majority of which was eluted in fraction five.

T.l.c. showed that all the fractions were complex mixtures of components as had been expected from the preliminary

results, and the active groups of fractions B to E were therefore combined. This material was further combined with similar material from approximately 3200 nymphs to give the pooled extract X. This was assayed at doses of two and one nymph equivalents per abdomen and showed activities of 66% and 67% respectively. The overall purification factor achieved at this stage was 690 times.

When a further batch of 2521 nymphs were extracted only four fractions were collected from the alumina column as shown in Table 8 (see page 211), and the material with moulting activity was confined to the last three fractions, by eluting the first fraction with a reduced volume of solvent. This gave an improved separation of active and inactive material. When the results in Table 8 are compared with those given in Table 7 (see page 209), it can be seen that the level of moulting activity was lower in this extract than in the one previously discussed. Such differences in the activity of corresponding fractions obtained from different extractions were observed but this kind of variability in biological material was to be expected.

The control assays for the results in Table 8 were 20% and 14% active respectively, and control assays generally showed a range of activity. This however was also to be

expected with biological material, and the level of 20% activity represented the average control value obtained during the course of this work. Although the control levels ranged from 0% to 33% activity they rarely exceeded 25% activity.

The efficiency of the modified method used to prepare the soluble portion of the aqueous extract from the light petroleum-water partition for chromatography on alumina was checked using a new aqueous extract (VIIIb) from 4140 nymphs. Portions of this material were triturated with butanol and ethyl acetate (see page 212) using varying proportions of butanol and ethyl acetate as shown in Table 9 (see page 213). The bioassay results showed that the active material was incompletely extracted from the insoluble residue if a greater proportion of ethyl acetate - butanol than (5:1) was used in the trituration. The insoluble material was however inactive when the extract had been triturated with ethyl acetate - butanol (5:1), and it was concluded that these proportions gave the most efficient separation of active and inactive components when using this solvent system.

The combined active fractions from alumina chromatography, extract X, representing 5500 nymphs were chromatographed on Davison silica gel, and seven fractions were collected (see Table 10, page 215), as compared with only six in the preliminary extraction, in an attempt to improve the separation. The assay

results given in Table 10 showed that the bulk of the active material had been eluted in fraction three. Collection of the benzene methanol (19:1) fraction in two portions had improved the separation, and fraction two contained mostly inactive material while fraction 2a showed high moulting activity. Active material was also eluted in fractions four and five, and this corresponded to a wider spread of activity into the polar fractions than detected by Kaplanis *et al.* in the extraction of tobacco hornworm pupae.

A portion of fraction three, the major active fraction, was subjected to t.l.c. and the results showed that the material was a complex mixture of components (see Figure 62, page 216). A reasonable separation of the major components visible under U.V. was however achieved using a solvent system of chloroform - 95% ethanol (4:1).[27]

The active fractions 2a to 5 from the silica gel column were combined to give extract XI which represented the extract from 5100 nymphs and corresponded to an overall purification factor at this stage of 1070 times. The extract showed moulting activity with an ED_{50}* between 1000 and 100 µg.

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*The ED_{50} of an extract is defined as the dose per abdomen which gives 50% moulting in a group of abdomens after subtraction of the control activity. The values given are only approximate and were used as a guide to the recovery of active material in the purification.

The active fractions two to four in Table 8 (see page 211) which originated from the second alumina column discussed (see page 40) were also combined and chromatographed on Davison silica gel, and the results are given in Table 11 (see page 218). These were similar to those discussed above for the corresponding material in extract X and the bulk of the weight and active material was again eluted in fraction three.

Column chromatography had been the last stage in the preliminary investigation, but this had given a poor separation of the active and inactive components. A portion of the combined active extract XI from the Davison silica gel column was however chromatographed on a column of Bio-sil silicic acid as a check on the earlier result. T.l.c. of the eluted materials showed that this absorbant had again given a poor separation of components, and the results given in Table 12 (see page 219) indicated that the bulk of the material had been eluted in the first two fractions, and that all four fractions contained material with moulting activity.

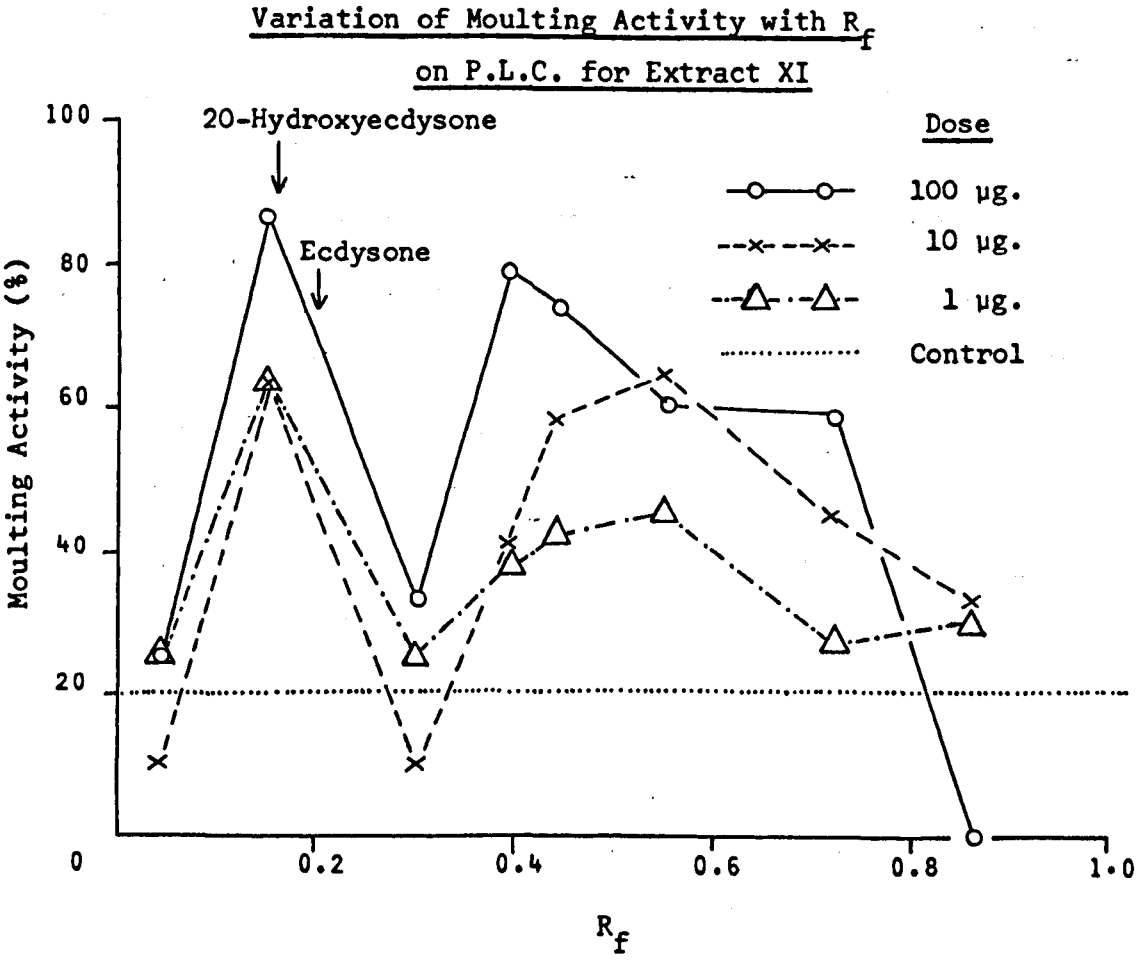
Preparative layer chromatography (P.L.C.) had been used on several previous occasions for the successful separation of ecdysones,[35,36,74] and it was recalled that t.l.c. using the solvent system chloroform - 95% ethanol (4:1) had given a

reasonable separation of the major components visible under U.V. in fraction three, the major active fraction, eluted from the Davison silica gel column described on page 213.

A portion of the extract XI, representing the material combined after chromatography on Davison silica gel, was therefore subjected to P.L.C. eluting with chloroform - 95% ethanol (4:1). A number of bands were seen under U.V. with faint absorption between them, and these are shown diagrammatically in Figure 63 (see page 221). The silica gel on the plate from R_f 0.00 to 1.00 was divided into eight areas as shown in Table 13, accompanying Figure 63, and the absorbed material was eluted with an overall recovery of about 86%.

The eluted materials were assayed for moulting activity, and the results given in Table 14 (see page 222) showed a separation of active components which indicated the presence of at least two active substances in desert locust nymphs. The variation of moulting activity with R_f at each of the three doses assayed is given in Figure 5 which shows the two peaks of activity.

FIGURE 5



The lower peak of activity which was represented by a narrow band on the plate was centred at about R_f 0.15, and this corresponded closely to the values of R_f 0.20 and 0.17 determined experimentally for ecdysone and 20-hydroxyecdysone respectively. The upper peak of activity which was represented by a broad band on the plate was centred in the region R_f 0.40 to 0.70 and must therefore have corresponded to at least one active substance less polar than ecdysone in absorption

chromatography. A high value of R_f 0.37 had been reported for ecdysone by Thompson *et al.*,[25] but the experimental value of R_f 0.20 determined during the course of this work was confirmed by Horn *et al.*[108] in 1970.

T.l.c. of the separated materials run as for P.L.C. showed only a partial separation of components, and all the fractions contained small amounts of overlapping material. The complexity of the original mixture, which was evident from the number of bands visible on the P.L.C. plate, had probably caused this partial entrainment of components. It was therefore decided to look for a method which would give a preliminary separation of components in extract XI before using P.L.C. to give a separation of active components.

When portions of extract XI had been subjected to column chromatography on silicic acid and P.L.C. on silica gel, a small amount of material in the extract was found to be sparingly soluble in methanol. As it was expected that any substances with moulting activity in the extract would be soluble in methanol, this provided a method for the removal of the insoluble material which might interfere with the dissolution of the extract in any further chromatography. A portion of the extract XI was therefore triturated with small volumes of methanol (see page 222), and the extracts obtained were assayed for

moulting activity. The results (see Table 15, page 223) showed that one trituration with methanol left only essentially inactive material undissolved, and sparingly soluble solid which represented about 5% of the extract could be removed as an inactive residue by three such triturations.

The procedure just discussed was carried out on the remainder of extract XI, and the soluble portion obtained was combined with similar material originating from fractions three and four in Table 11 (see page 218) to give extract XII. This material represented the active extract from approximately 6400 nymphs, and the assay results (see page 224) showed that it had an ED_{50} between 1000 and 100 µg. per abdomen. The overall purification factor at this stage was 1310 times.

In an attempt to find a method which would give a preliminary separation of the components in the active extract before P.L.C. was used, portions of extract XII were subjected to column chromatography on different absorbants, and the fractions eluted were monitored by t.l.c.

The bulk of the material had been eluted in the first two fractions when extract XI was chromatographed on silicic acid, and it was suspected at this stage that the spread of activity into all the fractions eluted from this column was due to the presence of more than one substance with moulting activity.

It was therefore decided to chromatograph a portion of extract XII on the silicic acid absorbant, and to begin eluting the column with solvent mixtures less polar in the eluotropic series than those previously used.

To enable the use of less polar solvent mixtures than those able to dissolve the sample in a reasonable volume, the extract to be chromatographed was absorbed onto about 6% of the column packing to give a free flowing powder (see page 224)*. This was then packed onto the top of the column before elution.

The results given in Table 16 (see page 226) showed that the absorbant had again given an unsatisfactory separation, and that materials with a wide range of R_f values (0.20 to 0.90) had been eluted in the early fractions. All the material was therefore eluted from the column and the fractions were recombined. The combined material was chromatographed on a column of floridin earth, but the results given in Table 17 (see page 227) showed that the separation of the components was again unsatisfactory.

Resolution in absorption chromatography is increased with decreasing particle size.[109] As the recovery of the active substances from the silica gel PF_{254} absorbant used for P.L.C.

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*Bioassay results during the course of this work had shown a good recovery of active material from the column using this technique.

had been satisfactory, and this had a particle size in the range 10 to 40 μ., it was decided to chromatograph a portion of extract XII on a column of this absorbant. All the material eluted from the floridin earth column was combined with a fresh portion of extract XII and this was absorbed onto silica gel PF_{254} to give a free flowing powder for chromatography. To give a satisfactory flow rate pressure was applied to the top of the column. The results showed that the separation of components was much better than for either of the two previous columns.

To determine the conditions required to give a good separation of components in extract XII using the silica gel PF_{254} absorbant, a second column the same as the first was prepared using a fresh portion of this extract. The column was eluted as shown in Table 18 (see page 229) and no one fraction was found to contain the bulk of the eluted material.

All the fractions were spotted on a single t.l.c. plate, and the results obtained on visualising the spots under U.V. are shown diagrammatically in Figure 64, page 230. The first four fractions eluted showed components in the region R_f 0.28 to 0.90, and although this overlapped with the R_f range (0.10 to 0.70) shown by fractions five to seven the appearance of the t.l.c. spots was essentially different. This was also

the case when fractions 8 to 11 were compared with fractions 12 and 13. The latter two fractions showed intense violet spots at R_f 0.07 and 0.13 as the major components visualised under U.V., and they were the only fractions that contained these materials.

As a column of silica gel PF_{254} had given a separation of the components in extract XII on the basis of their R_f values on t.l.c., a portion of this extract, representing about half the material which remained, was chromatographed on a similar but larger column. Four fractions were eluted as shown in Table 19 (see page 231) and these all exhibited moulting activity. T.l.c. confirmed a satisfactory separation of components and the result is shown diagrammatically in Figure 65 (see page 232). Only fraction four showed the intense violet spots under U.V. at R_f 0.07 and 0.13. The remaining portion of extract XII was also chromatographed on a column of silica gel PF_{254} to give the fractions 1a to 4a.

As a preliminary separation of the components in extract XII had been achieved, P.L.C. could be used for each of the simpler mixtures of components represented by fractions 1 to 4 and 1a to 4a from the silica gel PF_{254} columns. Fractions 1 to 4 were therefore subjected to P.L.C. using more polar solvents for the more polar later fractions from the column.

This enabled a greater area of the P.L.C. plates to be used for the separation of the mixtures than would have been possible eluting only with chloroform - 95% ethanol (4:1), and as a consequence reduced the overlap of the absorbed materials.

The bands detected by U.V. for each of the four fractions showed that each fraction was still a mixture of many components (see Figures 66 to 69, pages 234 to 235). The silica gel on the plates was cut into areas corresponding to the major bands as shown in Tables 20 to 23 accompanying Figures 66 to 69, and the absorbed material was eluted with an overall recovery which exceeded 90% for each column fraction chromatographed. T.l.c. of these materials showed a good separation, and only slight overlap was observed for material recovered from adjacent bands on the same plate.

Assay of moulting activity on the separated materials, together with the t.l.c. results on those found to be active showed that the moulting activity was concentrated into three areas.

All of the materials isolated were assayed at three dose levels, and the results which are given in Tables 24 to 27 (see pages 236 to 238) showed a variation of moulting activity with R_f. This is plotted in Figures 6 to 9.

FIGURE 6 Variation of Moulting Activity with R_f on P.L.C. for Fraction 1 from the Silica Gel PF254 Column

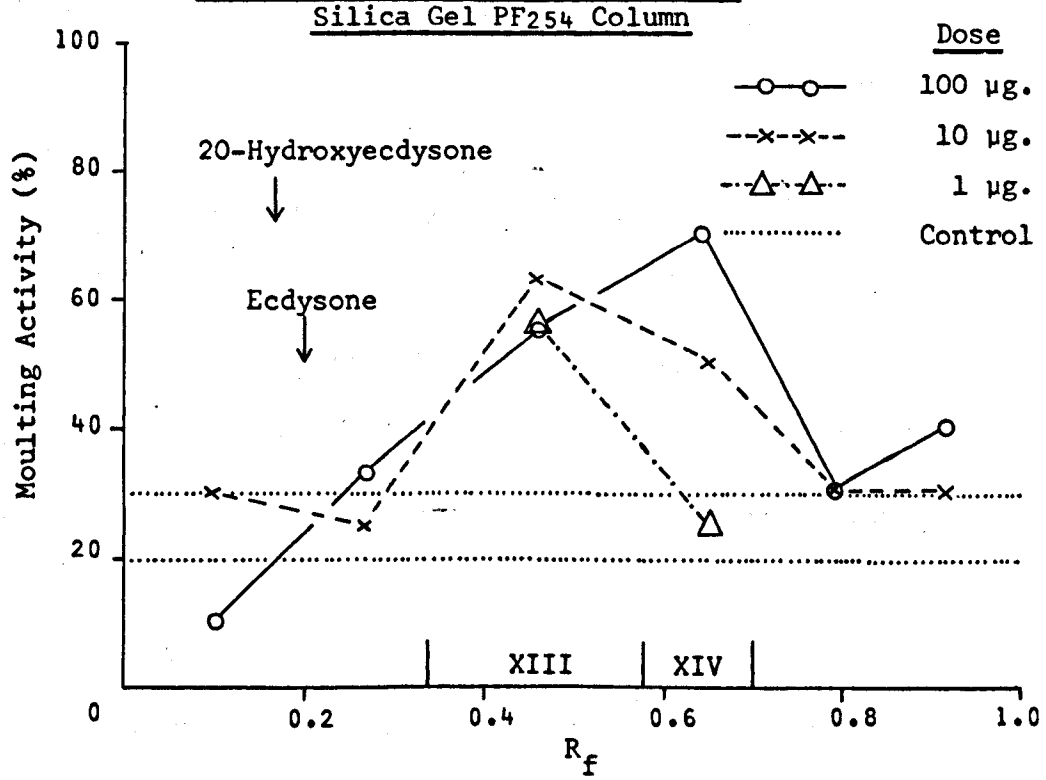


FIGURE 7 Variation of Moulting Activity with R_f on P.L.C. for Fraction 2 from the Silica Gel PF254 Column

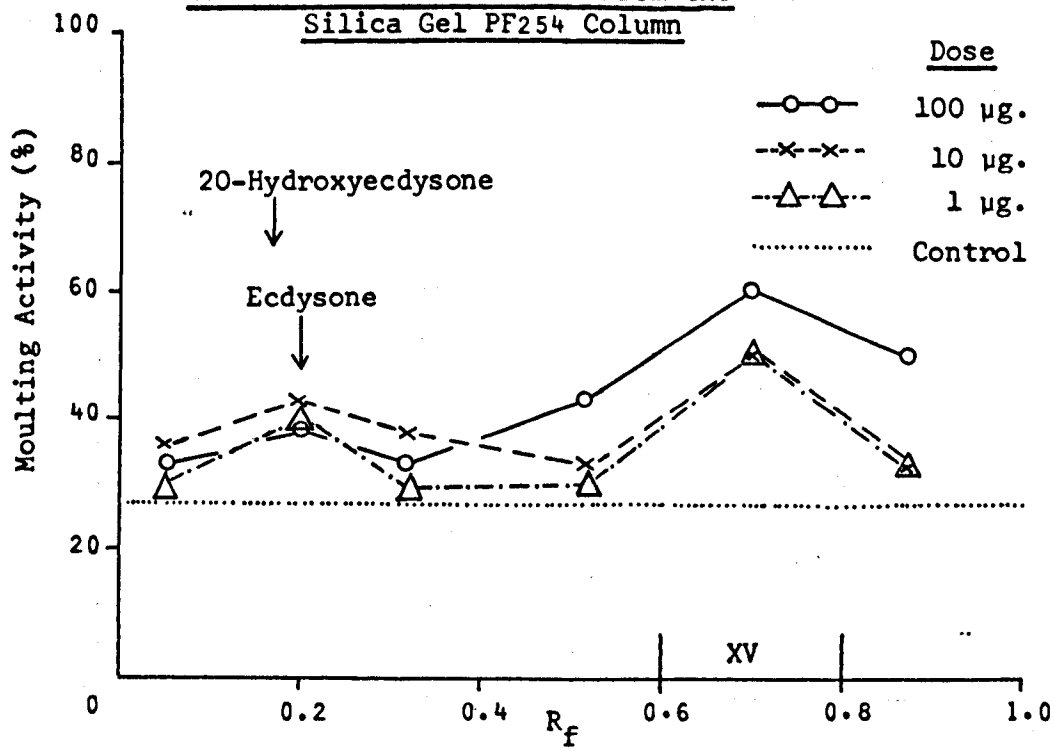


FIGURE 8 Variation of Moulting Activity with R_f on P.L.C. for Fraction 3 from the Silica Gel PF_{254} Column

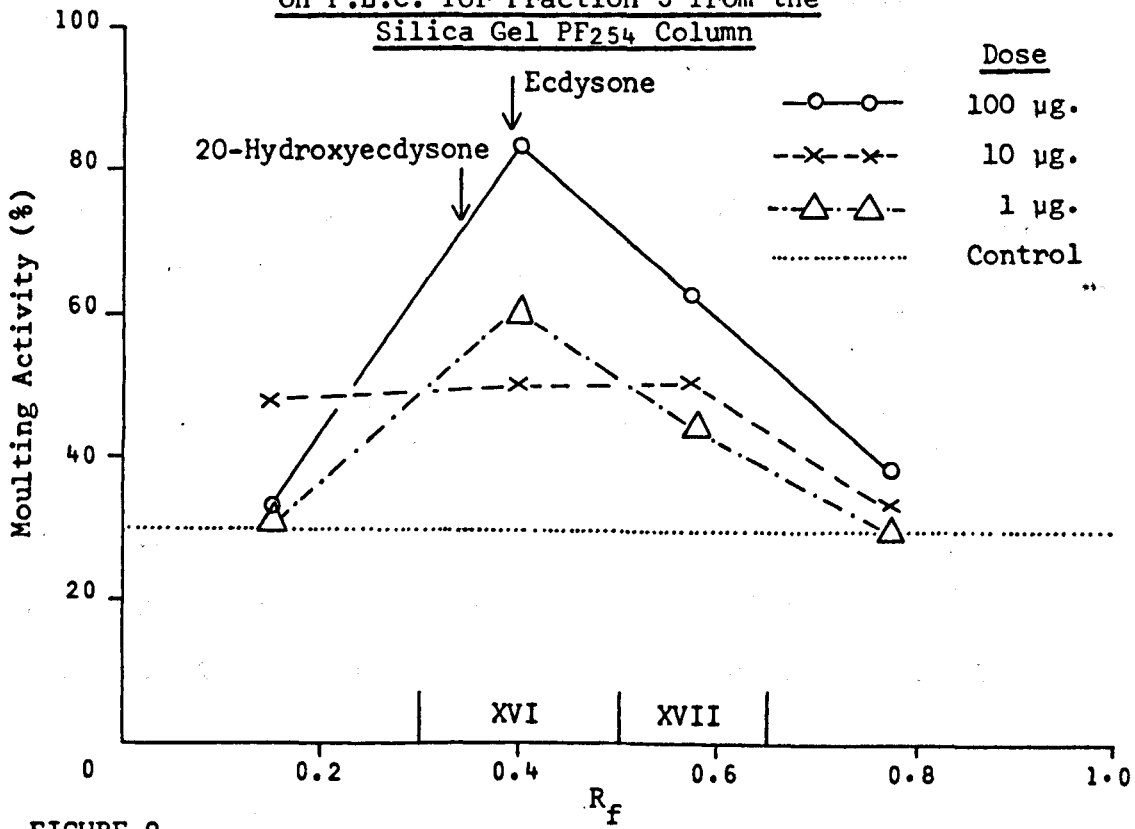
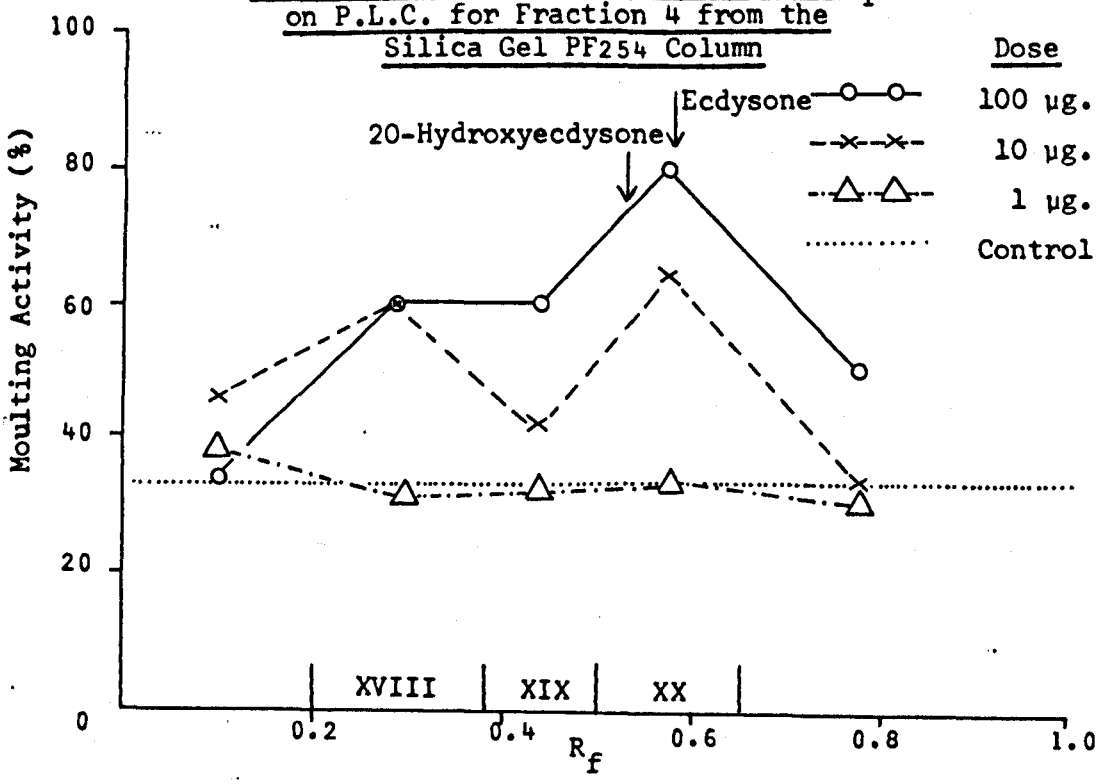


FIGURE 9 Variation of Moulting Activity with R_f on P.L.C. for Fraction 4 from the Silica Gel PF_{254} Column



The materials exhibiting relatively high moulting activity were selected for further investigation, and these active materials were numbered XIII to XX (see Table 28, page 239). The R_f regions from which these active extracts were eluted are indicated in Figures 6 to 9.

The extracts XIII to XV, which from their R_f ranges in P.L.C. corresponded to the upper peak of activity shown in Figure 5 (see page 45), were on t.l.c. in chloroform - 95% ethanol (4:1) all found to contain components visible under U.V. in the region R_f 0.50 to 0.60. The remaining material from extracts XIII to XV was therefore combined as extract XXI which was called the *upper active band.*

Extracts XVI to XX were also compared on t.l.c. (see Table 29, page 240) and the results showed that these materials corresponded to the lower peak of activity in Figure 5 (see page 45). The extracts could however be combined into two groups with different R_f ranges. Extracts XVI, XVII and XX were combined to give extract XXII which had a R_f range of 0.15 to 0.28 using chloroform - 95% ethanol (4:1), and this was called the *middle active band.* The remaining two extracts XVIII and XIX which corresponded to the major components in column fraction four visible on t.l.c. under U.V. as violet spots, gave extract XXIII. This had a R_f range of 0.05 to 0.18 using chloroform - 95% ethanol

(4:1), and was called the *lower active band.*

Comparison of the R_f ranges for the three active bands with the experimental R_f values for ecydsone and 20-hydroxy-ecdysone (see Figures 6 to 9 and Table 29) showed that the upper active band, which had originated from the column fractions one and two, could correspond to neither of these two hormones and must have contained active material less polar than ecdysone. The middle active band, which had originated from column fractions three and four, corresponded to the R_f range expected for the two known hormones and could possibly have contained either of them. The lower active band which only just overlapped with the R_f region for 20-hydroxyecdysone could possibly have contained this hormone, but as this material had only just been resolved from the middle band material (see Figure 9), these two bands could be expected to share active material in common.

P.L.C. was also carried out on fractions 1a to 4a from the second portion of extract XII, and the active materials isolated were combined with extracts XXI to XXIII. This gave three new extracts, upper active band XXIa, middle active band XXIIa, and lower active band XXIIIa, each representing active material from approximately 4500 nymphs.

T.l.c. of the upper and middle band materials under a

wide range of conditions failed to give any satisfactory resolution of the components. T.l.c. on polyamide had given unsatisfactory separation and therefore this support was unsuitable for column chromatography of these extracts, although it had previously been used in the isolation of ecdysones from plant extracts.[57] An attempt to grind amberlite XAD-2 resin and prepare a column as used by Hori[64] for the isolation of ecdysones from plant extracts and Moriyama *et al.*[65] for the isolation of ecdysones from insect extracts was unsuccessful, as the column would not run even under pressure. The use of dry column chromatography[110] on silica gel PF_{254} to resolve components in the upper band material also failed.

At this stage in the work two more batches of homogenate prepared from a total of 1.3×10^4 nymphs were extracted. An isolation procedure based on the one discussed above was used, but chromatography on alumina was avoided. Horn had reported a 75% loss of ecdysone activity on deactivated alumina in his extraction of crayfish waste,[42] and Kaplanis *et al.* had found that neutral, activity V alumina, prepared a considerable time before use, or which had formed clumps after deactivation, was destructive to ecdysones.[24] This stage in the locust extraction had given a highly active extract (see Table 7, page 209) but little purification, and as the possibility existed that ecdysones

could be deactivated it was avoided in all further isolations.

The purification of homogenate prepared from approximately 7000 nymphs gave an active extract from the aqueous phase of the light petroleum-water partition. This was triturated with butanol and ethyl acetate, and the soluble portion was isolated and chromatographed on Davison silica gel (see Table 30, page 244). The bioassay results showed that fraction three contained the bulk of the active material as had been expected from previous extraction results (see Table 10, page 215). The corresponding fraction from the extraction of approximately 6000 nymphs also contained the bulk of the active material at this stage, and both extracts had an ED_{50} between 1000 and 100 µg. Combination of these two fractions gave an extract originating from approximately 1.3×10^4 nymphs, and this was triturated with methanol and the soluble material isolated to give extract XXIV. This extract was chromatographed in two portions on columns of silica gel PF_{254}. Each column was eluted to give five fractions in an attempt to separate completely at this stage the material corresponding to the middle and lower active bands (the results for one portion of extract XXIV are given in Table 31, page 246). A larger proportion of solvent was used to elute the first fraction in an attempt to confine all the active material corresponding

to the upper band in this fraction. The relatively low activity in fraction two shows that this was partially successful.

The fractions one to five from the column were subjected to P.L.C. and the materials were isolated from the bands detected under U.V. Assay for moulting activity on the separated materials together with t.l.c. results showed that the moulting activity was concentrated into the same three areas as found for the earlier extraction. The assay results which are given in Tables 32 to 36 (see pages 247 to 249) show a variation of moulting activity with R_f, and this is plotted in Figures 10 to 14. The positions for samples of ecdysone and 20-hydroxyecdysone chromatographed under the same conditions are shown in each figure.

FIGURE 10 Variation of Moulting Activity with R_f on P.L.C. for Fraction 1 from the Silica Gel PF_{254} Column

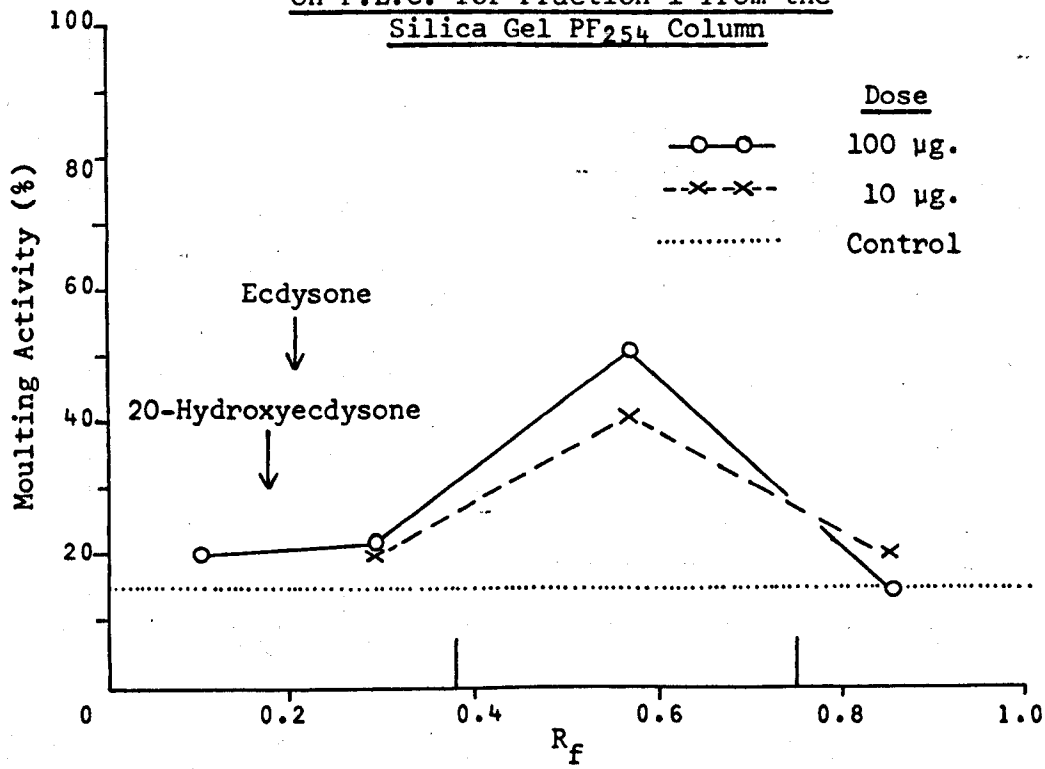


FIGURE 11 Variation of Moulting Activity with R_f on P.L.C. for Fraction 2 from the Silica Gel PF_{254} Column

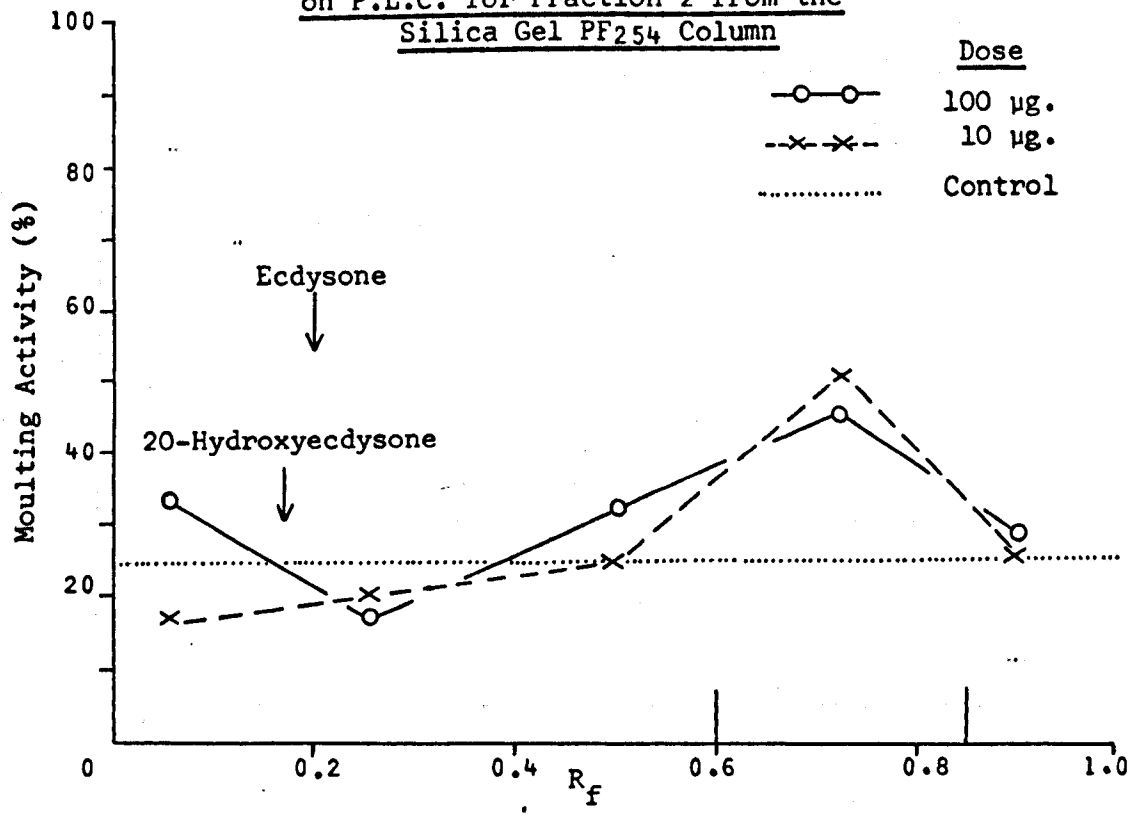


FIGURE 12 Variation of Moulting Activity with R_f on P.L.C. for Fraction 3 from the Silica Gel PF_{254} Column

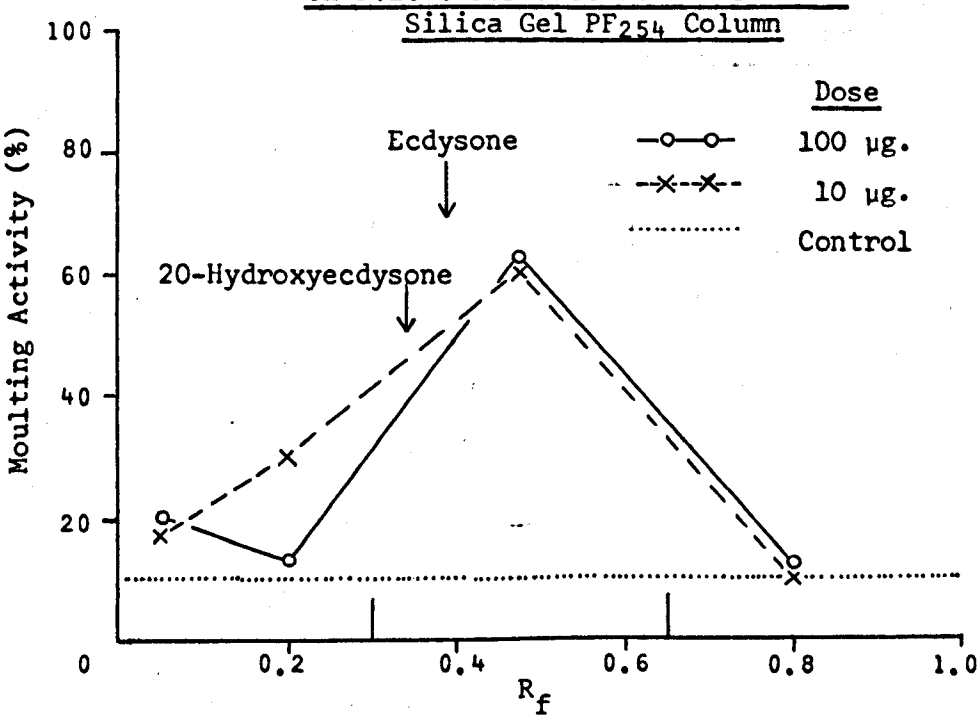


FIGURE 13 Variation of Moulting Activity with R_f on P.L.C. for Fraction 4 from the Silica Gel PF_{254} Column

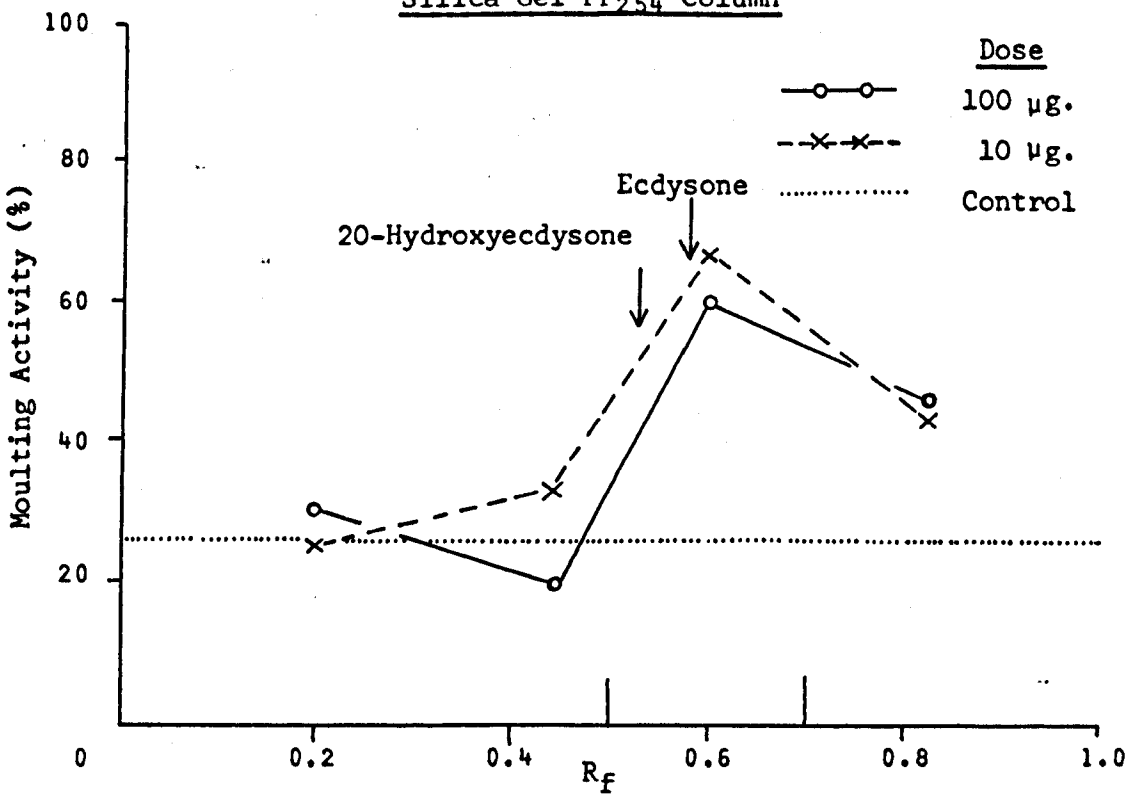
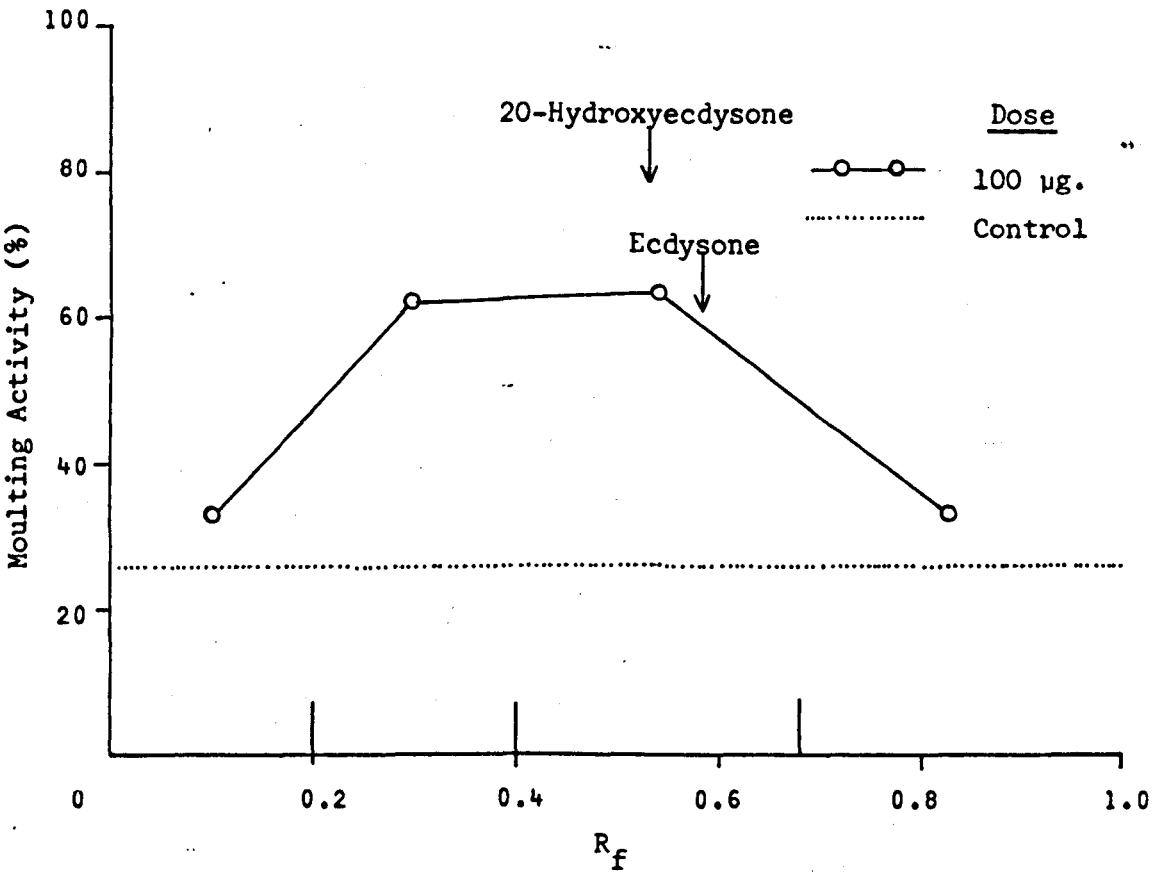


FIGURE 14 Variation of Moulting Activity with R_f on P.L.C. for Fraction 5 from the Silica Gel PF_{254} Column



A peak of activity corresponding to the upper active band is observed in Figure 10, and in Figure 11 where it represents the residual active material remaining in column fraction two. Figures 12 and 13 both show a peak of activity corresponding to middle active band material. This is also observed in Figure 14, but appears unresolved from the peak of

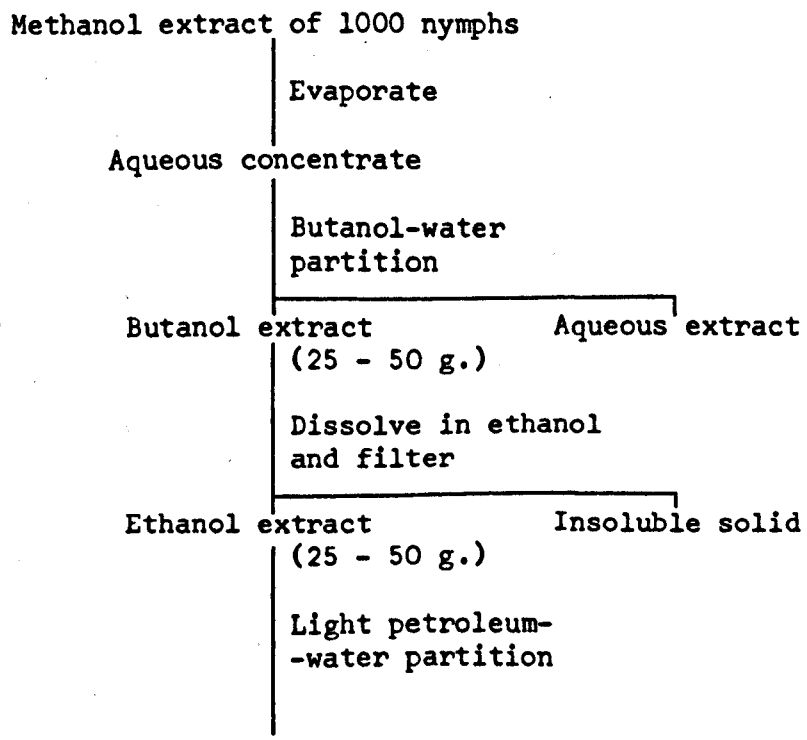
activity corresponding to the material from the lower active band. The degree of resolution can not be determined as these active materials were eluted from adjacent bands on P.L.C. The double peak of activity in Figure 14 shows that eluting five fractions from the column still failed to resolve the active components from the middle and lower active bands.

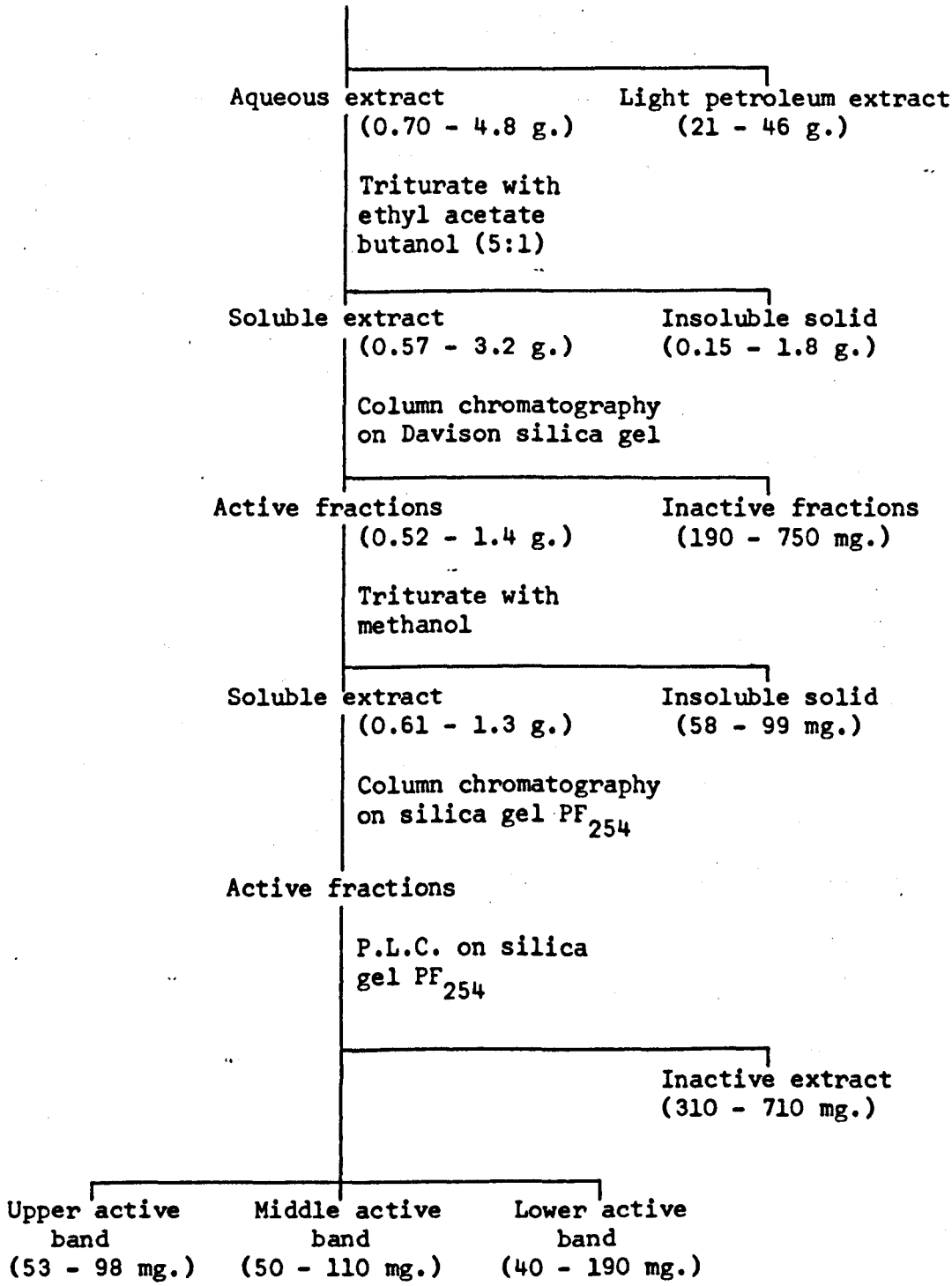
Fractions 1a to 5a originating from the second portion of extract XXIV were similarly subjected to P.L.C. The active materials isolated from the P.L.C. of both portions of extract XXIV were then combined to give upper, middle and lower active bands, and these were further combined with their active counterparts XXIa, XXIIa and XXIIIa which originated from the previous extraction. This gave three new active extracts each representing approximately 1.7×10^4 nymphs: Extract XXV as the upper active band (purification factor approximately 1.7×10^4 times), extract XXVI as the middle active band (purification factor approximately 2.3×10^4 times), and extract XXVII as the lower active band (purification factor approximately 2.1×10^4 times). These extracts were assayed for moulting activity and all three had an ED_{50} of approximately 100 µg.

A flow sheet showing the steps in the standard extraction procedure, developed as discussed above is given on the next page. The range of weights given for extracts at each

stage in the procedure is based on the extraction of 1000 nymphs and was calculated from the results of the several extractions carried out. The values given after the ethyl acetate - butanol (5:1) trituration stage were estimated from the results of all the extractions including those involving chromatography on alumina.

The Standard Extraction Procedure for Desert Locust Nymphs




Aqueous extract
(0.70 - 4.8 g.)
Light petroleum extract
(21 - 46 g.)
Triturate with ethyl acetate butanol (5:1)
Soluble extract
(0.57 - 3.2 g.)
Insoluble solid
(0.15 - 1.8 g.)
Column chromatography on Davison silica gel
Active fractions
(0.52 - 1.4 g.)
Inactive fractions
(190 - 750 mg.)
Triturate with methanol
Soluble extract
(0.61 - 1.3 g.)
Insoluble solid
(58 - 99 mg.)
Column chromatography on silica gel PF254
Active fractions
P.L.C. on silica gel PF254
Inactive extract
(310 - 710 mg.)
Upper active band
(53 - 98 mg.)
Middle active band
(50 - 110 mg.)
Lower active band
(40 - 190 mg.)

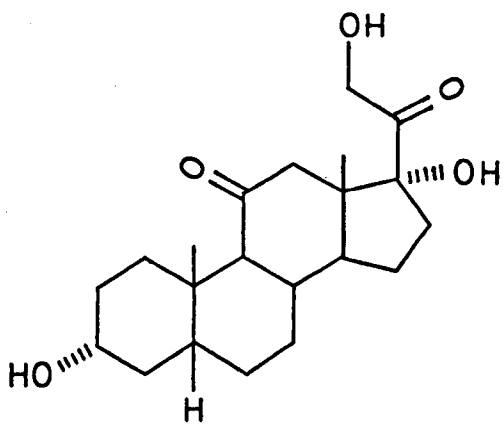
SYNTHESIS, GAS-LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY OF DERIVATIVES OF ECDYSONES

Throughout all previous moulting hormone isolations only bioassay techniques were available for the detection and quantitative determination of substances with moulting activity. Thin layer methods have been reported for the detection of ecdysones, but these are only suitable for highly purified extracts from insects[28,29] and crustaceans;[42] a result confirmed by the present studies. It was therefore important to develop a reliable chemical method to detect small amounts of substances with moulting activity which could be used on crude extracts for their determination.

Gas-liquid chromatography (g.l.c.) is an important quantitative method for the determination of small amounts of natural products in biological materials. Methods were available for the gas liquid chromatographic determination of steroids in mixtures,[111,112] but none had been applied to compounds of the ecdysone type. Samples of ecdysone (2.4 mg.) and 20-hydroxyecdysone (10 mg.), two hormones suspected as active components in the desert locust extract, were available. As all the insect moulting hormones previously isolated were polyhydroxy-steroids of the ecdysone type (see Introduction), it was decided to investigate the conditions necessary to give

high yields of derivatives of these hormones that were sufficiently volatile for g.l.c.

Chambaz and Horning had reported one of the few methods available to prepare volatile derivatives for use in the gas-liquid chromatographic identification of polar steroids.[113] They converted steroids in the pregnane series, such as tetrahydrocortisone (*9*), into their methoxime-trimethylsilyl (MO-TMSi) derivatives using O-methyl hydroxylamine hydrochloride and a range of silylating reagents.



(*9*)

Trimethylsilyl (TMSi) derivatives have been widely used for the gas-liquid chromatographic separation of steroids since their introduction into the field in 1961.[111,113] Their behaviour in mass spectrometry is valuable for identification

purposes,[114] and their 'hydrocarbon like' polarity is useful in t.l.c. for the separation of easily silylated hydroxy-steroids and compounds remaining unchanged.[115] The use of methoxime (MO) derivatives of steroid ketones for analytical and structural studies was recommended by Fales and Luukkainen[116] in 1965. They reported that these derivatives were stable under ordinary conditions of laboratory manipulation, and that they had good g.l.c. properties with lack of adsorption. Chambaz *et al.* found that when steroids containing keto groups were subjected directly to silylation conditions enol-trimethylsilyl ethers could be formed in certain cases, and they recommended that MO-TMSi derivatives be prepared for these compounds,[117] but they did not report on the 7-en-6-one grouping characteristic of the ecdysones.

Steroids with ecdysone structure are labile except under comparatively mild reaction conditions.[72] MO derivatives were therefore prepared using O-methyl hydroxylamine hydrochloride, but a wide range of reagents with different abilities to silylate sterically hindered hydroxyl groups were available.[117] The use of bis(trimethylsilyl)acetamide (B.S.A.) had been reported for quantitative silylation in many silyl-proton exchange reactions,[118] and this reagent was selected for the preparation of the trimethylsilyl ether derivatives, although it was known

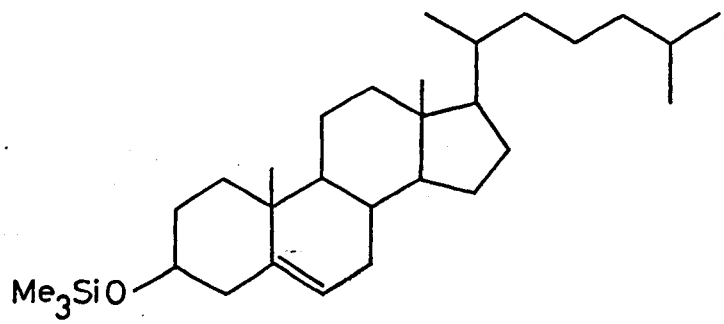
that it would not silylate the highly hindered 11β- and 17α-steroid hydroxyl groups.[113] Chlorosilanes were avoided as they produce HCl under silylation conditions.

Synthesis of Compounds for Gas-Liquid Chromatography

Since only small quantities of ecdysone (2.4 mg.) and 20-hydroxyecdysone (10 mg.) were available, it was decided to investigate derivative formation with model steroids, so that conditions for the formation and isolation of derivatives of the ecdysones could first be established. The derivatives of these model compounds could also be used as chromatographic standards, and as reference compounds for the interpretation of data from g.l.c. and mass spectrometry (m.s.).

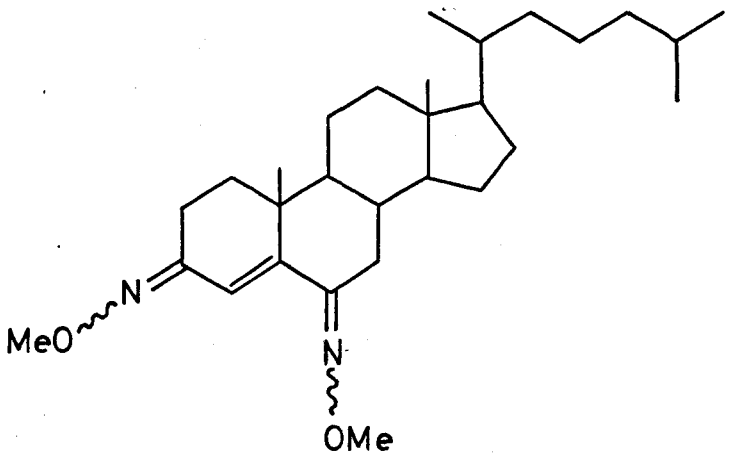
TMSi derivatives of non-phenolic hydroxy-steroids have been reported to be stable under t.l.c. conditions,[115] but the ease of hydrolysis of such derivatives has also been reported.[111] TMSi-cholesterol (*10*) was therefore prepared under dry conditions, and a few crystals were exposed to the atmosphere over saturated sodium chloride solution. T.l.c. after two weeks showed the material to be unchanged, and as the 3β-hydroxyl group in cholesterol is in a relatively exposed position, this confirmed the stability of TMSi derivatives to hydrolysis under ordinary laboratory conditions.

Care was however taken to avoid any potentially hydrolysing conditions when working with TMSi derivatives.



(10)

4-Cholesten-3,6-dione was a readily available steroid containing only ketone functional groups, and this was treated with O-methyl hydroxylamine hydrochloride in pyridine. Two products were detected by t.l.c. after 24 hours at room temperature, and these were separated by P.L.C. The major product was identified as a mixture of two of the possible *syn-* and *anti*-isomers of 4-cholesten-3,6-dione dimethoxime (*11*). The nuclear magnetic resonance (n.m.r.) spectrum of the minor product showed a complex absorption τ5.8 - 6.2 indicating that it was a more complex mixture.

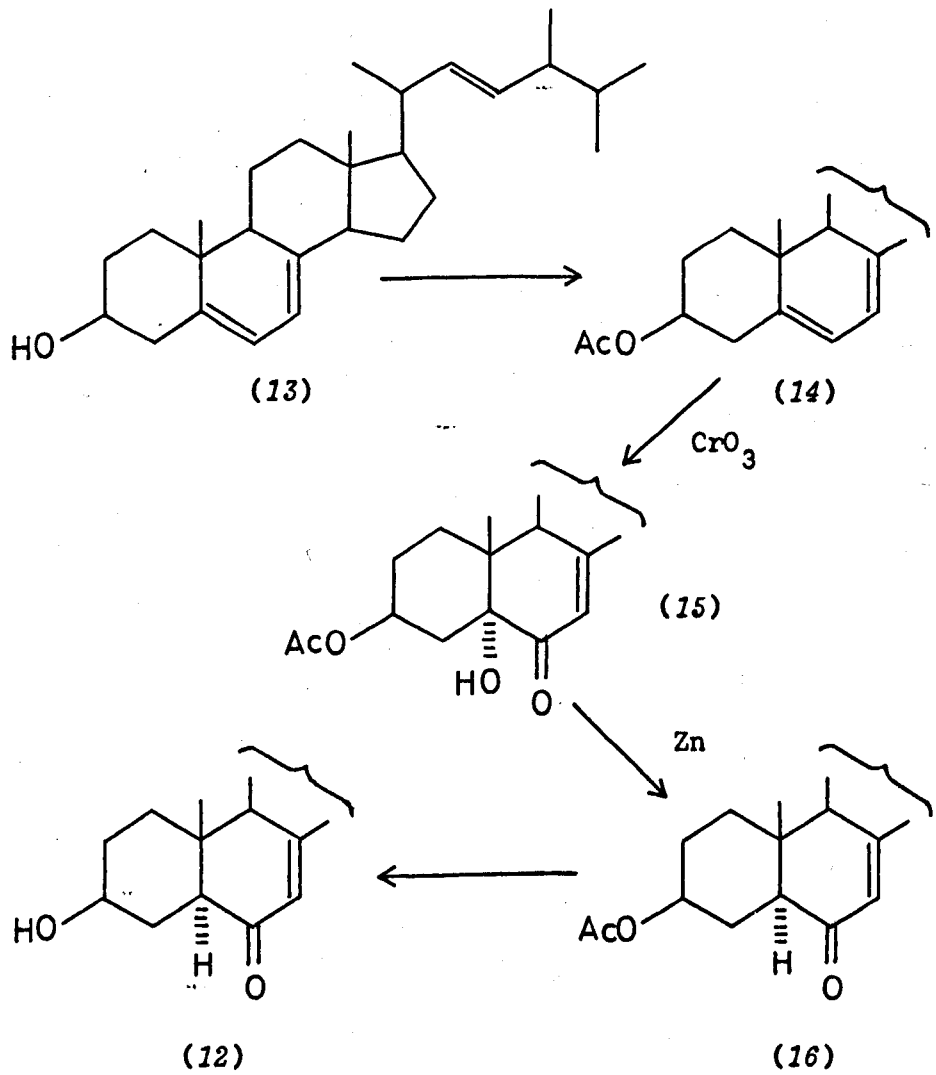


(*11*)

The formation of *syn*- and *anti*-isomers, separable by t.l.c. and g.l.c., had been observed by Horning *et al.*[112] in their preparation of methoxime derivatives of ketosteroids for g.l.c. Isomer formation however did not occur in several cases and this was correlated with steric effects. No 6--ketosteroids were used in their investigation, but a model of the ecdysone nucleus showed that this position was relatively unhindered. *Syn*- and *anti*-methoxime isomers might therefore be expected in this case.

No 7-en-6-one compounds were available to check for isomer formation or the reaction conditions for this grouping, but routes to such steroids had been used in the several syntheses of ecdysone. 3β-Hydroxy-5α-ergosta-7,22--dien-6-one (*12*) was chosen as the model compound to be prepared as it contained one hydroxyl group for silylation, the 7-en-6-one grouping of the ecdysones, and could be readily synthesised from ergosterol (*13*). The method of Burawoy[119] as modified by Barton and Robinson[120] was used and is outlined in the scheme on the next page.

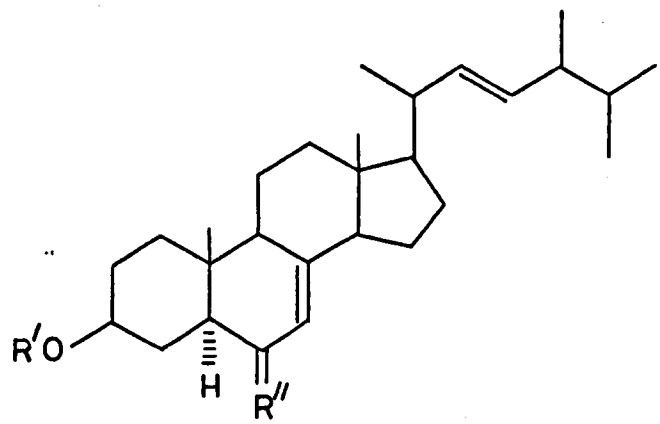
Ergosterol (*13*) was acetylated with acetic anhydride in pyridine to give ergosterol acetate (*14*), and this was selectively oxidised to 3β-acetoxy-5α-hydroxyergosta-7,22-dien--6-one (*15*) with chromium trioxide. The 3β-acetoxy-5α--hydroxyergosta-7,22-dien-6-one (*15*) was converted by reduction with zinc dust into 3β-acetoxy-5α-ergosta-7,22-dien-6-one (*16*), and this was hydrolysed to give 3β-hydroxy-5α-ergosta-7,22--dien-6-one (*12*).



(13) (14) (15) (12) (16)

3β-Hydroxy-5α-ergosta-7,22-dien-6-one (*12*) was treated at room temperature with O-methyl hydroxylamine hydrochloride and t.l.c. showed quantitative conversion to

a product after 60 hours. Heating at 60° for three hours caused a brown colour to develop in the reaction mixture but no significant increase in the reaction rate. In contrast Horning *et al.* found quantitative conversion of 3-, 16-, 17-, and 20-ketosteroids after only 24 hours at room temperature or three hours at 60°, but the hindered 11-keto group did not react under these conditions.[112] The product formed after 60 hours was isolated and identified as a mixture of *syn*- and *anti*-3β-hydroxy-5α-ergosta-7,22-dien-6-one methoxime (*17*), and this on treatment with B.S.A. in pyridine for 12 hours was converted quantitatively into a mixture of *syn*- and *anti*-3β--trimethylsilyloxy-5α-ergosta-7,22-dien-6-one methoxime (*18*).



(*17*) R' = H, R'' = N∿OMe

(*18*) R' = $SiMe_3$, R'' = N∿OMe

(*19*) R' = $SiMe_3$, R'' = O

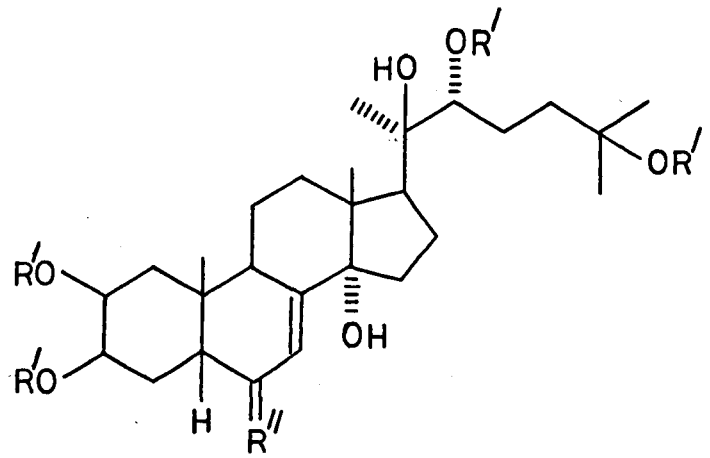
Treatment of 3β-hydroxy-5α-ergosta-7,22-dien-6-one (*12*) directly with B.S.A. also gave a single product, and its mass spectrum indicated this to be 3β-trimethylsilyloxy-5α-ergosta-7,22-dien--6-one (*19*).

As several phytoecdysones contain 5β-hydroxyl groups,[121,122] the 5α-hydroxy-compound (*15*), prepared as an intermediate in the synthesis of 3β-hydroxy-5α-ergosta-7,22-dien-6-one (*12*), was subjected to methoximating and silylating conditions as a guide to the possible behaviour of 5-hydroxy-steroids. A range of conditions were used for the reactions but both the 5α-hydroxyl and the 6-keto groups were resistant to derivative formation. The 5α-hydroxyl group was only partially silylated with a mixture of B.S.A. and trimethylchlorosilane (4:1), and vigorous conditions gave mixtures of products for both reactions. This resistance to reaction was probably caused by steric factors resulting from the introduction of the 5α-hydroxyl group.

With the conditions established for reaction of the model compounds and the isolation of their derivatives (and the information from g.l.c. discussed in the following section) as a guide, the preparation of derivatives of the ecdysones was undertaken. All the reactions were carried out on quantities of 2.5 mg. or less, and the products were identified from their g.l.c. traces and mass spectra which are discussed in the

following sections (see pages 79 to 115).

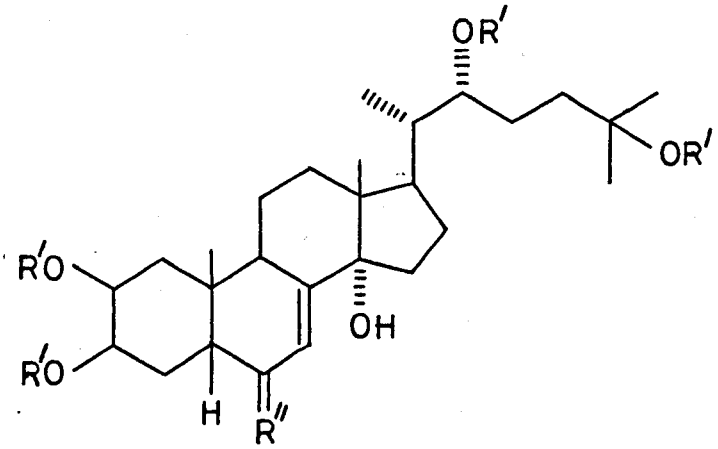
20-Hydroxyecdysone was quantitatively converted into a mixture of *syn*- and *anti*-20-hydroxyecdysone methoxime (*20*) with O-methyl hydroxylamine hydrochloride at room temperature for 100 hours, and treatment of this mixture with B.S.A. at room temperature for 70 hours gave tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*). Separation of part of the methoxime isomer mixture (*20*) by preparative thin layer chromatography (p.t.l.c.) gave the more polar isomer (*21*) and the less polar isomer (*22*), and these were separately treated with B.S.A. at room temperature for 48 hours to give the corresponding isomers of tetrakis-TMSi-20-hydroxyecdysone methoxime (*21a* and *22a*). These products also contained a small proportion of the tris-TMSi derivatives (see g.l.c. traces in Figure 22, page 84). In a second reaction with O-methyl hydroxylamine hydrochloride, 20-hydroxyecdysone yielded a product mixture identical to (*20*), but this mixture on standing for 48 hours on the open bench in ethyl acetate solution showed a conversion (approximately 80%) of the less polar isomer (*22*) to a third component (*23*). This was isolated, and on treatment with B.S.A. at room temperature for 70 hours gave another isomer of tetrakis-TMSi-20-hydroxyecdysone methoxime (*23a*).



(*20* to *22*) R' = H, R'' = N∿OMe

(*20a* to *22a*) R' = $SiMe_3$, R'' = N∿OMe

Ecdysone was also converted into a mixture of *syn*- and *anti*-ecdysone methoxime (*24*) on treatment with O-methyl hydroxylamine hydrochloride for 100 hours at room temperature, and treatment of this mixture with B.S.A. at room temperature for 70 hours gave tetrakis-TMSi-ecdysone methoxime (*24a*). Separation of part of the methoxime isomer mixture (*24*) by p.t.l.c. gave the more polar isomer (*25*) and the less polar isomer (*26*), and these were separately treated with B.S.A. at room temperature for 70 hours to give the corresponding isomers of tetrakis-TMSi-ecdysone methoxime (*25a* and *26a*).



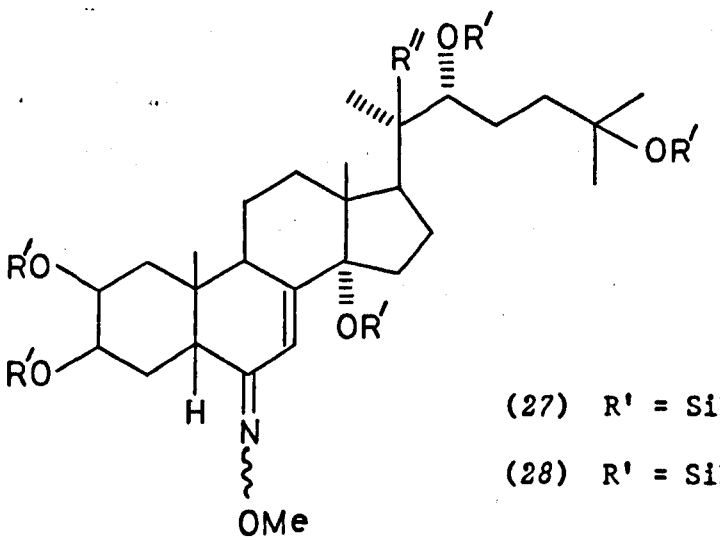
(*24* to *26*) R' = H, R'' = N∿OMe

(*24a* to *26a*) R' = $SiMe_3$, R'' = N∿OMe

Similar reaction times had been found for the methoximation stage with the model compound 3β-hydroxy-5α--ergosta-7,22-dien-6-one (*12*) and with the ecdysones, but the latter required a longer time for silylation. Tris- and tetrakis-TMSi-20-hydroxyecdysone methoxime were formed as mixtures (*21a* and *22a*) after 48 hours, but only the tetrakis-TMSi derivative (*20a*) remained after 70 hours. Silylation of the model compounds showed that the secondary 3β-hydroxyl group was rapidly silylated with B.S.A. at room temperature, and it was probable that the 25-hydroxyl group, the only tertiary hydroxyl group silylated in the ecdysones

under the conditions used (discussed in a following section see page 101), had shown a lower rate of reaction because of steric factors.

More vigorous reaction conditions were required to silylate the tertiary 14α-[81] and 20-hydroxyl[123] groups. Tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) on treatment with B.S.A. in dimethylformamide for 15 hours at 80° by the method of Thompson *et al.*[81] gave a mixture of the tetrakis-, pentakis-, and hexakis-TMSi derivatives. Treatment for a further 72 hours converted this latter mixture into hexakis--TMSi-20-hydroxyecdysone methoxime (*27*). Treatment of tetrakis-TMSi-ecdysone methoxime (*24a*) by Thompson's procedure gave a mixture of the tetrakis- and pentakis-TMSi derivatives after 15 hours and pentakis-TMSi-ecdysone methoxime (*28*) after 72 hours.



(*27*) R' = $SiMe_3$, R'' = $OSiMe_3$

(*28*) R' = $SiMe_3$, R'' = H

Gas-Liquid Chromatography of the Synthetic Compounds

Gas-liquid chromatography of steroids and their derivatives using a number of stationary phases has been reported,[111] and silicone phases from the recently available OV-range have been used to chromatograph and separate MO-TMSi derivatives of polar steroids.[112,113] Three phases in the OV-range were used for g.l.c. of the derivatives of the model compounds and the ecdysones. Two were polar phases, OV-210 (QF-1) which shows selective retention effects for alcohols, ketones and esters, but not for carbon-carbon unsaturation, and OV-17 which shows selective retention effects for carbon-carbon unsaturation.[111] The other was OV-101, a non-polar phase selective only by boiling point.[111]

An important problem in the analysis of relatively polar steroids and their derivatives by g.l.c. is the loss of material by irreversible adsorption at some point in the system.[111] To minimise this possibility, all separations were carried out using a silylated support and silylated glass columns. Decomposition of steroids can occur on g.l.c. at elevated column temperatures, and temperatures in the range 190 to 220° have been recommended with higher temperatures for polar steroids.[111] Values upto 260° have been used for

MO-TMSi derivatives of polar steroids.[113] All separations in the present work were carried out isothermally at 232°.

The g.l.c. results showed that it was possible to chromatograph the MO-TMSi derivatives of the model compounds and the ecdysones on the three OV-stationary phases, and corrected retention times and relative retentions with respect to an internal standard of TMSi-cholesterol are given in the Experimental section (see pages 272 to 275, and Tables 38 to 40 for the results on OV-210, OV-17 and OV-101 respectively).

The stationary phase OV-210 was used first during the course of this work, and was subsequently found to give much shorter retention times for the MO-TMSi derivatives of the model compounds and the ecdysones, than the other two phases when used at a 3% loading.

G.l.c. of 3β-acetoxy-5α-hydroxyergosta-7,22-dien-6-one (*15*) and 3β-hydroxy-5α-ergosta-7,22-dien-6-one (*12*) on OV-210 was unsuccessful and resulted in decomposition (see Figures 15 and 16, where TMSi-cholesterol was used as an internal standard the peak produced is indicated by S in the g.l.c. figures), but 4-cholesten-3,6-dione dimethoxime (*11*) gave two distinct peaks (see Figure 17). The peak corresponding to the minor component probably resulted from an incomplete separation on P.L.C.

FIGURE 15

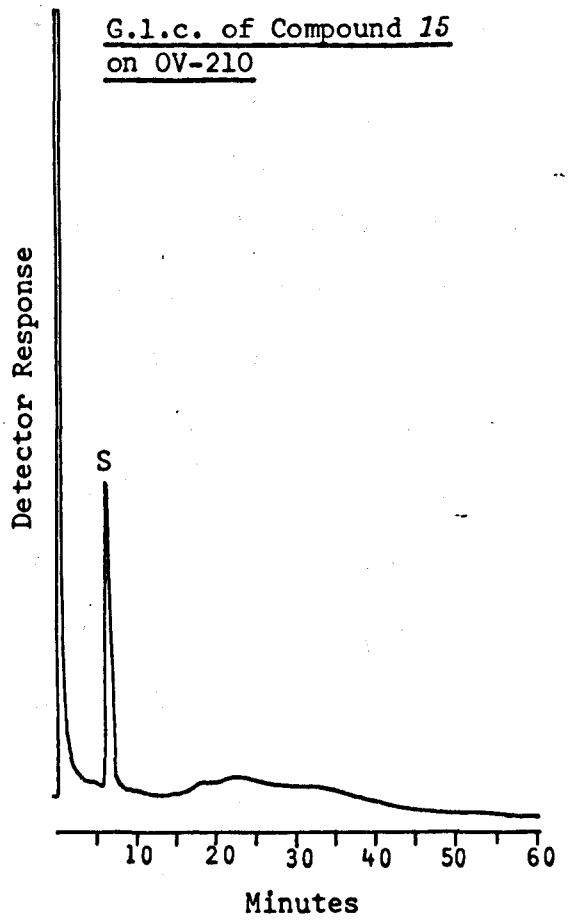


FIGURE 16

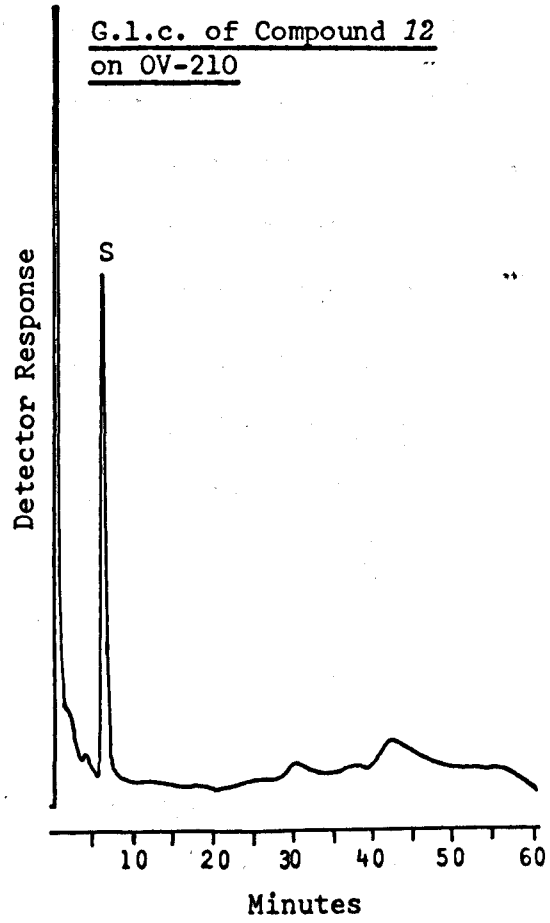
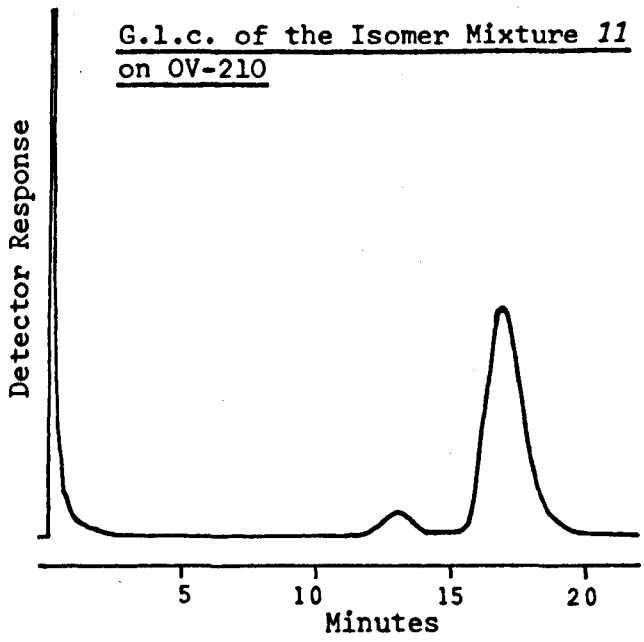


FIGURE 17



Chromatography of 3β-hydroxy-5α-ergosta-7,22-dien--6-one as the methoxime (*17*) was however successful on OV-210 and two peaks, each shown by linked g.l.c.-m.s. (see following section) to represent one of the *syn*- and *anti*-methoxime isomers, were observed (see Figure 18). This separation of methoxime isomers on OV-phases had been reported by Horning *et al.* and was more common on polar phases than on non-polar phases.[112] Silylation of 3β-hydroxy-5α-ergosta-7,22-dien-6-one methoxime (*17*) resulted in a reduction in the retention times of the two isomers, as expected, but these still remained separated on OV-210 (see Figure 19).

FIGURE 18

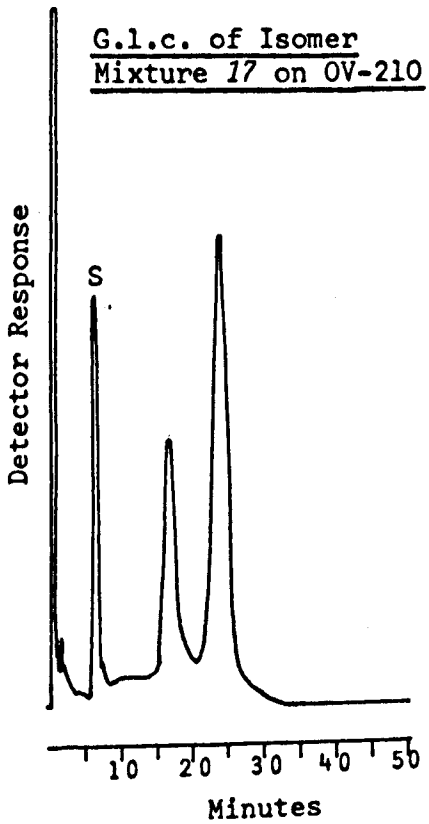
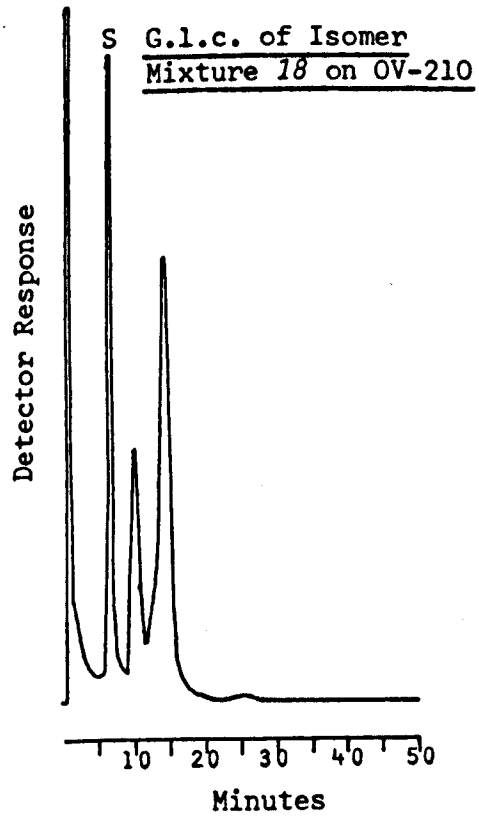
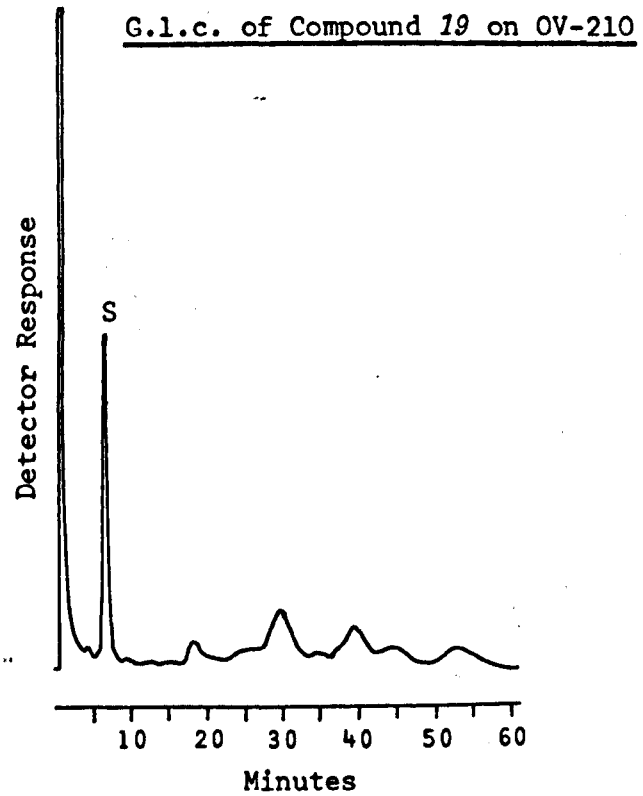


FIGURE 19



The product resulting from the silylation of 3β-hydroxy-5α-ergosta-7,22-dien-6-one (*12*), which was shown to be 3β-trimethylsilyloxy-5α-ergosta-7,22-dien-6-one (*19*) by its mass spectrum, gave a complex g.l.c. pattern of several peaks indicating its decomposition on the column (see Figure 20).

FIGURE 20



Chromatography of tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) on OV-210 gave a single skewed peak (see Figure 21) resulting from the overlap of the peaks of the two methoxime isomers (*21a* and *22a*). This result was confirmed

by the g.l.c. results for the separated isomers (*21a* and *22a*) which also showed that the separation by p.t.l.c. had been incomplete (see Figure 22).

FIGURE 21

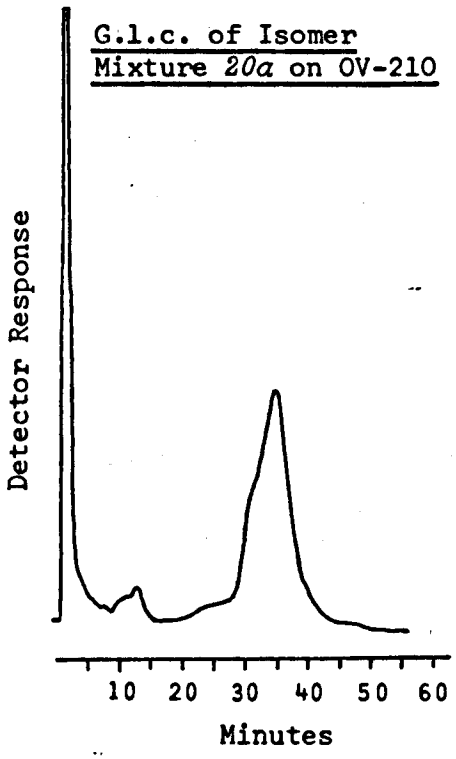
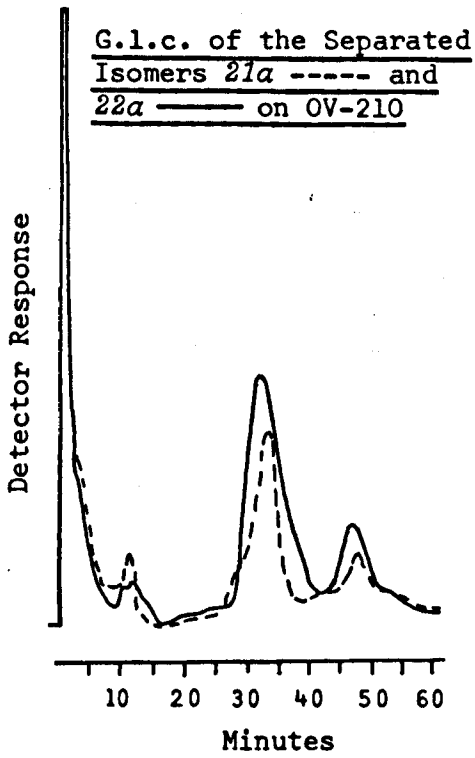


FIGURE 22



The 20-hydroxyecdysone derivatives each had a longer retention time (t_R) than either of the two isomers of 3β-trimethylsilyloxy-5α-ergosta-7,22-dien-6-one methoxime (*18*) due to their increased molecular size and the presence of two free hydroxyl groups. The greater overlap of the isomers (*21a* and *22a*) derived from 20-hydroxyecdysone was probably due to their relatively long retention times. The peaks between t_R 45 and

55 minutes in Figure 22 probably represented tris-TMSi derivatives still remaining in *21a* and *22a* after a reaction time of only 48 hours, and this may be compared with a reaction time of 70 hours for *20a* in which these components are absent (see Figure 21). The third isomer of tetrakis--TMSi-20-hydroxyecdysone methoxime (*23a*, see page 75) also gave a single peak on OV-210.

The g.l.c. of tetrakis-TMSi-ecdysone methoxime (*24a*) on OV-210 gave a similar result (see Figure 23) to that just discussed for tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*). The isomers however had a shorter retention time, and therefore an improved resolution, as only one free hydroxyl group was present, but the time was still longer than that found for 3β-trimethylsilyloxy-5α-ergosta-7,22-dien-6-one methoxime (*18*). The g.l.c. results for the separated isomers (*25a* and *26a*) again showed that the separation by p.t.l.c. had been incomplete (see Figure 24).

FIGURE 23

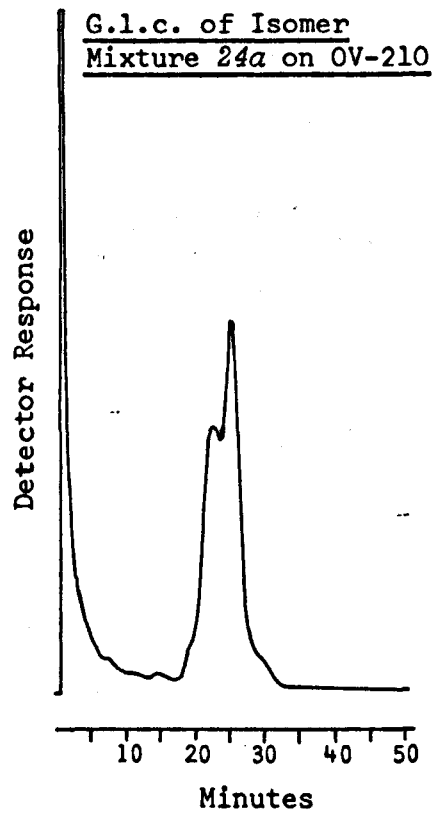
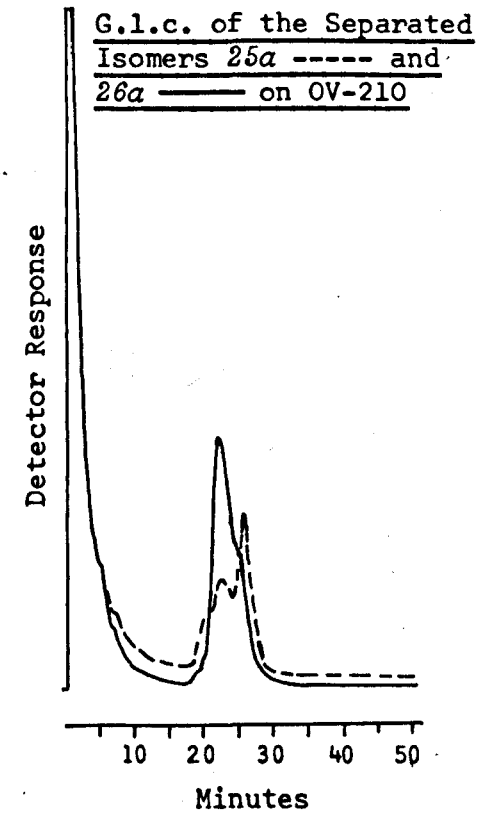


FIGURE 24



More vigorous reaction conditions had been required to silylate the 14α- and 20-hydroxyl groups, and the products from the further silylation of tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) and tetrakis-TMSi-ecdysone methoxime (*24a*) by B.S.A. in dimethylformamide for 15 hours at 80° were identified as mixtures from their g.l.c. traces. Tetrakis-TMSi-20--hydroxyecdysone methoxime (*20a*) under these conditions gave a mixture of the tetrakis-, pentakis-, and hexakis-TMSi derivatives, and the reaction could not have been stopped at any stage to give only the pentakis-product (see Figure 25,

the peaks corresponding to these three products are indicated in the Figure by T, P, and H respectively). Continued silylation for a further 72 hours converted this mixture into the hexakis-TMSi derivatives (see Figure 26).

FIGURE 25

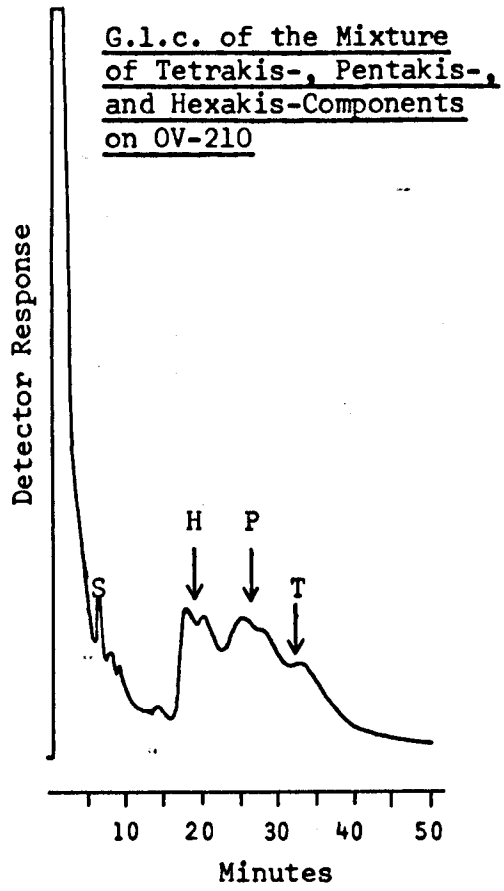
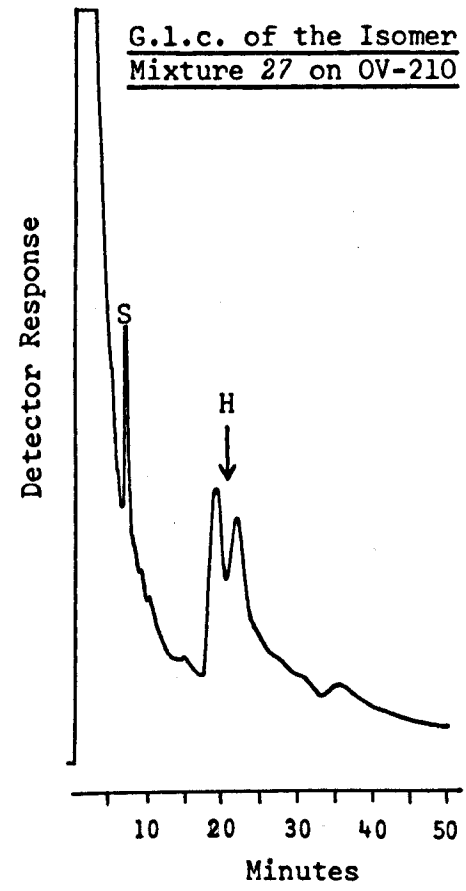


FIGURE 26



Tetrakis-TMSi-ecdysone methoxime (*24a*) gave a mixture of tetrakis- and pentakis-derivatives (see Figure 27) and this mixture was also converted to the fully silylated derivatives on silylation for a further 72 hours (see Figure 28).

FIGURE 27

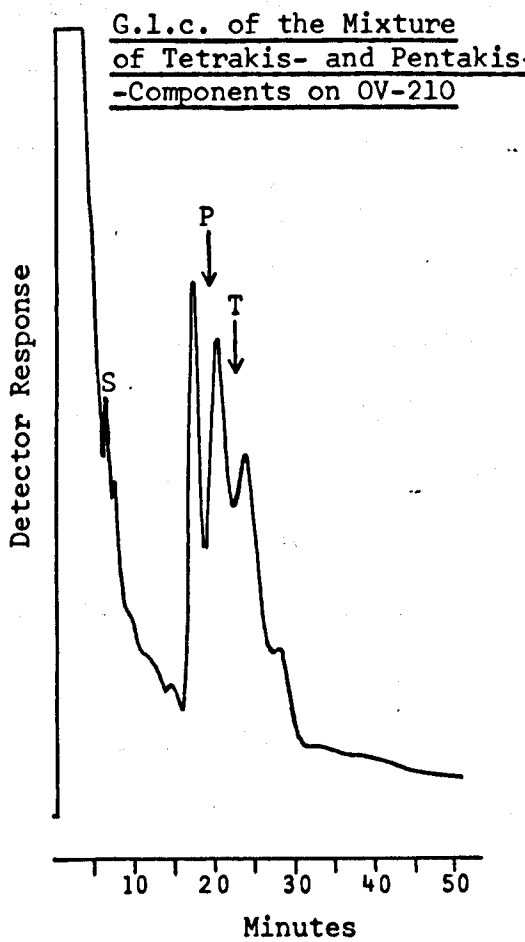
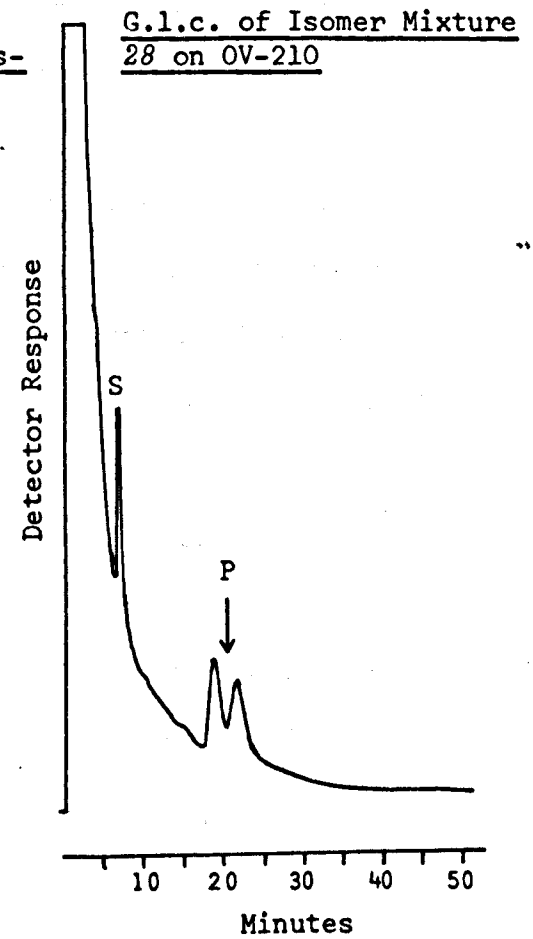


FIGURE 28



Hexakis-TMSi-20-hydroxyecdysone methoxime (*27*) and pentakis-TMSi-ecdysone methoxime (*28*) have no free hydroxyl groups, and differ only by one relatively non-polar TMSi group. This difference is small compared with the overall size of the molecules, and comparison of their retention times shows that a mixture of these derivatives would remain unresolved on OV-210 under the g.l.c. conditions used. The

retention times for these derivatives were still longer than that found for 3β-trimethylsilyloxy-5α-ergosta-7,22-dien-6--one methoxime (*18*), and this was now more representative of differences in molecular size.

Chromatography of TMSi-cholesterol on OV-17 and OV-101, the other two stationary phases used during the course of this work, gave retention times of 22 minutes and 16 minutes respectively, even when the phases were used at a 1% loading, and much longer retention times were observed for the MO-TMSi derivatives of the model compounds and the ecdysones than on the OV-210 column.

The longest retention times for compounds were observed on OV-17 which was expected since they all possess a carbon-carbon double bond, for which this phase has a selective retention effect (see Table 39, page 274). Methoxime isomers were separated in all cases and the g.l.c. traces for 3β-hydroxy-5α-ergosta-7,22-dien-6-one methoxime (*17*) and its silyl ether (*18*) are shown in Figure 29, and those for tetrakis--TMSi-20-hydroxyecdysone methoxime (*20a*) and tetrakis-TMSi--ecdysone methoxime (*24a*) are given in Figure 30.

FIGURE 29

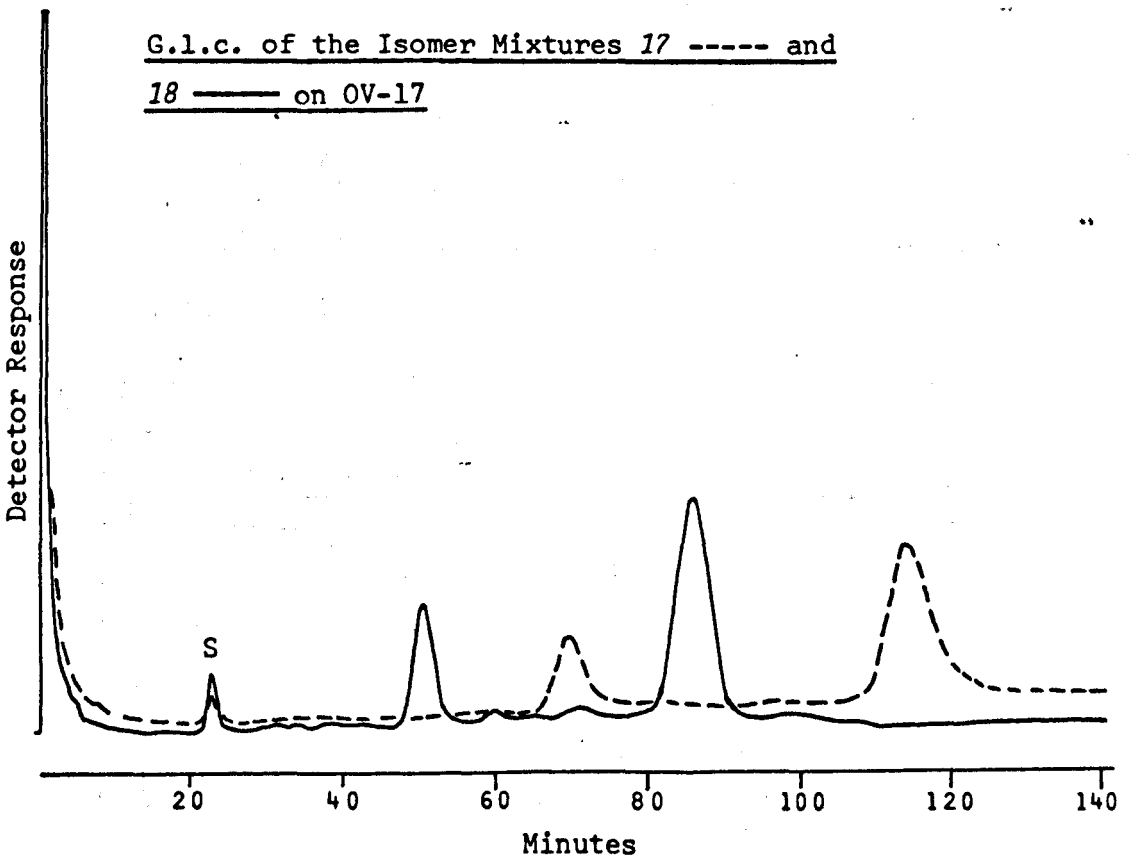
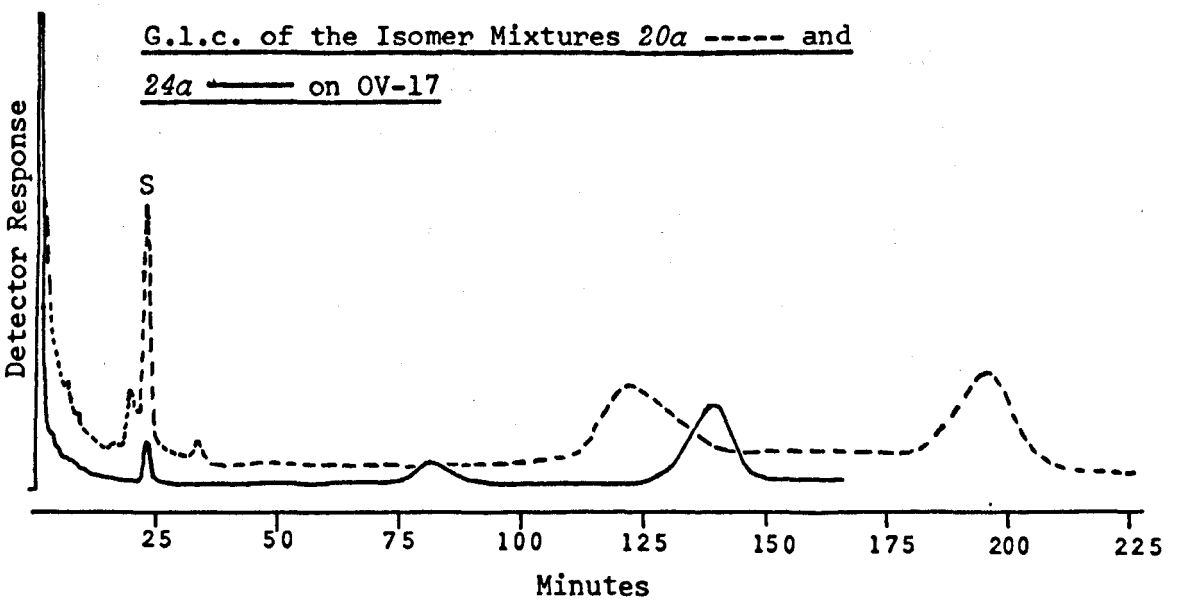
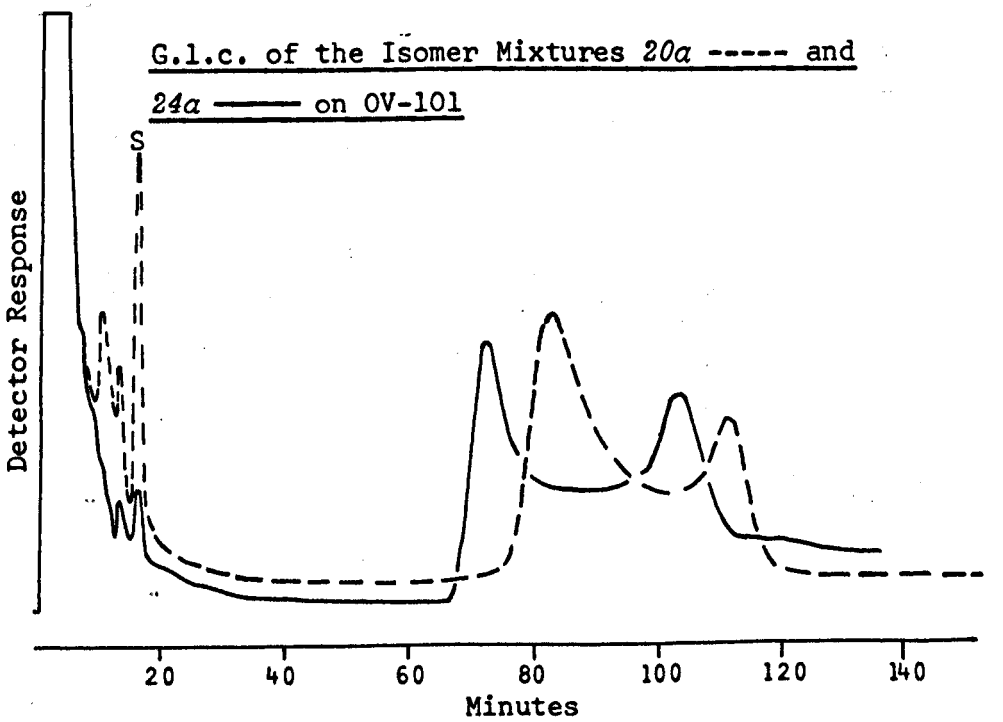


FIGURE 30



Shorter retention times than those found for derivatives on OV-17 were observed on OV-101 (see Table 40, page 275). This is a non-polar phase selective only by boiling point, but methoxime isomers were still separated. The g.l.c. traces for tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) and tetrakis-TMSi-ecdysone methoxime (*24a*) obtained on OV-101 are shown in Figure 31.

FIGURE 31

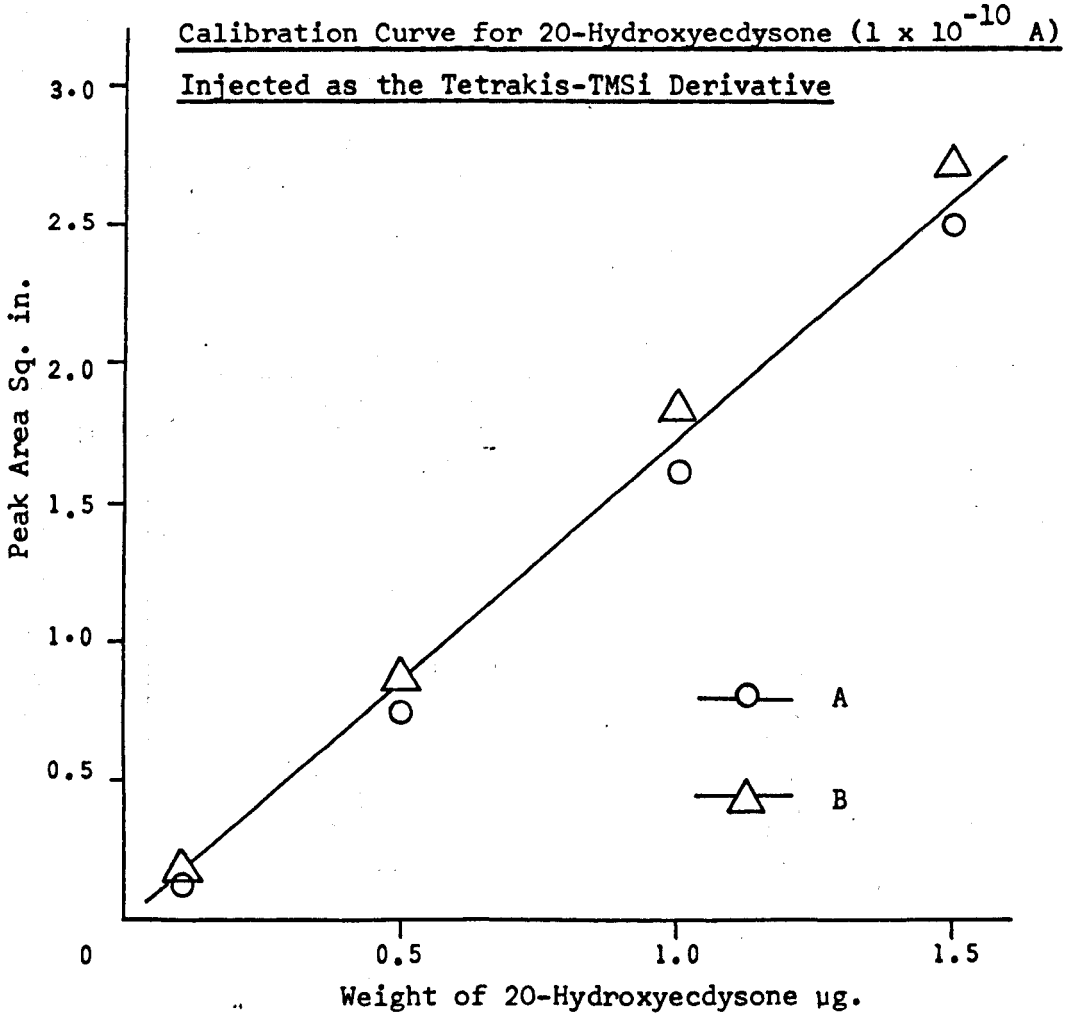


Tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) and tetrakis-TMSi-ecdysone methoxime (*24a*) were chosen for the quantitative determination of the ecdysones in biological materials, as they had sufficiently different retention times

on OV-210 to be separated in a mixture. This stationary phase, which gave shorter retention times and less separation of isomer pairs than either OV-17 or OV-101, was also used for the quantitative work. The minimum separation of isomer pairs[112] and the relatively short retention times are desirable for practical purposes.

No ecdysone remained at this stage in the work, but a method of direct injection[124] was used to construct a calibration curve for 20-hydroxyecdysone. Tetrakis-TMSi-20--hydroxyecdysone methoxime (*20a*) was prepared from two separate weighed portions of 20-hydroxyecdysone A and B, and known amounts of these two mixtures were injected onto the g.l.c. using OV-210 columns. The areas of the resulting peaks were measured (see Table 41, page 277) and the variation of peak area with weight of 20-hydroxyecdysone from which the peak was derived is plotted in Figure 32. This showed a linear relationship in the range 0.1 to 1.5 µg., and at these levels there was no evidence of loss due to adsorption.

FIGURE 32

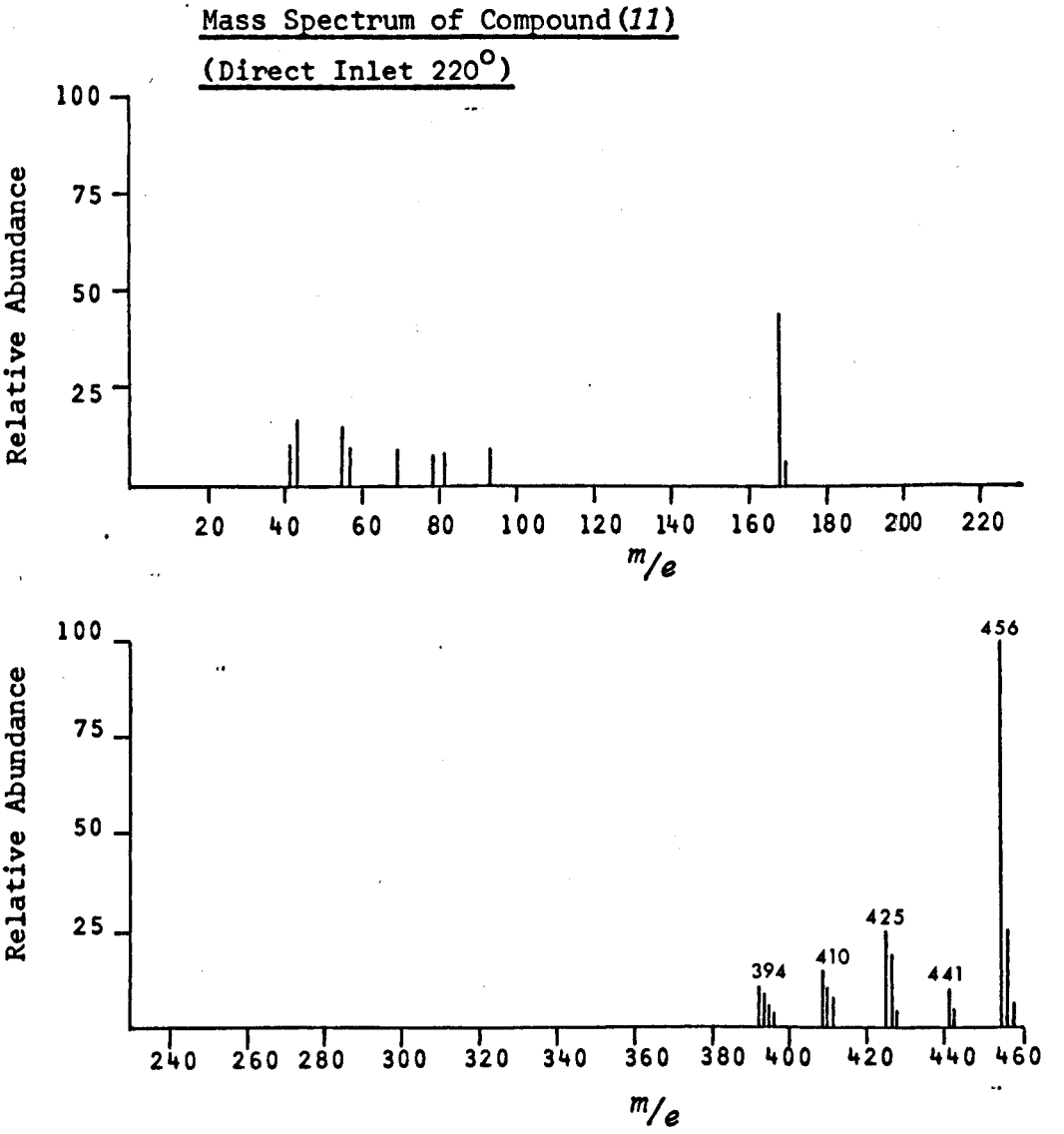


Mass Spectrometry of the Synthetic Compounds

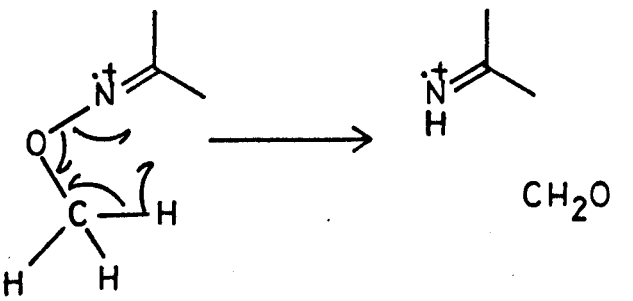
Mass spectrometry was used in the identification of the MO and TMSi derivatives of the model steroids and the ecdysones, and is an important technique for identification purposes when only small amounts of material are available.

The relatively simple mass spectrum of 4-cholesten--3,6-dione dimethoxime *(11)* clearly shows the characteristic fragmentation pattern due to the two O-methyl oxime functions[125] (see Figure 33, peaks of less than 5% relative abundance are not shown).

FIGURE 33

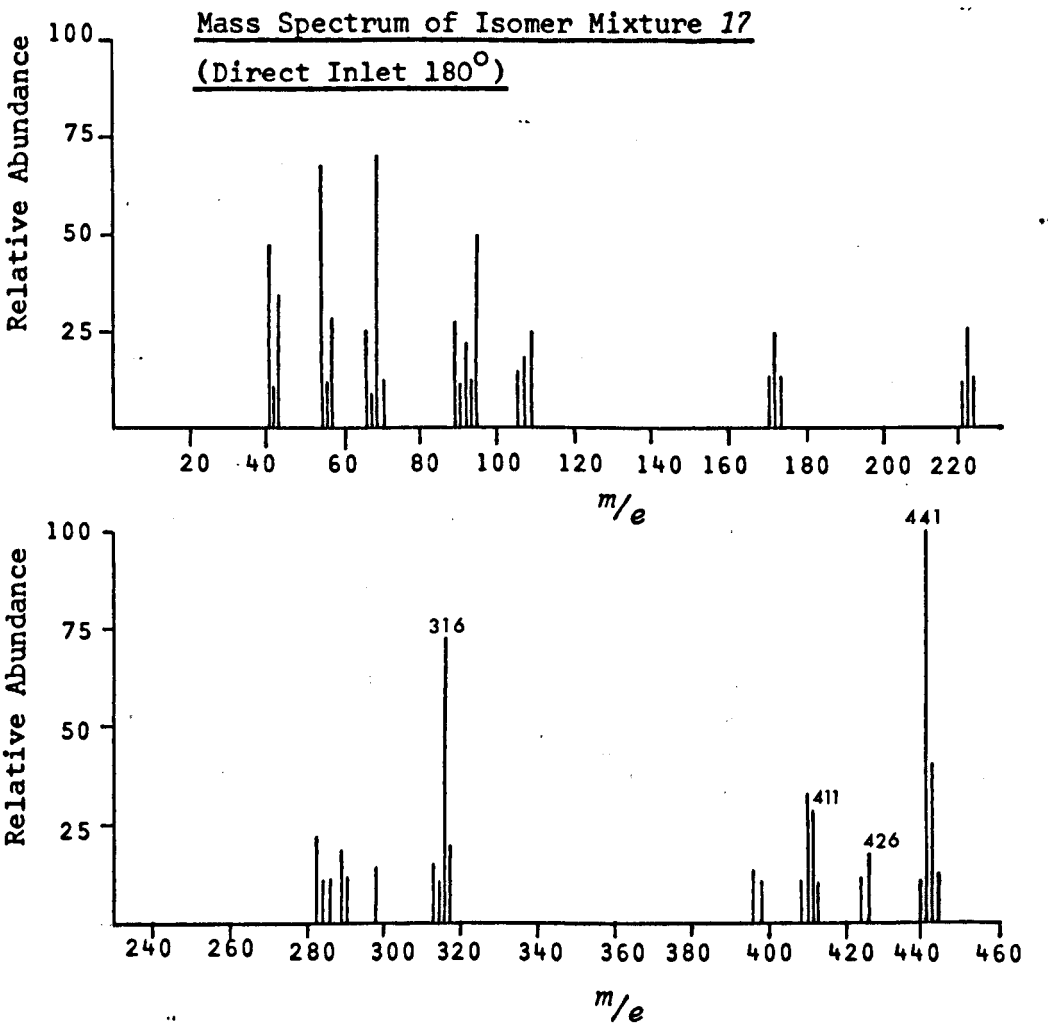


The molecular ion, $^{m}/_{e}$ 456, is the base peak and prominent peaks are observed at $^{m}/_{e}$ 441 (M - CH_3), 425 (M - OCH_3), 410 (M - CH_3 + OCH_3) and 394 (M - 2 x OCH_3). Superimposed onto this pattern is the loss of CH_2O, which can occur by a cyclic process as shown below, and the subsequent loss of a hydrogen atom from the charged fragment formed.[116] The loss of CH_3 from ions other than the molecular ion is also evident.



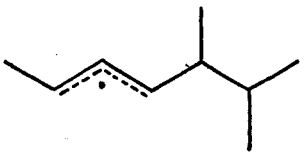
A similar pattern of fragmentation due to a single O-methyl oxime function is found in the mass spectrum of 3β-hydroxy-5α-ergosta-7,22-dien-6-one methoxime (*17*, see Figure 34).

FIGURE 34



The molecular ion, $^{m}/_{e}$ 441, is again the base peak and ions are observed at $^{m}/_{e}$ 426 (M - CH_3), 411 (M - CH_2O) and 410 (M - OCH_3). The prominent ion at $^{m}/_{e}$ 316 is almost certainly due to loss of the side chain by cleavage of the C17-C20 bond with formation of the resonance stabilised allylic fragment (*29*),

and this is supported by the presence of a strong metastable peak at $^{m}/_{e}$ 226.



(*29*)

The mass spectrum of 3β-trimethylsiloxy-5α-ergosta--7,22-dien-6-one methoxime (*18*) again shows the characteristic fragmentation pattern of the O-methyl oxime function (see Figure 35) with ions at $^{m}/_{e}$ 498 (M - CH_3), 483 (M - CH_2O) and 482 (M - OCH_3), and the loss of the allylic fragment (*29*) giving a peak at $^{m}/_{e}$ 388 with a metastable peak at $^{m}/_{e}$ 294, but also present are peaks arising from fragmentation of the TMSi group.

FIGURE 35

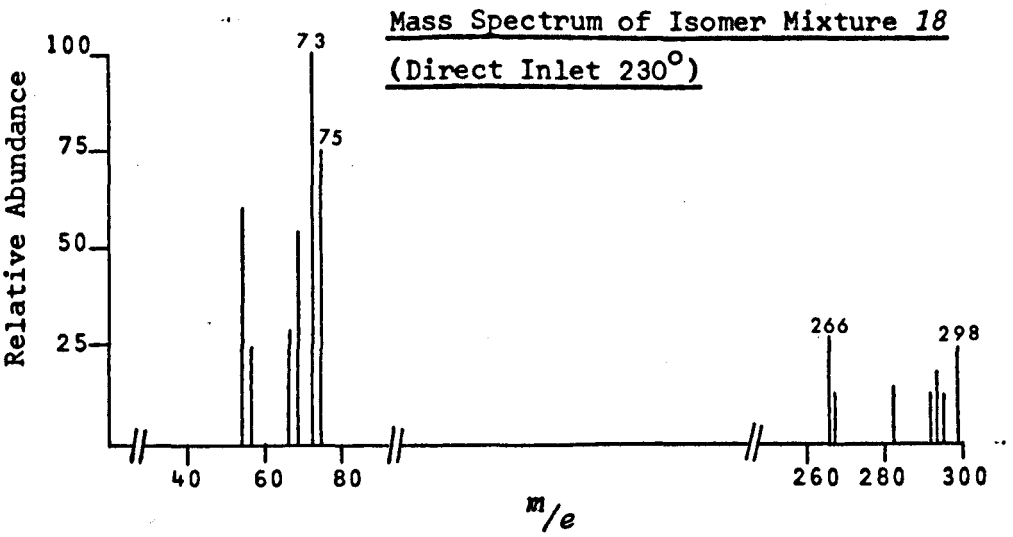
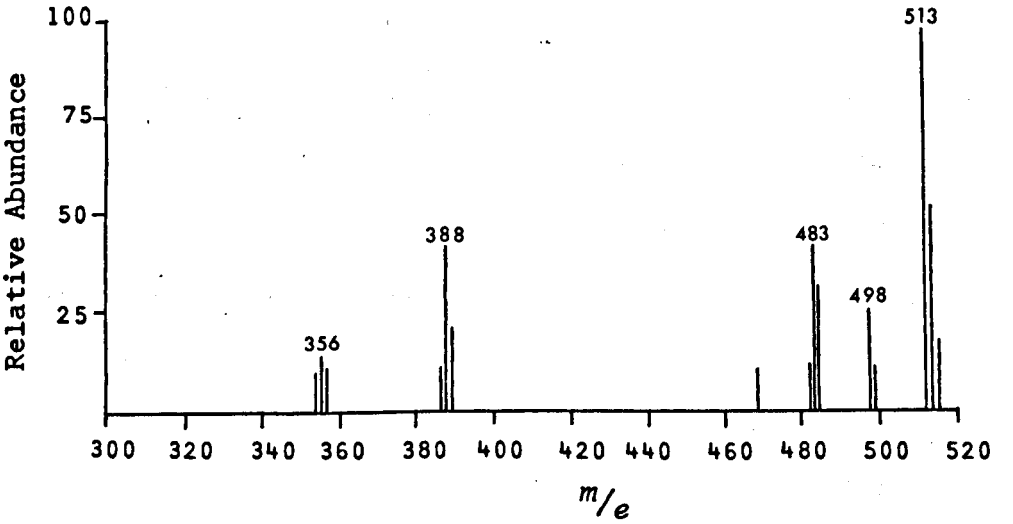


FIGURE 35 CONTINUED



The peaks observed at $^{m}/_{e}$ 73 (Me_3Si^+) and 75 (Me_2SiOH^+) are characteristic of many TMSi derivatives and are frequently of considerable abundance in the mass spectra of steroids with TMSi groups.[126] Pairs of peaks at $^{m}/_{e}$ 388 and 298, and $^{m}/_{e}$ 356 and 266 are related by loss of 90 mass units, and this probably represents the loss of trimethylsilanol which is another characteristic of TMSi derivatives.[126] Loss of CH_3 also occurs for TMSi groups.

The two components in 3β-trimethylsilyloxy-5α--ergosta-7,22-dien-6-one methoxime (*18*), formed in the methoximation reaction and designated as *syn*- and *anti*-isomers, were shown to be isomeric by comparison of their mass spectra

which were obtained by linked g.l.c.-m.s. (described in the following section, see Figures 44 and 45, pages 113 and 114). Comparison of these spectra with the spectrum of the mixture (see Figure 35) shows that all three are the same except for the relative intensities of some of the peaks and this would be expected due to the variation in the conditions used.

The TMSi derivative prepared from 20-hydroxyecdysone methoxime (*20*) by reaction with B.S.A. at room temperature for 70 hours was shown to be tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) from its mass spectrum. This showed a weak molecular ion at $^{m}/_{e}$ 797 (see Figure 36 for $^{m}/_{e}$ 380 to 800 and Figure 48 page 124 for a more complete spectrum).

FIGURE 36

Mass Spectrum of Isomer Mixture *20a*
(Direct Inlet 250°)

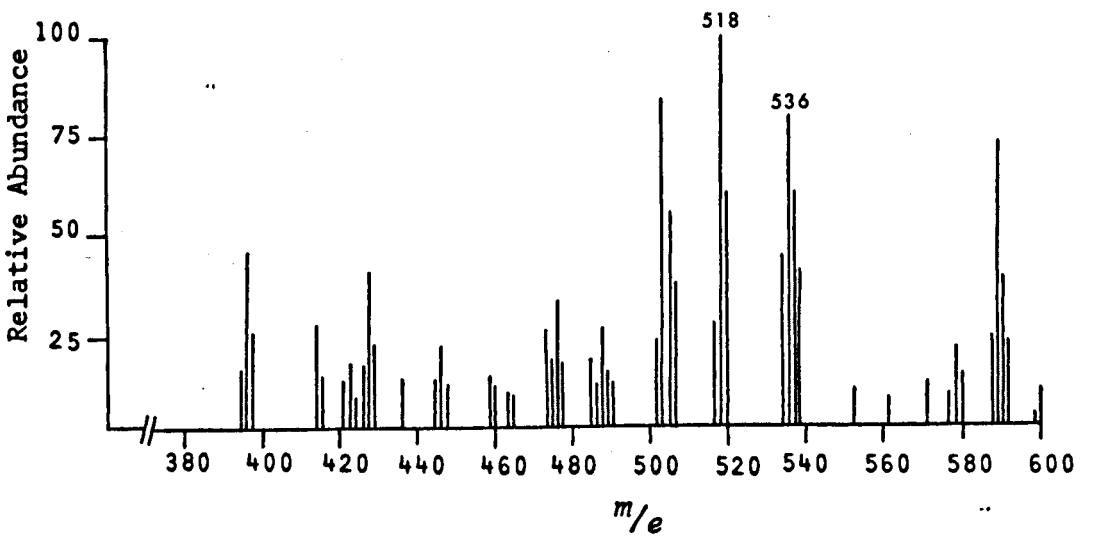
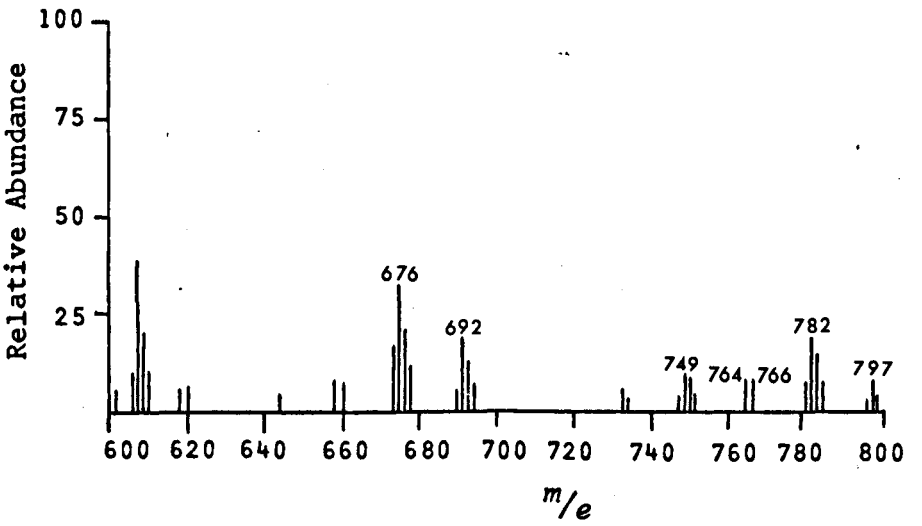


FIGURE 36 CONTINUED

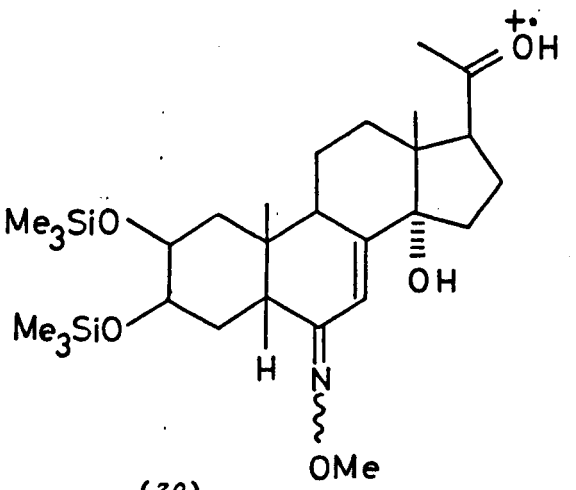


Steroidal TMSi derivatives frequently give molecular ions of considerable abundance as shown by the model compounds, and the low intensity of the molecular ion in this case indicates the greater number of fragmentation routes available. This may be correlated with the detection of molecular ions for ecdysones themselves which is extremely difficult.[61]

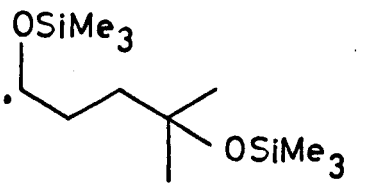
The characteristic fragmentation pattern expected for the O-methyl oxime function and the loss of water can be seen in the ions at high $^{m}/_{e}$ values with peaks at $^{m}/_{e}$ 782 ($M - CH_3$), 766 ($M - OCH_3$), 764 ($M - CH_3 + H_2O$) and 749 ($M - CH_2O + H_2O$). These fragmentations are only suggested ones, and loss of 2 x CH_3 from the TMSi groups would be equivalent to

loss of CH_2O. Pairs of peaks at $^{m}/_{e}$ 782 and 692, and $^{m}/_{e}$ 766 and 676 related by loss of 90 mass units probably represent the loss of trimethylsilanol as found in the spectrum of 3β-trimethylsilyloxy-5α-ergosta-7,22-dien-6-one methoxime (*18*).

The prominent ion (*30*) at $^{m}/_{e}$ 536 (M - 261) corresponds to the cleavage of the C20-C22 bond with loss of the fully silylated fragment (*31*), and this is followed by loss of water to give the more abundant ion at $^{m}/_{e}$ 518. This fragmentation pattern is exactly comparable to the case of 20-hydroxyecdysone where cleavage of the C20-C22 bond also gives rise to a prominent ion which subsequently looses water to give the base peak.[61]



(*30*)



(*31*)

Loss of the side chain fragment (*31*) showed that the 22- and 25-hydroxyl groups were silylated in tetrakis-TMSi-20-hydroxy-

ecdysone methoxime (*20a*). Tetrakis-TMSi-ecdysone methoxime (*24a*) was also identified from its mass spectrum (see Figures 40 and 41, pages 107 and 108) which showed a molecular ion at $^{m}/_{e}$ 781. As these tetrakis-TMSi derivatives differed only by a tertiary 20-hydroxyl group, this group must have remained unsilylated under the mild reaction conditions used, and this is consistent with the formation of the ion (*30*).

The vigorous reaction conditions required to silylate the 14α-hydroxyl group compared with the milder conditions required for the 2β- and 3β-hydroxyl groups[81] indicated that these would be the other two hydroxyl groups silylated in the tetrakis-TMSi derivatives. Tetrakis-TMSi-20--hydroxyecdysone methoxime (*20a*) is therefore 14α,20-dihydroxy--2β,3β,22,25-tetrakis(trimethylsilyloxy)-5β-cholest-7-en-6-one methoxime, and tetrakis-TMSi-ecdysone methoxime (*24a*) is 14α-hydroxy-2β,3β,22,25-tetrakis(trimethylsilyloxy)-5β-cholest--7-en-6-one methoxime. These structures were consistent with the g.l.c. results which showed that vigorous silylation conditions could give a mixture of tetrakis-, pentakis- and hexakis-TMSi derivatives in the 20-hydroxyecdysone case and a mixture of tetrakis- and pentakis-TMSi derivatives in the ecdysone case (see Figures 25 and 27, pages 87 and 88).

The two components in tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*), formed in the methoximation reaction and

separated at this stage by p.t.l.c., which had been designated as *syn*- and *anti*-isomers, were shown to be isomeric by comparison of their mass spectra (see Figures 37 and 38).

FIGURE 37

Mass Spectrum of the Isomer *21a*
(Direct Inlet 250°)

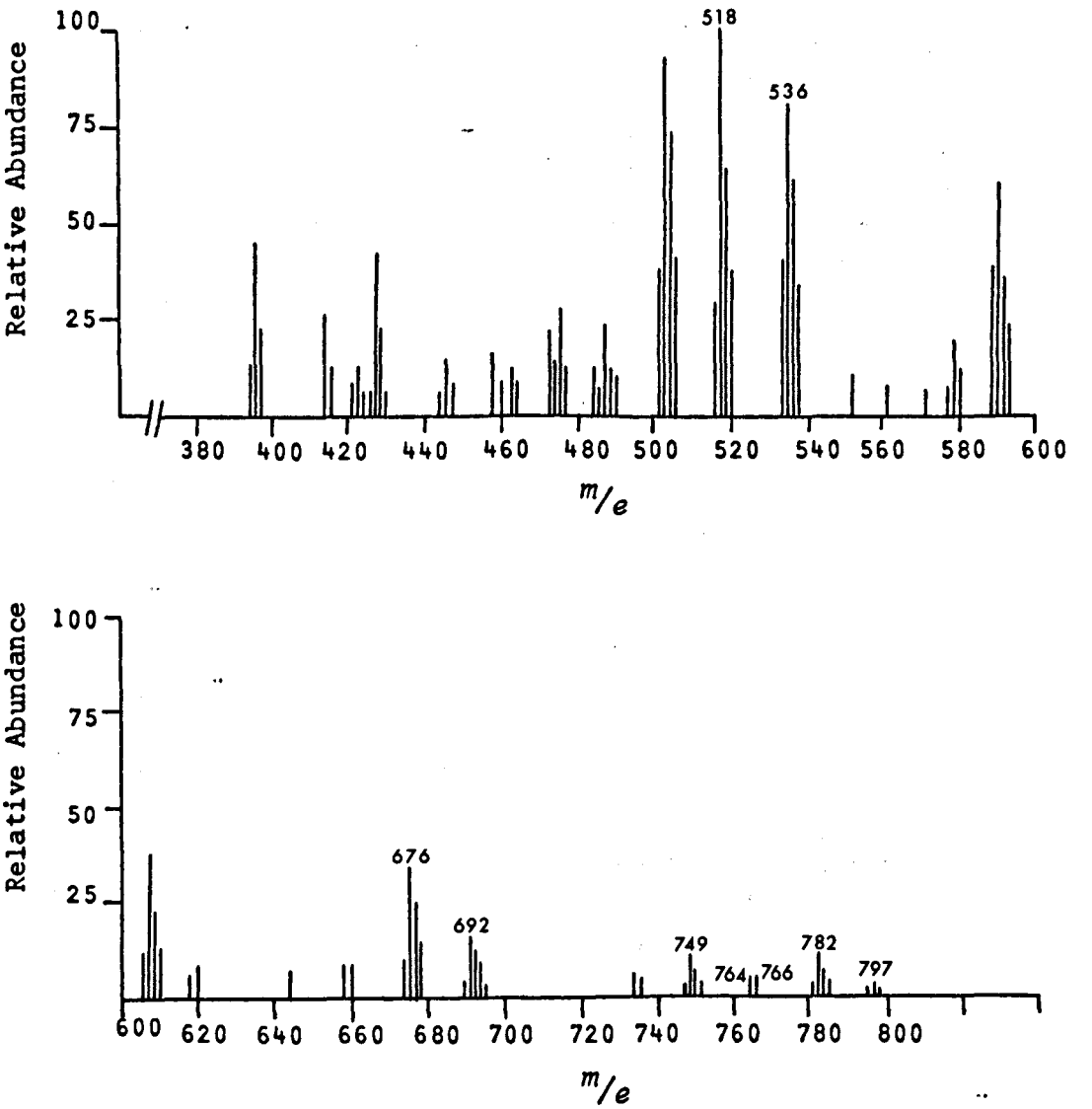
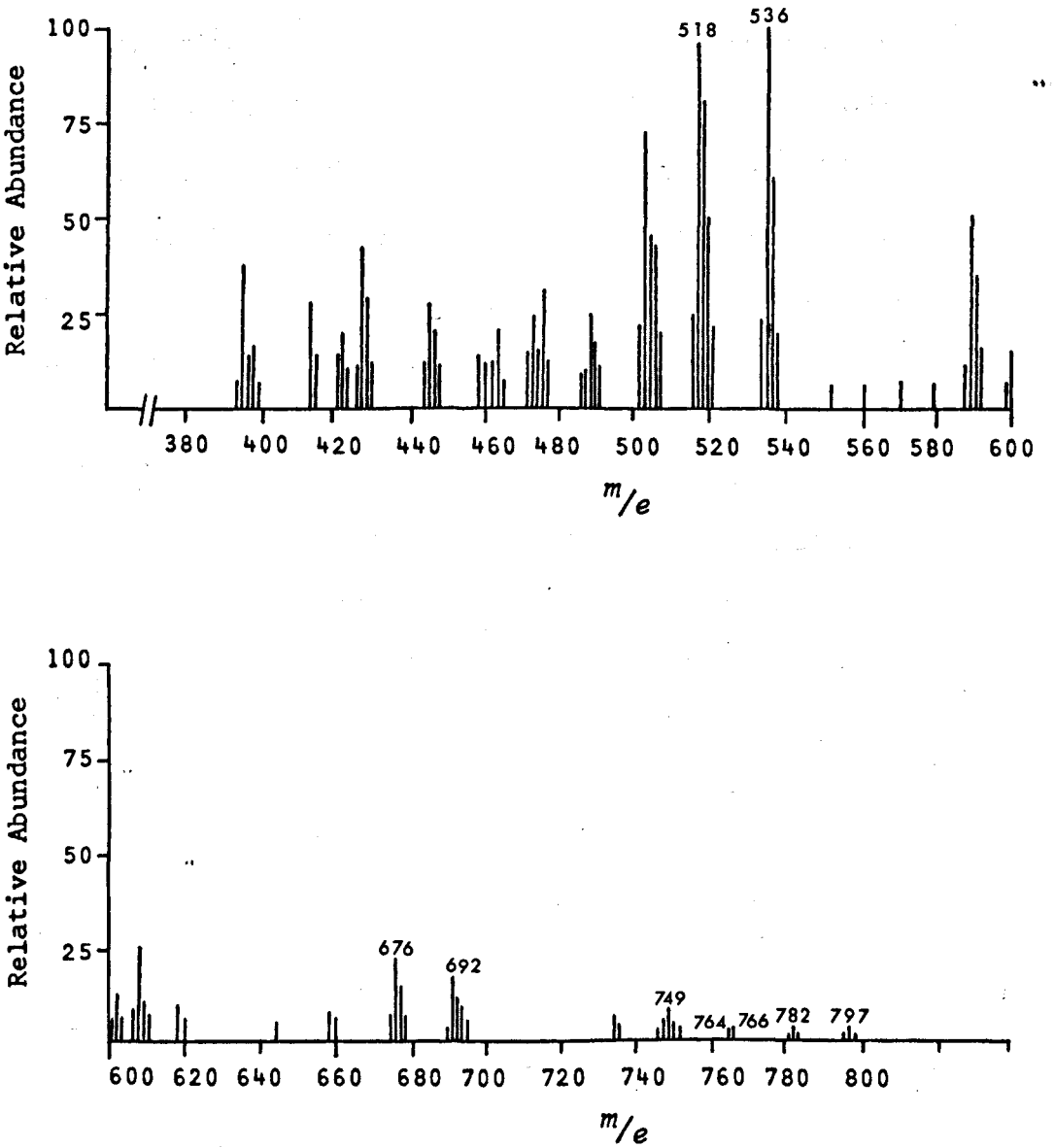


FIGURE 38

Mass Spectrum of the Isomer *22a*
(Direct Inlet 245°)



These were almost identical and were the same as the spectrum of the mixture (see Figure 36). The rearranged product (*23*), formed by allowing the least polar isomer of 20-hydroxyecdysone methoxime (*22*) to stand in ethyl acetate solution, also gave a TMSi derivative (*23a*). This was shown to be an isomer of tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) from its mass spectrum which showed a molecular ion at $^{m}/_{e}$ 797 (see Figure 39). The fragmentation pattern of the rearranged isomer was not however the same as that of the other two isomers (compare Figures 37 to 39).

FIGURE 39

Mass Spectrum of the Rearranged Isomer *23a*
(Direct Inlet 250°)

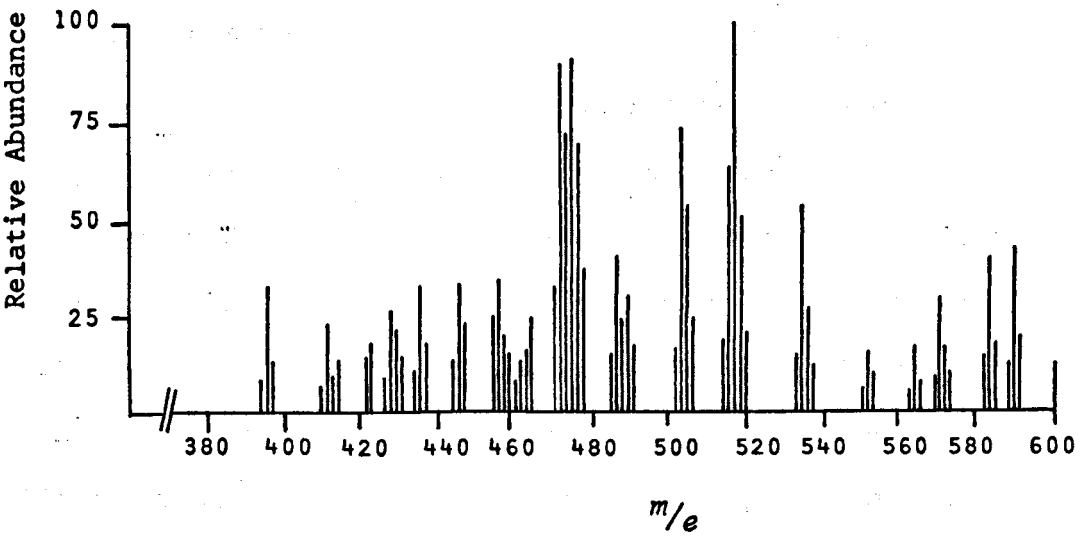
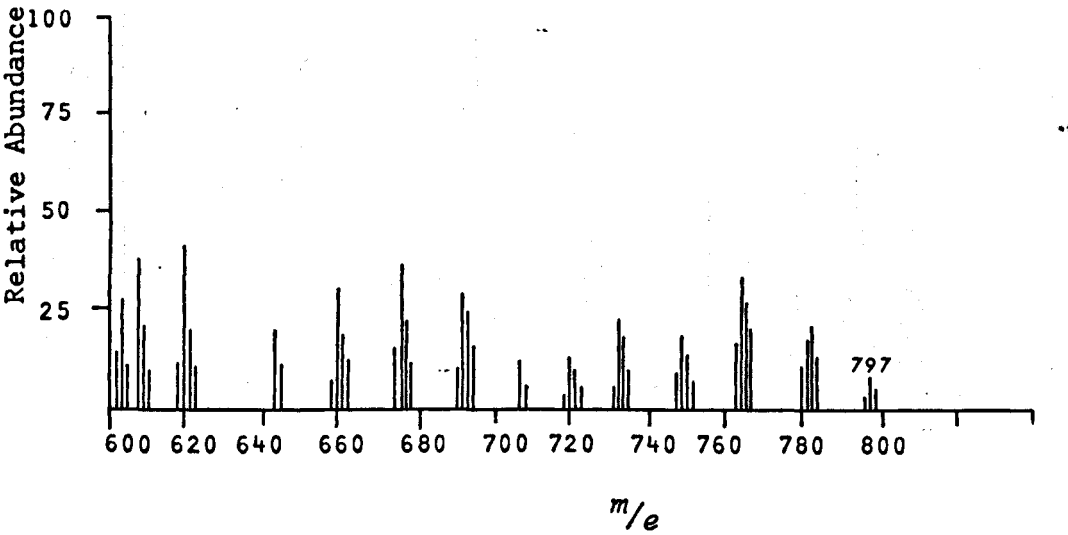


FIGURE 39 CONTINUED

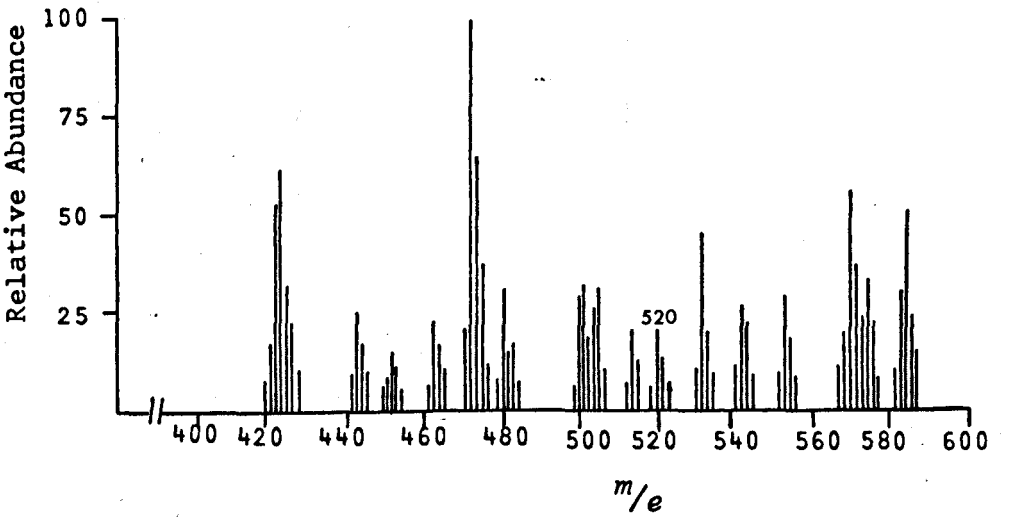


The mass spectrum of tetrakis-TMSi-ecdysone methoxime (*24a*) shows a relatively more abundant molecular ion ($^{m}/_{e}$ 781) than in the case of tetrakis-TMSi-20-hydroxy-ecdysone methoxime (*20a*) as the absence of the 20-hydroxyl group gives fewer possible fragmentation routes. The scission of the C20-C22 bond to give the uncharged side chain fragment (*31*) is now no longer favourable as indicated by the weak ion at $^{m}/_{e}$ 520 (see Figure 40 for $^{m}/_{e}$ 400 to 800, and Figure 41 for a more complete spectrum with prominent ions below $^{m}/_{e}$ 400).

FIGURE 40

Mass Spectrum of the Isomer Mixture *24a*

(Direct Inlet 240°)



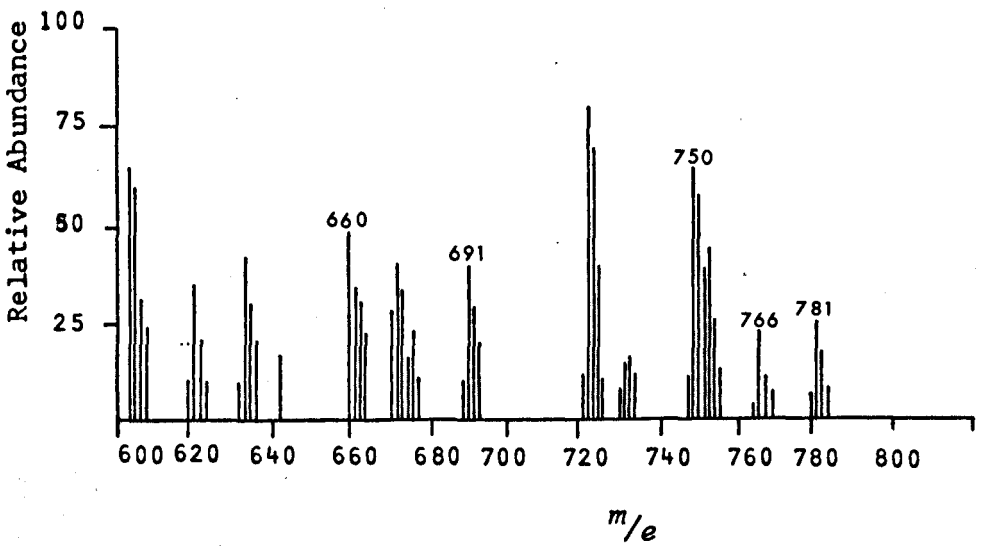
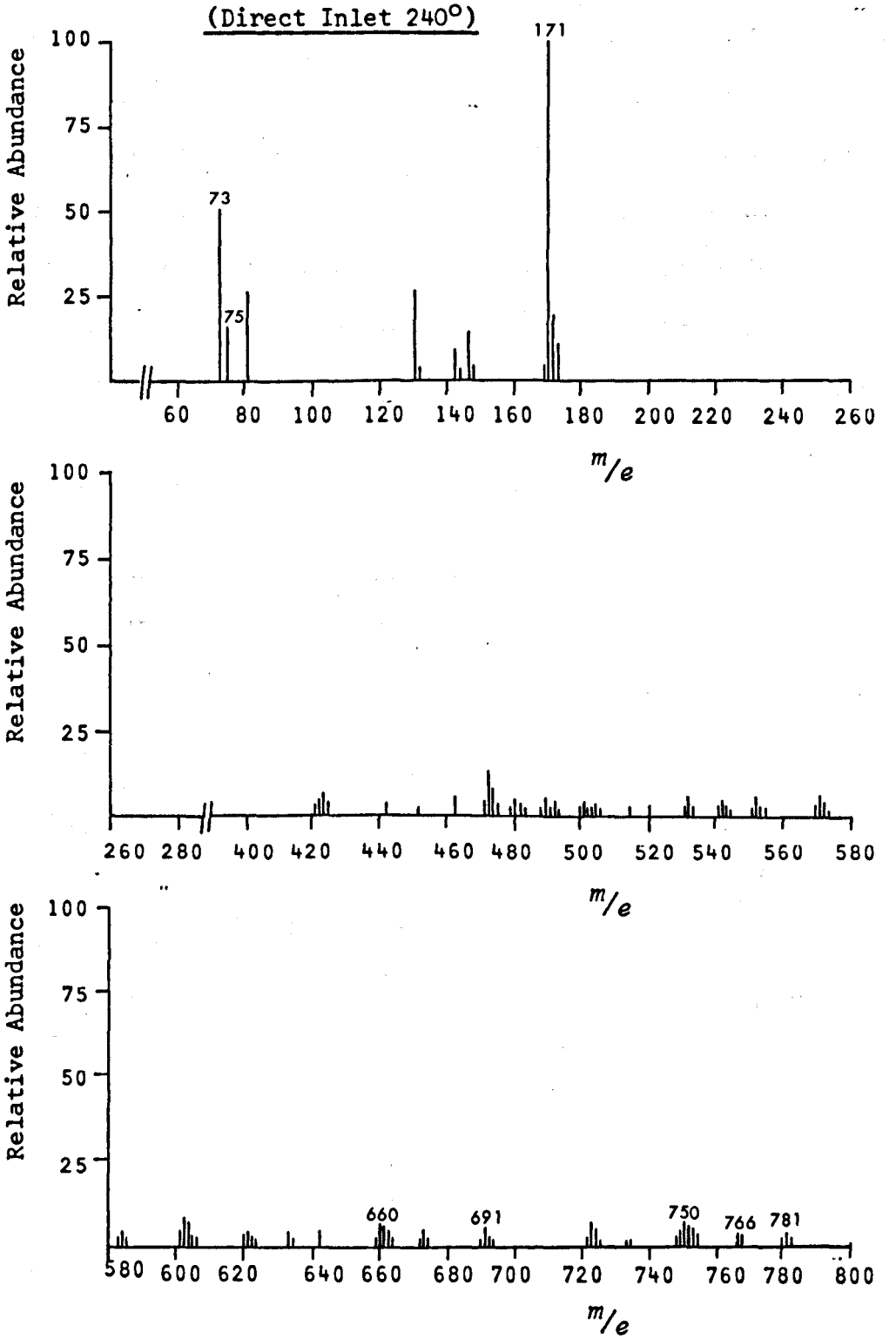


FIGURE 41

Mass Spectrum of the Isomer Mixture *24a*

(Direct Inlet 240°)



The fragmentation pattern due to the O-methyl oxime group can again be seen in the ions at high $^{m}/_{e}$ values with peaks at $^{m}/_{e}$ 766 (M - CH_3), 751 (M - CH_2O) and 750 (M - OCH_3). Pairs of peaks at $^{m}/_{e}$ 781 and 691, and $^{m}/_{e}$ 750 and 660 related by probable loss of trimethylsilanol (90 mass units) are also found. The mass spectra of the separated *syn*- and *anti*-isomers of tetrakis-TMSi-ecdysone methoxime (*25a* and *26a*, see Figures 42 and 43) confirmed their identity as isomers and showed that they had almost identical fragmentation patterns.

FIGURE 42

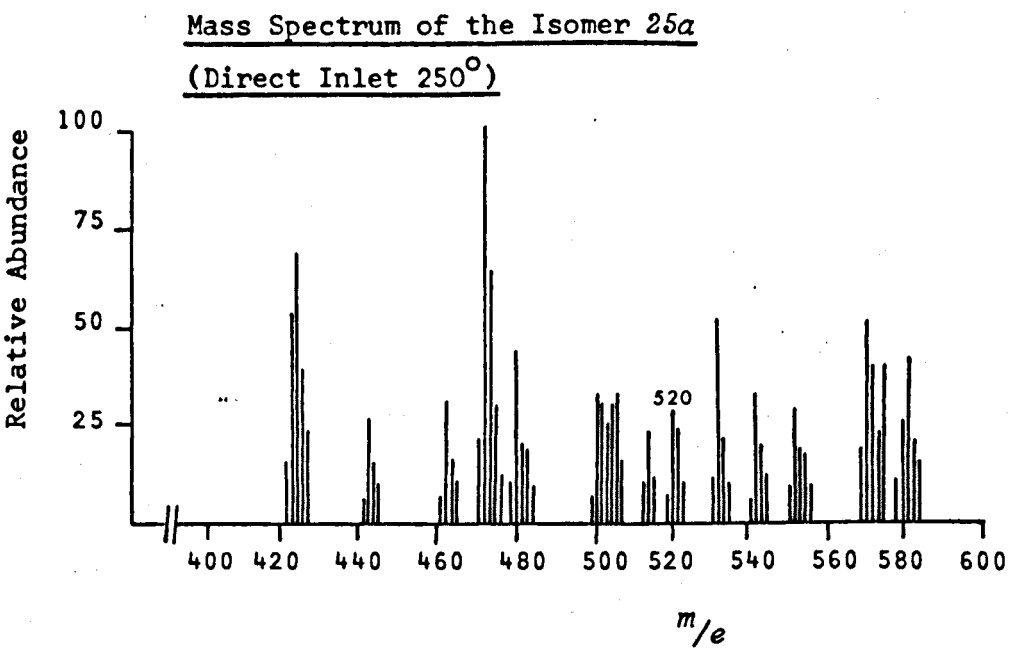


FIGURE 42 CONTINUED

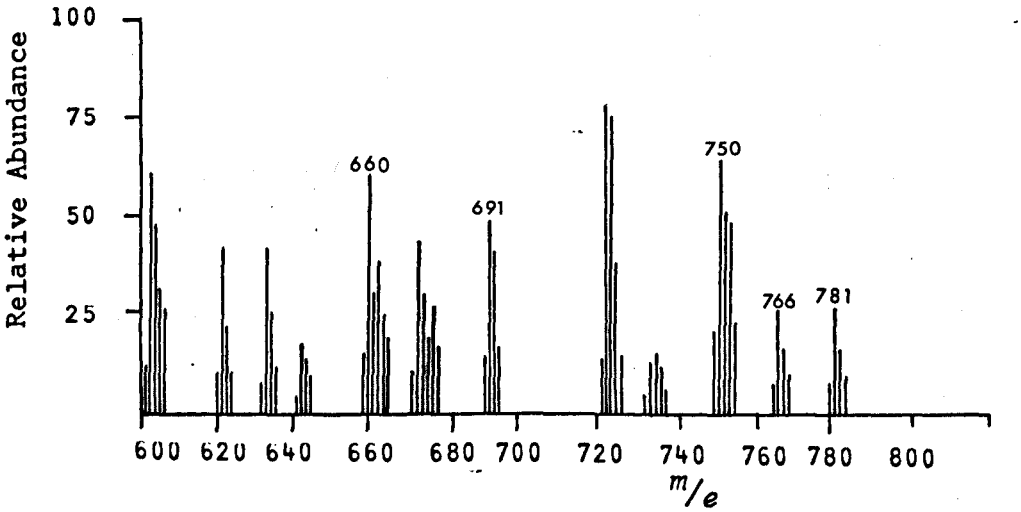
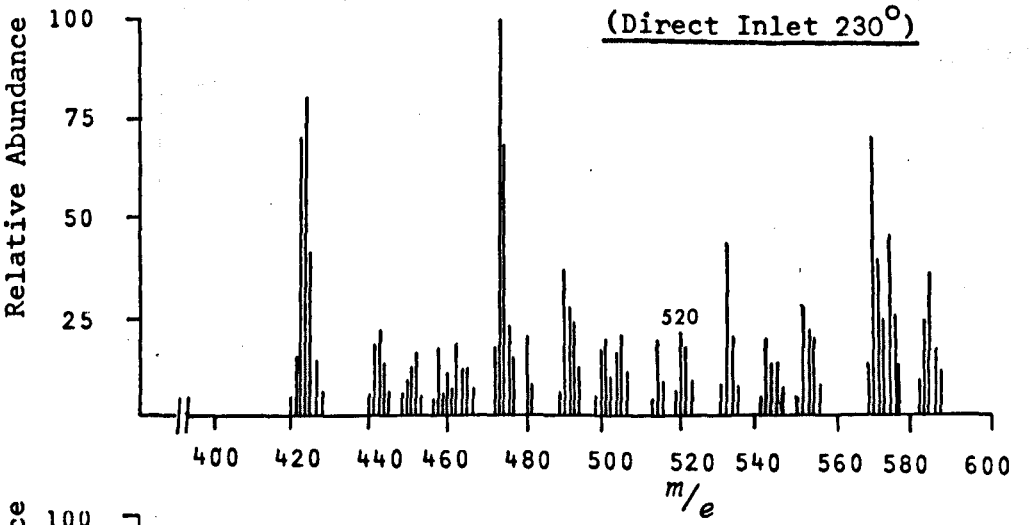
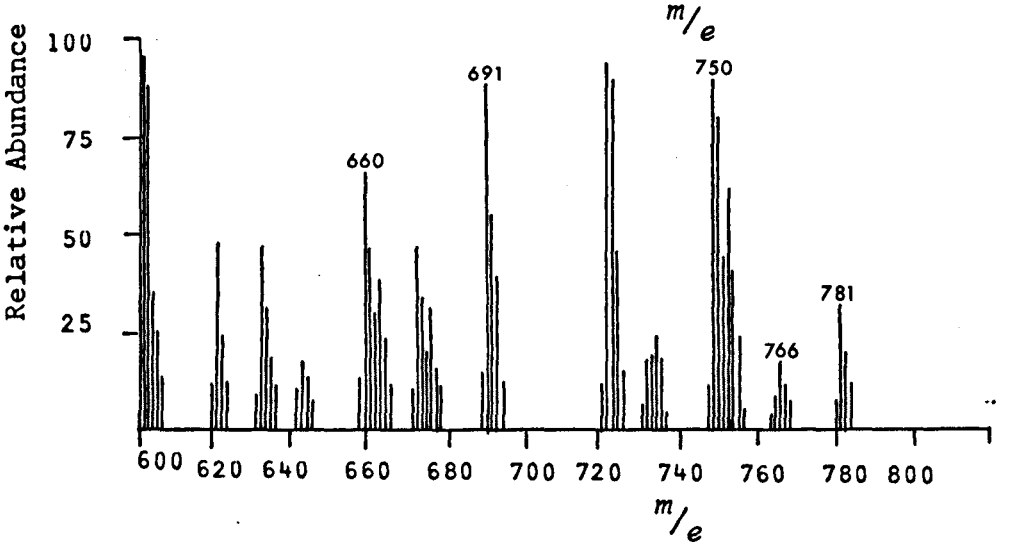
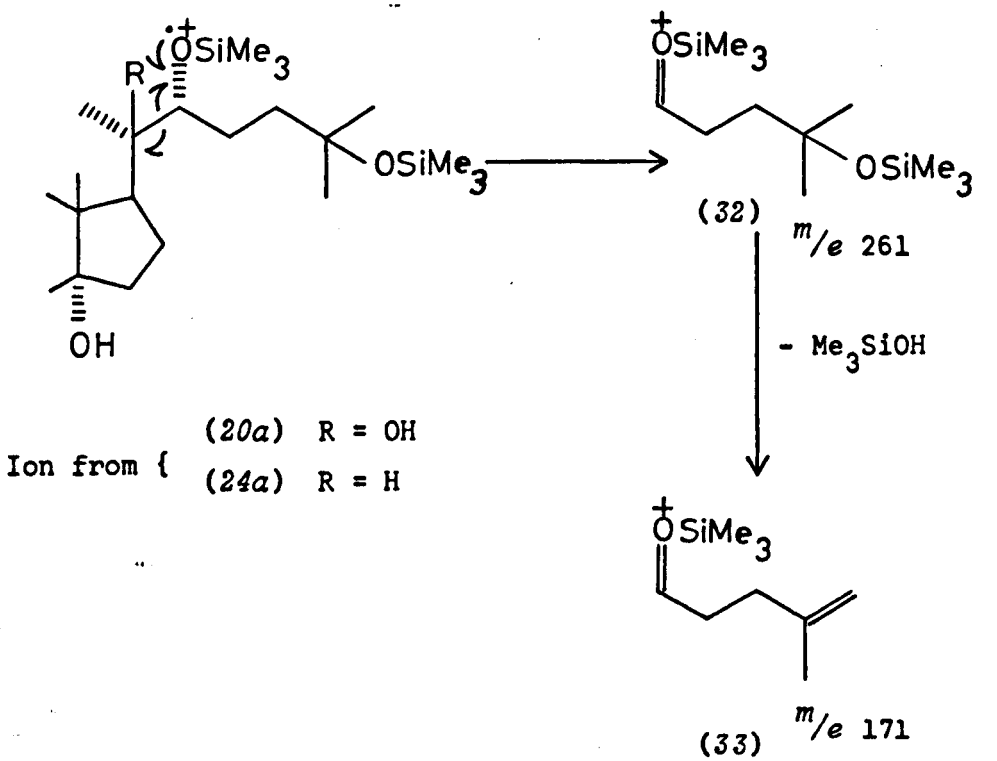


FIGURE 43 Mass Spectrum of the Isomer *26a*



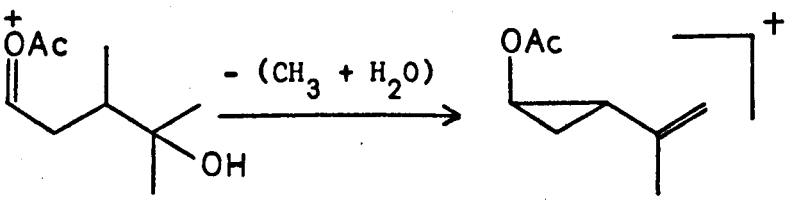


The base peak in the mass spectrum of tetrakis-TMSi--20-hydroxyecdysone methoxime (*20a*) and tetrakis-TMSi-ecdysone methoxime (*24a*) is at $^{m}/_{e}$ 171 in each case. This possibly arises from localisation of the positive charge on the C22 oxygen atom, followed by cleavage of the C20-C22 bond to give the ion (*32*) of $^{m}/_{e}$ 261 which undergoes subsequent loss of trimethylsilanol to give the ion (*33*) as the base peak at $^{m}/_{e}$ 171.



Ion from { (*20a*) R = OH
(*24a*) R = H

The ion of $^{m}/_{e}$ 261 is present in the spectrum of both ecdysone derivatives and is the most prominent ion (approximately 5% relative abundance) in the region $^{m}/_{e}$ 180 to 390 in each case. This fragmentation can be compared with the one suggested for an acetylated phytoecdysone makisterone A triacetate (2β,3β,22-triacetoxy-14α,20,25-trihydroxy-24--methyl-5β-cholest-7-en-6-one) where the ion *34* is formed (relative abundance less than 10%), and then decays to the prominent ion *35* (relative abundance approximately 55%).[127]



(*34*) (*35*)

Gas-Liquid Chromatography-Mass Spectrometry of the Synthetic Compounds

Linked g.l.c.-m.s. using the system described in the Experimental section was successful for the model compounds, TMSi-cholesterol (Mol. wt. 458) and 3β-trimethylsilyloxy-5α-

-ergosta-7,22-dien-6-one methoxime (Mol. wt. 513), but was unsuccessful for tetrakis-TMSi-20-hydroxyecdysone methoxime (Mol. wt. 797).

The results showed that the *syn*- and *anti*-isomers of 3β-trimethylsilyloxy-5α-ergosta-7,22-dien-6-one methoxime (*18*) were well separated and both had relatively short retention times. A satisfactory spectrum for each isomer could be obtained on injecting as little as 8 µg. of the mixture onto the column (see Figures 44 and 45).

FIGURE 44

Mass Spectrum of the Short t_R Isomer from *18*

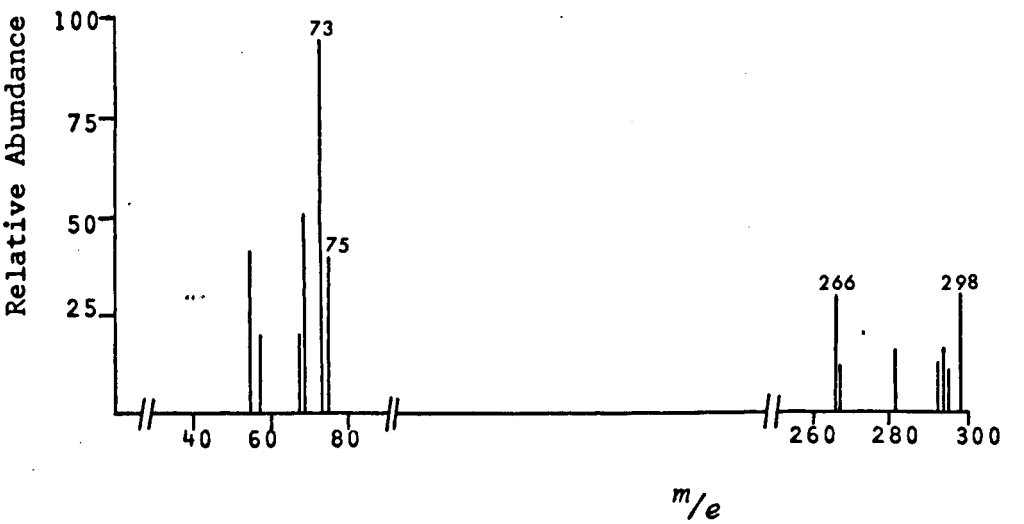


FIGURE 44 CONTINUED

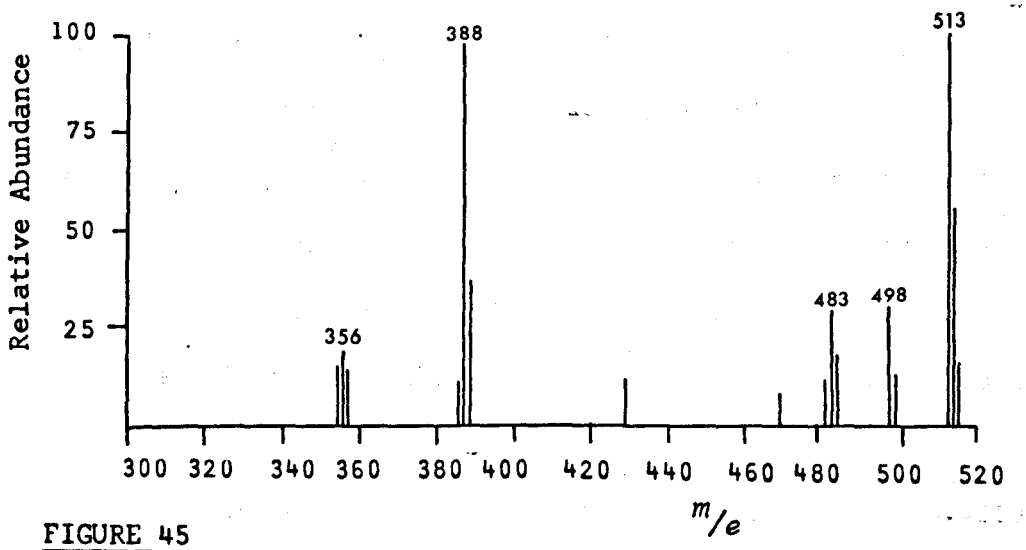
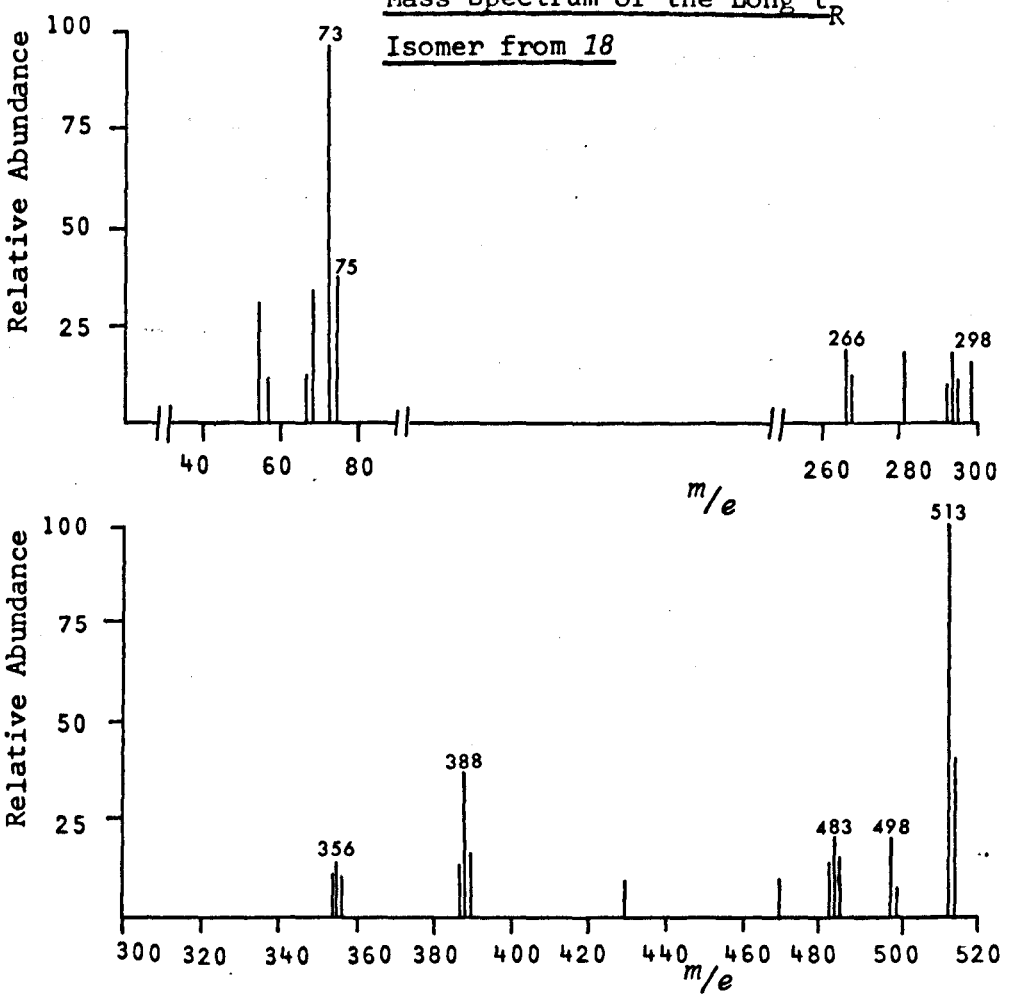


FIGURE 45

Mass Spectrum of the Long t_R Isomer from *18*



Although tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) was successfully chromatographed, the two isomers were incompletely separated under the linked g.l.c.-m.s. conditions used. They had relatively long retention times which gave broad flattened peaks unsatisfactory for m.s., and at no time was there any evidence of material in the mass spectrometer to give a spectrum, even when 40 µg. of the mixture was injected onto the column (see Table 42, page 279 for the g.l.c. results).

One possible answer to this problem would be to attempt g.l.c. of the ecdysone derivatives using a 1% OV-210 column to reduce the retention times of the isomers. This would give sharper g.l.c. peaks and therefore more material per unit time crossing the g.l.c.-m.s. interface. This might be successful providing there was no adsorption of the material onto the glass walls of the system after the compounds had been eluted from the column.

INVESTIGATION OF THE ACTIVE EXTRACTS XXV TO XXVII BY GAS-LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

With a technique developed to detect and determine ecdysone and 20-hydroxyecdysone in mixtures by g.l.c., the extracts XXV (upper active band), XXVI (middle active band) and XXVII (lower active band) were investigated for these two hormones. Materials were subjected to g.l.c. on an OV-210 column under the conditions described in the Experimental section.

G.l.c. of the extracts XXV to XXVII gave, in each case, a complex pattern of incompletely resolved peaks with retention times of less than two minutes. Extract XXVI showed the only other major peaks to be observed, at t_R 11.9 minutes and 12.6 minutes, and no other peaks were observed up to t_R 50 minutes.

Further portions of the three extracts were treated with O-methyl hydroxylamine hydrochloride as previously described for the ecdysones, but t.l.c. showed that these were recovered almost unchanged (see Figure 70, page 282). This indicated that the extracts contained little ecdysone like material. The products from the methoximation reactions were treated with B.S.A. under the conditions required to give the

tetrakis-TMSi derivatives of the ecdysones, and the materials obtained were subjected to p.t.l.c. using chloroform - 95% ethanol (4:1), followed by g.l.c. of the components from the separated bands visible under U.V.

The silylated material derived from extract XXV (upper active band) gave a strong band at R_f 0.82 in the region expected for the derivatives of ecdysone and 20-hydroxyecdysone (see Figure 71, page 283 and Table 37, page 268), but these could not be detected by g.l.c. The absence of these two hormones at detectable levels in the upper band extract was consistent with its previously determined R_f range (0.39 to 0.71), and showed that the active material in this extract must represent another hormone less polar than ecdysone. The g.l.c. trace showed a complex pattern of peaks t_R less than five minutes, and linked g.l.c.-m.s. indicated that the components corresponding to these were relatively small molecules with molecular weights in the range 260 to 350. Only traces of components with longer retention times were detected.

The material remaining on the plate in the R_f range for the untreated upper band extract (0.39 to 0.71) was isolated as a solid residue (XXVa), which although it had appeared as weak bands on the plate represented over 70% of

the original active extract. Comparison of this residue and the upper band extract by mass spectrometry (direct inlet) showed that they were essentially similar, and as residue XXVa was probably unchanged starting material it was tested for moulting activity. The result showed that it was essentially inactive (see page 284) and therefore the active material which must have been changed under the reaction conditions probably represented only a small proportion of the original extract.

The silylated material derived from extract XXVI (middle active band) also gave a band at R_f 0.82 on p.t.l.c. (see Figure 72, page 285), and g.l.c. of the material isolated from this band indicated the presence of 20-hydroxyecdysone in the original extract. Two peaks were observed at t_R 31.3 minutes and 32.8 minutes as an overlapping pair (see Figure 46) and these had the same characteristic appearance as the two peaks obtained from the isomer mixture tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) under identical conditions.

FIGURE 46

Measurement of the area of the double peak indicated that the total amount of 20-hydroxyecdysone in extract XXVI (middle active band, isolated from approximately 1.7×10^4 nymphs) was about 0.76 mg. The presence of 20-hydroxyecdysone (R_f 0.17) in the middle active band was consistent with the R_f range (0.15 to 0.25) of this extract as was the possible presence of ecdysone R_f 0.20). No peaks corresponding to ecdysone derivatives were however detected in g.l.c. and as a peak area 1% of that

due to the 20-hydroxyecdysone derivatives would have been detected, the amount of ecdysone, if present, must have been less than 1% of that of the 20-hydroxyecdysone.

A complex pattern of peaks t_R less than nine minutes was also observed, and linked g.l.c.-m.s. showed that the components corresponding to these were again relatively small molecules with molecular weights in the range 250 to 370.

The material remaining on the plate in the R_f range for the untreated middle band extract (0.15 to 0.25) was also isolated as a solid residue (XXVIa). This again represented approximately 70% of the original extract, but appeared on the plate as an intense band at R_f 0.20. G.l.c. of this material showed components at t_R 11.9 minutes and 12.6 minutes and these corresponded to the major components observed in extract XXVI when subjected directly to g.l.c. Comparison of the residue and the middle band extract by mass spectrometry (direct inlet) showed that they were still essentially similar, and as the residue XXVIa was therefore mainly starting material it was tested for moulting activity. The result showed that it was inactive (see page 287) and therefore if any active material other than 20-hydroxyecdysone was present in extract XXVI it must also have been changed under the reaction conditions.

The silylated material from extract XXVII (lower

active band) gave bands at R_f 0.86, 0.80 and 0.72, but g.l.c. of the materials isolated from these bands failed to indicate the presence of ecdysone or 20-hydroxyecdysone in the lower band extract, and only a complex pattern of peaks t_R less than 10 minutes was observed in each case with traces of components in the range t_R 10 to 45 minutes for the band at R_f 0.80. The absence of ecdysone (R_f 0.20) was consistent with the R_f range (0.05 to 0.18) for extact XXVII, but the presence of 20-hydroxyecdysone (R_f 0.17) had been suspected as the middle active band had been incompletely resolved from the lower active band on P.L.C. The 20-hydroxyecdysone must however have been isolated in the middle active band, and if present in extract XXVII its content probably represented less than 1% of that in the middle active band. This result showed that the active material in extract XXVII therefore represented another hormone, and this was more polar than 20-hydroxyecdysone.

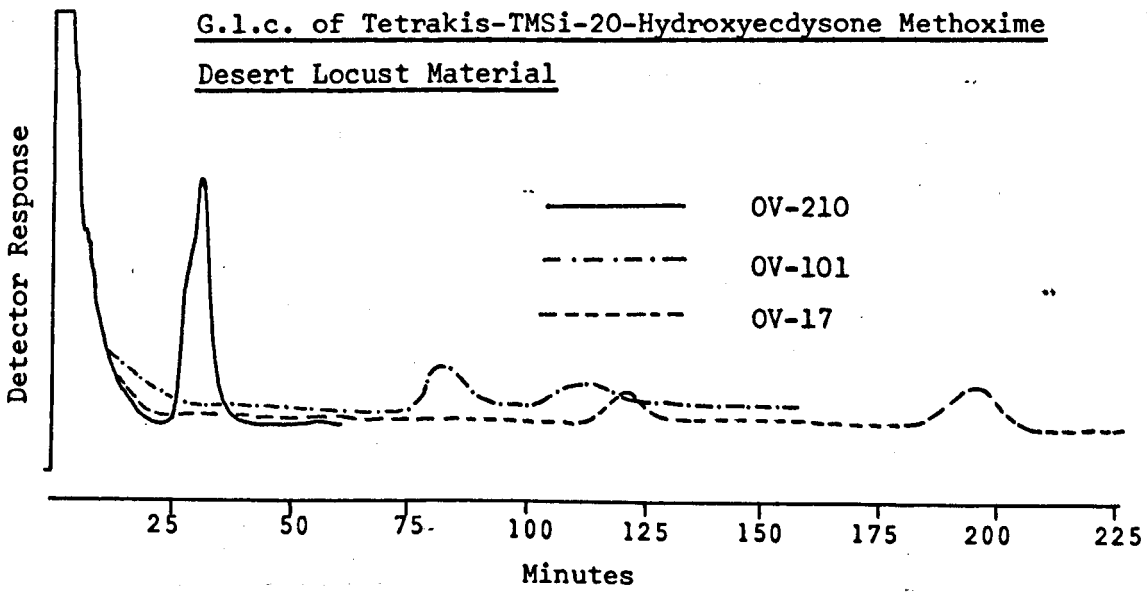
Ecdysone had not been detected in any of the three active extracts XXV to XXVII. If it is present in desert locust nymphs, it must therefore represent less than 1% of the 20-hydroxyecdysone content.

The material remaining on the p.t.l.c. plate in the R_f range for the untreated lower band extract (0.05 to 0.18) was isolated as a solid residue XXVIIa. This represented

approximately 50% of the original extract, and mass spectrometry (direct inlet) showed that it was still essentially similar to the lower band extract. As the residue XXVIIa also appeared to be mainly starting material it was tested for moulting activity. The result showed that it was essentially inactive (see page 289) and therefore the active material must have been changed under the reaction conditions.

Extracts XXV to XXVII were further investigated for ecdysone and 20-hydroxyecdysone using a two dimensional p.t.l.c. method developed during the investigation of locust prothoracic glands for 20-hydroxyecdysone (described in detail on page 324). The silylated material prepared from a further portion of extract XXVI by the method previously described gave a complex pattern of spots on two dimensional p.t.l.c. (see Figure 74, page 290). The components of the two spots at co-ordinates R_f (0.82, 0.10) and (0.82, 0.21), which corresponded to the expected position of two methoxime isomers of tetrakis-TMSi-20--hydroxyecdysone methoxime (*20a*) chromatographed under the same conditions (see Table 37, page 268), were isolated together. G.l.c. of this material using three different stationary phases showed that it was a simple mixture containing *syn*- and *anti*--tetrakis-TMSi-20-hydroxyecdysone methoxime as the only major components (see Figure 47).

FIGURE 47

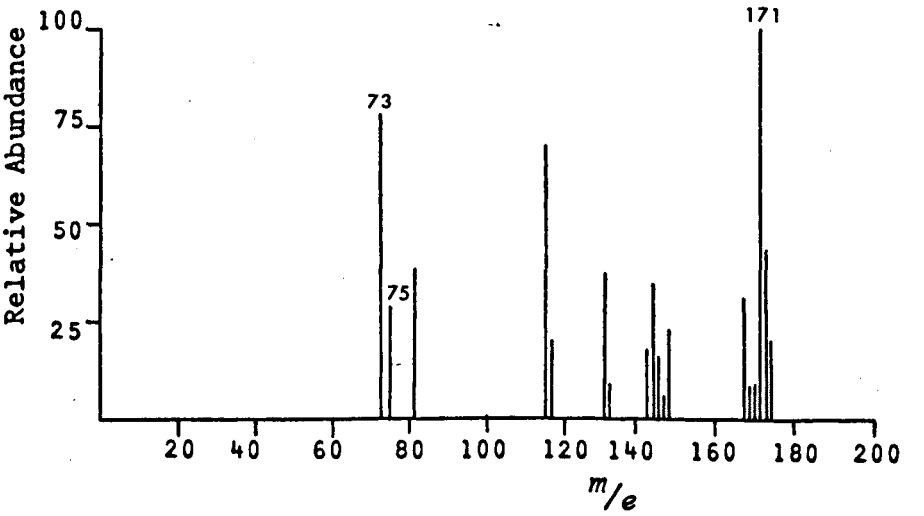


Measurement of the area of the double peak produced, using OV-210, indicated that the total amount of 20-hydroxy-ecdysone in extract XXVI was about 0.92 mg., and this was in close agreement with the value of 0.76 mg. previously determined by g.l.c.

The mass spectrum (direct inlet) of the tetrakis-TMSi--20-hydroxyecdysone methoxime derived from the locust extract XXVI (middle active band) and the mass spectrum of the authentic sample (*20a*) were compared and were found to be identical (see Figure 48). The identity of the desert locust material and the authentic 20-hydroxyecdysone derivative in mass spectrometry, and on g.l.c. using the three different stationary phases, proved conclusively that the desert locust extract contained the moulting hormone 20-hydroxyecdysone.

FIGURE 48

Mass Spectrum of Tetrakis-TMSi-20-Hydroxyecdysone Methoxime, Desert Locust Material (Direct Inlet 250°)



Mass Spectrum of Tetrakis-TMSi-20-Hydroxyecdysone Methoxime (*20a*), From Authentic 20-Hydroxyecdysone (Direct Inlet 250°)

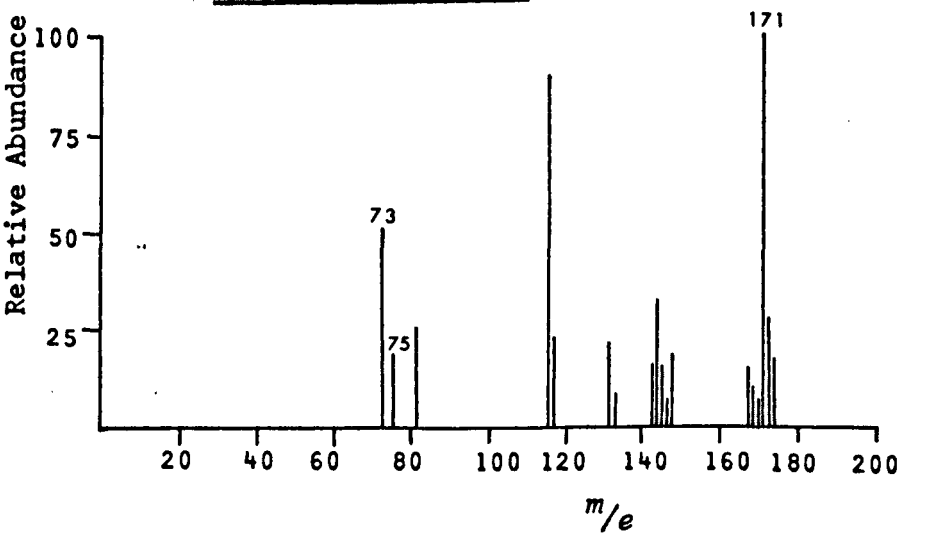
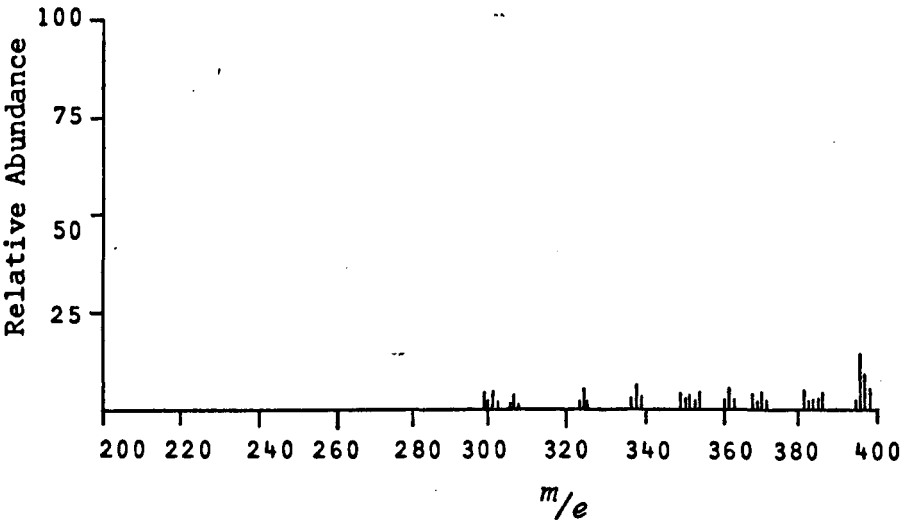


FIGURE 48 CONTINUED

Desert Locust Material



Authentic Material

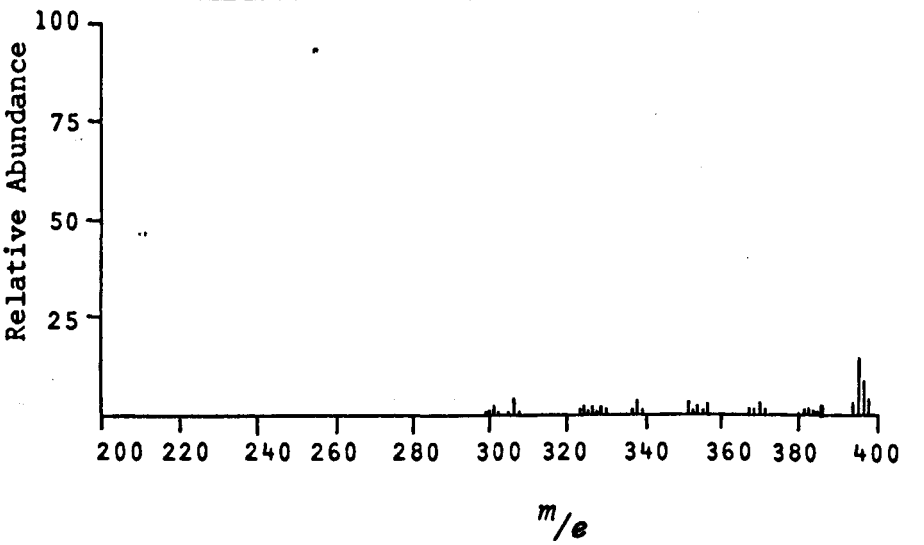


FIGURE 48 CONTINUED

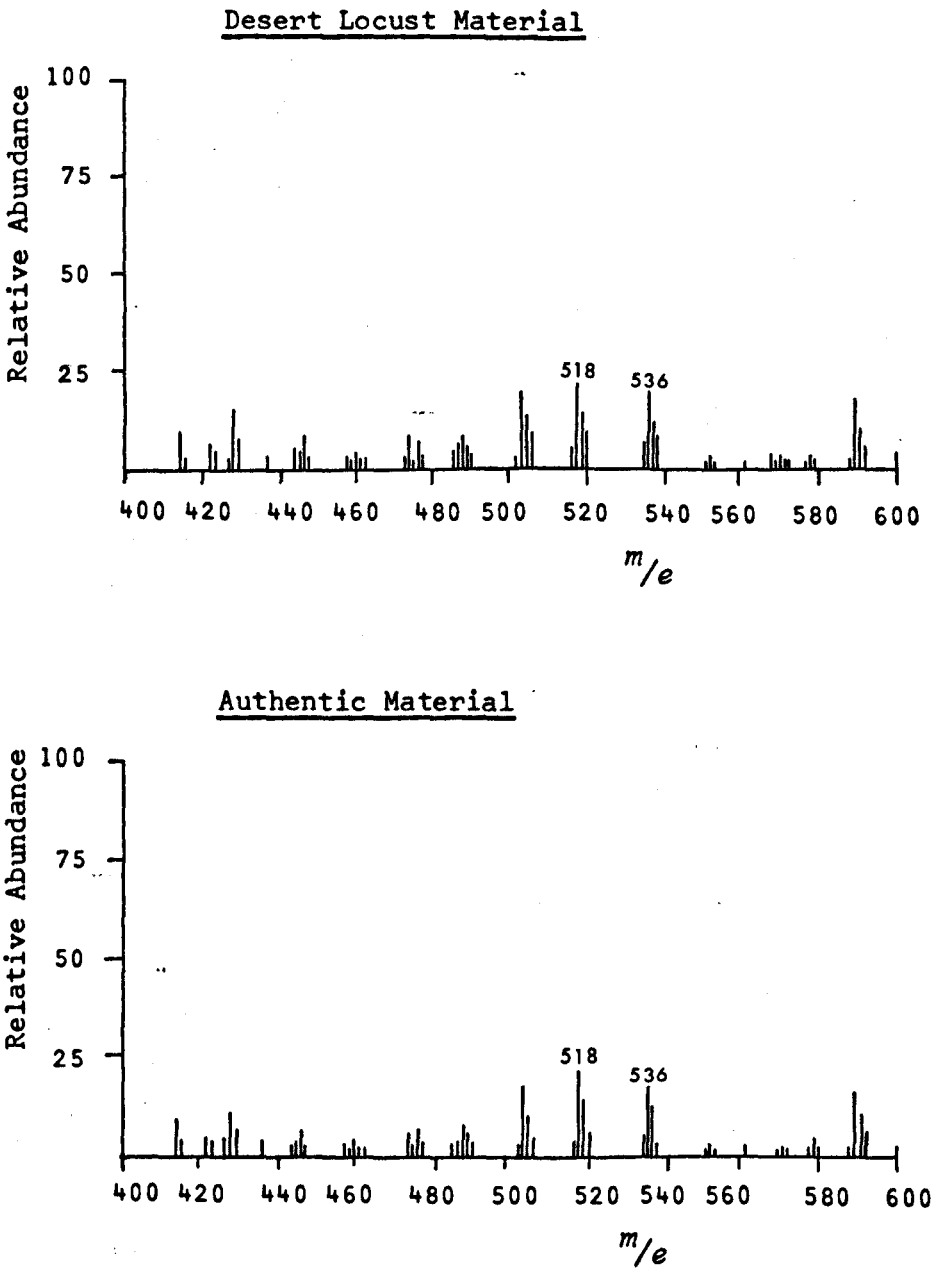
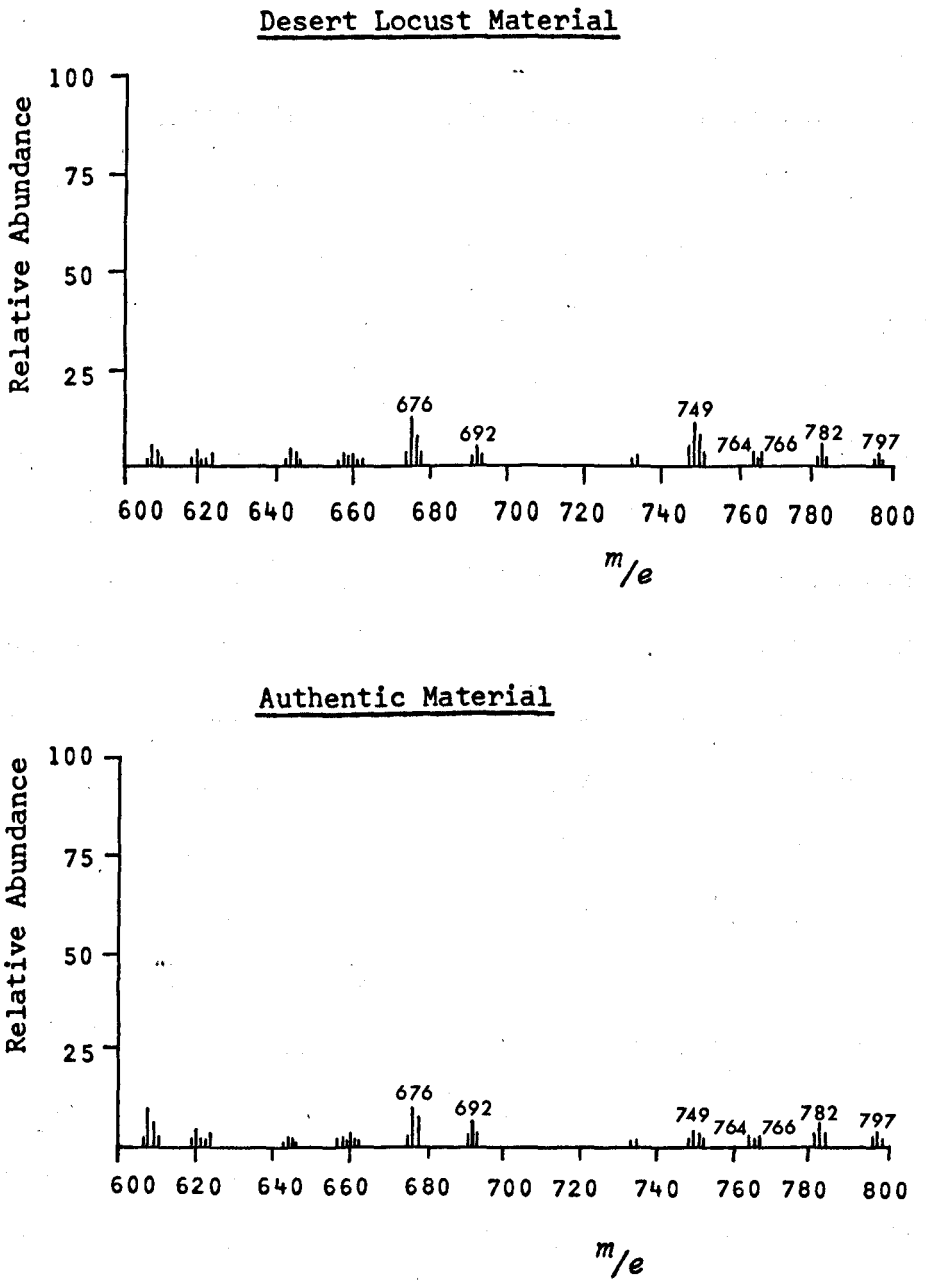


FIGURE 48 CONTINUED



The components of the spots at co-ordinates R_f (0.79, 0.26), (0.82, 0.32) and (0.82, 0.60) on the two dimensional p.t.l.c. plate were also subjected to g.l.c. This showed that they corresponded to the components giving a complex pattern of peaks t_R less than nine minutes. Derivatives of ecdysone were again not detected.

The silylated materials from further portions of extracts XXV and XXVII (upper and lower active bands) were also subjected to two dimensional p.t.l.c. but even with the improved method of separation no derivatives of ecdysone or 20-hydroxyecdysone could be detected by g.l.c. in either case.

The materials remaining at the R_f positions of the upper and lower active bands after treatment of these latter two extracts with O-methylhydroxylamine hydrochloride and B.S.A. were no longer active in bioassay, and therefore the active substances must have been changed by the reagents. Examination of the treated extract by p.t.l.c. followed by g.l.c. however failed to reveal detectable amounts of any components with retention times similar to those of derivatives of ecdysone or 20-hydroxyecdysone. The unidentified active substances were therefore either present in quantities less than about 1% of the amount of 20-hydroxyecdysone (with a correspondingly higher level of activity in the bioassay), or they have structures which are somewhat different from those of the two known hormones.

ISOLATION OF 20-HYDROXYECDYSONE FROM THE MIDDLE ACTIVE BAND (XXVI)

As 20-hydroxyecdysone had been detected by g.l.c. in the middle active band (XXVI), it was decided to attempt the isolation of this hormone to see whether it was the only detectable substance with moulting activity in this extract.

Because the amount of 20-hydroxyecdysone detected in extract XXVI was less than 1 mg., a fresh batch of homogenate, corresponding to 1.8×10^4 nymphs, was extracted by the standard desert locust extraction procedure to give more material for the isolation. The material which corresponded to extract XXVI was isolated, and the g.l.c. procedure showed that it contained approximately 1.2 mg. of 20-hydroxyecdysone. The new middle band material was combined with extract XXVI to give extract XXVIII which corresponded to the middle active band from approximately 3.2×10^4 nymphs (purification factor 3.3×10^4 times).

Solvent partition methods were selected for the initial purification as these give good recovery of material, and extract XXVIII only contained about 2 mg. of 20-hydroxy-ecdysone. Extract XXVIII had originated from polar fractions in the locust extraction, and therefore the first separation

was carried out using a countercurrent distribution system of chloroform - ethanol - aqueous potassium hydrogen carbonate (1:1:1) in which 20-hydroxyecdysone is partitioned into the less polar phase.[42]

Countercurrent distribution of extract XXVIII in five separating funnels using the double withdrawal procedure outlined in the Experimental section gave two new extracts, and these were examined by bioassay for moulting activity and by g.l.c. for 20-hydroxyecdysone. Extract XXIX from the combined lower layers was active and contained all the 20--hydroxyecdysone (approximately 1.99 mg.), whereas extract XXX from the combined upper layers contained no detectable 20-hydroxyecdysone and was inactive (see page 296). The overall purification factor for the active material in extract XXIX was about 8.7×10^4 times at this stage, but the 20-hydroxyecdysone was still not detected by t.l.c. using vanillin spray.

Extract XXIX was therefore subjected to a second countercurrent distribution using a system of ethyl acetate - water (1:1) as described by Kaplanis *et al.*, in which 20-hydroxyecdysone is preferentially partitioned into the polar phase ($K = 0.32$ at 23-24°).[24] The double withdrawal procedure using eight separating funnels gave two extracts for examination

by bioassay and g.l.c. Extract XXXI from the combined lower layers was active in the bioassay and contained all the 20-hydroxyecdysone (approximately 1.78 mg.) whereas extract XXXII from the combined upper layers contained no detectable 20-hydroxyecdysone and was inactive in the bioassay (see page 298). The overall purification factor for the active material in extract XXXI was about 1.6×10^5 times but the 20-hydroxyecdysone was still undetected by t.l.c. using vanillin spray.

The active extract(XXXI) which contained the 20-hydroxyecdysone was now sufficiently soluble in water for gel filtration chromatography on a column of CM-Sephadex.[42] A column was prepared and was eluted as described in the Experimental section, to give 71 fractions. These were monitored by t.l.c. eluting with chloroform - 95% ethanol (4:1) and the spots were visualised with vanillin spray. 20-Hydroxyecdysone was detected in appreciable quantities in fractions 18 to 21, and these were recombined to give extract XXXIII. The fractions eluted before and after these four fractions were also recombined, fractions 1 to 17 gave extract XXXIV and fractions 22 to 71 gave extract XXXV. The latter two extracts showed only slight moulting activity, but extract XXXIII, which contained most of the 20-hydroxyecdysone, was

highly active with an ED_{50} between 100 and 10 µg. The overall purification factor for the material in this active extract was about 1.36×10^6 times at this stage in the isolation.

Portions of extract XXXIII were compared with authentic 20-hydroxyecdysone on t.l.c. under the conditions described in the Experimental section, and in each case visualisation under U.V. showed a major spot which had the same R_f as the standard, and showed the colour reactions of 20-hydroxyecdysone with vanillin spray (see Table 37, page 268). The U.V. spectrum of extract XXXIII however showed no $\lambda_{max.}$ at 244 nm. characteristic of the 7-en-6-one chromophore of 20-hydroxyecdysone[61] (see Figure 49, page 135) and this indicated that 20-hydroxyecdysone could not be the major component in the extract. This was consistent with the level of moulting activity found for extract XXXIII (compare the assay results for extract XXXIII, see page 300, and authentic 20-hydroxyecdysone, see Figure 50, page 136), and with the amount of 20-hydroxyecdysone (less than 2 mg.) expected in this extract from the g.l.c. results previously described.

Charring of the t.l.c. plates which had been sprayed with vanillin reagent revealed another component resolved from 20-hydroxyecdysone (see Figure 75, page 301), and this was well separated on the chromatogram eluted with

ethyl acetate - ethanol (8:2). The remaining extract was therefore subjected to p.t.l.c. eluting with ethyl acetate - ethanol (8:2). The band corresponding to the R_f (0.32) of 20-hydroxyecdysone was located under U.V. (see Figure 76, page 302), and the absorbed material was isolated from this band (extract XXXVI) and from the region R_f 0.00 to 0.30 (extract XXXVII) which included the material only visualised on charring with vanillin spray. The two new extracts were assayed for moulting activity (see Table 43 accompanying Figure 76) and extract XXXVI which contained the 20-hydroxyecdysone was highly active with an ED_{50} between 10 and 1 µg. Extract XXXVII was relatively inactive with an ED_{50} greater than 100 µg., and this low activity was probably only due to traces of entrained 20-hydroxyecdysone.

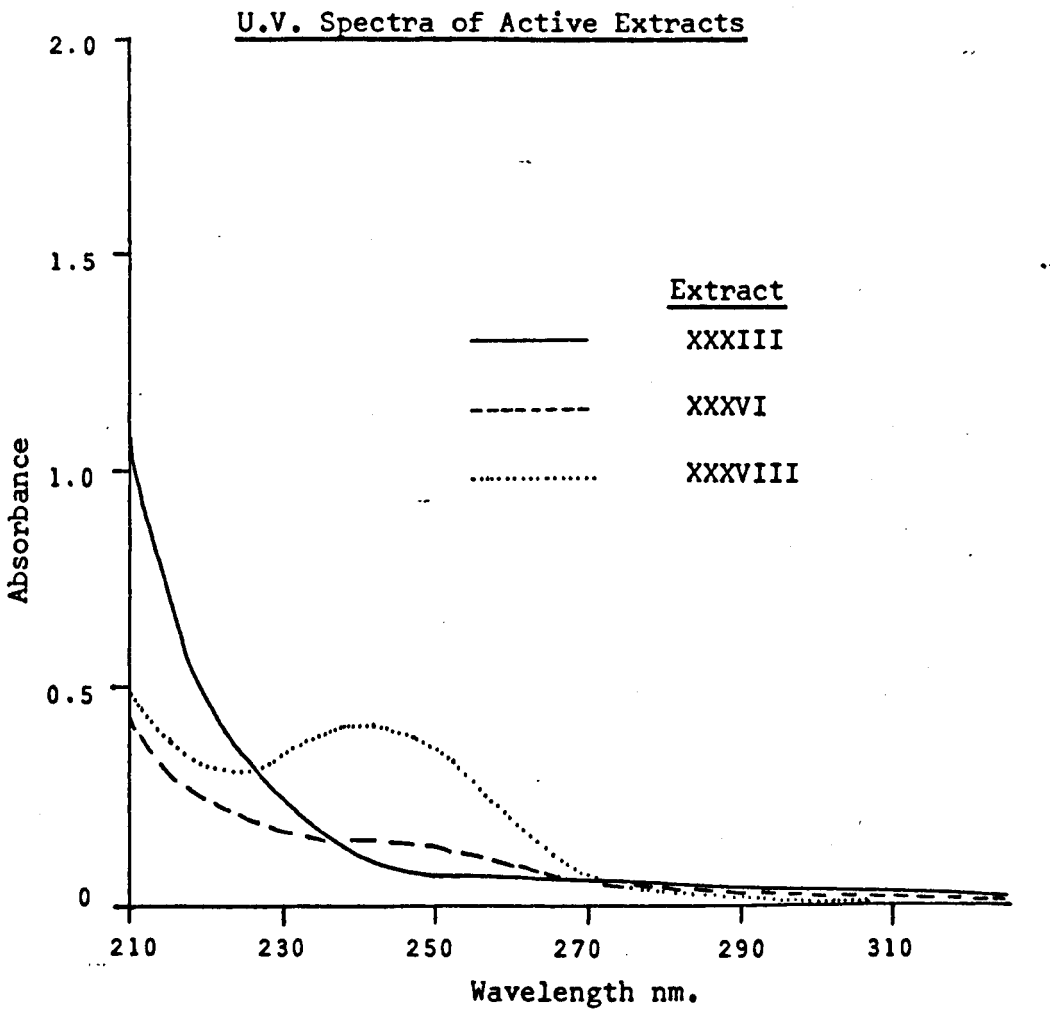
T.l.c. of the active extract XXXVI showed that the p.t.l.c. had given a good separation, but the U.V. spectrum of the material (see Figure 49, page 135) showed only a shoulder at 244 nm., and the extinction coefficient indicated that the extract contained about 16% 20-hydroxyecdysone (about 1.2 mg.). This was consistent with the level of moulting activity found for extract XXXVI (compare the assay results for extract XXXVI, page 302, and authentic 20-hydroxyecdysone, Figure 50, page 136), and with the amount of 20-hydroxyecdysone

previously estimated by g.l.c. Further investigation of chromatographic conditions showed that a separation of components in extract XXXVI was possible using aluminium oxide sheet and eluting with chloroform - methanol - acetone (6:2:1, see Figure 77, page 304).

The remaining extract XXXVI was therefore subjected to p.t.l.c. under the above conditions. The band corresponding to the R_f (0.16) of 20-hydroxyecdysone was located under U.V. (see Figure 78, page 305), and the absorbed material was isolated from this band (extract XXXVIII) and the regions R_f 0.00 to 0.14 (extract XXXIX) and 0.18 to 0.65 (extract XL) on either side.

The U.V. spectrum of extract XXXVIII (see Figure 49) showed a $\lambda_{max.}$ at 244 nm. characteristic of the 7-en-6-one chromophore of 20-hydroxyecdysone, and the 20-hydroxyecdysone content of this extract was estimated to be about 64% from the extinction coefficient. When extract XXXVIII was compared with authentic 20-hydroxyecdysone on t.l.c. both materials gave identical results on visualisation with vanillin spray. As no components other than 20-hydroxyecdysone were detected in extract XXXVIII on t.l.c., the remaining material in the extract was probably due to traces of alumina or binder from the aluminium oxide layer used in the previous p.t.l.c.

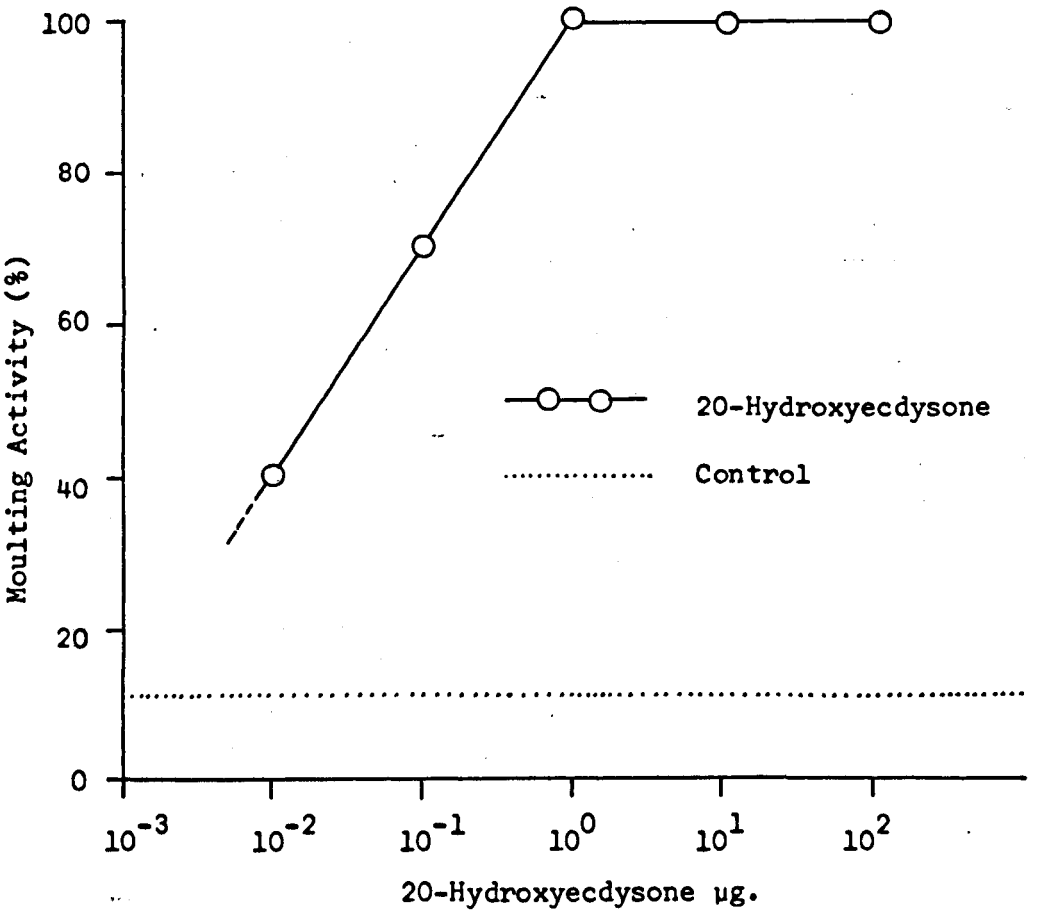
FIGURE 49



Extract XXXVIII was tested for activity in the moulting hormone bioassay and was found to have a high level of activity similar to that shown by authentic 20-hydroxyecdysone which has an ED_{50} of less than 0.1 µg. The dose response curve for authentic 20-hydroxyecdysone is plotted in Figure 50, and the assay results for this hormone and extract XXXVIII are compared below.

FIGURE 50

Dose Response Curve for 20-Hydroxyecdysone



	Activity at dose:					
	100 µg.	40 µg.	10 µg.	1 µg.	0.1 µg.	0.01 µg.
XXXVIII*		100%	100%	100%	75%	50%
20-Hydroxyecdysone†	100%		100%	100%	70%	40%

* Control 17%
† Control 11%

Crystalline 20-hydroxyecdysone was obtained from the remaining portion of extract XXXVIII by crystallisation from ethyl acetate, and this material was shown to be essentially the pure hormone from its melting point, and mixed melting point with authentic 20-hydroxyecdysone.

Extracts XXXIX and XL were also active, but were less active than extract XXXVIII. These two extracts had an ED_{50} of about 10 µg., and this was probably due to traces of entrained 20-hydroxyecdysone (weakly visible on t.l.c. visualising with vanillin spray).

The isolation of 20-hydroxyecdysone from the middle active band confirmed the presence of this hormone in 5th instar desert locust nymphs, and the amount isolated was consistent with that determined by g.l.c. in the middle active band and other subsequent active fractions. The 20-hydroxyecdysone titre per nymph estimated for each of the extracts examined by g.l.c. and found to contain this hormone is listed below, also included is the value for extract XXXVIII determined from its U.V. extinction coefficient.

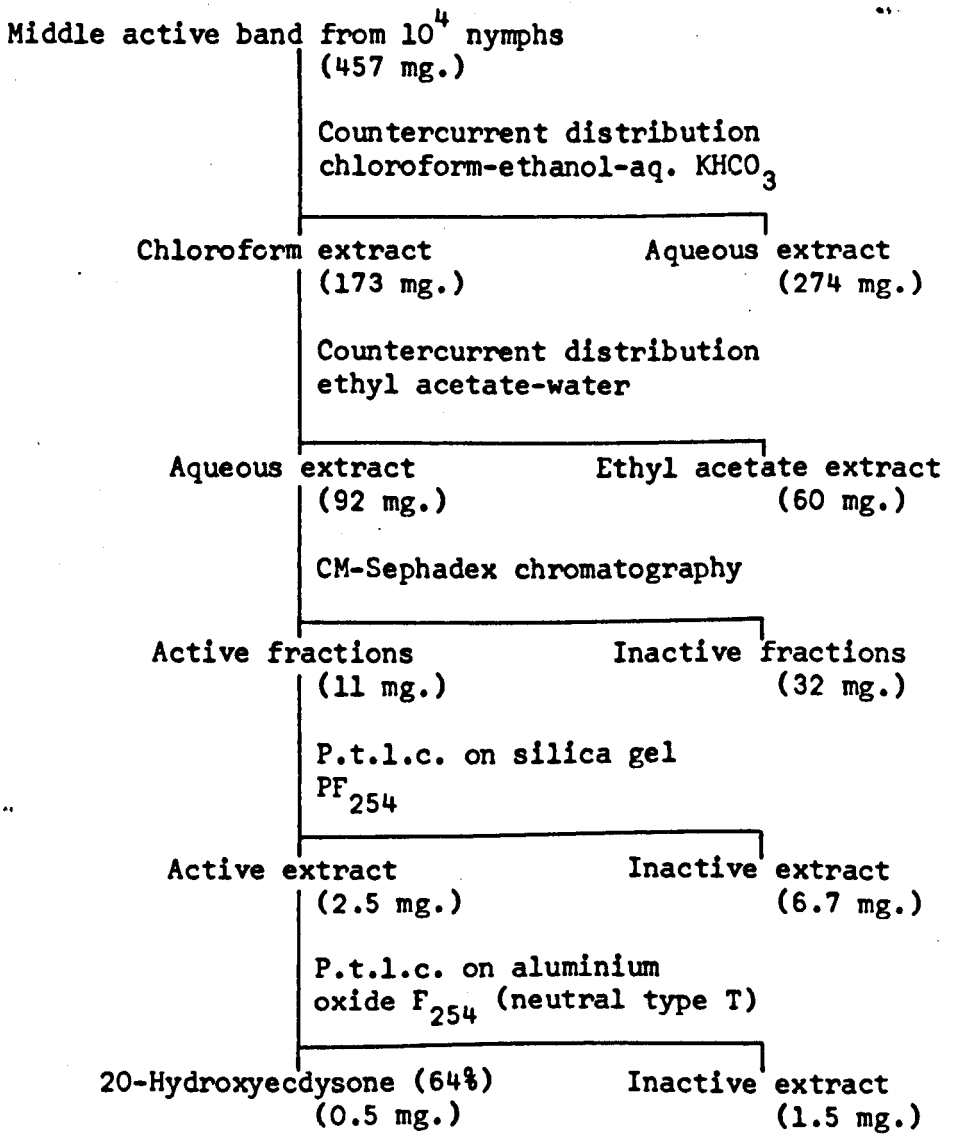
	Titre of 20-hydroxyecdysone ng./nymph
XXVI (middle active band)	46 54
XXIX	62
XXXI	56
XXXVIII	31

The results showed that the average 20-hydroxyecdysone titre per nymph (days 8 to 11 of the 5th instar) detected by g.l.c. in extracts from the latter part of the isolation was about 55 ng. per nymph.

The extracts with highest moulting activity obtained at each stage during the isolation of 20-hydroxyecdysone from the middle active band were those found to contain this hormone, and the lower activity in the other extracts obtained at each stage appeared to correspond to entrained 20-hydroxyecdysone. It is therefore concluded that 20-hydroxyecdysone was the hormone responsible for the moulting activity of the middle active band extract. A flow sheet showing the steps in the extraction procedure used to isolate 20-hydroxyecdysone from the middle active band is shown below. The weights given for

extracts at each stage in the procedure are based on the extraction of 10^4 nymphs.

Procedure for the Extraction of 20-hydroxyecdysone from the Middle Active Band Extract (XXVI)



DETERMINATION OF THE 20-HYDROXYECDYSONE CONTENT OF NYMPHS AT DAILY INTERVALS THROUGHOUT THE 5TH INSTAR BY GAS-LIQUID CHROMATOGRAPHY

The determination of 20-hydroxyecdysone in the middle active band and other more highly purified fractions by g.l.c. had shown that this hormone was present in extracts of late 5th instar desert locust nymphs (days 8 to 11 of the 5th instar) at an average level of approximately 55 ng. per nymph. It was therefore decided to investigate the variation in the titre of this hormone throughout the 5th instar by the g.l.c. method. As some material is usually lost during any large scale extraction, 20-hydroxyecdysone titres exceeding 55 ng. per nymph were expected, particularly if nymphs exhibited a maximum titre of this hormone on any given day.

A limitation of the two dimensional p.t.l.c. method used in the purification of silylated extracts before g.l.c. is that amounts of material greater than about 10 mg. in weight cannot be successfully applied to the corner of the p.t.l.c. plate.

Groups of 8 to 10 nymphs were therefore selected for the extractions at daily intervals as their 20-hydroxyecdysone content was expected to be satisfactory for the g.l.c.

determination of this hormone, and numbers of nymphs in this range had been found to give 10 mg. or less of active extract after the solvent partition stages in the standard extraction procedure.

Each group of nymphs was extracted using this method, but as the amount of extract was relatively small the enthanol stage was omitted. Aqueous methanol (4:1) was also substituted for water in the final solvent partition in an attempt to prevent emulsification and entrainment of 20-hydroxyecdysone in the light petroleum phase. The weights of material obtained from the butanol and aqueous methanol (4:1) phases in the extraction of nymphs from day eight in the 4th instar to day four of the adult stage and on day 14 of the adult stage are given in Table 45 (see page 309).

The aqueous methanol extract obtained for each group of nymphs was examined for 20-hydroxyecdysone by the procedure of derivative formation and two dimensional p.t.l.c. followed by g.l.c. as described in the Experimental section. The results showed that 20-hydroxyecdysone was present in extracts isolated from nymphs throughout the 5th instar and that the titre was a maximum on the day of ecdysis.

The appearance of the p.t.l.c. plates under U.V. were similar for the extracts of whole nymphs from each day

investigated, and a typical plate at each of the three stages of the separation is shown in Figure 79 (see page 310, day three of the 5th instar). The g.l.c. traces also contained several features in common and two typical traces are shown in Figures 51 and 52 below. Both of these show the characteristic double peak (arrowed) centred at about t_R 32 minutes which corresponded to *syn*- and *anti*-tetrakis-TMSi-20--hydroxyecdysone methoxime and indicated the presence of 20-hydroxyecdysone in the initial extracts.

FIGURE 51

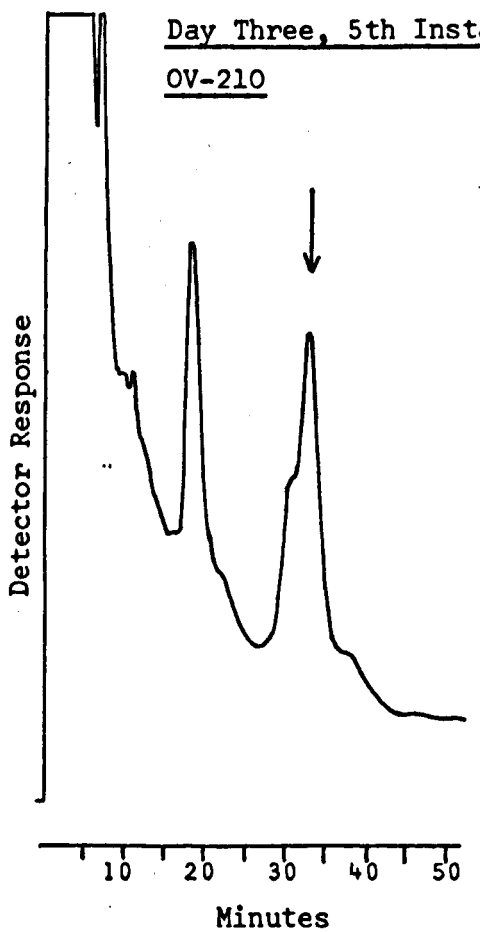
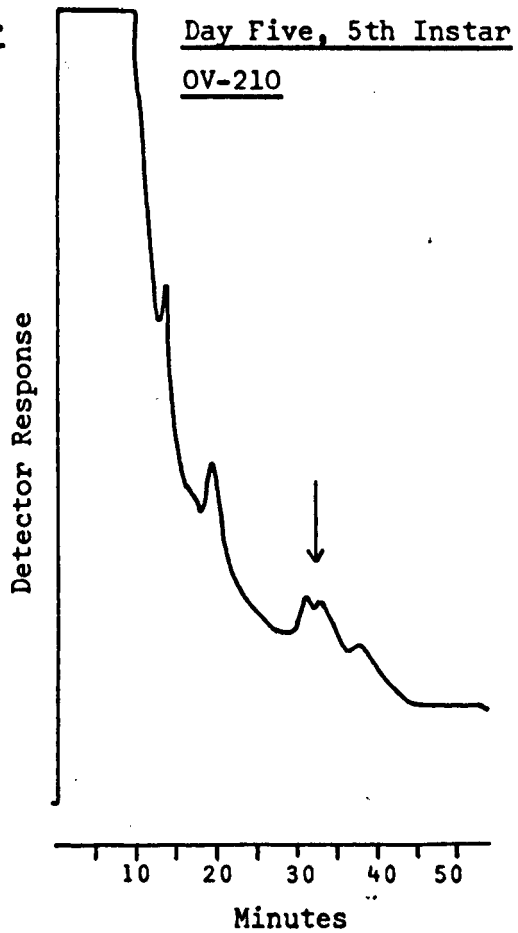
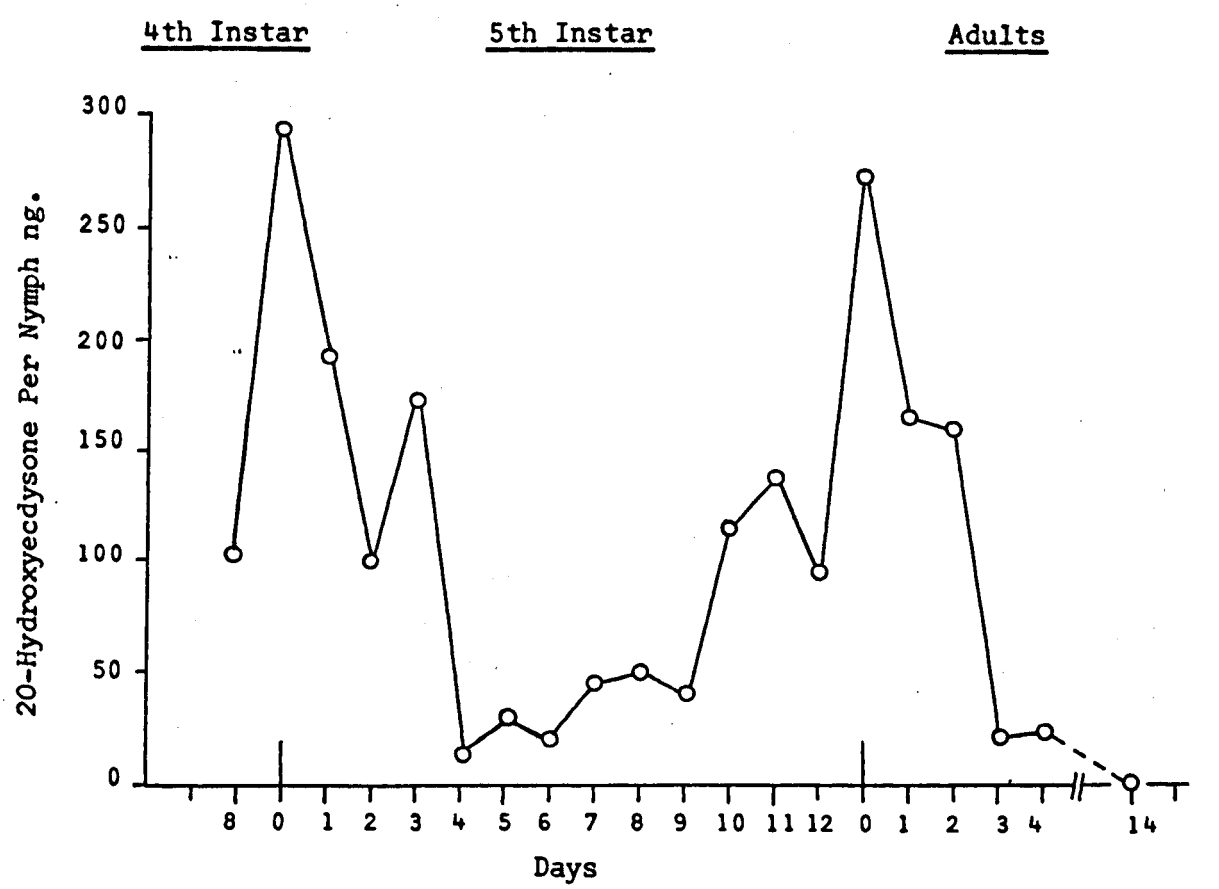


FIGURE 52



The area of the characteristic double peak was measured for each group of nymphs extracted when one third of the total material from p.t.l.c. was subjected to g.l.c. These results were used to estimate the amount of 20-hydroxy-ecdysone in each extract and also the amount of this hormone per nymph for each of the days selected (see Table 46, page 313). No ecdysone could be detected on any of the days investigated. The variation of the titre of 20-hydroxyecdysone per nymph from day eight in the 4th instar to day four of the adult stage is plotted in Figure 53.

FIGURE 53



This showed an increase in the titre of 20-hydroxyecdysone at the end of the 4th instar to a maximum (294 ng. per nymph) at the time of ecdysis.* The titre then decreased at the start of the 5th instar to a minimum level from days four to nine (13 to 50 ng. per nymph) in the middle of the 5th instar followed by an increase to another maximum (270 ng. per nymph) at the time of ecdysis to give the adult. The titre then again decreased, to a level of 20 ng. per nymph on day three of adult development, and no 20-hydroxyecdysone could be detected by day 14.

If 20-hydroxyecdysone controls the moulting process it is surprising to find the maximum titre on the actual day of ecdysis. Horn *et al.* however found a high titre of this hormone (280 ng. per gram fresh weight) in the marine crab *Callinectes sapidus* just after ecdysis,[46] and they suggested that 20-hydroxyecdysone was therefore not only associated with cuticle shedding but also with cuticle hardening. The possibility exists that this is the case in the desert locust.

Previous determinations of the moulting hormone titre at different stages in arthropod development had been carried

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*The time of ecdysis (day 0 in Figure 53) was taken at the stage when a newly fledged locust had just emerged from its old cuticle and the new cuticle was still soft and limp. This time probably represented less than a day after the last day of each instar.

out by bioassay of partially purified extracts.[24,128,129] This method has the disadvantage that if two or more substances with moulting activity are present their total effect is measured. The g.l.c. method can however independently determine one of several substances with moulting activity in a mixture when the substance has been identified. Identification is important in the case of arthropods in which more than one moulting hormone has been detected, for the possibility still exists that different hormones control different processes.[46]

Comparison of the maximum moulting hormone titres for the different arthropods so far investigated shows that they cover a wide range. The values for these titres expressed in nanograms of ecdysone are given in Table 1.

TABLE 1

Arthropod	Max. titre, ng. of ecdysone per insect	per g.§	Reference
Silkworm* *Bombyx mori*	150	140	128
Tobacco hornworm* *Manduca sexta*	12600	3000	24
Blue blowfly* *Calliphora erythrocephala*	5	75	130
Shore crab** *Carcinus maenas*		110	129
Milkweed bug† *Oncopeltus fasciatus*	2.4	43	131
Desert locust† *Schistocerca gregaria*	294††	200††	

* Holometabolous insects
** Crustaceans
† Hemimetabolous insects
†† Determined by g.l.c., ng. of 20-hydroxyecdysone
§ Fresh weight

The maximum titre of 20-hydroxyecdysone determined for the desert locust is also included in Table 1, and this falls within the range of values (expressed in nanograms of ecdysone) found for the other arthropods.

Holometabolous insects have been used in most previous determinations of the moulting hormone level at different stages of development. Shaaya and Karlson detected a number of peaks of activity in the silkworm *Bombyx mori*, one at the last larval moult, one during spinning, and two large peaks in the pupal stage.[128] Large variations however occur even in the holometabolous group of insects and these authors reported only two peaks during the last larval and the pupal stages of development of the blue blowfly *Calliphora erythrocephala*. One was on the day of pupation and the other was just before the middle of the pupal stage. Thompson *et al.* examined only the pupal stage of the tobacco hornworm *Manduca sexta* and found a single peak after a third of the pupal time had elapsed.[24] It was from insects taken at this time that they isolated three different ecdysones (see Introduction).

Comparison between the developmental stages of holometabolous and hemimetabolous insects is also difficult. The milkweed bug *Oncopeltus fasciatus* is the only hemimetabolous

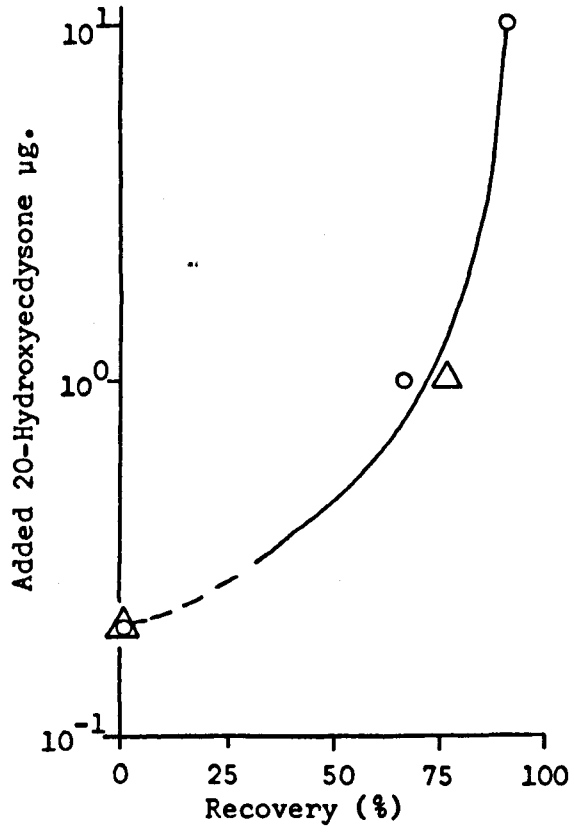
insect to have been investigated by the bioassay method, and only a relatively low hormone titre was detected. No appreciable maximum was found and the level of activity per gram of fresh weight was constant across the 5th instar. However the shore crab *Carcinus maenas* which has a direct mode of development similar to a hemimetabolous insect, such as the desert locust, has a number of peaks of activity during its final intermoult period. The two largest peaks occur early on the two days of ecdysis which bound this intermoult stage, and the similarity between this result and the positions of maximum 20-hydroxyecdysone titre in the desert locust is striking.

In all the arthropods listed in Table 1 (see page 146) the titre of moulting hormones decreased to a low level in the days following the emergence of the adult. This was also found to be the case with the 20-hydroxyecdysone titre in the desert locust which had decreased to a low level (20 ng. per nymph) by day three of the adult stage, and could not be detected on day 14 (see Table 46, page 313). Karlson has suggested that the low levels of activity found during the adult development represent hormone residues.[12]

As no 20-hydroxyecdysone had been detected in the desert locust on day 14 of adult development, nymphs at this

stage were used in an attempt to determine the limit of detection of the overall procedure used in the g.l.c. determination of this hormone at daily intervals. Groups of five nymphs from this day were extracted by the procedure used in the earlier determinations, but at the start of each extraction a known weight of 20-hydroxyecdysone in the range 0.2 to 10 µg. was added to the initial methanol extract. The weight of added 20-hydroxyecdysone was then determined by the g.l.c. method, and the results showed a good recovery of added hormone in the range 1 to 10 µg., but no recovery when 0.2 µg. was added (see Table 47, page 314). The weight of added hormone plotted against the percentage recovery is shown in Figure 54.

FIGURE 54 Recovery of 20-Hydroxyecdysone in the G.l.c. Procedure



The results in Figure 54 show that only about 50% of the hormone would have been recovered if 0.35 µg. had been added, and that below this amount the recovery decreased sharply. This indicated that the minimum titres of 20-hydroxyecdysone determined for the desert locust at daily intervals were too low, but that approximately 80% of the hormone had been recovered at the times of maximum titre. However the results shown in Figure 53 (see page 143) for the daily variations in the 20-hydroxyecdysone titre should be taken as the general trend as only one set of determinations are at present available. Ecdysone could not be detected on any of the days investigated, and therefore if this hormone was present its maximum titre could not have been more than about 10% of that of 20-hydroxyecdysone under the conditions of the experiment.

The average titre for late 5th instar nymphs (the 5th instar lasted 11 days at the Anti-Locust Research Centre), estimated from the results at daily intervals was approximately 150 ng. per nymph. As 20-hydroxyecdysone was isolated from late 5th instar nymphs at a level of about 31 ng. per nymph, it is concluded that approximately one fifth of this hormone initially present in late 5th instar nymphs had been isolated in the large scale extraction. If homogenates had been prepared from nymphs on the day of ecdysis, they would have contained about twice as much 20-hydroxyecdysone as the bulk extracts used in the course of this work.

INVESTIGATION OF PROTHORACIC GLANDS FOR SUBSTANCES WITH MOULTING ACTIVITY

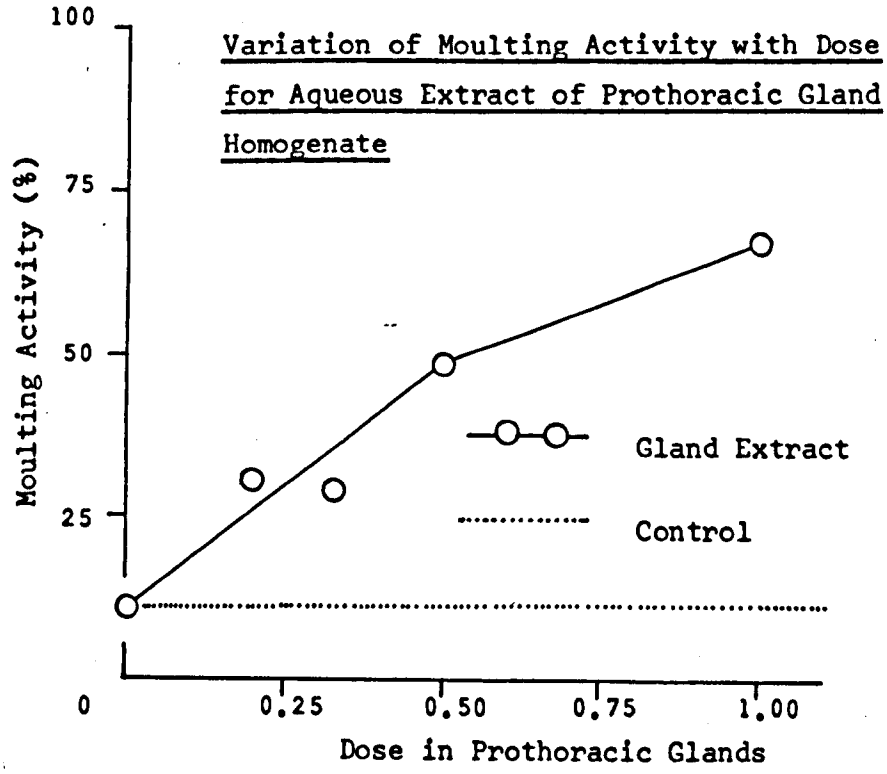
The early work of Wigglesworth and others demonstrated that the prothoracic gland of insects produced a hormone that was necessary for moulting to take place.[12] The steroid hormones ecdysone and 20-hydroxyecdysone which have been isolated from whole insects stimulate moulting, and it is commonly assumed that the prothoracic glands control moulting by synthesising ecdysone.[12,132] There is however no evidence for the latter,[132] and therefore the hormone from the prothoracic gland may not yet have been identified.

The desert locust has a pair of relatively large prothoracic glands that are easily dissected out of the body, and Carlisle and Ellis have demonstrated that fresh homogenates of these glands contain a moult inducing substance.[22,30] Prothoracic glands isolated from desert locusts were therefore investigated for substances with moulting activity.

Freeze-dried extracts were prepared at the Anti-Locust Research Centre, London, from groups of 300 to 400 prothoracic glands which had been dissected from desert locust nymphs in the 8th to 11th day of the 5th instar. These were homogenised using either water, aqueous ethanol (1:1) or ethanol. Extracts prepared by this procedure exhibited

positive moulting activity, and Figure 55 shows the variation of moulting activity with dose for an aqueous extract of prothoracic gland homogenate.

FIGURE 55



The freeze-dried extracts were examined for substances with moulting activity by partition between butanol and water. Only material which was inactive in bioassay was isolated from the aqueous phase in each case, and this was discarded. The material from the butanol phase was subjected directly to p.t.l.c. eluting with chloroform - 95% ethanol (4:1). The p.t.l.c. plates showed a number of bands visible under U.V. for each extract, and the appearance of the plates for material

isolated from glands homogenised with water, aqueous ethanol (1:1) and ethanol is shown in Figures 80 to 82 respectively (see pages 317 to 319).

The silica gel on each plate from R_f 0.00 to 1.00 was divided into five areas as shown in Tables 48 to 50 accompanying Figures 80 to 82. The division was made on the basis of the bands observed under U.V. for each of the prothoracic gland extracts, and the R_f regions found for the two peaks of activity detected when material from the Davison silica gel column stage in the extraction of whole nymphs was subjected to P.L.C. (see Figure 5, page 45). Division of the silica gel into the minimum number of areas possible on the above basis was done in an attempt to prevent losses, when the small quantities of absorbed material were isolated.

The eluted materials were dissolved in 400 µl. of water and were assayed for moulting activity at a dose of 20 µl. (equivalent to 20 prothoracic glands per abdomen), and one fifth of this dose. The results for each of the three extracts (see Tables 48 to 50) showed a similar separation of active components, and this indicated the presence of at least two active substances in the prothoracic glands of 5th instar desert locust nymphs. The variation of moulting activity with R_f for the three extracts chromatographed is plotted in Figures 56 to 58.

FIGURE 56 Variation of Moulting Activity with R_f on P.t.l.c. for the Material from Prothoracic Glands Homogenised with Water

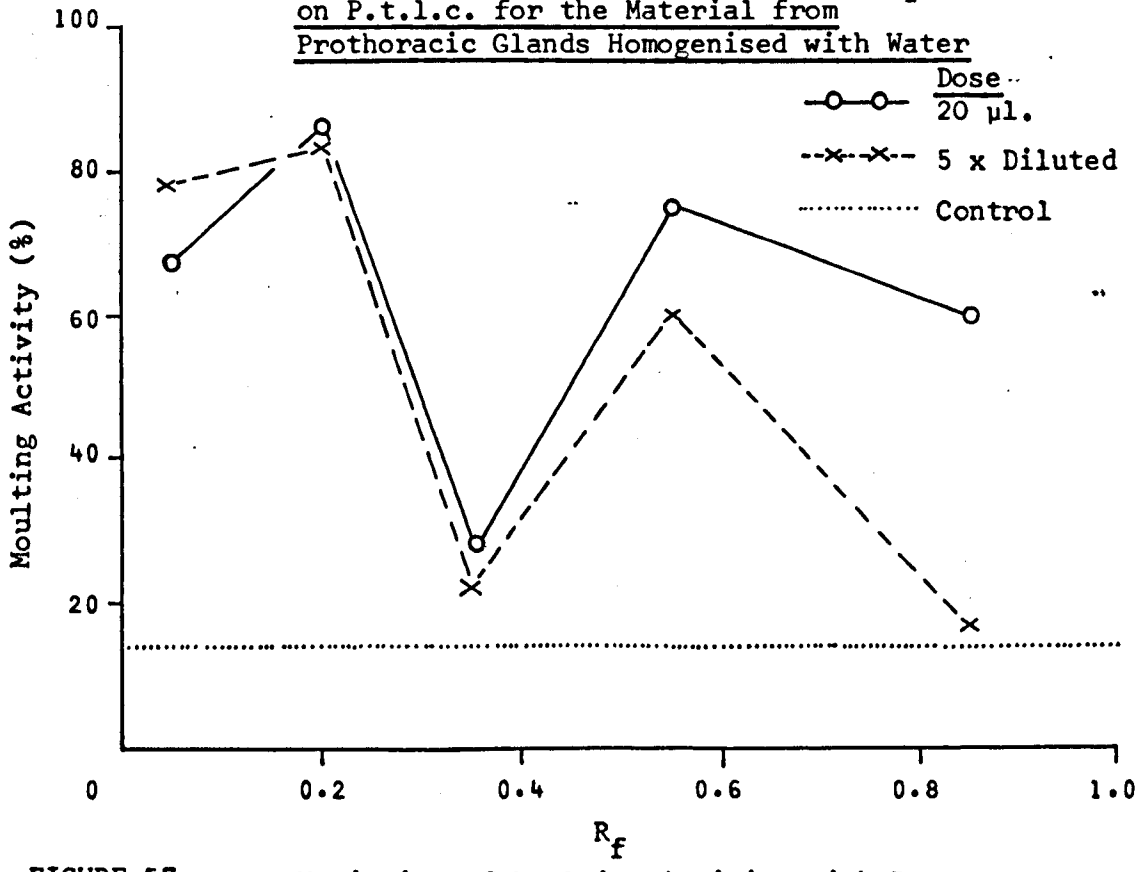


FIGURE 57 Variation of Moulting Activity with R_f on P.t.l.c. for the Material from Prothoracic Glands Homogenised with Aqueous-ethanol (1:1)

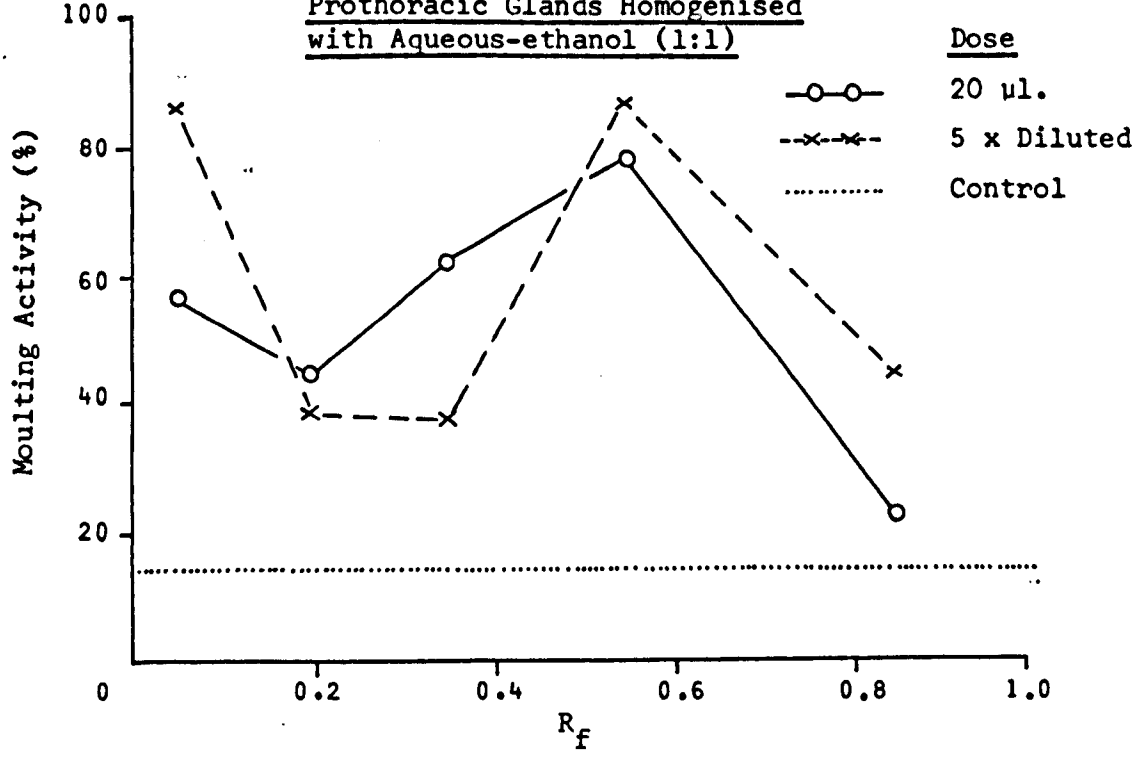
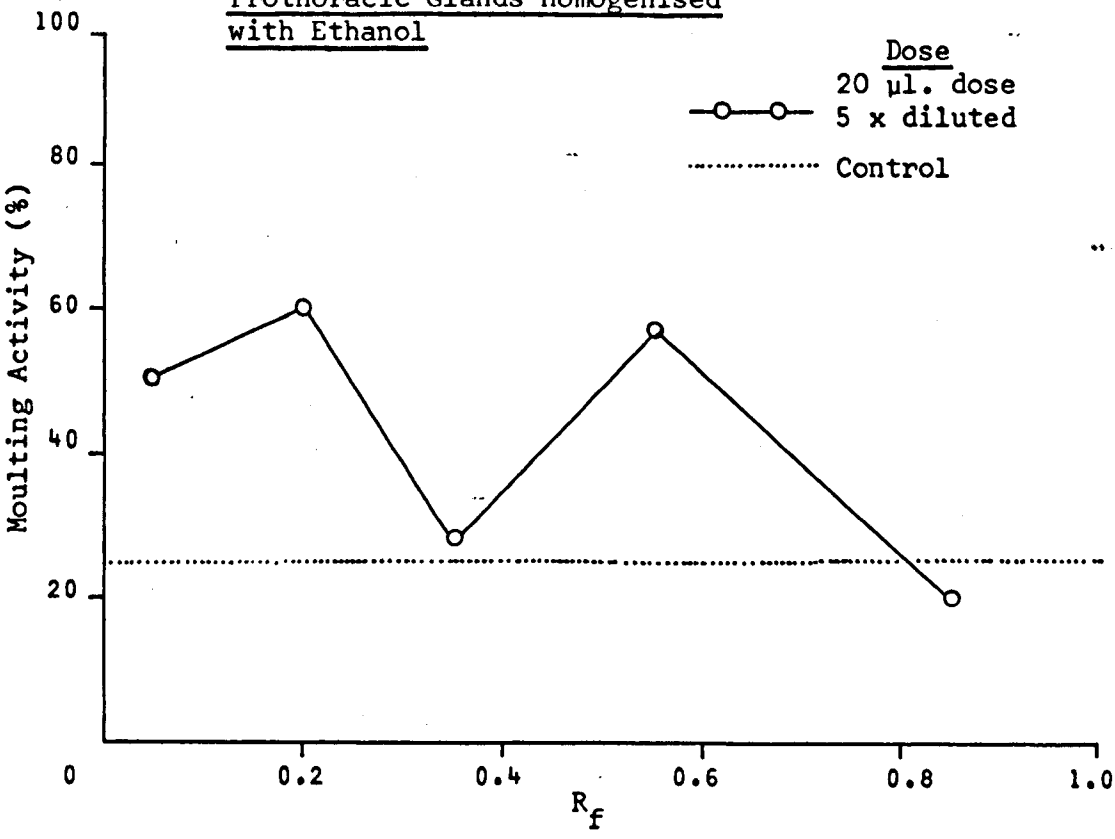


FIGURE 58 Variation of Moulting Activity with R_f on P.t.l.c. for the Material from Prothoracic Glands Homogenised with Ethanol



These three Figures each show two peaks of activity. The lower peak in each case is centred in the region R_f 0.00 to 0.30 and this corresponds to the R_f region found for the lower and middle active bands detected in the whole locust extract (see Figure 5, page 45). The upper peak is centred in the region R_f 0.40 to 0.70 and this corresponds to the R_f region found for the upper active band detected for whole nymphs. The differences in the exact positions of the peaks of activity in the three Figures probably reflects variation in the experimental conditions.

As the substances with moulting activity isolated from whole nymphs and prothoracic glands had similar chromatographic properties, the possibility that some of these substances are produced in the glands was considered. To examine this possibility a calculation of the concentration of active substances in prothoracic gland tissue compared with the average concentration of similar substances in whole nymphs was attempted.

Comparison of the levels of activity for the lower R_f region shown in Figure 5 (see page 45) for whole nymphs and in Figure 56 for prothoracic glands shows that these are similar at the highest doses assayed. The levels for the upper R_f region are also similar at these doses. The active material from the lower R_f region is considered first. The prothoracic gland material derived from 400 glands, was dissolved in 400 µl. of water and assayed at 20 µl. per abdomen as the highest dose. This dose therefore represented the active material from 20 glands. The whole nymph material was derived from a portion of extract XI (see page220) which was equivalent to 165 whole nymphs, and gave 69 mg. of this active material (lower R_f region) when separated by P.L.C. The whole nymph material was assayed at 100 µg. per abdomen as the highest dose, which therefore corresponded to the active material from 0.24 nymphs.

The extract derived from 20 glands therefore showed a

similar level of activity to that from 0.24 nymphs. Hence for similar active substances in glands and whole nymphs, one nymph contained as much active material (lower R_f region) as 83 glands. As a single fresh gland weighs about 140 µg.* and a whole nymph (days 8 to 11 of the 5th instar) weighs approximately 1.5 g. (see page 33), the concentration of active substance (lower R_f region) in prothoracic gland tissue is about 130 times that of the average concentration in a whole nymph at this stage of development.

A similar calculation for the active substance in the upper R_f region showed that the concentration in the prothoracic gland tissue was about 280 times that of the average concentration in a whole nymph at this stage of development.

The prothoracic gland results used in this calculation were those for the aqueous extract, but similar concentration values would have been obtained for the glands extracted with aqueous ethanol (1:1) as the levels of activity detected in the extracts after p.t.l.c. were similar in both cases.

This method of comparing the relative amounts of active substances in different extracts can however at best be only semi-quantitative, as it involves variations in the experimental conditions and differences in the purity of the extracts under

---

*The mean weight of a single fresh prothoracic gland was determined from the weight of 20 glands dissected from nymphs in the 8th to 11th day of the 5th instar.

comparison. The method is also dependent on bioassay results which are subject to unexpected variation (compare the two dose levels in Figure 56, see page 154). However using the calculated values as a semi-quantitative guide it can be seen that the concentration of substances with moulting activity in the prothoracic gland tissue was found to be substantially greater than the average concentration of similar substances in a whole nymph, at this stage of development. This result is consistent with the postulate that the prothoracic glands are the site of production of some of the substances with moulting activity detected in whole nymphs.

Dr. Ellis reports that in the locust abdomen bioassay, it is possible to distinguish between substances causing apolysis (separation of cuticle from the underlying epidermis) and others which in addition stimulate the formation of new cuticle. These can be distinguished by lifting the cuticle on the dorsal part of the abdomen. The effect was confirmed by histological examination of the abdomens.[31] Pure 20-hydroxy-ecdysone* and purified nymph extracts containing this hormone produced the latter effect. Upper and lower active band extracts

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*Pure 20-hydroxyecdysone gave the following results in the locust abdomen assay. At a dose of 1 µg., 100% activity was obtained, with 90% of abdomens showing new cuticle formation; at a dose of 10 ng., 40% activity was obtained, with one quarter of these having new cuticle formation. In the control group only 11% of abdomens showed evidence of moulting and these showed only apolysis.

from whole nymphs and all active extracts from prothoracic glands produced apolysis only, when assayed at relatively high doses.

The material from the upper R_f region of activity (upper active band) detected in whole nymphs has not been further resolved, but if only one active substance is present it appears probable that its site of production is the prothoracic glands. The lower R_f region of activity detected in whole nymphs has been further resolved into lower and middle active band components. The active material in the lower band has not been further resolved or isolated, but the middle band has yielded 20-hydroxyecdysone as the active substance (see section beginning on page 129). From the evidence at this stage it was therefore possible that both of the active substances detected in the lower R_f region were produced in the prothoracic gland.

INVESTIGATION OF PROTHORACIC GLANDS FOR 20-HYDROXYECDYSONE BY GAS-LIQUID CHROMATOGRAPHY

The detection of material with moulting activity in the same R_f region as that expected for a mixture of the middle and lower active bands from whole locusts prompted the investigation of desert locust prothoracic glands for 20-hydroxyecdysone by g.l.c.

The freeze-dried extract of 100 prothoracic glands (dissected from nymphs in the 8th to 11th day of the 5th instar) was partitioned between butanol and water, and the material from the butanol phase was partitioned directly between light petroleum and aqueous methanol (7:3). These latter two phases were evaporated to give the lipophilic and hydrophilic extracts respectively. The hydrophilic extract was subjected to the procedure of derivative formation for g.l.c. and a portion (10%) of the silylated residue was examined for derivatives of 20-hydroxyecdysone by g.l.c., but none were detected. The remainder of the residue was therefore subjected to the previously described procedure of one dimensional p.t.l.c. The plate was visualised under U.V. and its appearance is shown diagrammatically in Figure 83 (see page 321). The material represented by the three bands in the R_f region 0.70 to 0.84 was isolated for g.l.c., but derivatives of 20-hydroxyecdysone

could still not be detected. Peaks corresponding to ecdysone derivatives were also absent.

As 20-hydroxyecdysone had not been detected in the first prothoracic gland extract, it was decided to examine a second extract of 100 glands by g.l.c., but to use a shorter extraction procedure, which omitted the removal of lipids with light petroleum, in an attempt to reduce any possible loss of hormone material. For the second solvent partition to be omitted it was necessary to be able to separate the 20-hydroxyecdysone derivatives from the lipids at the p.t.l.c. stage in the extraction, and this was found to be possible using a solvent system of light petroleum - ether (4:1). In this solvent system the *syn*- and *anti*-isomers of tetrakis-TMSi-20--hydroxyecdysone methoxime (*20a*) were completely separated. The more polar isomer (*21a*) had a R_f of 0.10 and the less polar isomer (*22a*) a R_f of 0.21.

The butanol extract from the second group of 100 prothoracic glands was subjected directly to derivative formation and the silylated material obtained was run on p.t.l.c. using the light petroleum - ether (4:1) solvent system. The material absorbed onto the silica gel in the region R_f 0.08 to 0.25 was isolated and examined by g.l.c., but derivatives of 20-hydroxyecdysone were again not detected. The methoxime isomers

of tetrakis-TMSi-ecdysone methoxime (*24a*) have R_f values of 0.20 and 0.31 in light petroleum - ether (4:1), and the silylated material was also examined for these derivatives, the more polar of which would have been isolated with the derivatives of 20-hydroxyecdysone, but neither of the ecdysone derivatives could be detected by g.l.c.

As no 20-hydroxyecdysone or ecdysone could be detected by g.l.c. in the aqueous extract of 100 prothoracic glands (dissected from nymphs in the 8th to 11th day of the 5th instar) using the above procedure, it was decided to examine similar extracts prepared from glands dissected from nymphs at daily intervals throughout the 5th instar, as there was a possibility that a time of maximum hormone titre might be found.

Two solvent systems, chloroform - 95% ethanol (4:1) and light petroleum - ether (4:1), in which the R_f values for the derivatives of ecdysone and 20-hydroxyecdysone were known (see Table 37, page 268), were available for p.t.l.c. It was therefore decided to perform a two dimensional p.t.l.c. on extracts using these two solvent systems to obtain an improved separation of components.

Freeze-dried extracts of 100 prothoracic glands prepared at daily intervals throughout the 5th instar (under

the conditions used to rear nymphs at the Anti-Locust Research Centre, the 5th instar lasted only 10 to 11 days, see page 324) were subjected directly to the conditions for derivative formation. The silylated materials obtained were subjected to two dimensional p.t.l.c., and this was carried out in three stages as described in the Experimental section. The appearance of the p.t.l.c. plates under U.V. were similar for the extracts of glands from each day of the instar, and a typical plate at each of the three stages of the separation is shown in Figure 84 (see page 325, day three of the 5th instar).

The absorbed material in the area expected for derivatives of 20-hydroxyecdysone was isolated from the silica gel in each case, and then examined by g.l.c., but no peaks corresponding to derivatives of 20-hydroxyecdysone were detected for any of the materials chromatographed. The appearance of the g.l.c. traces was similar for each day of the instar, and two typical traces (days three and five of the 5th instar) are shown in Figures 59 and 60.

FIGURE 59

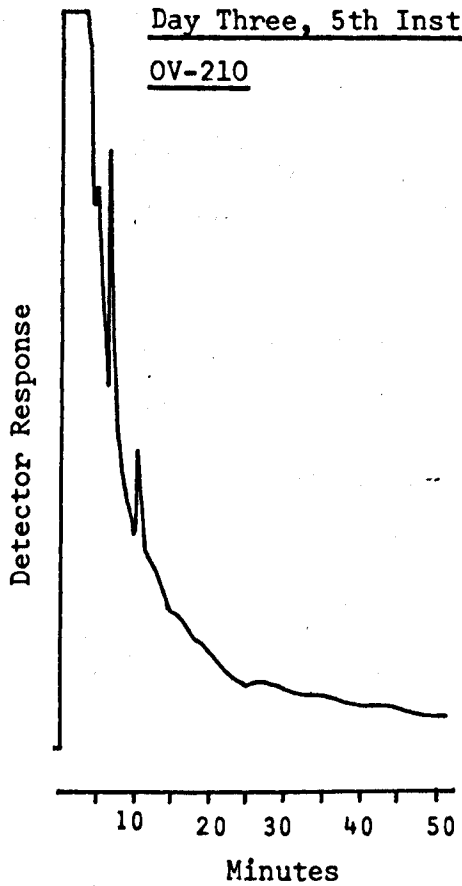
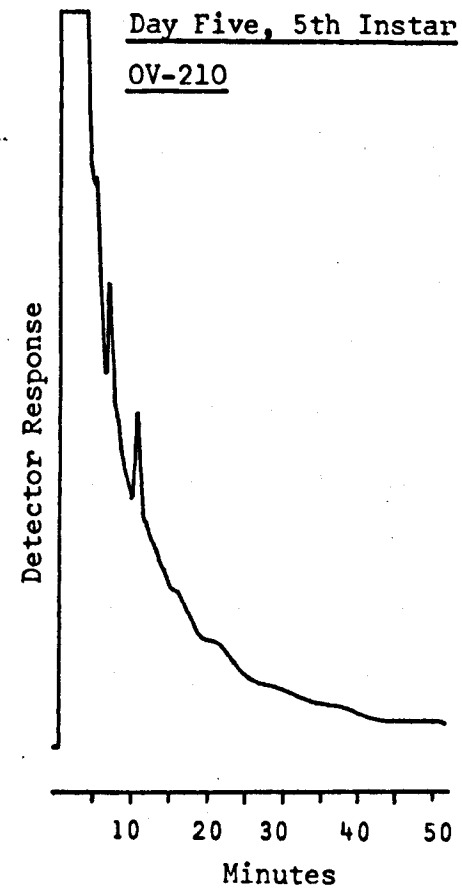


FIGURE 60



All the traces showed a complex pattern of peaks t_R less than 10 minutes, and only minute amounts of components having longer retention times. The silylated extracts were also examined for derivatives of ecdysone but again none were detected.

As no 20-hydroxyecdysone had been detected in any of the prothoracic gland extracts examined by g.l.c., the amount of this hormone, if present, must have been below

the limit of detection in every case. This limit was determined as approximately 200 ng. of 20-hydroxyecdysone in earlier experiments on whole nymph extracts. Below this level none of the added 20-hydroxyecdysone was recovered (see Figure 54, page 149). As a similar but shorter procedure had been used to prepare the prothoracic gland extracts for g.l.c., the value of 200 ng. of 20-hydroxyecdysone can be used as a guide to the limit of detection for this hormone in gland extracts. The prothoracic gland extracts examined by g.l.c. had each been prepared from 100 glands, and this therefore put an upper limit of 2 ng. of 20-hydroxyecdysone per gland on the maximum titre of this hormone for any day throughout the 5th instar.

A maximum 20-hydroxyecdysone titre of 2 ng. per gland is however too low to satisfactorily account for the level of activity found in the lower R_f region when prothoracic gland extracts were separated by p.t.l.c. The activity (86%, control 14%) found for material in this R_f region from glands extracted with water (see Figure 56, page 154) was produced by the 20 µl. dose, and this corresponded to 20 prothoracic glands. This number of glands could contain a maximum of 40 ng. of 20-hydroxyecdysone (by g.l.c.) which would be expected to show a lower level of activity in the bioassay (53%, control

11%, see Figure 50, page 136) than that detected for the gland extract. A similar conclusion is also reached if the results for glands extracted in aqueous ethanol (1:1) and ethanol are considered.

The active material from prothoracic glands detected in the lower R_f region on p.t.l.c. could therefore correspond in part to 20-hydroxyecdysone, but the presence of a moult-inducing substance other than 20-hydroxyecdysone was also indicated, and this material is probably the same as that detected in the lower active band isolated from whole nymphs with which it is chromatographically similar.

Although no 20-hydroxyecdysone was detected in prothoracic gland extracts by g.l.c., the upper limit of 2 ng. of this hormone per gland (about 15 µg./g. fresh weight) does not exclude the prothoracic gland as the site of production of 20-hydroxyecdysone. The maximum titre of 20-hydroxyecdysone in a desert locust nymph at the end of the 5th instar was found to be about 270 ng. (180 ng./g. fresh weight). The concentration of 20-hydroxyecdysone in gland tissue could therefore be up to 80 times that of its average concentration in a whole nymph at this stage of development. If other days in the instar were chosen the permitted ratio of concentration in the gland tissue to the whole locust would

rise even higher as the titre in the whole insect fell.

To determine, by the g.l.c. method, whether 20-hydroxyecdysone is produced in the prothoracic glands, either a greater number of glands would be required (over 10^4) or the overall g.l.c. procedure must be improved to reduce the limit of detection.

INVESTIGATION OF BLOOD FROM 5TH INSTAR NYMPHS FOR MOULTING ACTIVITY

The early work of Fraenkel (1935) had demonstrated that a hormone responsible for pupation in the blowfly *Calliphora erythrocephala* was transported in the blood, and that blood from larvae about to pupate could promote pupation in the posterior portions of ligated larvae[7] (which would normally never have pupated).

This latter result was re-investigated by Williams *et al.* using larvae of the fleshfly *Sarcophaga peregrina*, but they found that blood from larvae about to pupate was toxic and inactive on injection using the Sarcophaga bioassay. They were however able to detect a low hormone titre in the blood (15% of a Sarcophaga unit per insect, equivalent to 5 ng. of ecdysone, about 25% of the ecdysone titre in the whole insect at this stage of development) after preliminary extraction of the blood to concentrate any moulting hormones present by a factor of 10 times. They therefore concluded that Fraenkel's results on the promotion of pupation by injection of whole blood were "false positives".[133]

A recent re-examination of *Calliphora erythrocephala* by Shaaya (1969),[134] using the calliphora assay, showed that

the blood of larvae about to pupate contained only about 15% of a Calliphora unit per insect (equivalent to 1.5 ng. of ecdysone, about 30% of the ecdysone titre in the whole insect at this stage), and this result supported the conclusion of Williams *et al.*

Although the moulting hormone titre determined by bioassay for the insect blood so far investigated was relatively low, it represented about one quarter of the titre in the whole insect (at the stage of development discussed). Blood from 5th instar desert locust nymphs was therefore examined to see if substances with moulting activity could be detected. Freeze-dried samples of blood supernatant were prepared from groups of 40 nymphs collected early (days one to five) and late (days 6 to 11) in the 5th instar. These two samples were subjected to partition between butanol and water, and the extract from the butanol phase was partitioned between light petroleum and aqueous methanol (4:1) using a similar procedure to that described for groups of 10 whole nymphs. Similar results were obtained for both of the blood samples in the solvent partitions, and these are listed in the Experimental section (see pages 328 and 330). The extract from the butanol phase was assayed for moulting activity in each case and both extracts were of low activity (approximately 10% above

the control level).

The aqueous methanol (4:1) extracts were examined by p.t.l.c. eluting with chloroform - 95% ethanol (4:1), and the silica gel on each plate was divided into five areas on the same basis as that discussed for the prothoracic gland extracts (see page 153). The materials absorbed onto each area of silica were isolated, dissolved in water (400 µl.), and assayed for moulting activity at a dose of 20 µl. (equivalent to the blood from two nymphs per abdomen). The results for the two blood samples investigated were similar, and all of the materials isolated from the plates were of low activity (none were greater than 17% above the control level). The appearance of the p.t.l.c. plates under U.V. is shown in Figures 85 and 86, and the bioassay results are given in Tables 51 and 52 accompanying these Figures (see pages 329 and 331). The variation of moulting activity with R_f for each blood sample is plotted in Figure 61.

FIGURE 61

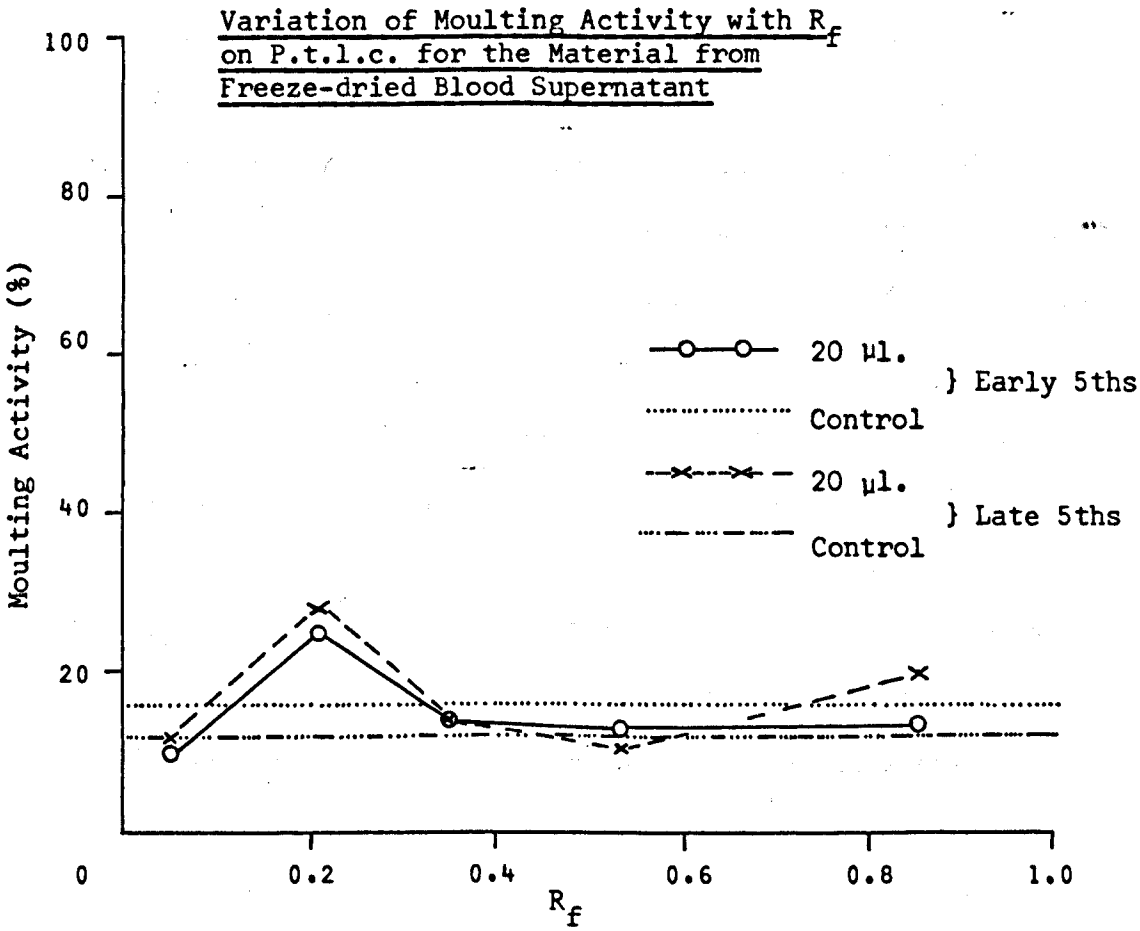


Figure 61 showed a small peak of activity centred in the region R_f 0.10 to 0.30 which corresponded to the R_f region found for the middle and lower active bands detected in whole nymph extracts, and the lower peak of activity detected in prothoracic gland extracts. No peak of activity was however observed in the region of R_f (0.40 - 0.70) which corresponded to the R_f region for the upper active band detected in whole nymph extracts, and the upper peak of activity in prothoracic

gland extracts. These results should however be treated with caution as none of the materials assayed showed a level of activity more than 17% above the control level, and only one set of results, with materials assayed at one dose, is available for each of the two blood samples investigated.

All the fractions from the blood supernatant separated by p.t.l.c. showed low activity when assayed at a dose of 20 µl. (equivalent to the blood from two nymphs). When whole nymph extracts were similarly separated by p.t.l.c. (extract XI, Figure 5, see page 45), material from the lower R_f region was highly active (86% activity, control 20%, dose equivalent to 0.24 nymphs) and material from the upper R_f region (although broad) showed a similar level of activity. The blood supernatant from nymphs, both in the early and late stages of the 5th instar, therefore contained only a small proportion of the moult-inducing substances found in whole nymphs (days 8 to 11 of the 5th instar), and this proportion was much lower than in the case of *Calliphora* or *Sarcophaga* larvae just before puparium formation. The possibility of a peak in the titre of moult-inducing substances in the blood of locust nymphs should not however be overlooked, and this could be investigated by the examination of blood supernatant from nymphs at daily intervals throughout the 5th instar. Blood cells and suspended material could also be examined.

INVESTIGATION OF BLOOD FROM 5TH INSTAR NYMPHS FOR 20-HYDROXYECDYSONE BY GAS-LIQUID CHROMATOGRAPHY

The maximum titre of 20-hydroxyecdysone at the end of the 5th instar was detected in nymphs on the day of ecdysis. It was therefore decided to investigate blood, taken from 5th instar nymphs the day before ecdysis, for 20-hydroxyecdysone by g.l.c. Although only low moulting activity had been detected in the blood supernatant from the groups of 40 early and late 5th instar nymphs, a maximum titre of 20-hydroxyecdysone in the blood might be expected just before the time of maximum titre in the whole insect, if at this stage the hormone is being transported to the target tissues.

Freeze-dried blood supernatants and freeze-dried aqueous ethanol (1:1) extracts of blood cells were prepared from two groups of 25 nymphs in the 10th day of the 5th instar (the 5th instar only lasted about 11 days under the conditions at the Anti-Locust Research Centre, see page 324).

The freeze-dried materials were partitioned between butanol and water, and the weights of the extracts obtained are given in Table 53 (see page 333). The butanol extract in each case was examined for 20-hydroxyecdysone by the procedure of derivative formation and two dimensional p.t.l.c. followed by

g.l.c., but no peaks corresponding to derivatives of 20-hydroxyecdysone or ecdysone were detected for any of the materials chromatographed. Similar results were obtained when the above procedure was repeated for the freeze-dried blood supernatants.

As no 20-hydroxyecdysone had been detected in any of the blood extracts examined by g.l.c., the amount of this hormone, if present, must have been below the limit of detection in every case. This limit had been determined as approximately 200 ng. of 20-hydroxyecdysone from earlier experiments on whole nymph extracts, in which a similar procedure to that described for the blood extracts was used to prepare the materials for g.l.c. The blood extracts examined by g.l.c. had each been prepared from 25 nymphs, and this therefore put an upper limit of 8 ng. of 20-hydroxyecdysone on the amount of this hormone in either the blood supernatant or the blood cell material from one 5th instar nymph the day before ecdysis.

The activity expected from 8 ng. of 20-hydroxyecdysone in the bioassay would be about 35% (control 11%, see Figure 50, page 136), and this value is consistent with the level of activity detected when blood supernatant was separated by p.t.l.c. (see peak centred at R_f 0.20 in Figure 61, page 171). The titre of 20-hydroxyecdysone in a desert locust nymph the

day before ecdysis was found to be about 200 ng. (estimated on the basis of a steady increase in the 20-hydroxyecdysone titre at the end of the 5th instar, see Figure 53, page 143). The amount of this hormone in the blood supernatant or the blood cell material could therefore be up to 4% of that present in the whole nymph at this stage. As the amount of blood in a 5th instar nymph the day before ecdysis is about 170 mg.*, the permitted concentration of 20-hydroxyecdysone in the blood extracts could be upto about one third of the average concentration in the whole nymph. Using this value as a guide, the permitted concentration of 20-hydroxyecdysone in blood is lower than the average concentration in the whole nymph at this stage. This could indicate that either there is a high concentration of this hormone at the site of production, or that 20-hydroxyecdysone is strongly bound in the target tissues and is therefore concentrated at the latter site. Alternatively both of these processes might operate.

To determine, by the g.l.c. method, whether 20-hydroxyecdysone is present in locust blood at a lower level, either the blood from a greater number of nymphs would have to be extracted or the overall g.l.c. procedure must be improved to reduce the limit of detection.

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*The mean weight of blood in a desert locust nymph the day before ecdysis was determined from the weight of blood removed by syringe from four nymphs.

INVESTIGATION OF OENOCYTE BEARING TISSUE FROM 5TH INSTAR NYMPHS FOR 20-HYDROXYECDYSONE BY GAS-LIQUID CHROMATOGRAPHY

Recent work by Weir (1970) with ligated larvae of *Calpodes ethlius* has shown that the prothoracic glands which are normally competent to cause pupation in the headless larva cannot induce pupation in an isolated thorax, although the thoracic tissues themselves can respond to ecdysones. He therefore postulated an additional step in the control of moulting; interaction of the prothoracic glands with some abdominal tissue, and he further suggested that the oenocytes (sometimes called abdominal endocrine glands) were the cells most probably interacting with the prothoracic glands.[132]

This latter suggestion was also made by Locke (1969) after his examination of the ultrastructure of oenocytes in *Calpodes* larvae, which he found was appropriate for steroid synthesis, and he proposed the oenocytes as centres for ecdysone synthesis and that these were activated by the prothoracic glands.[135] Locke also pointed out that oenocytes were universally present not only in insects, but also in all the material for bioassays purporting to test the action of the prothoracic gland upon the epidermis. This latter observation is also true for the desert locust bioassay.

Substances with moulting activity had been detected in the prothoracic glands of desert locust nymphs by bioassay, and this could in part have been due to 20-hydroxyecdysone (see page 166). No 20-hydroxyecdysone could however be detected in the prothoracic glands by g.l.c., but the limit of detection for the method was not low enough to exclude the gland as the site of production of this hormone. As 20-hydroxyecdysone had been found in whole nymphs, it was possible that a site other than the prothoracic glands was responsible for its production, and on the basis of the results of Locke and Weir it appeared that this site might be the oenocytes.

Oenocytes are not as localised in desert locust nymphs as they are in *Calpodes* larvae where they form clusters close to the spiracles. Sections prepared from 5th instar locust nymphs show that they spread over the whole of the epidermis in the form of a sheet as ecdysis approaches, and are also present in the fat body.[31] Oenocyte bearing epidermal tissue and fat body were therefore examined for 20-hydroxyecdysone by g.l.c.

Freeze-dried aqueous ethanol (1:1) extracts were prepared from fat bodies and abdominal strips of the following epidermal tissues: heart, upper lateral, spiracle, lower lateral and nerve cord, isolated from 20 nymphs in the 10th day

of the 5th instar.

The freeze-dried materials were extracted, by the procedure discussed for the aqueous concentrates derived from groups of nymphs taken at daily intervals throughout the 5th instar, to give aqueous methanol (4:1) extracts from the second solvent partition. The weights of material obtained at each stage in the extraction are given in Table 54 (see page 336). The aqueous methanol (4:1) extract in each case was examined for 20-hydroxyecdysone by the procedure of two dimensional p.t.l.c. followed by g.l.c., but no peaks corresponding to derivatives of 20-hydroxyecdysone could be identified for any of the materials chromatographed. Similar results were obtained when the above procedure was repeated for spiracle and lower lateral tissue extracts.

As no 20-hydroxyecdysone had been detected in any of the oenocyte bearing tissues examined by g.l.c., the amount of this hormone, if present, must have been below the limit of detection in every case. A similar calculation to that for the blood extracts (see page 174) shows that this result places an upper limit of 10 ng. of 20-hydroxyecdysone on the amount of this hormone in any of the oenocyte bearing tissues isolated from one 5th instar nymph the day before ecdysis. This amount corresponds to about 5% of the 20-hydroxyecdysone present in the

whole nymph at this stage of development. To determine, by the g.l.c. method, whether 20-hydroxyecdysone is present in the oenocyte bearing tissues at a lower level either the tissue from a greater number of nymphs would have to be extracted or, as in the case of blood and prothoracic gland extracts, the overall g.l.c. procedure would have to be improved to reduce the limit of detection. As the proportion of oenocytes to other cells in the strips of abdominal tissue so far examined was low, a more promising solution to the problem, than preparing extracts from a greater number of nymphs, would be to strip off the sheet of oenocytes from the epidermis to give an oenocyte preparation containing a high proportion of oenocyte cells. This problem is at present under investigation by Dr. Ellis.[31]

CONCLUSION

Desert locust nymphs from days 8 to 11 of the 5th instar have been found to contain at least three moult--inducing substances active in the locust abdomen bioassay, and one of these has been identified as the known arthropod moulting hormone 20-hydroxyecdysone. The presence of 20--hydroxyecdysone in late 5th instar nymphs was first confirmed by g.l.c., and the titre of this hormone in purified extracts was determined as about 55 ng. per nymph. Subsequent isolation of 20-hydroxyecdysone confirmed its identity.

The other two active substances, although they exhibit similar properties to 20-hydroxyecdysone in the early stages of the isolation can be separated from this hormone, and each other, at the P.L.C. stage. They have so far been obtained in the form of partially purified extracts after P.L.C., and these were called upper and lower active bands. The former is less polar and the latter more polar than either ecdysone or 20-hydroxyecdysone in absorption chromatography.

The active components of the upper and lower active bands are either somewhat different in chemical properties from 20-hydroxyecdysone, or, if similar, are present in much smaller quantities and consequently have higher moulting activity. The

nature of this activity is different from that of 20-hydroxyecdysone in that the two unidentified components induce apolysis but not new cuticle formation.

The titre of 20-hydroxyecdysone in nymphs at daily intervals throughout the 5th instar, determined by g.l.c., was found to be a maximum on the days of ecdysis at the beginning and end of the instar, a minimum in the middle of the instar, and had decreased to a low level by day three of the adult stage. No 20-hydroxyecdysone was detected in adults on day 14. From these results it was estimated that approximately one fifth of the 20-hydroxyecdysone initially present in late 5th instar nymphs had been isolated in the large scale extraction.

The detection of 20-hydroxyecdysone on the day of ecdysis is surprising if this hormone controls the moulting process. The possibility however exists that 20-hydroxyecdysone is not only associated with cuticle shedding but also with cuticle hardening.[46] Alternatively, the slowly rising titre of this hormone during the last four days of the 5th instar may sequentally trigger different stages in the moulting process as threshold titres are reached.[133] The decrease in the titre of 20-hydroxyecdysone to a low level following the emergence of the adult would be expected, as no further moulting takes place, unless the hormone had another role in adult development.

No ecdysone was detected in any of the locust extracts obtained during the course of this work. The amount of this hormone contained in whole late 5th instar nymphs must therefore be less than about 1% of the amount of 20-hydroxyecdysone present, and the ecdysone titre on any day in the 5th instar could not have exceeded about 10% of the maximum titre of 20--hydroxyecdysone detected on the day of ecdysis. The possibility however exists that very small amounts of ecdysone would have been lost during the large scale extraction.

Extracts of prothoracic glands isolated from late 5th instar nymphs have been found to contain at least two moult--inducing substances. These could be separated on p.t.l.c. and were found to be chromatographically similar to the two unidentified moult-inducing substances detected in whole nymph extracts. Both of the active substances from prothoracic glands cause apolysis but not new cuticle formation as was the case for the unidentified active substances from whole nymphs. It is therefore suggested that the two unidentified active components detected in the prothoracic glands are the same as those detected in whole nymphs. This is a reasonable hypothesis as whole nymph extracts were prepared from insects containing their prothoracic glands.

Comparison of the number of glands and whole nymphs

required to produce similar levels of activity in the bioassay showed that the concentration of each of the two active substances found in glands must be over 100 times greater in the prothoracic gland than in the whole bodies. It is therefore further suggested that the two unidentified moult-inducing substances found in whole nymphs are produced in the prothoracic glands.

No ecdysone or 20-hydroxyecdysone was detected in prothoracic glands by g.l.c. At the time of maximum 20-hydroxyecdysone titre however, as little as 2% of the amount of this hormone present in whole 5th instar nymphs would have been detected in the prothoracic glands. Because of the small size of the gland, this limit could still permit the hormone to be upto 80 times more concentrated in the gland than in the whole body. However this amount of 20-hydroxyecdysone (less than 2 ng.) would be too little to account for the level of activity produced by the more polar active prothoracic gland material after p.t.l.c. As this latter extract showed only apolysis in bioassay and no evidence for new cuticle formation it is probable that 20-hydroxyecdysone was absent.

It is therefore suggested that the prothoracic gland does not produce 20-hydroxyecdysone but instead produces another substance or substances which trigger the formation of 20-hydroxy-

ecdysone in another part of the body.[65] From the recent work of Weir,[132] Locke,[135] and Romer[136] it appears that this site might well be the oenocytes of the abdomen.

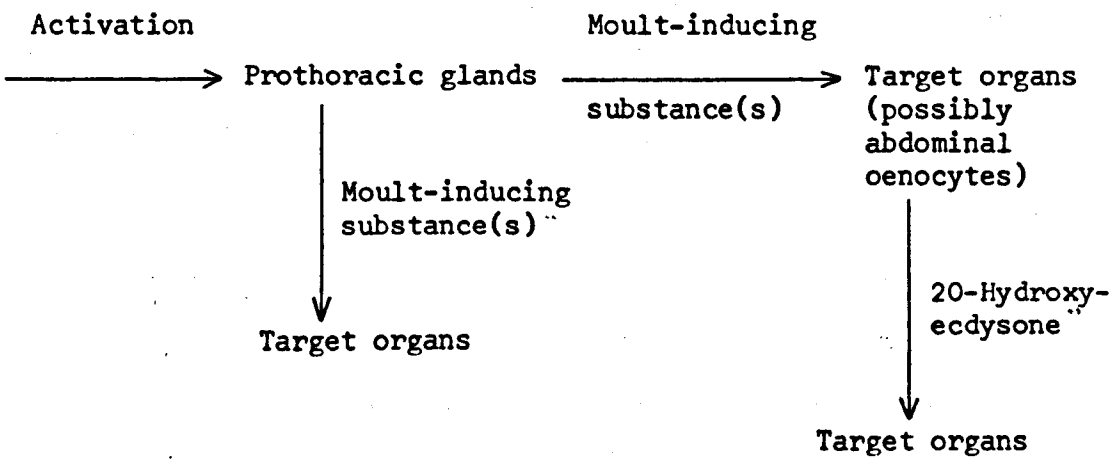
Oenocyte bearing abdominal tissues from 5th instar locusts the day before ecdysis were examined by g.l.c., but no 20-hydroxyecdysone was detected. At this stage of development 5% of the amount of 20-hydroxyecdysone in whole nymphs would have been detected in each tissue. This limiting value is however too high to be used as a guide to the possible presence of this hormone in the oenocytes as the proportion of these cells in the tissues examined was low. Further investigation is required to test the hypothesis that 20-hydroxyecdysone is produced in the oenocytes.

Blood extracted from early and late 5th instar locusts had relatively low activity in bioassay, which showed that it contained only a small proportion of the moult-inducing substances found in late 5th instar nymphs. This would seem to indicate that these substances are concentrated in certain tissues, as found in the case of the prothoracic glands, and are transported from the site of production to the target organs without any large build up in the blood. The possibility of a sharp peak in the titre of moult-inducing substances in blood should not however be overlooked, and could be investigated by

examining the blood from nymphs at daily intervals throughout the instar. Blood cells and suspended material should also be examined.

No 20-hydroxyecdysone could be detected, by g.l.c., in blood from 5th instar nymphs the day before ecdysis. At this stage of development 4% of the amount of this hormone present in whole nymphs would have been detected. Using this value as a guide the permitted concentration of 20-hydroxyecdysone in blood is lower than the average concentration in the whole nymph at this stage. This could indicate that either there is a high concentration of this hormone at the site of production, or that 20-hydroxyecdysone is strongly bound in the target tissues and is therefore concentrated at the latter site. Alternatively both of these processes might operate.

From the evidence presently available the following tentative scheme, which is in agreement with current theories for the control of moulting in insects,[132] is suggested for the sequence controlling moulting in the 5th instar desert locust.



It is now important to isolate the two unidentified moult-inducing substances from whole nymphs in pure form for characterisation. These substances could then be examined to see if they increase 20-hydroxyecdysone production in whole nymphs, isolated abdomens or other tissues. The two new substances should also be characterised by g.l.c., if possible, or perhaps by liquid-liquid column chromatography. The titre of these materials could then be selectively determined throughout the 5th instar and compared with the titre of 20--hydroxyecdysone. If any peaks in the titre of the new active materials were detected these might be related to particular stages in the moulting process, and also to the possible role of one or both of these substances in the production of 20-hydroxyecdysone.

If the two new active substances from whole nymphs could be detected in the prothoracic glands by g.l.c., the identity of these substances with the two active components presently detected in glands by bioassay could be established. It might then be possible to confirm that the site of production of these substances is the prothoracic glands. The titre of each of the two active substances in the glands could be determined throughout the 5th instar and any variations might be related to the secretory activity and histological changes in the glands.[138] The titre of these substances in the glands and whole nymphs could also be compared.

The determination of the titre of 20-hydroxyecdysone in nymphs at daily intervals throughout the 5th instar by g.l.c. should be repeated to confirm the present results and to obtain a mean value for the titre of this hormone on each day. Oenocytes should also be re-examined for 20-hydroxyecdysone by g.l.c., so that it can be decided whether this tissue is the site of production of this hormone. Oenocyte preparations containing a much higher proportion of oenocytes than strips of abdominal tissue would have to be used, and if possible isolated oenocytes themselves should be investigated at different stages.

The variation in composite titre of moult-inducing substances in whole nymphs and prothoracic glands could be

determined by bioassay, and this could be compared with the variations found for the titre of the individual active components when the latter had been determined. These investigations could also be extended to the other instars, and would make possible the verification of the scheme proposed for the control of moulting by the prothoracic glands.

EXPERIMENTAL

PRELIMINARY NOTES

General Procedures

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

Infrared (I.R.) absorption spectra were measured on a Perkin Elmer 257 grating spectrophotometer. The spectra were determined in solution (e.g. $CHCl_3$) or as a KBr disc (KBr).

Ultraviolet (U.V.) absorption spectra were recorded on a Unicam SP800 spectrophotometer using 1 cm. cells and commercial 95% ethanol as solvent.

Nuclear magnetic resonance (n.m.r.) spectra, unless stated otherwise, were recorded on a Perkin Elmer R10 60 MHz instrument and are quoted as 'tau' (τ) values in p.p.m. from an internal standard of tetramethylsilane (τ10.00 p.p.m.).

Mass spectra were measured with a Hitachi-Perkin Elmer RMU-6 mass spectrometer using an electron accelerating voltage of 80 eV.

Pyridine was purified by distillation from barium oxide powder. All water used was deionised. Light petroleum

refers to light petroleum 60 - 80°, and B.S.A. refers to the silylation reagent bis(trimethylsilyl)acetamide. Solutions were evaporated *in vacuo* unless stated otherwise at temperatures below 40° using a Büchi rotary evaporator. Butanol was evaporated from solutions by azeotropic distillation with water.

Extracts after evaporation were reduced to dryness *in vacuo* (0.05 - 0.1 mm. of Hg.) and stored under an atmosphere of nitrogen at temperatures below 5°.

Solvents used in chromatography were dried and then purified by distillation. Purified chloroform was stabilised with 1% of absolute ethanol.

Woelm alumina deactivated by the addition of water was used in column chromatography.

The method used to prepare columns for absorption chromatography depended upon the particle size of the absorbant. The column was plugged at the outlet with glass wool and part filled with solvent, coarse absorbants (particle size less than 100 mesh, e.g. Woelm alumina) were poured into the column as a slow steady stream, fine absorbants (particle size greater than 100 mesh, e.g. Davison silica gel) were packed in the form of a slurry.

Analytical thin layer chromatography (t.l.c.) unless

stated otherwise was carried out on 5 x 20 cm. and 20 x 20 cm. glass plates coated with an 0.25 mm. layer of silica gel PF_{254} (Merck). Plates were eluted to a distance of 10 cm. from the origin. Components were visualised using one or more of the following techniques: illumination with an ultraviolet lamp (called U.V.), development in iodine vapour (called iodine) or treatment with a spray of either vanillin - concentrated sulphuric acid - ethanol (5:70:25, w/v/v, called vanillin spray) or 2,4-dinitrophenylhydrazine in 2N methanolic hydrochloric acid (called 2,4-D.N.P.), followed by heating at 100 - 120° for 5 to 10 minutes.[137]

Preparative thin layer chromatography (p.t.l.c.) unless stated otherwise was carried out on 5 x 20 cm. and 20 x 20 cm. glass plates coated with an 0.25 mm. layer of silica gel PF_{254}. The plates were dried in a stream of nitrogen after elution. The separated components visualised under an ultraviolet lamp were isolated by scraping off the silica, crushing and packing it into a small column and eluting with either methanol or acetone.

Preparative layer chromatography (P.L.C.) was carried out as described above for p.t.l.c., but using 20 x 40 cm. plates coated with a 1.5 mm. layer of silica gel PF_{254}. Plates were developed to a distance of approximately

17 cm. from the origin. Eluted components containing silica were dissolved in methanol; the methanol was centrifuged and evaporated.

All chromatography plates unless stated otherwise were equilibrated to a standard activity over saturated aqueous sodium chloride solution before use. Where R_f values are quoted for ecdysone and 20-hydroxyecdysone as standards these were obtained by running authentic samples unless literature references are quoted. The relative intensity of spots and bands on chromatography plates was estimated visually and is indicated in figures by the density of the lines and in tables by the abbreviations: W = weak, M = medium, and S = strong.

Analytical gas-liquid chromatography (g.l.c.) was performed on a Pye model 64 instrument using 1.5 m. x 4 mm. bore helical glass columns. The operating conditions, the method of column preparation, and the technique of sample injection are described on pages 269 and 270.

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 m x 2 mm. bore helical glass column linked to the RMU-6 mass spectrometer through a Watson-Biemann separator as the interface. The operating conditions and details of the system are given on page 278.

Moulting Hormone Bioassay[138]

Moulting activity was determined using a bioassay developed and carried out by Dr. P.E. Ellis at the Anti-Locust Research Centre, London. The assay was performed on isolated abdomens of 4th instar nymphs of the desert locust *Schistocerca gregaria*. Nymphs from 27 to 56 hours after ecdysis were starved for two hours, the head was then crushed, the tip of the abdomen cut off and the head pulled away from the rest of the body. The neck membranes were torn, but the gut remained attached to the head and was neatly removed. A ligature was tied around the anterior part of the abdomen with sterilised sewing 'Silko'. The legs and thorax were cut off. A ligature at the hind end of the body completed the preparation.

Materials for testing were dissolved in fresh, twice distilled water containing antibiotic at the rate of 15 to 25 µg. of streptomycin and 20 to 30 µg. penicillin per abdomen. Injection was made with a Hamilton syringe and No. 30 needle, via an intersegmental membrane. Each abdomen received 20 µl. of test solution and was then placed in a sterilised petri dish containing a few drops of distilled water and incubated at 32° for 48 hours. Healthy abdomens remained plump and normal in appearance and the heart continued to beat. Ten to 15 abdomens were used for each assay and deaths rarely exceeded 20%.

Abdomens were scored as follows. Moulting was detected by tearing the cuticle on the dorsal part of the abdomen in a posterior - anterior direction. Histological examination showed that the ability to peel away the cuticle from the underlying epidermis indicated that the moulting process had started. Abdomens showing indications of moulting were given a positive score. Cuticle that did not peel away indicated the absence of moulting and such abdomens were given a negative score.

The quantitative results are quoted as the percentage of abdomens given a positive score (the percentage activity of the extract) at a particular dose. In each case a group of abdomens injected only with distilled water and antibiotic acted as a control. A few qualitative results from the early part of the work are expressed only in terms of positive or negative moulting activity. In the early stages of the isolation, levels of activity in extracts were unknown, so doses were related to number of nymphs. For example; if 1000 nymphs gave 1 g. of extract and this was tested at a dose of 1 mg. per abdomen, the dose per abdomen would be equivalent to 1 nymph. The dose always refers to the amount injected per abdomen.

Whole Nymphs for Extraction

Nymphs of the desert locust from days 8 to 11 of the 5th instar (as determined from the previous ecdysis) were collected when available at the Anti-Locust Research Centre, homogenised in methanol and the homogenate stored in five gallon drums at a concentration of approximately 150 nymphs per litre. This homogenate was used for the large scale extraction of whole insects.

PRELIMINARY EXTRACTION OF WHOLE NYMPHS FOR MOULTING HORMONE

Solvent Extractions[13]

The methanol homogenate prepared from approximately 350 nymphs (2.5 litres) was filtered under vacuum and the filter-cake washed consecutively with three volumes of methanol (600 ml., 450 ml., 450 ml.) to yield an inhomogeneous residue (approximately 150 g.) which was discarded, and a clear yellow-orange extract. Evaporation of the methanol gave the viscous dark brown aqueous concentrate I (approximately 200 ml.).

Butanol - Water Partition The aqueous concentrate (I) was diluted with water (100 ml.) and shaken with butanol (4 x 40 ml.), the phases separated without emulsification. Evaporation of the aqueous phase gave a dark brown semi-solid residue (58.1 g.). The butanol phases were combined to give a clear green-brown solution.

Butanol - Aqueous Acid and Base Partitions The combined butanol phase was extracted consecutively with the following reagents: iced water (20 ml.), cold aqueous 1% sulphuric acid (20 ml.), cold aqueous 10% sodium carbonate (3 x 20 ml.), iced water (20 ml.), cold aqueous 5% acetic acid (20 ml.), and iced water (35 ml.). Evaporation of the butanol after extraction

gave the dark yellow-brown semi-solid oil II (21.0 g.) having positive activity in the moulting hormone bioassay.

Light Petroleum - Water Partition Attempted partition of the active oil (II) between light petroleum (100 ml.) and deionised water (250 ml.) resulted in an emulsion which did not separate on standing. The emulsion was broken by evaporation to remove the solvent. The solvent-free oil was shaken with absolute ethanol (130 ml.) and the suspension was filtered to remove a pale brown insoluble solid (0.450 g.). The ethanol was evaporated to leave an oil (20.5 g.) which was then successfully partitioned between water (160 ml.) and light petroleum (3 x 80 ml.). A small amount of emulsion and insoluble solid at the interface of the two phases was evaporated to dryness before repartitioning between deionised water (20 ml.) and light petroleum (20 ml.), insoluble solid at the interface between these two phases was separated by filtration and gave the light brown powder IV (0.500 g.).

The combined light petroleum phases gave a green-brown oil (18.9 g.) on evaporation, and the combined aqueous phases produced the light brown tacky gum III (0.710 g.) which showed positive activity in the moulting hormone bioassay.

Alumina Column Chromatography[13]

A column of alumina (activity V, 15 g., internal diameter 1.5 cm.) was made up in ethyl acetate. The powder (IV) and the active gum (III) were combined and the portion of combined extract soluble in ethyl acetate (15 ml.) was absorbed onto the top of the alumina and the column was eluted as shown in Table 2. The insoluble residue which originated mainly from the powder (IV) was triturated in turn with each eluting solvent before being discarded.

TABLE 2

Group	Fraction	Eluting solvent	Volume ml.	Weight g.
A	1	EtAc	30	0.074
B	2	EtAc-BuOH (5:1)	40	0.105
	3	" " (")	40	0.024
C	4	" " (")	40	0.014
	5	EtAc-MeOH (3:1)	40	0.087
	6	MeOH	40	0.102
D	7	"	40	0.035
	8	"	40	0.031
	9	"	40	0.020

The fractions obtained were combined into four groups as shown in Table 2, and these were further combined with corresponding fractions originating from the extraction of 1050 nymphs. Groups A and B were inactive but groups C and D showed positive activity in the moulting hormone bioassay. The total active

extract was combined to give the pale brown resin V (1.81 g.).

Davison Silica Gel Column Chromatography[24]

Davison silica gel (grade 950, 60-200 mesh) was deactivated by equilibration with water (10% w/w) in a stoppered flask for two hours. A solution of the resin (V) in butanol (10 ml.) was diluted with benzene (90 ml.) and absorbed onto a column of the deactivated silica gel (57 g. internal diameter 2 cm.) prepared and washed before use with benzene. The column was eluted and the fractions collected were tested for moulting activity; the results are given in Table 3.

TABLE 3

Fraction	Eluting solvent	Volume ml.	Weight g.	Dose* mg.	Activity
1	Benzene-BuOH (9:1)	500	0.347	1.6	0%
2	Benzene-MeOH (19:1)	525	0.234	1.5	40%
3	Benzene-MeOH (4:1)	800	0.432	1.4	57%
4	Benzene-MeOH (1:1)	500	0.408	1.5	0%
5	MeOH	500	0.245	1.5	0%
6	MeOH-water (85:15)	250	0.074	0.2	0%
Control					0%

*The dose per abdomen was 20 µl. of a 5% solution prepared by dissolving the bioassay sample in water.

T.l.c. was carried out on the fractions using the following conditions.

Silica	Eluting solvent	Detection method
G	Chloroform-MeOH (9:1)*	U.V.
G	Chloroform-MeOH (4:1)	U.V.
G and PF_{254}	Dichloromethane-acetone--MeOH (2:1:1)†	U.V. Vanillin spray 2,4-D.N.P.

Ecdysone	*R_f 0.10[74]	†R_f 0.69[137]
20-Hydroxyecdysone	*R_f 0.07	†R_f 0.62

The results showed that all fractions were complex mixtures which gave an incompletely resolved streak of components from the origin of the plate to the solvent front. The predominant components in the least polar fractions one and two were observed at R_f greater than 0.5 while those in the polar fractions five and six were observed at R_f less than 0.5. The identification of any ecdysones in such complex mixtures was not possible.

Silicic Acid Column Chromatography[24]

The remaining active material from the silica gel column (fractions two and three) was combined and chromatographed on a column of silicic acid (Bio-Sil A, 100-200 mesh, 42 g., internal

diameter 2.5 cm.) made up and washed with benzene. A solution of the sample in methanol (4.5 ml.) was diluted with benzene (40.5 ml.) and absorbed onto the column. The column was eluted and the fractions collected were tested for moulting activity; the results are shown in Table 4.

TABLE 4

Fraction	Eluting solvent	Volume ml.	Weight g.	Dose mg.	Activity
1	Benzene-MeOH (9:1)	460	0.329	0.250*	100%
				0.051†	80%
2	Benzene-MeOH (4:1)	710	0.247	0.190*	14%
				0.038†	33%
3	MeOH	460	0.084	0.065*	33%
				0.013†	70%
4	MeOH-water (85:15)	230	0.008	0.006*	33%
Control*					0%
Control†					0%

*Dose equivalent to 1 nymph per abdomen.
†Dose equivalent to 0.2 nymphs per abdomen.

MODIFIED EXTRACTION PROCEDURE

Nymphs were extracted as each batch of methanol homogenate became available, and the scale was varied according to the number of insects in a particular batch. The stages at which freshly isolated extracts were added into the main extraction sequence are indicated.

Where results of activity in the moulting hormone bioassay are available for more than one batch at any particular stage in the extraction they are quoted with the weights of the extracts and the number of locusts from which the extracts originated.

When the percentage of the active extract put aside for analytical and bioassay studies was significant (more than 1%) the equivalent number of nymphs are subtracted from the total carried forward to the next stage of the extraction.

Solvent Extraction

The methanol homogenate (12.5 litres) prepared from 2271 nymphs was filtered as described in the preliminary extraction procedure. The methanol extract (approximately 20 litres) was evaporated at a temperature below 40° in a 20 litre vacuum distillation apparatus equipped with a nitrogen

bleed, to give an aqueous methanol concentrate (approximately 3 litres). The remaining methanol was removed using a rotary evaporator to produce the aqueous concentrate VI (approximately 900 ml.).

Butanol - Water Partition The aqueous concentrate (VI) was diluted with water (400 ml.) and partitioned consecutively with four volumes of butanol (800 ml., 600 ml., 400 ml., 400 ml.). After the first partition the phases were filtered to remove suspended pale brown insoluble residue (XLI, 1.51 g.). The combined butanol phases were washed consecutively with water (400 ml., 200 ml.) and the aqueous wash finally extracted with butanol (80 ml.). Evaporation of the aqueous phase gave the dark brown semi-solid residue XLII (146 g.), and the combined butanol phases yielded a brown oil (VII, 60.0 g.) which contained suspended solid material.

The extracts XLI, XLII and VII were tested for moulting activity with the following results (Table 5).

TABLE 5

Extract	Dose mg.*	Activity
XLI	0.13	0%
XLII	12.86	0%
VII	5.29	100%
Control		0%

*Dose equivalent to 0.2 nymphs per abdomen.

Light Petroleum - Water Partition The active oil (VII) was treated with absolute ethanol (550 ml.) as described in the preliminary extraction procedure to give a dark brown amorphous solid (XLIII, 0.959 g.) and the brown semi-solid oil XLIV (58.8 g.). The oil was partitioned between water (600 ml.) and light petroleum (300 ml.) and the aqueous phase was further partitioned with two consecutive volumes of light petroleum (200 ml., 150 ml.). The combined light petroleum phases were washed with water (200 ml.) and the aqueous wash finally extracted with light petroleum (40 ml.). The small amounts of emulsion and suspended solid were treated as described for emulsified solids in the preliminary extraction procedure. The phases were

evaporated to yield a brown oil (XLV, 48.1 g.) from the light petroleum and a pale brown gum (VIII, 8.15 g.) from the water. A small amount of insoluble residue (XLVI, 1.80 g.) was obtained as described above.

The extracts XLIII to XLVI and VIII were tested for moulting activity and the results are given in Table 6.

TABLE 6

Extract	Dose mg.*	Activity
XLIII	0.09	0%
XLIV	5.29	100%
XLV	4.22	0%
VIII	0.72	90%
XLVI	0.16	0%
Control		0%

*Dose equivalent to 0.2 nymphs per abdomen.

The extraction of a further 2300 nymphs by the above procedure gave the following extracts at this stage, a brown oil (XLVa, 93.4 g.) from the light petroleum phase, a pale brown gum (VIIIa, 12.7 g.) from the aqueous phase, and a brown

residue (XLVIa, 2.71 g.) which was insoluble in the light petroleum and water. The gum VIIIa showed positive activity in the moulting hormone bioassay but the oil (XLVa) and the residue (XLVIa) were inactive.

Alumina Column Chromatography

(i) The active extract VIIIa (12.7 g.) was chromatographed as follows on a column of alumina (activity V, 160 g., internal diameter 2.5 cm.) made up in ethyl acetate. The gum was triturated with ethyl acetate (160 ml.) and the soluble portion was absorbed directly onto the top of the alumina. The insoluble residue was dissolved in butanol (71.6 ml.) and the resulting clear brown solution was diluted with ethyl acetate (358.4 ml.) to give an ethyl acetate - butanol mixture (5:1) containing precipitated solid. The solid was removed by filtration and the clear yellow filtrate was absorbed onto the column. The precipitated solid was treated twice more as described above and the resulting filtrates were absorbed onto the alumina as before. The insoluble residue finally remaining was a dark brown amorphous powder (IX, 4.34 g.). The column was eluted and the fractions obtained were divided into six groups which were tested for moulting activity, the results are shown in

Table 7. The bioassay result for the powder (IX) is also included.

TABLE 7

Group	Fraction	Eluting solvent	Volume ml.	Weight g.	Dose* mg.	Activity
A	1	EtAc	160	0.219	0.190	0%
	2	EtAc-BuOH (5:1)	430	1.686		
B	3	" " "	430	1.091	2.54	35%
	4	" " "	430	0.151		
C	5	EtAc-MeOH (3:1)	430	1.206	1.05	100%
	6	MeOH	430	0.859		
	7	"	430	0.359		
D	8	"	430	0.114	1.25	100%
	9	"	430	0.108		
E	10	MeOH-water (4:1)	500	0.766	0.668	90%
F	11	MeOH-AcOH (9:1)	500	0.764	0.664	0%
IX						0%
Control						0%

*Dose equivalent to 2 nymphs per abdomen.

The fractions were subjected to t.l.c. on plates coated with silica gel G using dichloromethane - acetone - methanol (2:1:1) as the eluting solvent and the components were visualised with U.V., vanillin spray, and 2,4-D.N.P. The results showed that all fractions were complex mixtures which gave an incompletely resolved streak of components from the origin of the plate to the solvent front.

The active groups of fractions B to E in Table 7 were combined. This material, which after removing samples for bioassay and t.l.c. corresponded to the extract from approximately 2300 nymphs, was further combined with a similar extract from approximately 3200 nymphs to give the pale brown gum X (11.9 g.) which exhibited the moulting activity shown below.

	Dose mg.	Activity
X	2.15*	66%
	1.08**	67%
Control		0%

*Dose equivalent to 2 nymphs per abdomen.
**Dose equivalent to 1 nymph per abdomen.

(ii) A further 2521 nymphs were extracted according to the modified procedure so far described. The aqueous phase from the light petroleum - water partition gave the extract VIIIc (8.75 g.) as a pale brown gum. When subjected to the ethyl acetate - butanol treatment before alumina chromatography, the gum yielded an insoluble dark brown amorphous powder (IXa, 2.62 g.). The results from the alumina chromatography of the soluble portion are given in Table 8.

TABLE 8

Fraction	Eluting solvent	Volume ml.	Weight g.	Activity at dose: 1 mg.	0.5 mg.
1	EtAc-BuOH (5:1)	300	2.005	10%	0%
2	EtAc-BuOH (5:1)	600	0.116	58%	0%
3	EtAc-MeOH (3:1)	300	0.881	48%	27%
4	MeOH	1200	2.313	37%	25%
	MeOH-water (4:1)	340			
Control				20%	14%

Efficiency of the Ethyl Acetate - Butanol Treatment

The aqueous phase obtained from the light petroleum - water partition of a new extract of 4140 nymphs gave the pale brown gum VIIIb (9.86 g.) on evaporation. Samples of this were subjected to the ethyl acetate - butanol treatment as described above but using varying proportions of ethyl acetate and butanol. Each sample was triturated three times consecutively with the volume of solvent stated in Table 9 and the soluble material was obtained in each case by combination of the three solutions obtained by filtration followed by evaporation. All the results are summarised in Table 9.

TABLE 9

Triturating solvent	Sample g.	Material	Weight g.	Activity at dose: 1 mg.
	9.86	VIIIb		78%
EtAc (20 ml.)	0.400	Soluble	0.091	56%
		Insoluble	0.254	70%
EtAc-BuOH (8:1) (15 ml.)	0.293	Soluble	0.195	67%
		Insoluble	0.099	56%
EtAc-BuOH (6:1) (15 ml.)	0.304	Soluble	0.160	75%
		Insoluble	0.092	80%
EtAc-BuOH (5:1) (300 ml.)	8.86	Soluble	5.06	70%
		Insoluble	3.70	22%
		Control		20%

Davison Silica Gel Column Chromatography

(i) The active extract (X) was dissolved in butanol (65 ml.) and the solution diluted with benzene (585 ml.), this was

absorbed onto a column of Davison silica gel (grade 950, 60-200 mesh, 370 g., internal diameter 3.5 cm.) deactivated with water (10% w/w) and prepared and washed with benzene before use. The column was eluted and the fractions were tested for moulting activity. The results are given in Table 10.

TABLE 10

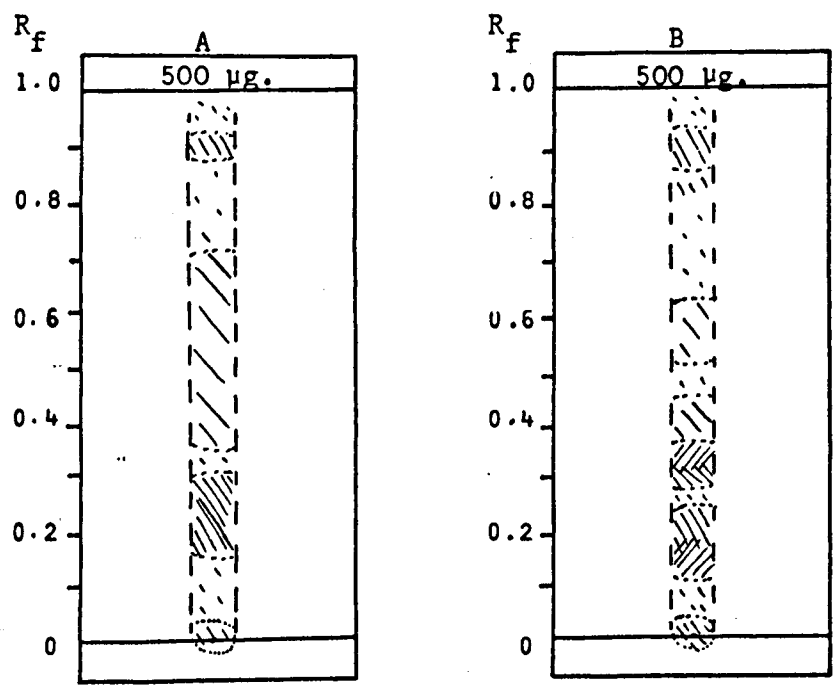
Fraction	Eluting solvent	Volume ml.	Weight g.	Dose mg.	Activity
1	Benzene-BuOH (9:1)	2275	1.936	0.352*	10%
2	Benzene-MeOH (19:1)	2786	1.951	0.353*	25%
				0.071†	33%
2a	" " "	600	0.628	0.114*	89%
				0.023†	33%
3	Benzene-MeOH (4:1)	5200	5.846	1.063*	100%
				0.212†	63%
4	Benzene-MeOH (1:1)	3250	0.944	0.176*	43%
				0.035†	29%
5	MeOH	3250	0.249	0.045*	35%
				0.010†	11%
6	MeOH-water (85:15)	1630	0.273	0.050*	0%
Control*					0%
Control†					0%

*Dose equivalent to 1 nymph per abdomen.
†Dose equivalent to 0.2 nymphs per abdomen.

A portion of fraction three, the major active fraction, was dissolved in a mixture of benzene - methanol at a concentration of 50 mg. ml.$^{-1}$ and was subjected to t.l.c. A known weight of material was applied to each plate with a 10 µl. Hamilton syringe. The conditions used, and the results obtained under U.V. are summarised diagrammatically in Figure 62.

FIGURE 62

Thin Layer Chromatograms of Fraction Three from the Davison Silica Gel Column



A Dichloromethane - acetone - methanol (2:1:1)

B Chloroform - 95% ethanol (4:1)

Similar results showing streaks of components from the origin to R_f 0.90 were obtained using silica gel G plates and visualising components with U.V. and vanillin spray. The best resolution of components was obtained with the chloroform - 95% ethanol (4:1) solvent system[27] (Figure 62B).

The active fractions 2a to 5 in Table 10 were combined to give the pale brown gum XI (7.18 g.). As approximately 6% of the active material had been used for bioassay and t.l.c., this now corresponded to the extract from approximately 5100 nymphs. The gum was tested for moulting activity and the results are given below.

	Dose µg.	Activity
XI	1000	86%
	100	64%
	10	57%, 30%
	1	20%
Control		20%

(ii) The active fractions two to four in Table 8 which originated from the second alumina column described (see page 211) were combined to give the pale brown gum Xa (3.19 g.). As approximately 3.5% of the active material had been used this now corresponded to the extract from approximately 2430 nymphs. The gum was dissolved in butanol (17 ml.), the solution diluted with benzene (153 ml.) and then chromatographed on a column of Davison silica gel (grade 950, 60-200 mesh, 100 g., internal diameter 2.5 cm.) prepared as described before. The column was eluted and the results are shown in Table 11.

TABLE 11

Fraction	Eluting solvent	Volume ml.	Weight g.	Activity at dose: 100 µg.
1	Benzene-BuOH (9:1)	870	0.155	
2	Benzene-MeOH (19:1)	770	0.178	13%
3	Benzene-MeOH (19:1)	150	1.890	67%
	Benzene-MeOH (4:1)	1390		
4	MeOH	870	0.286	33%
5	MeOH-water (85:15)	500	0.100	
Control				10%

Investigations Leading to Further Purification of the Active Extract

(i) Chromatography on Silicic Acid A portion of the extract XI (500 mg.) representing the active material combined after chromatography on Davison silica gel was absorbed onto a column of silicic acid (Bio-Sil A, 100-200 mesh, 38 g., internal diameter 2.5 cm.) as described in the preliminary procedure but using methanol (4 ml.) and benzene (36 ml.). A small amount of solid material (approximately 10 mg.) did not dissolve and was not chromatographed. The results of eluting the column and assaying the fractions collected for moulting activity are given in Table 12.

TABLE 12

Fraction	Eluting solvent	Volume ml.	Weight mg.	Activity at dose: 100 µg.	10 µg.	1 µg.
1	Benzene-MeOH (9:1)	410	239	47%	67% 50%	41%
2	Benzene-MeOH (4:1)	630	257	80%	67% 31%	58%
3	MeOH	410	29	40%	64% 46%	50%
4	MeOH-water (85:15)	210	17	62%	55% 36%	*
Control (all doses)					20%	

*Several abdomens died.

T.l.c. eluting with chloroform - 95% ethanol (4:1) under identical conditions to those described in Figure 62B (see page 216) showed that the fractions contained major components in the regions, R_f 0.25 - 0.90 for fraction one, R_f 0.00 - 0.50 for fraction two, and R_f 0.00 - 0.30 for fractions three and four.

(ii) Preparative Layer Chromatography on Silica Gel PF_{254} A further portion of the active extract XI (232 mg.) was applied to the origin of a P.L.C. plate using minimum volumes of methanol and benzene as solvents. A small amount of sparingly soluble solid (approximately 5 mg.) was not chromatographed. The plate was eluted with chloroform - 95% ethanol (4:1); the bands were detected by U.V. and the areas of silica gel indicated in Figure 63 were scraped off and the absorbed material eluted with methanol. The results are given in Table 13. As the gum XI was a complex mixture bands overlapped.

FIGURE 63

P.L.C. of Extract XI

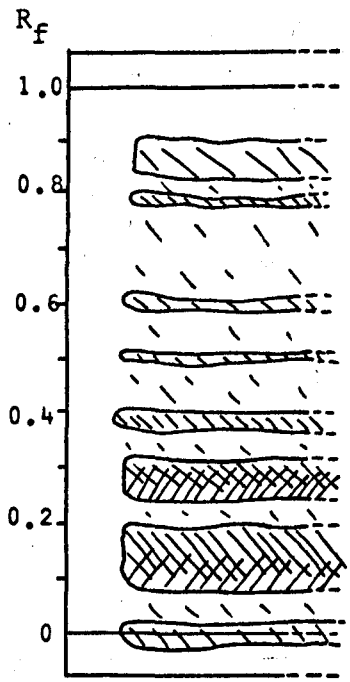


TABLE 13

Cut	Area	R_f range	Recovered mg.
	8	0.80 - 0.92	31
	7	0.62 - 0.80	5
	6	0.48 - 0.62	9
	5	0.41 - 0.48	12
	4	0.38 - 0.41	16
	3	0.22 - 0.38	32
	2	0.08 - 0.22	69
	1	0.00 - 0.08	22

The separated materials eluted from the silica gel were tested for moulting activity and the results are given in Table 14 and Figure 5 (see page 45).

TABLE 14

Area	R_f range	Activity at dose: 100 µg.	10 µg.	1 µg.
1	0.00 - 0.08	25%	10%	25%
2	0.08 - 0.22	86%	63%	63%
3	0.22 - 0.38	33%	10%	25%
4	0.38 - 0.41	78%	40%	38%
5	0.41 - 0.48	73%	58%	42%
6	0.48 - 0.62	60%	64%	45%
7	0.62 - 0.80	58%	45%	27%
8	0.80 - 0.92	0%	33%	30%
Control (all doses)			20%	

T.l.c. of the separated materials under the same conditions as those used for the P.L.C. showed that only a partial separation had been achieved and all fractions contained small amounts of other components running outside the R_f range cut for the fraction.

(iii) Removal of Sparingly Soluble Material A portion of the gum XI (116 mg.) was triturated with methanol (0.5 ml.) until only a fine suspension of solid remained undissolved, this was separated with the use of a centrifuge and treated with two

volumes of methanol (0.3 ml., 0.2 ml.) by the same method. The three solutions were evaporated separately and the residues obtained were tested for moulting activity; the results are given in Table 15.

TABLE 15

Residue	Weight mg.	Activity at dose: 100 µg.	10 µg.	1 µg.
1st Centrifugate	72	90%	80%	60%
2nd Centrifugate	18	40%	30%	30%
3rd Centrifugate	5	40%	35%	30%
Sparingly soluble solid*	6			
Control (all doses)			30%	

*The sparingly soluble material was not assayed because of its insolubility.

The above procedure was carried out on the remaining 79% of the extract XI (5.68 g.), this gave a pale yellow insoluble solid (0.394 g.) and a pale brown gum (XIa, 5.31 g.) which corresponded to approximately 4000 nymphs.

(iv) Combination of Active Extracts The extract XIa (5.31 g.) was combined with similar material originating from fractions three and four in Table 11 (see page 218) to give extract XII (7.33 g.) as a pale brown gum, this represented the extract from approximately 6400 nymphs. This material was tested in the moulting hormone bioassay and the results are given below.

	Dose µg.	Activity
	1000	71%
XII	100	57%
	10	38%
Control		20%

(v) Modification of the Chromatography on Silicic Acid A portion of extract XII (500 mg.) dissolved in methanol (5 ml.) was absorbed onto silicic acid (Bio-Sil A, 100-200 mesh) by addition of absorbant to the solution followed by evaporation of the methanol under vacuum while the flask was gently rotated. Three different ratios of absorbant to gum were tried as shown below.

Absorbant-gum ratio (w/w)	Consistency of residue
1:1	Tacky lumps
1.5:1	" "
2:1	Free flowing powder

The free flowing powder was placed on the top of a silicic acid column (14 g. internal diameter 1.5 cm.) prepared as previously described. The column was eluted and fractions (50 ml.) were collected and monitored by t.l.c. using the chloroform - 95% ethanol solvent system. The results are given in Table 16.

TABLE 16

Fraction (50 ml.)	Eluting solvent	Weight mg.	R_f, (U.V.)
1 - 2	Benzene	<1	
3 - 5	Benzene-MeOH (1%)	<1	
6 - 8	Benzene-MeOH (3%)	10	0.70 - 0.90
9 - 11	Benzene-MeOH (5%)	69	0.70 - 0.90 (W) 0.20 - 0.45 (M)
12 - 14	Benzene-MeOH (7%)	67	0.70 - 0.90 (W) 0.22 - 0.45 (M)
15 - 17	Benzene-MeOH (10%)	114	0.70 - 0.90 (W) 0.20 - 0.45 (S)
18*	Methanol-water (15%)	206	0.25 - 0.45 (M) 0.00 - 0.25 (S)

*Fraction volume (250 ml.).

(vi) Chromatography on Floridin Earth All the fractions (Table 16) were recombined and the residue (463 mg.) was

absorbed onto floridin earth (B.D.H. Florex XXS, 1 g.) by the procedure described above. The free flowing powder obtained was placed on the top of a column of floridin earth (14 g., internal diameter 1.5 cm.) made up and washed with benzene. The column was eluted with benzene - ether, and ether - acetone mixtures, and fractions (50 ml.) were collected and monitored by t.l.c. The results are given in Table 17.

TABLE 17

Fraction (50 ml.)	Eluting Solvent	Weight mg.	R_f, (U.V.)
1 - 31	Benzene-ether (1-100%) Ether-acetone (1-25%)	29	0.70 - 0.90
32 - 34	Ether-acetone (50%)	8	" "
35 - 37	Ether-acetone (75%)	25	0.70 - 0.90 (W) 0.10 - 0.65 (M)
38 - 39	Acetone	51	" "
40*	MeOH-water (15%)	250	0.00 - 0.65

*Fraction volume (200 ml.).

(vii) Column Chromatography on Silica Gel PF_{254}

The fractions 1 - 40 were recombined and the residue (360 mg.) was combined with a further portion of extract XII (127 mg.)

This combined material (487 mg.) was absorbed onto silica gel PF_{254} (1 g.) which had been equilibrated to a standard activity over saturated aqueous sodium chloride solution. The free flowing powder obtained was placed on the top of a column of silica gel PF_{254} (11.5 g., internal diameter 1.5 cm.) prepared and washed with ether. The column was eluted under pressure (10 lb. sq. $in.^{-1}$) at a flow rate of 2 ml. $min.^{-1}$, ether - acetone mixtures were used as eluting solvents, and material was only eluted by ether containing more than 10% acetone. The fractions (50 ml.) were monitored by t.l.c. as described for the columns in (v) and (vi) above, and the results indicated a much better separation of components.

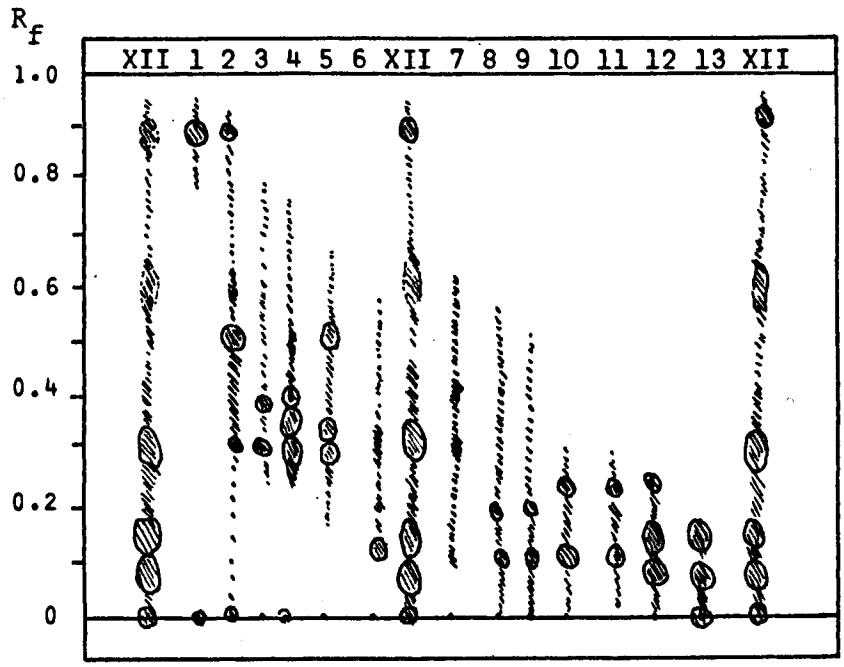
An identical column to the one just described was prepared and a fresh portion of extract XII (486 mg.) was chromatographed in an attempt to determine the exact conditions required to give a good separation. The column was eluted by a slightly modified procedure, and monitored by t.l.c. using the chloroform - 95% ethanol (4:1) solvent system (all the fractions were spotted on a single plate along side samples of extract XII for comparison purposes), the results are shown in Table 18 and Figure 64.

TABLE 18

Fraction	Eluting solvent	Volume ml.	Weight mg.
1	Ether-acetone (10%)	100	2
2	Ether-acetone (25%)	150	8
3	" " "	50	2
4	" " "	50	18
5	Ether-acetone (50%)	150	116
6	" " "	100	32
7	" " "	100	17
8	Ether-acetone (75%)	100	22
9	" " "	50	2
10	" " "	50	7
11	" " "	50	5
12	Acetone	50	34
13	Methanol-water (15%)	250	195

FIGURE 64

Thin Layer Chromatogram of the Silica Gel PF_{254} Column Fractions in Table 18 (visualised under U.V.)



A similar result was obtained when the plate was visualised with iodine.

Silic Gel PF_{254} Column Chromatography

(i) A portion of the active extract XII (3.00 g.) representing approximately 2650 nymphs was absorbed onto silica gel PF_{254} (6 g.) and chromatographed on a column of silica gel PF_{254} (69 g., internal diameter 3 cm.) prepared as previously

described. The column was eluted and the fractions were tested for moulting activity with the results shown in Table 19.

TABLE 19

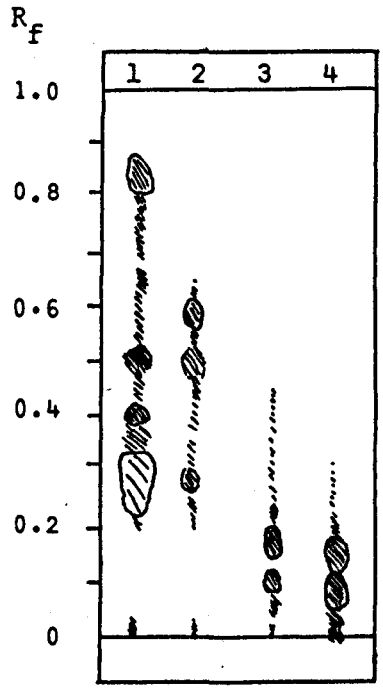
Fraction	Eluting solvent	Volume ml.	Weight g.	Activity at dose: 100 µg.	10 µg.
1	Ether-acetone (25%)	1500	0.212	50% 50% 40%	30% 50% 40%
2	Ether-acetone (50%)	1200	1.063	56% 42% 60%	50% 50% 38%
3	Ether-acetone (75%)	1200	0.396	33% 50% 43%	42% * 40%
4	MeOH-water (15%)	750	1.143	56% 40% 63% 25%	25% 25% 35%
Control (both doses)				20%	

*Several abdomens died

Fractions one to four were subjected to t.l.c. using the chloroform - 95% ethanol (4:1) solvent system. The results are illustrated in Figure 65.

FIGURE 65

Thin Layer Chromatogram of the Silica Gel PF_{254} Column Fractions in Table 19 (visualised under U.V.)



The components visible as intense violet overlapping spots at R_f 0.07 and 0.13 under the above conditions were only present in fraction four. The components in fraction three did not show the characteristic violet colour.

(ii) The remaining portion of the active extract XII (2.88 g.) representing approximately 2550 nymphs was also chromatographed on silica gel PF_{254} with similar results to those described above to give fractions 1a - 4a.

Preparative Layer Chromatography on Silica Gel PF_{254}

Each of the fractions one to four from the silica gel PF_{254} column were separately chromatographed on preparative plates of silica gel PF_{254} eluting as shown below.

Fraction	Number of plates	Eluting solvent
1	1	Chloroform-95% ethanol (4:1)
2	2	" " " (4:1)
3	4	" " " (7:3)
4	5	" " " (3:2)

Bands were detected by U.V. and had the appearance as shown in Figures 66 to 69. The silica gel on the plates was cut into areas as shown in the accompanying tables (20 to 23), and the absorbed material was eluted with methanol.

FIGURE 66

P.L.C. of Column

Fraction 1

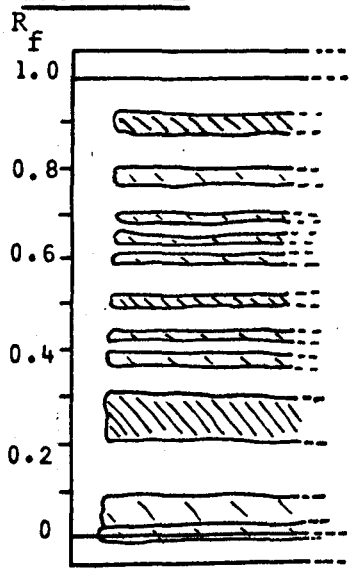


TABLE 20

Cut	Area	R_f range	Recovered mg.
	6	0.88 - 0.95	22
	5	0.70 - 0.88	9
	4	0.58 - 0.70	18
	3	0.34 - 0.58	64
	2	0.20 - 0.34	63
	1	0.00 - 0.20	22

FIGURE 67

P.L.C. of Column

Fraction 2

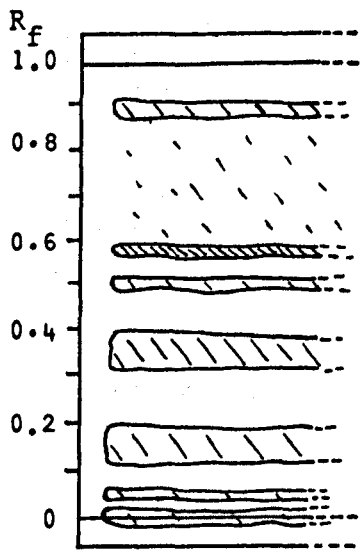


TABLE 21

Cut	Area	R_f range	Recovered mg.
	6	0.80 - 0.95	23
	5	0.60 - 0.80	24
	4	0.43 - 0.60	358
	3	0.30 - 0.43	95
	2	0.10 - 0.30	68
	1	0.00 - 0.10	407

FIGURE 68

P.L.C. of Column

Fraction 3

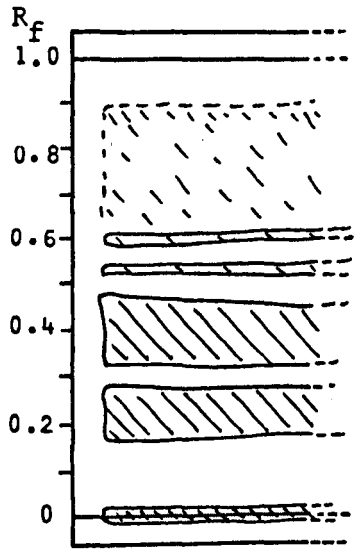


TABLE 22

Cut	Area	R_f range	Recovered mg.
	4	0.65 - 0.90	45
	3	0.50 - 0.65	60
	2	0.30 - 0.50	147
	1	0.00 - 0.30	153

FIGURE 69

P.L.C. of Column

Fraction 4

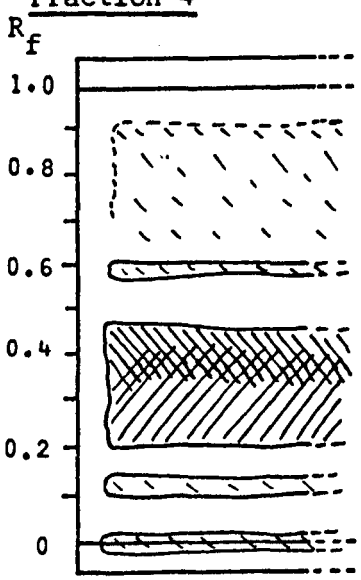


TABLE 23

Cut	Area	R_f range	Recovered mg.
	5	0.65 - 0.90	243
	4	0.48 - 0.65	108
	3	0.38 - 0.48	167
	2	0.20 - 0.38	324
	1	0.00 - 0.20	373

The materials recovered from the silica gel as shown in Tables 20 to 23 were tested for moulting activity and the results are given in Tables 24 to 27 and in Figures 6 to 9 (pages 52 to 53) respectively.

TABLE 24

Area	R_f range	Activity at dose: 100 µg.	10 µg.	1 µg.
1	0.00 - 0.20	10%	30%	
2	0.20 - 0.34	33%	25%	
3	0.34 - 0.58	55%	63%	56%
4	0.58 - 0.70	70%	50%	25%
5	0.70 - 0.88	30%	30%	
6	0.88 - 0.95	40%	30%	
Control A	(all doses)		20%	
Control B	(all doses)		30%	

TABLE 25

Area	R_f range	Activity at dose: 100 µg.	10 µg.	1 µg.
1	0.00 - 0.10	33%	36%	30%
2	0.10 - 0.30	38%	43%	40%
3	0.30 - 0.43	33%	38%	30%
4	0.43 - 0.60	43%	33%	30%
5	0.60 - 0.80	60%	50%	50%
6	0.80 - 0.95	50%	33%	33%
Control (all doses)			27%	

TABLE 26

Area	R_f range	Activity at dose: 100 µg.	10 µg.	1 µg.
1	0.00 - 0.30	33%	48%	32%
2	0.30 - 0.50	83%	50%	60%
3	0.50 - 0.65	63%	50%	44%
4	0.65 - 0.90	38%	33%	30%
Control (all doses)			30%	

TABLE 27

Area	R_f range	Activity at dose: 100 µg.	10 µg.	1 µg.
1	0.00 - 0.20	34%	46%	38%
2	0.20 - 0.38	60%	60%	28%
3	0.38 - 0.48	60%	42%	32%
4	0.48 - 0.65	80%	64%	33%
5	0.65 - 0.90	50%	33%	30%
Control (all doses)			33%	

To determine whether a good separation had been achieved by the above procedure the isolated materials were subjected to t.l.c. under identical conditions to those used for the P.L.C. The spots were visualised under U.V. and the t.l.c. results showed only slight overlap for those spots which represented material recovered from adjacent areas on the same P.L.C. plate. As a good separation had been achieved, samples exhibiting appreciable moulting activity were selected for further investigation, and these are summarised and given extract numbers as shown in Table 28.

TABLE 28

Column fraction	Figure	Area	Extract number
1	6	3	XIII
		4	XIV
2	7	5	XV
3	8	2	XVI
		3	XVII
4	9	2	XVIII
		3	XIX
		4	XX

Combination of Active Extracts

Comparison of extracts XIII to XV by t.l.c. using chloroform - 95% ethanol (4:1) as eluting solvent showed that they all contained components visible under U.V. in the region R_f 0.50 to 0.60. The remaining material from these extracts was therefore combined to give a pale yellow solid (XXI, 79 mg.) which was called the *upper active band*. As approximately 25%

of the material had been used for bioassay and t.l.c., extract XXI corresponded to the upper active band material isolated from approximately 2000 nymphs.

Extracts XVI to XX were similarly compared but using chloroform - 95% ethanol (4:1) and (7:3), and ethyl acetate - acetone (6:4) as eluting solvents. The plates were eluted three times in the ethyl acetate - acetone solvent system. The results giving the regions of R_f showing major components visible under U.V. are shown in Table 29. The R_f values obtained for ecdysone and 20-hydroxyecdysone under identical conditions are given for comparison.

TABLE 29

Extract	Chloroform - 95% ethanol (7:3) R_f	(4:1) R_f	Ethyl acetate - acetone (3:2) R_f
XVI	0.30 - 0.40	0.15 - 0.25	0.15 - 0.30
XVII	0.35 - 0.50	0.20 - 0.28	0.20 - 0.35
XVIII	0.18 - 0.28	0.05 - 0.18	
XIX	0.18 - 0.28	0.05 - 0.18	
XX	0.28 - 0.40	0.15 - 0.25	0.10 - 0.25
Ecdysone	0.39	0.20	
20-Hydroxy-ecdysone	0.34	0.17	

On the basis of these results the extracts were combined as follows. Extracts XVI, XVII and XX were combined to give extract XXII (243 mg.) as a light brown gum which was called the *middle active band.* This extract corresponded, as in the case of the upper active band material, to the extract from approximately 2000 nymphs. The remaining two extracts XVIII and XIX were also combined and they produced a yellow brown resin (XXIII, 398 mg.) which was called the *lower active band.* This extract also corresponded to the extract from approximately 2000 nymphs.

The fractions 1a - 4a (see page 232) were similarly subjected to P.L.C. and the areas of silica gel which corresponded to the active materials described above were scraped off the plates and eluted. The materials isolated gave the same results on t.l.c. as those obtained for the corresponding materials from column fractions one to four, they were therefore combined with the appropriate active band. This gave upper active band (XXIa, 240 mg.), middle active band (XXIIa, 509 mg.), and lower active band (XXIIIa, 867 mg.), each representing an active extract from approximately 4500 nymphs.

Portions of the upper and middle active band materials were subjected to t.l.c. on silica gel PF_{254} plates, and Eastman polyamide (K541V) and Polycarbonate (K511V) chromagram

sheet. The following eluting solvents were used for the upper band samples: benzene - methanol (4:1), dichloromethane - methanol (9:1), chloroform - methanol (9:1), ether - acetone (1:4), ethyl acetate - acetone (3:2), ether - ethyl acetate (3:7), ethyl acetate - methanol (9:1), acetone - 95% ethanol (9:1), ether - methanol (9:1). The following eluting solvents were used for the middle band samples: benzene - methanol (7:3), dichloromethane - methanol (7:3), chloroform - methanol (7:3), ether - ethyl acetate (1:4), ether - acetone (3:7), ethyl acetate - acetone (3:2) three times eluted, ethyl acetate - methanol (7:3), acetone - 95% ethanol (7:3), acetonitrile - methanol (7:3), ether - methanol (7:3), ether - 95% ethanol (7:3), butanol, ethyl acetate - methanol - acetonitrile (1:1:2). The solvent systems containing alcohols were unsuitable for use with the polyamide sheet and those containing chlorinated solvents were unsuitable for use with the polycarbonate sheet. These combinations were therefore avoided, but all others were used.

The components were visualised on the silica gel PF_{254} with U.V., iodine, and vanillin spray, and with U.V. and iodine on the other two absorbants. The results showed that each of the active materials chromatographed was a mixture of several components and none of the conditions listed above gave a satisfactory separation.

STANDARD EXTRACTION PROCEDURE

The extraction of approximately 7000 nymphs using the modified extraction procedure gave a pale brown gum (XLVII, 10.5 g.) from the aqueous phase of the light petroleum - water partition. This was triturated with butanol - ethyl acetate (5:1), by the method previously described, to yield a dark brown amorphous powder (1.21 g.) and the gum XLVIII (9.20 g.). The extracts XLVII and XLVIII were tested for moulting activity with the following results.

	Activity at 1 mg. dose
XLVII	54%
XLVIII	63%
Control	20%

Davison Silica Gel Column Chromatography

The active gum XLVIII was dissolved in butanol (50 ml.) and the solution diluted with benzene (550 ml.), this was absorbed onto a column of Davison silica gel (grade 950, 60-200 mesh, 286 g., internal diameter 3.5 cm.) prepared as described in the modified extraction procedure. The column was eluted and

the fractions were tested for moulting activity as shown in Table 30.

TABLE 30

Fraction	Eluting solvent	Volume ml.	Weight g.	Activity at dose: 1000 µg.	100 µg.	10 µg.
1	Benzene-BuOH (9:1)	1755	0.368	19%		
2	Benzene-MeOH (19:1)	2615	0.590	22%	25%	
3	Benzene-MeOH (4:1)	4000	5.889	60%	40%	57%
4	MeOH	2500	0.392	17%	20%	
5	MeOH-water (85:15)	1260				
Control (all doses)					10%	

The extraction of a further 6000 nymphs by the standard procedure so far described gave the pale brown gum XLIX (3.15 g.) which corresponded to fraction three in Table 30. Extract XLIX was tested for moulting activity with the result shown below.

	Activity at dose: 1000 µg.	100 µg.	10 µg.
XLIX	78%	43%	50%
Control (all doses)		10%	

Fraction three (Table 30) and extract XLIX were combined after bioassay to give extract L (8.98 g.), and this material was triturated with methanol, by the method described in the modified procedure, to yield a pale yellow insoluble solid (0.754 g.) and brown gum (XXIV, 8.21 g.). This corresponded to the extract from approximately 1.3×10^4 nymphs.

Silica Gel PF_{254} Column Chromatography

(i) A portion of the extract XXIV (3.50 g.) representing approximately 5550 nymphs was absorbed onto silica gel PF_{254} (7 g.) and chromatographed on a column of silica gel PF_{254} (87 g. internal diameter 3 cm.) prepared as described in the modified extraction procedure. The column was eluted and the fractions were tested for moulting activity; the results are given in Table 31.

TABLE 31

Fraction	Eluting solvent	Volume ml.	Weight g.	Activity at dose: 500 µg.	100 µg.
1	Ether-acetone (25%)	2420	0.986	*	50%
2	Ether-acetone (50%)	1460	0.628	*	22%
3	Ether-acetone (75%)	1460	0.346	*	43%
4	Acetone	915	0.342	*	57%
5	MeOH-water (15%)	915	0.411	*	77%
Control					10%

*Many abdomens died because the distilled water used was impure.

(ii) A second portion of the extract XXIV (4.46 g.) representing approximately 7050 nymphs was chromatographed on silica gel PF_{254} as described above, with similar results, to give fractions 1a - 5a.

Preparative Layer Chromatography on Silica Gel PF_{254}

Each of the fractions one to five from the silica gel PF_{254} column were separately chromatographed on preparative plates of silica gel PF_{254} by a similar procedure to that described on page 233; fractions four and five were both eluted

with the chloroform - 95% ethanol (3:2) solvent system. The pattern of bands observed under U.V. was similar to that shown diagrammatically in Figures 66 to 69 (see pages 234 and 235), and areas of silica were scraped off and treated by the method previously described to give the results summarised in Tables 32 to 36, and Figures 10 to 14 (see pages 59 to 61).

TABLE 32 (Column fraction 1)

Area	Recovered mg.	R_f range	Activity at dose: 100 µg.	10 µg.
1	267	0.00 - 0.20	20%	*
2	147	0.20 - 0.38	22%	20%
3	540	0.38 - 0.75	50%	40%
4	39	0.75 - 0.95	14%	20%
Control (both doses)			15%	

*Several abdomens died.

TABLE 33 (Column fraction 2)

Area	Recovered mg.	R_f range	Activity at dose: 100 µg.	10 µg.
1	147	0.00 - 0.11	33%	17%
2	169	0.11 - 0.40	17%	20%
3	308	0.40 - 0.60	32%	25%
4	33	0.60 - 0.85	45%	50%
5	24	0.85 - 0.95	28%	25%
Control (both doses)			25%	

TABLE 34 (Column fraction 3)

Area	Recovered mg.	R_f range	Activity at dose: 100 µg.	10 µg.
1	26	0.00 - 0.10	20%	17%
2	77	0.10 - 0.30	13%	30%
3	185	0.30 - 0.65	62%	60%
4	39	0.65 - 0.95	12%	10%
Control (both doses)			10%	

TABLE 35 (Column fraction 4)

Area	Recovered mg.	R_f range	Activity at dose: 100 µg.	10 µg.
1	298	0.00 - 0.40	30%	25%
2	11	0.40 - 0.50	20%	33%
3	30	0.50 - 0.70	60%	67%
4	26	0.70 - 0.95	46%	43%
Control (both doses)			26%	

TABLE 36 (Column fraction 5)

Area	Recovered mg.	R_f range	Activity at dose: 100 µg.
1	81	0.00 - 0.20	33%
2	216	0.20 - 0.40	62%
3	57	0.40 - 0.68	62%
4	38	0.68 - 0.95	33%
Control			26%

The column fractions 1a - 5a (see page 246) were similarly subjected to P.L.C. and the areas of silica gel which corresponded to the active extracts in the above tables were scraped off and eluted. The materials isolated gave the same results on t.l.c. as those obtained for their active counterparts in Tables 32 to 36.

Combination of Active Extracts

The P.L.C. results above and results from t.l.c. in the solvent systems described in Table 29 (see page 240) showed that the new active extracts isolated in the standard extraction could be combined into three groups similar to those obtained using the modified extraction procedure. The appropriate extracts were therefore combined, and the three groups obtained were further combined with their active counterparts XXIa, XXIIa, and XXIIIa (see page 241). This gave upper active band XXV (1.50 g.) as a pale yellow solid, middle active band XXVI (1.12 g.) as a brown gum, and lower active band XXVII (1.23 g.) as a light brown resin. Each band represented an active extract from approximately 1.7×10^4 nymphs. The bands were tested for moulting activity and the results are given below.

	R_f region§	Activity at dose 100 µg.	10 µg.	1 µg.
XXV	0.39 - 0.71	70%†	60%†	38%**
XXVI	0.15 - 0.25	63%*	53%*	
XXVII	0.05 - 0.18	50%*	73%*	
Control		14%*	30%†	0%**

§Eluting with chloroform - 95% ethanol (4:1) and visualising under U.V.

SYNTHESIS OF COMPOUNDS FOR GAS-LIQUID CHROMATOGRAPHY

Isolation of Methoxime Derivatives

Methoxime (MO) derivatives of steroids were prepared in pyridine and were isolated by modification of the methods of Horning *et al.*[112]

(i) For steroids readily soluble in organic solvents, the reaction mixture, for example, pyridine solution (0.5 ml.) was diluted with water (2 ml.) and extracted consecutively with three volumes of benzene (3 ml.). The benzene extracts were combined and evaporated; the last traces of pyridine were removed under high vacuum (0.1 mm. of Hg).

(ii) For polar steroids with several hydroxyl groups in the molecule, the reaction mixture, for example, pyridine solution (0.5 ml.) was diluted with saturated sodium chloride solution (2 ml.) and extracted consecutively with three volumes of ethyl acetate (3 ml.) in a centrifuge tube. The ethyl acetate phases removed after centrifugation were combined and evaporated; the last traces of pyridine were removed under high vacuum (0.1 mm. of Hg).

Isolation of Trimethylsilyl Derivatives

Trimethylsilyl (TMSi) derivatives of steroids were

isolated by removal of the volatile components from the reaction mixture under vacuum (0.1 mm. of Hg.).

Reaction Conditions

All methoximation and silylation reactions carried out at room temperature were allowed to react in the dark. When reactions were carried out at elevated temperatures an atmosphere of nitrogen was used.

TMSi-Cholesterol (*10*)

A quantitative yield of TMSi-cholesterol (*10*) was obtained by adding excess bis(trimethylsilyl)acetamide (B.S.A., 1 ml.) to a solution of cholesterol (250 mg.) in chloroform (1 ml.) and allowing the mixture to stand at room temperature for ten minutes. T.l.c. eluting with benzene - ethyl acetate (9:1) and visualising with aqueous 20% sulphuric acid spray showed the absence of starting material R_f 0.20 and the presence of a single product R_f 0.80. The volatile components were evaporated (3 hours, 0.1 mm. of Hg) to leave a white solid which on crystallisation from acetone gave TMSi-cholesterol (*10*) as colourless needles m.p. 129 - 130° (lit.,[139] m.p. 129 - 130°). N.m.r. ($CDCl_3$) τ9.85 (singlet, 9H, Me_3Si), mass spectrum M^+ 458

($C_{30}H_{54}OSi$ requires 458).

The g.l.c. results are given in Tables 38 to 40 (pages 272 to 275).

A few crystals of TMSi-cholesterol (*10*) were exposed to the atmosphere over saturated sodium chloride solution, t.l.c. after two weeks showed no change.

Reaction of 4-Cholesten-3,6-dione with O-methyl hydroxylamine hydrochloride

O-methyl hydroxylamine hydrochloride (600 mg.) was added to a solution of 4-cholesten-3,6-dione (150 mg.) in pyridine[112] (6 ml.) and the solution was allowed to stand at ambient temperature for 24 hours. T.l.c. eluting with light petroleum - ethyl acetate (4:1) and visualising with aqueous 20% sulphuric acid spray showed the absence of starting material R_f 0.30 and the presence of a minor component R_f 0.52 and a major component R_f 0.62.

The products were isolated by method (i) (see page 252) and the mixture was subjected to P.L.C. eluting with the solvent system described above and visualising under U.V.

The material from band I at R_f 0.52 was isolated as a yellow gum (22 mg.); the n.m.r. spectrum showed a complex absorption τ5.8 - 6.2 indicating that this was a mixture.

The material from band II at R_f 0.62 was isolated as a yellow gum (102 mg.) which on recrystallisation from ethanol gave a mixture of two of the possible *syn*- and *anti*-isomers of 4-cholesten-3,6-dione dimethoxime (*11*) as yellow needles m.p. 105 - 107°. $\lambda_{max.}$ (95% EtOH) 279 nm. ($\log_{10}\varepsilon$, 4.25), n.m.r. ($CDCl_3$) τ6.15 (singlet 6H, 2 x CH_3O), mass spectrum M^+ 456 ($C_{29}H_{48}O_2N_2$ requires 456).

The mass spectrum is given in Figure 33 (page 94) and the g.l.c. results are given in Table 38 (page 272) and Figure 17 (page 81).

Ergosterol Acetate (*14*)

Ergosterol (*13*, 10 g.) was dissolved in a mixture of pyridine - acetic anhydride (redistilled, 200 ml.) and the solution was allowed to stand in the dark at room temperature for 48 hours. T.l.c. eluting with benzene - ether (4:1) and visualising with aqueous 20% sulphuric acid spray indicated the absence of starting material R_f 0.21, and the presence of a single product R_f 0.60. Dilution of the reaction mixture by the addition of ice cold water (1 litre), with vigorous stirring, gave a white precipitate which on isolation followed by recrystallisation from methanol gave ergosterol acetate (*14*) as white needles m.p. 167 - 169° (lit.,[140] m.p. 170 - 171),

$\nu_{max.}$ (KBr) 1730 cm.$^{-1}$ (strong), n.m.r. ($CDCl_3$) τ7.98 (singlet, 9H, CH_3CO), mass spectrum M^+ 438 ($C_{30}H_{46}O_2$ requires 438).

3β-Acetoxy-5α-hydroxyergosta-7,22-dien-6-one (*15*)

Ergosterol acetate (*14*) was selectively oxidised with chromium trioxide by the method of Burawoy[119] as modified by Barton and Robinson.[120] The reaction was carried out in the dark to give a white solid which on crystallisation from ethyl acetate gave 3β-acetoxy-5α-hydroxyergosta-7,22-dien-6-one (*15*) as white plates (23%) m.p. 261 - 264° (lit.[120] 263 - 265°). $\nu_{max.}$ (KBr) 3400 (broad), 1680 - 1730 cm.$^{-1}$ (strong), $\lambda_{max.}$ (95% EtOH) 250 nm. ($\log_{10}\varepsilon$, 4.06), mass spectrum M^+ 470 ($C_{30}H_{46}O_4$ requires 470).

The g.l.c. results are given in Table 38 (page 272) and Figure 15 (page 81).

3β-Acetoxy-5α-ergosta-7,22-dien-6-one (*16*)

3β-Acetoxy-5α-hydroxyergosta-7,22-dien-6-one (*15*) was reduced with zinc dust by the method of Barton and Robinson[120] to give 3β-acetoxy-5α-ergosta-7,22-dien-6-one (*16*, 10%). Recrystallisation from methanol gave white needles m.p. 182 - 185° (lit.,[120] 184 - 186°). $\nu_{max.}$ (KBr) 1730 (strong), 1670 cm.$^{-1}$

(weak), $\lambda_{max.}$ (95% EtOH) 245 nm. ($\log_{10}\varepsilon$, 4.10), mass spectrum M^+ 454 ($C_{30}H_{46}O_3$ requires 454).

3β-Hydroxy-5α-ergosta-7,22-dien-6-one (*12*)

3β-Acetoxy-5α-ergosta-7,22-dien-6-one (*16*) was hydrolysed by the method of Dory and Geri[141] to give *3β-hydroxy-5α-ergosta-7,22-dien-6-one* (*12*, 85%). Recrystallisation from methanol gave white needles m.p. 157 - 159° $\nu_{max.}$ (KBr) 3400 (broad), 1660 $cm.^{-1}$ (weak), $\lambda_{max.}$ 245 ($\log_{10}\varepsilon$, 4.04), mass spectrum M^+ 412 ($C_{28}H_{44}O_2$ requires 412).

The g.l.c. results are given in Table 38 (page 272) and Figure 16 (page 81).

Reaction of 3β-Hydroxy-5α-ergosta-7,22-dien-6-one (*12*) with O-methyl hydroxylamine hydrochloride

(i) O-methyl hydroxylamine hydrochloride (8 mg.) was added to a solution of 3β-hydroxy-5α-ergosta-7,22-dien-6-one (*12*) (2 mg.) in pyridine (0.5 ml.). The extent of reaction was monitored by t.l.c. eluting with ether - benzene (3:1), this showed a quantitative conversion of starting material R_f 0.22 to a component R_f 0.44 after a period of 60 hours.

(ii) The reaction was repeated and the solution was heated to 60° for three hours,[112] this caused a brown colour to

develop but caused no significant increase in the rate of reaction as observed by t.l.c.

(iii) The reaction was again repeated but using O-methyl hydroxylamine hydrochloride (320 mg.) and 3β-hydroxy--5α-ergosta-7,22-dien-6-one (*12*, 80 mg.) in pyridine (10 ml.). The reaction mixture was allowed to stand at room temperature for 60 hours, and the product was then extracted by method (i) to give a yellow residue (88 mg.). This was subjected to P.L.C. under the same conditions as those used for t.l.c. The material from the band at R_f 0.44 was isolated to give a mixture of *syn*- and *anti*-3β-hydroxy-5α-ergosta-7,22-dien-6-one methoxime (*17*) as a pale yellow gum (56 mg., 60%) which failed to crystallise. $\nu_{max.}$ (KBr) 3400 $cm.^{-1}$ (strong), $\lambda_{max.}$ (95% EtOH) 251 nm. ($\log_{10}\varepsilon$, 3.99), n.m.r. ($CDCl_3$) τ*6.15 and τ6.17 (two singlets, 3H, CH_3O), τ:τ* approximately 3:1, mass spectrum M^+ 441 ($C_{29}H_{47}NO_2$ requires 441).

The mass spectrum is given in Figure 34 (page 96) and the g.l.c. results are given in Tables 38 and 39 (pages 272 and 274), and Figures 18 and 29 (see pages 82 and 90).

Reaction of the Mixture of *Syn-* and *Anti*-3β-Hydroxy-5α--ergosta-7,22-dien-6-one methoxime (*17*) with B.S.A.

Bis(trimethylsilyl)acetamide (2 ml.) was added to a solution of *syn-* and *anti*-3β-hydroxy-5α-ergosta-7,22-dien--6-one methoxime (*17*, 50 mg.) in pyridine (2 ml.) and the reaction mixture was allowed to stand at room temperature for 12 hours. T.l.c. eluting with ether - benzene (3:1) showed a single spot at R_f 0.82. The volatile components were removed under vacuum and the residue was applied to one P.L.C. plate and eluted with ether - benzene (3:1). The band at R_f 0.82 was isolated to give a mixture of *syn-* and *anti*-3β-trimethylsilyloxy-5α-ergosta-7,22-dien-6-one methoxime (*18*) as a pale yellow gum (45 mg., 78%) which failed to crystallise. $\lambda_{max.}$ (95% EtOH) 251 nm. ($\log_{10}\varepsilon$, 3.98), n.m.r. ($CDCl_3$) τ*6.15 and τ6.17 (two singlets, 3H, CH_3O), τ:τ* approximately 3:1, τ9.85 (singlet, 9H, Me_3Si), mass spectrum M^+ 513 ($C_{32}H_{55}NO_2Si$ requires 513).

The mass spectrum is given in Figure 35 (pages 97 and 98) the g.l.c. results are given in Tables 38 and 39 (pages 272 and 274) and in Figures 19 and 29 (pages 82 and 90).

Reaction of 3β-Hydroxy-5α-ergosta-7,22-dien-6-one (*12*) with B.S.A.

To a solution of 3β-hydroxy-5α-ergosta-7,22-dien-6-one (*12*, 2 mg.) in pyridine (0.2 ml.) was added B.S.A. (0.5 ml.) and the reaction was allowed to stand at room temperature for 24 hours. T.l.c. eluting with ether - benzene (3:1) showed a single spot at R_f 0.82. The volatile components were evaporated to give 3β-trimethylsilyloxy-5α-ergosta-7,22-dien-6-one (*19*). Mass spectrum M^+ 484 ($C_{31}H_{52}O_2Si$ required 484).

The g.l.c. results are given in Table 38 (page 272) and Figure 20 (page 83).

Attempted Methoximation of 3β-Acetoxy-5α-hydroxyergosta-7,22--dien-6-one (*15*)

A solution of 3β-acetoxy-5α-hydroxyergosta-7,22-dien--6-one (*15*, 2 mg.) in pyridine (0.5 ml.) was treated with O-methyl hydroxylamine hydrochloride (8 mg.) at room temperature. T.l.c. eluting with benzene - ether (4:1) showed only starting material R_f 0.24 after 60 hours.

The reaction mixture was heated at 60° for three hours and t.l.c. showed a faint spot at R_f 0.52, heating for a further three hours at 100° caused this spot to become more intense

(approximately 10% of the starting material by visual estimation).

The mixture was heated under reflux for three hours, this caused a brown colour to develop in the solution, and t.l.c. showed the formation of a further product R_f 0.48, however visual estimation showed that approximately 70% of the starting material still remained.

Attempted Silylation of 3β-Acetoxy-5α-hydroxyergosta-7,22-dien--6-one (*15*)

To a solution of 3β-acetoxy-5α-hydroxyergosta-7,22--dien-6-one (*15*, 2 mg.) in pyridine (0.2 ml.) was added B.S.A. (0.4 ml.), this was allowed to stand at room temperature for 20 hours. Starting material, R_f 0.24 on t.l.c. eluting with benzene - ether (4:1), was recovered unchanged.

The unchanged starting material was subjected to the above conditions with the addition of trimethylchlorosilane (0.1 ml.); t.l.c. then showed a faint spot at R_f 0.56 (approximately 5% of the intensity of the starting material by visual estimation). The reaction mixture was heated to 60° for 20 hours and this caused a brown colour to develop in the solution. T.l.c. showed two minor spots at R_f 0.41 and 0.50 and a major spot at R_f 0.56, visual estimation showed that

approximately 30% of the starting material still remained.

Reaction of 20-Hydroxyecdysone with O-methyl hydroxylamine hydrochloride

(i) To a solution of 20-hydroxyecdysone (2.5 mg.) in pyridine (0.6 ml.) was added O-methyl hydroxylamine hydrochloride (10 mg.). The reaction was monitored by t.l.c. eluting with chloroform - 95% ethanol (4:1), and after 100 hours at room temperature starting material R_f 0.15 was absent and two major overlapping spots, I (R_f 0.18) and II (R_f 0.21) of approximately equal intensity were present.

The product mixture was extracted by method (ii) (see page 252), and divided into two equal portions. One portion (*20*) was retained for direct silylation, the other was subjected to preparative thin layer chromatography (p.t.l.c.) on a 5 x 20 cm. plate eluting three times with chloroform - 95% ethanol (4:1), two major bands were observed, I (R_f 0.35) and II (R_f 0.45) which corresponded to spots I and II above. Bands I and II were separately isolated and eluted with methanol directly into 5 ml. flasks. The methanol was evaporated to give one of the *syn*- and *anti*-isomers of 20-hydroxyecdysone methoxime from band I as residue *21* and the other isomer from band II as residue *22*.

(ii) 20-Hydroxyecdysone (2 mg.) yielded an identical product mixture to that described above when treated with O-methyl hydroxylamine hhydrochloride in pyridine under the same reaction conditions. After extraction by method (ii), the ethyl acetate solution was allowed to stand for 48 hours before evaporation. T.l.c. showed that the component corresponding to spot II (R_f 0.21) had undergone approximately 80% conversion (visual estimation) to give a third component R_f 0.32. The mixture was subjected to p.t.l.c. as above and the component III (R_f 0.70) was isolated using methanol to give the residue *23*.

Reaction of the 20-Hydroxyecdysone Methoxime Derivatives *20* to *23* with B.S.A.

The 20-hydroxyecdysone methoxime derivatives *20* to *23* were each dissolved in B.S.A. (0.5 ml.) and the mixtures were allowed to stand at room temperature for 48 hours in the case of *21* and *22* and for 70 hours in the case of *20* and *23*. The volatile components were evaporated from the mixtures to leave yellow residues, and these were individually subjected to p.t.l.c. eluting once with chloroform - 95% ethanol (4:1). All gave single bands at R_f 0.82 which were isolated using acetone and methanol to give the products *20a* to *23a*.

These were all subjected to g.l.c. and the results are shown as follows:

20a Tables 38 to 40 (pages 272 to 275) and Figures 21 (page 84), 30 (page 90) and 31 (page 91).

21a, *22a* Tables 38 and 39 (pages 272 to 274) and Figure 22 (page 84).

23a Table 38 (page 272).

Mass spectra are shown in the following Figures:

20a Figures 36 (pages 99 and 100) and 48 (pages 124 to 127).

21a Figure 37 (page 103).

22a Figure 38 (page 104).

23a Figure 39 (pages 105 and 106).

The evidence indicated that they were all isomers of *tetrakis-TMSi-20-hydroxyecdysone methoxime*.

Reaction of Ecdysone with O-methyl hydroxylamine hydrochloride

A solution of ecdysone (1.4 mg.) in pyridine (0.35 ml.) was treated with O-methyl hydroxylamine hydrochloride (6 mg.) for 100 hours at room temperature. T.l.c. eluting with chloroform - 95% ethanol (4:1) showed the absence of starting material R_f 0.20 and the presence of two overlapping spots at R_f 0.24 and 0.26. The reaction mixture was extracted by method

(ii), and the extract was divided into two halves, one half (*24*) was retained for direct silylation, the other half was subjected to p.t.l.c. on a 5 x 20 cm. plate eluting three times with chloroform - 95% ethanol (4:1), two bands were observed, I (R_f 0.47) and II (R_f 0.53), these were eluted as described in the case of 20-hydroxyecdysone to give one of the *syn-* and *anti*-isomers of ecdysone methoxime as residue *25* (band I) and the other isomer as residue *26* (band II).

Reaction of the Ecdysone Methoxime Derivatives *24* to *26* with B.S.A.

The ecdysone methoxime derivatives *24* to *26* were each dissolved in B.S.A. (0.5 ml.) and the reactions were allowed to stand for 70 hours at room temperature. The volatile components were evaporated to give yellow residues which on subjection to p.t.l.c. eluting once with chloroform - 95% ethanol (4:1) all gave single bands at R_f 0.82 which were isolated using acetone and methanol to give the products *24a* to *26a*. These were subjected to g.l.c. and the results are shown as follows:

24a Tables 38 to 40 (pages 273 to 275) and Figures 23 (page 86), 30 (page 90), and 31 (page 91).

25a, *26a* Tables 38 and 39 (pages 273 to 274) and Figure 24 (page 86).

Mass spectra are shown in the following Figures:

24a Figures 40 and 41 (pages 107 and 108).

25a Figure 42 (pages 109 and 110).

26a Figure 43 (page 110).

The evidence indicated that they were all isomers of *tetrakis-TMSi-ecdysone methoxime*.

Reaction of the Ecdysone and 20-Hydroxyecdysone Methoxime Derivatives with B.S.A. under more Forcing Conditions

Portions of ecdysone and 20-hydroxyecdysone each (500 µg.) were treated according to the following procedure. The sample was dissolved in pyridine (0.5 ml.) and treated with O-methyl hydroxylamine hydrochloride (8 mg.) as previously described, and the reaction mixture was extracted by method (ii). The isomer mixture obtained was dissolved in B.S.A. (0.5 ml.) and allowed to stand at room temperature for 70 hours. G.l.c. indicated the formation of the tetrakis-TMSi derivatives for both ecdysone and 20-hydroxyecdysone.

(i) Pyridine (0.1 ml.) was added and the mixture was heated at 80° for 1 minute[142] followed by rapid cooling to room temperature, the volatile components were evaporated to give a yellow residue. G.l.c. indicated no further change in either case.

(ii) The residue was dissolved in a mixture of B.S.A. (0.2 ml.) and dimethylformamide (1 ml.) and heated at 80° in a benzene vapour bath for 15 hours,[81] the solution became yellow in colour. The volatile components were evaporated to give a yellow residue. G.l.c. in each case as shown in Figures 25 and 27 (pages 87 and 88) showed that for ecdysone the residue was a mixture of the tetrakis- and pentakis-TMSi derivatives, where as for 20-hydroxyecdysone the residue was a mixture of the tetrakis-, pentakis-, and hexakis-TMSi derivatives.

(iii) The residue remaining from (ii) above was subjected to the same conditions as in (ii) for a further 72 hours and the volatile components were evaporated to leave for ecdysone the brown residue *28*, and for 20-hydroxyecdysone the brown residue *27*. These residues were each subjected to p.t.l.c. eluting once with chloroform - 95% ethanol (4:1), and each gave a band at R_f 0.87. The products were isolated using acetone and methanol. These were subjected to g.l.c. as shown in Table 38 (page 273) and Figures 26 and 28 (pages 87 and 88). The evidence indicated that both of these products were fully silylated compounds.

Thin Layer Chromatography of Derivatives of the Ecdysones

The MO-TMSi derivatives of ecdysone and 20-hydroxy-ecdysone were subjected to t.l.c. under a series of conditions

during the course of this work. The results of eluting the derivatives with chloroform - 95% ethanol (4:1) and light petroleum - ether (4:1) and visualising with vanillin spray are given in Table 37.

TABLE 37

Material	Eluting solvent		Colour with vanillin spray	
	Chloroform 95%--ethanol (4:1) R_f	Light Petroleum--ether (4:1) R_f	Cold	Heated*
Tetrakis-TMSi--20-hydroxyecdysone methoxime (M†, *20a*)	0.82	0.10 0.21	Prussian blue	Black
Hexakis-TMSi--20-hydroxyecdysone methoxime (M, *27*)	0.87	0.60 0.68	"	"
Tetrakis-TMSi--ecdysone methoxime (M, *24a*)	0.82	0.20 0.31	Colourless	Dark brown
Pentakis-TMSi--ecdysone methoxime (M, *28*)	0.87	0.60 0.68	"	"
20-Hydroxy-ecdysone	0.17	0.00	Turquois	Olive green
Ecdysone	0.20	0.00	Blue	Violet

*Heated at 100 to 120° for 5 to 10 minutes.
†Mixtures of methoxime isomers are denoted by (M).

GAS-LIQUID CHROMATOGRAPHY OF THE SYNTHETIC COMPOUNDS

Preparation of Gas-Liquid Chromatography Columns

The empty columns (1.5 m. x 4 mm.) were silylated before packing by treatment with a 5% solution of silyl-8 (Pierce Chemical Company) in toluene. The column packing was prepared and the columns were packed and pretreated by the method described in the operating manual for Pye series 104 chromatographs. The packing was held in place with plugs of glass wool also treated with a 5% solution of silyl-8 in toluene.

Five columns were prepared using CQ support (100-120 mesh, J.J.'s Chromatography Limited) coated with the following stationary phases at the loadings indicated OV-210 (QF-1, 3%), OV-101 (3% and 1%), OV-17 (3% and 1%).

Operating Conditions

The mobile phase was nitrogen with a flow rate of 50 ml. $min.^{-1}$ All separations were performed isothermally at 232° with an injection point temperature and a detector oven temperature of 250°.

Sample Injection

All samples were dissolved in acetone and were injected by the solvent flush technique[143] using a 10 µl. syringe (Terumo, type UMS-10, N-75). The solvent flush method was carried out as follows. The syringe was filled with acetone (0.1 µl.) followed by nitrogen (0.5 µl.) and then the sample solution to be injected (1 µl.). When an internal standard of TMSi-cholesterol was used, a further volume of nitrogen (0.5 µl.) was introduced into the syringe followed by an acetone solution of the standard (0.2 µl.). The injection when carried out by this method ensured complete introduction of the sample onto the column at the same time as the standard.

Retention Times

The following Tables (38 to 40) summarise the g.l.c. retention times observed for steroid derivatives under the conditions described above. Where samples were resolved to show more than one component, the retention times of the components are given. In cases where the resolution

(R_s)* was greater than about 1.0 (unless tailing was bad) the relative proportions of the components were estimated. Peak areas were measured using a planimeter as peak shapes were rarely Gaussian curves. Corrected retention times are quoted and relative retentions with respect to TMSi-cholesterol as an internal standard are given. In cases where the recorded g.l.c. trace is shown elsewhere the number of the Figure is given. Mixtures of methoxime isomers are denoted in the Tables by (M) after the name of the compound, and 'C' denotes that a complex pattern of several peaks was observed for the material.

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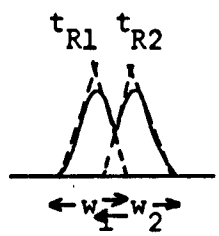
*Resolution (R_s)[124]

The separation of two components can be characterised in terms of their retention times and peak broadenings.

$$R_s = 2\frac{t_{R2} - t_{R1}}{w_1 + w_2}$$



where t_R is the retention time, and w the distance on the base line between the tangents at the inflection points. Index 1 refers to the more weakly and index 2 to the more strongly absorbed component.

TABLE 38

Stationary phase OV-210 (3%)

Sample	Corrected retention time min.	Relative retention	Figure
TMSi-cholesterol (*10*)	6.0 - 6.5	1.00	
4-Cholesten-3,6-dione dimethoxime (M, *11*)	12.7(5%), 16.8(95%)	2.12, 2.80	17
3β-Acetoxy-5α-hydroxy-ergosta-7,22-dien-6--one (*15*)	'C'		15
3β-Hydroxy-5α-ergosta--7,22-dien-6-one (*12*)	'C'		16
3β-Hydroxy-5α-ergosta--7,22-dien-6-one methoxime (M, *17*)	16.5(26%), 23.5(74%)	2.75, 3.92	18
3β-Trimethylsilyloxy--5α-ergosta-7,22-dien--6-one methoxime (M, *18*)	9.5(23%), 13.8(77%)	1.58, 2.30	19
3β-Trimethylsilyloxy--5α-ergosta-7,22-dien--6-one (*19*)	'C'		20
Isomers of tetrakis-TMSi--20-hydroxyecdysone methoxime	(*21a*) 33.0 (*22a*) 31.5 (*23a*) 29.0	5.50 5.25 4.84	22
Tetrakis-TMSi-20--hydroxyecdysone methoxime (M, *20a*)	33.0, 31.5	5.50, 5.25	21

TABLE 38 CONTINUED

Sample	Corrected retention time min.	Relative retention	Figure
Pentakis-TMSi-20--hydroxyecdysone methoxime (M)*	25.0, 27.5	4.16, 4.85	25
Hexakis-TMSi-20--hydroxyecdysone methoxime (M, *27*)	18.3, 21.0	2.82, 3.24	26
Isomers of Tetrakis-TMSi--ecdysone methoxime	(*25a*) 24.5 (*26a*) 22.0	4.08 3.66	24
Tetrakis-TMSi--ecdysone methoxime (M, *24a*)	24.5, 22.0	4.08, 3.66	23
Pentakis-TMSi--ecdysone methoxime (M, *28*)	18.3, 21.0	2.82, 3.24	28

*Results for this sample were taken from the g.l.c. trace of the mixture of tetrakis-, pentakis-, and hexakis-TMSi-20-hydroxy-ecdysone methoxime derivatives.

TABLE 39

Stationary phase OV-17 (1%)

Sample	Corrected retention time min.	Relative retention	Figure
TMSi-cholesterol (*10*)	21.5 - 23.0	1.00	
3β-Hydroxy-5α-ergosta--7,22-dien-6-one methoxime (M, *17*)	69.0(23%),114.5(77%)	3.14, 5.21	29
3β-Trimethylsilyloxy--5α-ergosta-7,22-dien--6-one methoxime (M, *18*)	49.5(24%), 85.0(76%)	2.25, 3.86	29
Isomers of tetrakis-TMSi--20-hydroxyecdysone methoxime	(*21a*) 200.0 (*22a*) 121.5	8.70 5.29	
Tetrakis-TMSi-20--hydroxyecdysone methoxime (M, *20a*)	121.0(39%),195.5(61%)	5.28, 8.69	30
Isomers of tetrakis-TMSi--ecdysone methoxime	(*25a*) 137.5 (*26a*) 82.0	6.09 3.64	
Tetrakis-TMSi--ecdysone methoxime (M, *24a*)	82.2(26%),138.0(74%)	3.64, 6.09	30

TABLE 40

Stationary phase OV-101 (1%)

Sample	Corrected retention time min.	Relative retention	Figure
TMSi-cholesterol (*10*)	15.5 - 16.5	1.00	
Tetrakis-TMSi-20--hydroxyecdysone methoxime (M, *20a*)	82.0, 110.5	5.29, 7.13	31
Tetrakis-TMSi--ecdysone methoxime (M, *24a*)	73.0, 103.5	4.71, 6.68	31

The other two columns which were prepared were only used for TMSi-cholesterol, the results are shown below.

Stationary phase	Retention time TMSi-cholesterol min.
OV-17 (3%)	61.0
OV-101 (3%)	51.5

Construction of a Calibration Curve for 20-Hydroxyecdysone

A method of direct injection[124] was used to construct a calibration curve for 20-hydroxyecdysone using OV-210 (3%) as stationary phase.

Portions of 20-hydroxyecdysone (A, 400 µg.) and (B, 525 µg.) were treated by the following standard procedure to produce derivatives suitable for g.l.c., for example, 20-hydroxyecdysone (1 mg.) was dissolved in pyridine (0.25 ml.) and treated with O-methyl hydroxylamine hydrochloride for 100 hours at room temperature. The product was extracted by method (ii) and was treated with B.S.A. (0.25 ml.) for 70 hours at room temperature. This yielded in the case of both A and B a mixture of *syn*- and *anti*-tetrakis-TMSi-20-hydroxyecdysone methoxime.

Known amounts of these mixtures were injected onto the g.l.c. in the form of acetone solutions and the areas of the resulting peaks (an overlapping pair in each case) were measured. The standard operating conditions previously described were used and the samples were injected at a sensitivity setting of 1×10^{-10} A (full scale). The results are shown in Table 41 and Figure 32 (see page 93).

TABLE 41

Weight of 20-hydroxyecdysone from which the measured peak was derived µg.	Area sq. in.	
	A	B
1.5	2.49	2.71
1.0	1.61	1.83
0.5	0.74	0.85
0.1	0.12	0.15

GAS-LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY OF THE SYNTHETIC COMPOUNDS

Operating Conditions

A g.l.c. column (1.5 m.x2 mm.) packed with OV-210 (3%) on CQ was prepared by the method previously described" (see page 269). This was used under the following operating conditions; the mobile phase was helium with a flow rate of 15 ml. $min.^{-1}$, and all separations were performed isothermally at 232° with an injection point temperature and a detector oven temperature of 250°.

The column outlet was connected through a glass T-piece to the mass spectrometer system and a flame ionisation detector. The connection to the mass spectrometer system via a glass capillary (length 2.5 cm., internal diameter 0.1 mm.) allowed 90% of the helium flow to pass into an evacuated glass line (1×10^{-3} mm. of Hg, length 60 cm., internal diameter 5.0 mm.), this was connected to a Watson-Biemann separator which was in turn connected to the source of the mass spectrometer. The glass T-piece, line and Watson-Biemann separator were all maintained at a temperature of 250°. The system from the column outlet to the mass spectrometer source was silylated at the operating temperature before use by the injection of silyl-8 onto the g.l.c. column. Samples were dissolved in acetone and

were injected by the solvent flush technique.

Retention Times and Molecular Ions

The following Table (42) summarises the retention times and molecular ions observed when the samples indicated were subjected to linked g.l.c.-m.s. under the conditions described above. The results are expressed in the same way as the g.l.c. results in Tables 38 to 40 (see pages 272 to 275).

TABLE 42

Stationary phase OV-210 (3%)

Sample	Corrected retention time min.	Relative retention	M^+	Figure
TMSi-cholesterol (*10*)	9.8 - 10.6	1.00	458	
3β-Trimethylsilyloxy--5α-ergosta-7,22-dien--6-one methoxime (M, *18*)	19.5 (25%) 30.0 (75%)	1.95 3.00	513 513	44 45
Tetrakis-TMSi-20--hydroxyecdysone methoxime (M, *20a*)	56.0 57.8	5.29 5.46	* *	*

*No mass spectrum was obtained. (Approximately 40 µg. of *20a* was injected onto the g.l.c. column.)

INVESTIGATION OF THE ACTIVE EXTRACTS XXV TO XXVII BY GAS-LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

Where the conditions are not specified in the following section materials were subjected to g.l.c. on a column of OV-210 (3%) on CQ using the operating conditions previously described (see page 269); materials were injected as solutions (1 µl.) using the solvent flush technique. The instrument sensitivities used were in the range 1 to 10 x 10^{-10} A for a full scale recorder deflection, and where it is stated that peaks were not observed materials were injected at the highest sensitivity and at a high concentration. Retention times, component percentages, and peak resolution are defined as previously described (see page 271).

Gas-Liquid Chromatography of Extracts XXV to XXVII

Portions of the active extracts XXV to XXVII (1.5 mg.) were individually dissolved in methanol (50 µl.) and were subjected to g.l.c. All the extracts showed a complex pattern of incompletely resolved peaks (t_R less than two minutes). Extract XXVI showed the only other major peaks to be observed. These were at t_R 11.9 minutes and 12.6 minutes; they were of similar proportions but were poorly resolved (R_s less than 0.5).

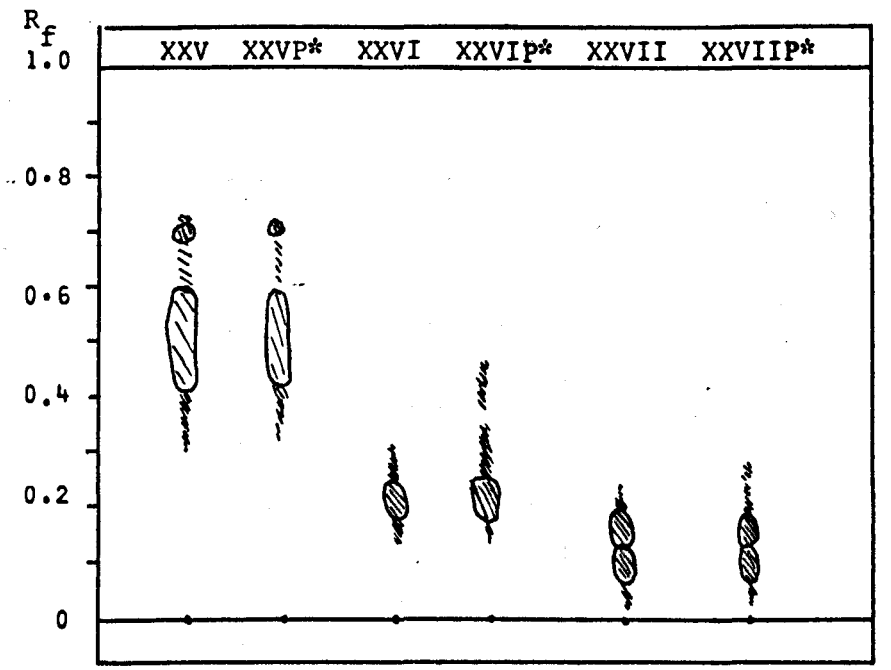
The two components which they represented corresponded to approximately 40% of the eluted material. No other peaks were observed up to t_R 50 minutes.

Preparation and Gas-Liquid Chromatography of Derivatives of Extracts XXV to XXVII

Further portions of the active extracts XXV to XXVII (each 4 mg.) were treated with O-methyl hydroxylamine hydrochloride in pyridine according to the standard procedure previously described (see page 276). T.l.c. of the products eluting with chloroform - 95% ethanol (4:1) showed that the samples appeared to be almost unchanged when compared with the original extracts, (see Figure 70).

FIGURE 70

Thin Layer Chromatogram of Extracts XXV to XXVII and Their Products after Reaction with O-methyl hydroxylamine hydrochloride in Pyridine



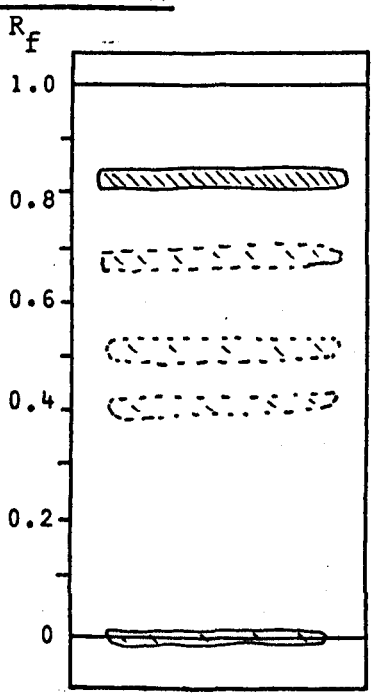
*P refers to the reaction product.

The products from the above reactions were silylated with B.S.A. according to the standard procedure, and the materials obtained were subjected to p.t.l.c., followed by g.l.c. of the components from the separated bands, as outlined in (i) to (iii) below.

(i) The silylated material from extract XXV (upper active band) was subjected to p.t.l.c. on a 5 x 20 cm. plate eluting with chloroform - 95% ethanol (4:1). Bands were detected by U.V. and had the appearance as shown in Figure 71.

FIGURE 71

Preparative Thin Layer Chromatogram of the Silylated Material from Extract XXV



The material in the intense band at R_f 0.82 was isolated using acetone and methanol. The g.l.c. of this material showed a complex pattern of peaks t_R less than five minutes and only traces of components having longer retention

times were detected. The material was subjected to g.l.c.-m.s. using a column of OV-17 (3%) on CQ prepared as described for the corresponding column of OV-210 (3%) on CQ (see page 278), this gave a complex pattern of incompletely resolved peaks t_R up to 10 minutes and indicated that the components had molecular weights in the range 260 to 350.

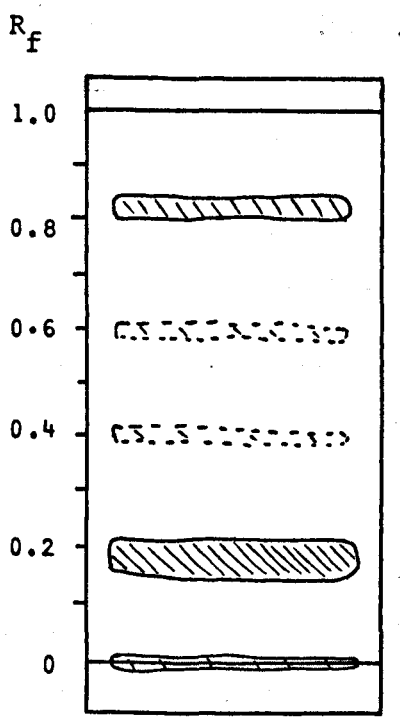
The area of silica in the region R_f 0.39 to 0.71 (R_f range for untreated extract XXV under the above conditions) was also scraped off the plate and the absorbed material was isolated with methanol to give a colourless crystalline solid XXVa, approximately 3 mg.). This represented over 70% of the original portion of extract XXV subjected to the above reaction conditions. Comparison of the mass spectra (direct inlet) of XXV and XXVa showed that they were essentially similar. As the residue XXVa was probably unchanged starting material it was tested for moulting activity with the result shown below.

	Dose µg.	Activity
XXVa	100	28%
	10	30%
Control		20%

(ii) The silylated material from the extract XXVI (middled active band) was subjected to p.t.l.c. under the conditions described in (i) above. The bands detected had the appearance as shown in Figure 72.

FIGURE 72

Preparative Thin Layer Chromatogram of the Silylated Material from Extract XXVI



The material in the band at R_f 0.82 was isolated using acetone and methanol. The g.l.c. of this material showed a complex pattern of peaks t_R less than nine minutes, and

g.l.c.-m.s. performed as described in (i) above for the corresponding band indicated that these peaks represented components having molecular weights in the range 250 to 370. The only other major peaks were at t_R 31.3 minutes and 32.8 minutes and were observed as an overlapping pair (see Figure 46, page 119), these had the same characteristic appearance as the peaks obtained for the methoxime isomer mixture of tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) under identical conditions. When 10% of the material from the band at R_f 0.82 was injected onto a column of OV-210 (3%) on CQ using a sensitivity setting of 1×10^{-10} A, the area under the two peaks (t_R 31.3 and 32.8 minutes) was 0.47 sq. in. This area corresponded to a 20-hydroxyecdysone content of approximately 0.76 mg. in extract XXVI as calculated from the calibration curve in Figure 32 (see page 93). Derivatives of ecdysone could not be detected.

The area of silica in the R_f region 0.15 to 0.25 (R_f range for untreated extract XXVI under the above conditions) was scraped off the plate and the absorbed material eluted with methanol to give an amorphous white solid, (XXVIa, approximately 3 mg.). This material was subjected to g.l.c. and showed components at t_R 11.9 minutes and 12.6 minutes. These corresponded to the major components observed in extract XXVI

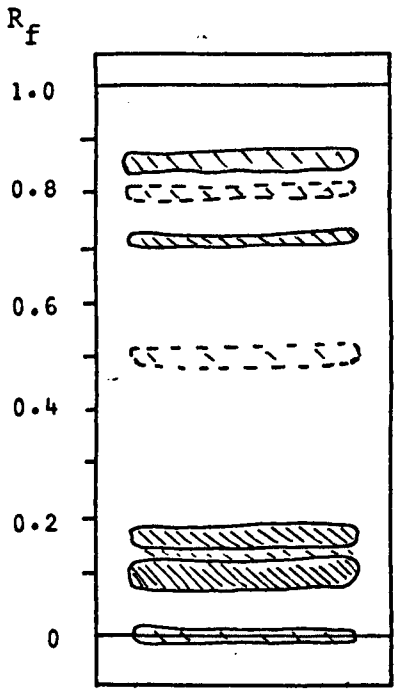
when subjected directly to g.l.c. Comparison of the mass spectra (direct inlet) of XXVI and XXVIa showed that they were essentially similar. As the residue XXVIa appeared to be unchanged starting material it was tested for activity in the moulting hormone bioassay, the results are given below.

	Dose µg.	Activity
XXVIa	100	25%
	10	24%
Control		20%

(iii) The residue originating from the extract XXVII (lower active band) was similarly subjected to p.t.l.c., and the bands detected had the appearance as shown in Figure 73.

FIGURE 73

Preparative Thin Layer Chromatogram of the Silylated Material from Extract XXVII



The bands at R_f 0.86, 0.80 and 0.72 were isolated using acetone and methanol, and the materials obtained were subjected to g.l.c. Complex patterns of peaks were observed t_R less than 10 minutes in each case, and no other peaks of any significance were observed for the bands at R_f 0.86 and 0.72. The band at R_f 0.80 showed traces of components in the range t_R 10 to 45 minutes when injected at high concentration, but derivatives of ecdysone and 20-hydroxyecdysone could not be

identified.

The material absorbed onto the area of silica in the R_f region 0.05 to 0.18 was isolated (R_f range for untreated extract XXVII under the above conditions) as an amorphous pale yellow solid (XXVIIa, approximately 2 mg.). The mass spectrum of this material and that of extract XXVII showed similar characteristics, and the material was tested for moulting activity with the result shown below.

	Dose µg.	Activity
XXVIIa	100	10%
	10	28%
Control		20%

Two Dimensional Preparative Thin Layer Chromatography of Derivatives of the Extracts XXV to XXVII

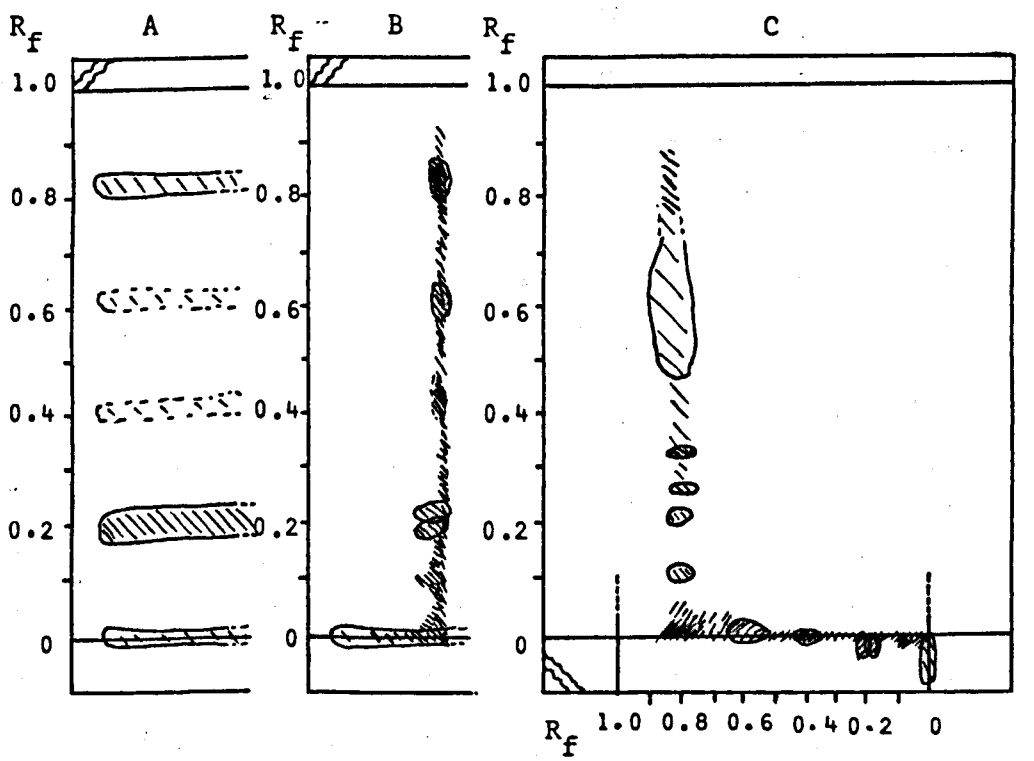
Portions of the extracts XXV to XXVII (6 mg.) were individually treated with O-methyl hydroxylamine hydrochloride in pyridine followed by B.S.A. as previously described. The silylated materials obtained in each case were subjected to two dimensional p.t.l.c. by the method described for gland extracts

(see page 324).

(i) The silylated material from extract XXVI (middle active band) was subjected to two dimensional p.t.l.c., and the appearance of the plate under U.V. after each of the three stages of elution A to C is shown in Figure 74.

FIGURE 74

Two Dimensional Preparative Thin Layer Chromatogram of the Silylated Material from Extract XXVI



The components of the two spots at co-ordinates R_f (.82, 0.10) and (0.82, 0.21), which corresponded to the expected positions of the two methoxime isomers of

tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) chromatographed under the same conditions, were isolated together using acetone and methanol. The material isolated was subjected to g.l.c. (see Figure 47, page 123) and this showed that it was a simple mixture containing only *syn*- and *anti*-tetrakis-TMSi-20--hydroxyecdysone methoxime as the major components. This was confirmed by g.l.c. using columns of OV-17 (1%) on CQ and OV-101 (1%) on CQ (see Figure 47), and by comparison of the mass spectrum (direct inlet) of the locust material with that of the authentic mixture of *syn*- and *anti*-tetrakis-TMSi-20--hydroxyecdysone methoxime (*20a*, see Figure 48 pages 124 to 127). From a measurement of the area under the peaks obtained using the column of OV-210 (3%) on CQ, the 20-hydroxyecdysone content of extract XXVI was recalculated to be approximately 0.92 mg.

The components of the spots at co-ordinates R_f (0.79, 0.26), (0.82, 0.32) and (0.82, 0.60) were also subjected to g.l.c. This showed that they corresponded to the components giving a complex pattern of peaks t_R less than nine minutes. Derivatives of ecdysone could not be detected at any stage.

(ii) The silylated materials from extracts XXV and XXVII (upper and lower active bands respectively) were also subjected to two dimensional p.t.l.c. as described above,

but even with the improved method of separation no derivatives of ecdysone or 20-hydroxyecdysone could be detected by g.l.c. in either case.

ISOLATION OF 20-HYDROXYECDYSONE FROM THE MIDDLE ACTIVE BAND XXVI

A further 1.8×10^4 nymphs were extracted using the standard extraction procedure to give a brown gum (LI, 16.1 g.) from the aqueous phase of the light petroleum - water partition. This was tested in the moulting hormone bioassay as a check on the activity of the extract and the results are given below.

	Dose µg.	Activity
	100	53%
LI	10	46%
	1	9%
Control		8%

Extract LI was subjected to column chromatography and P.L.C. as described in the standard extraction procedure and the material which corresponded to extract XXVI (middle active band) was isolated as a brown gum (LII, 0.544 g.). A portion of this extract (7 mg.) was treated with O-methyl hydroxylamine

hydrochloride in pyridine followed by B.S.A., and the mixture of reaction products was subjected to the procedure of two dimensional p.t.l.c. followed by g.l.c. using a column of OV-210 (3%) on CQ as described for extract XXVI. This showed that extract LII contained 20-hydroxyecdysone (approximately 1.23 mg.).

Extract LII was combined with the remaining 82% of extract XXVI (this had become discoloured on prolonged standing and was now dark red-brown in colour) to give the extract XXVIII (1.46 g.) which corresponded to the middle active band originating from approximately 3.2×10^4 nymphs.

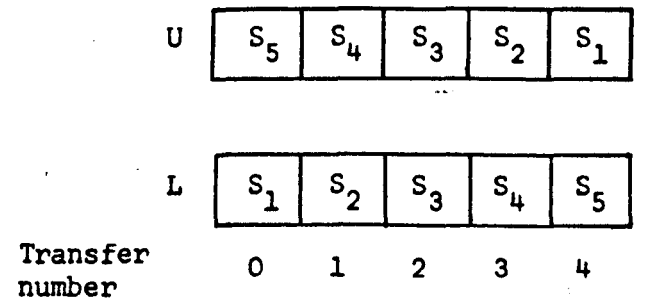
First Countercurrent Distribution

Extract XXVIII (1.46 g.) was subjected to countercurrent distribution using the solvent system described by Horn *et al.*[42]

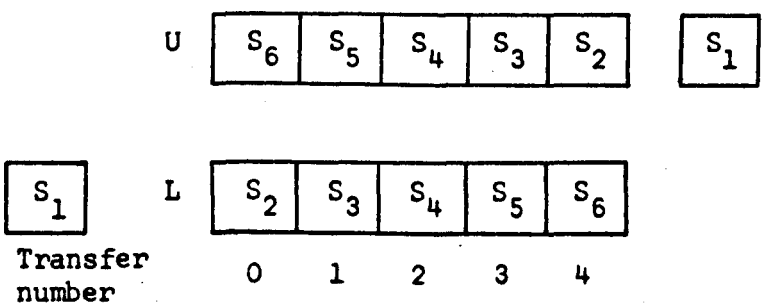
A solvent mixture of chloroform - ethanol - water (1:1:1) was prepared, and this gave two phases, upper phase and lower phase in the ratio 45:55 (v/v) at 23.5°. The extract was transferred in solution to the first of five separating funnels (100 ml.) using lower phase (44 ml.) and upper phase (36 ml.), potassium hydrogen carbonate (0.1 g.) was then added. The countercurrent distribution, using the double withdrawal procedure outlined diagrammatically below,[144] was then carried

out in the five funnels.

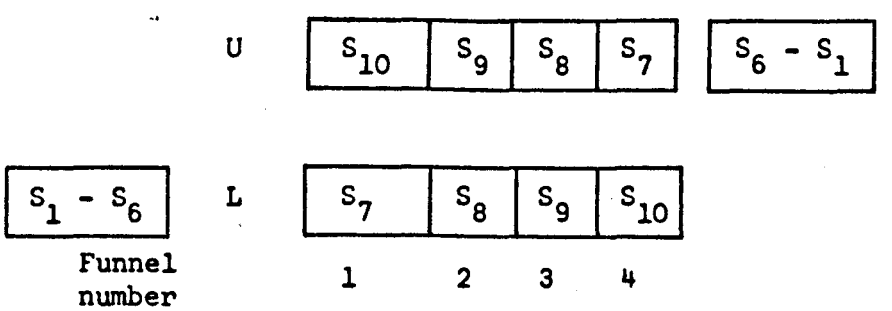
(i) Completed fundamental procedure



(ii) First double withdrawal



(iii) First separation of phases after five double withdrawals



S refers to the solvent introduced, and the index refers to the number of the stage at which the solvent was introduced.

The combined lower phases were evaporated to yield extract XXIX (553 mg.) as a yellow brown resin. Butanol (200 ml.) was added to the combined upper phases and the solution was evaporated until only butanol (approximately 20 ml.) remained as solvent, this was centrifuged and the insoluble residue which remained was washed with absolute ethanol (3 ml.). The butanol and ethanol solutions were combined and evaporated to give extract XXX (875 mg.) as a red-brown gum.

Portions of extracts XXIX and XXX were examined for 20-hydroxyecdysone by methoximation and silylation, followed by two dimensional p.t.l.c. and g.l.c. All the 20-hydroxyecdysone was detected in extract XXIX (determined by g.l.c. to be approximately 1.99 mg.) and none could be detected in extract XXX. 20-Hydroxyecdysone could not however be detected in either extract by t.l.c. using vanillin spray. The two extracts were tested for moulting activity and the results are shown below.

	Activity at dose: 100 µg.	10 µg.
XXIX	50%	59%
XXX	22%	14%
Control (both doses)	20%	

Second Countercurrent Distribution

The remaining portion of extract XXIX (545 mg. equivalent to approximately 3.15 x 10^4 nymphs) was subjected to countercurrent distribution using the solvent system described by Kaplanis *et al.*[24]

A solvent mixture of ethyl acetate - water (1:1) was prepared, and this gave two phases, upper phase and lower phase in the ratio approximately 1:1 (v/v) at 24°. The soluble portion of the extract was transferred to the first of eight separating funnels (100 ml.) using lower phase and upper phase (each 25 ml.). An insoluble residue remained as a red-brown powder (LIII, 58 mg.). The soluble material was subjected to countercurrent distribution using the procedure outlined for extract XXVIII. The combined lower phases were evaporated to yield extract XXXI (291 mg.) as a pale yellow resin, the combined upper phases gave extract XXXII (190 mg.) as a brown resin on evaporation.

The 20-hydroxyecdysone content of portions of extracts XXXI and XXXII was determined. All the 20-hydroxyecdysone was detected in extract XXXI (determined by g.l.c. to be approximately 1.78 mg.) and none could be detected in extract XXXII. 20-Hydroxyecdysone could not be detected in either extract by t.l.c. using vanillin spray.

The extracts XXXI and XXXII were tested for activity in the moulting hormone bioassay and the results are given below.

	Activity at dose: 100 µg.	10 µg.	1 µg.
XXXI	86%	50%	33%
XXXII	13%	27%	
Control (all doses)		20%	

Column Chromatography of Extract XXXI on CM-Sephadex[42]

CM-Sephadex (type C-25, 100 g.) was prepared for use according to the procedure given in the Pharmacia booklet 'Sephadex Ion Exchangers'. The prepared gel was equilibrated for 24 hours with water, then approximately 70% of it was packed as an aqueous slurry into a glass gel filtration column (length 85 cm. internal diameter 1.6 cm.). The column was calibrated and the homogeneity of the bed was checked by absorbing an aqueous solution of blue dextran 2000 (4 ml., 0.1%) onto the top of the column and eluting with water. The blue dextran 2000 was eluted as a compact zone (9 ml.), and indicated a void volume for the column of 62 ml.

Extract XXXI (285 mg., equivalent to approximately 3.08×10^4 nymphs) was dissolved in water (7 ml.) containing absolute ethanol (5%), and the solution was absorbed onto the top of the sephadex, the column was eluted with water and 71 fractions (10 ml.) were collected.

The fractions collected were monitored by t.l.c. eluting with chloroform - 95% ethanol (4:1), and the spots were visualised with vanillin spray. Fractions 18 to 21 were found to contain appreciable quantities of 20-hydroxyecdysone (R_f 0.17, an authentic sample of 20-hydroxyecdysone chromatographed on the same plate had an R_f of 0.17), and each gave the characteristic colour reactions for 20-hydroxyecdysone with vannilin spray (see Table 37, page 268). Traces of 20-hydroxyecdysone were also present in fractions 17 and 22. The following fractions were recombined and evaporated; fractions 1 to 17 gave extract XXXIV (29 mg.) as a colourless gum, fractions 18 to 21 which contained most of the 20-hydroxyecdysone gave extract XXXIII (35 mg.) as a pale yellow gum, and fractions 22 to 71 gave extract XXXV (69 mg.) as a bright yellow solid.

Extracts XXXIII to XXXV were tested for moulting activity with the result shown below.

	Activity at dose: 100 µg.	10 µg.	1 µg.
XXXIII	100%	60%	38%
XXXIV	40%	38%	22%
XXXV	34%	40%	25%
Control (all doses)		25%	

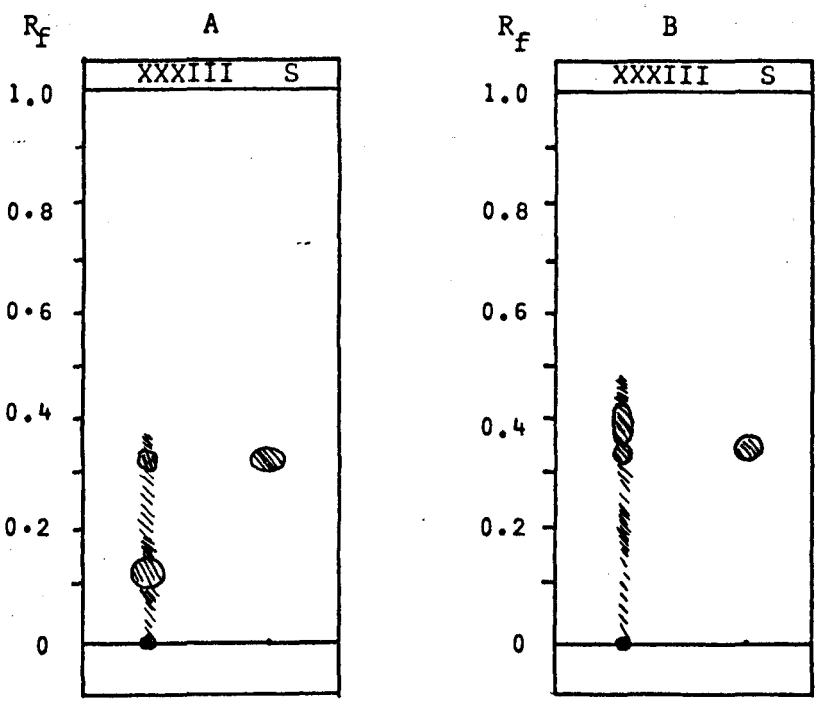
Extract XXXIII was compared with authentic 20-hydroxyecdysone on t.l.c. eluting with ethyl acetate - ethanol (8:2, 20-hydroxyecdysone R_f 0.32) and chloroform - methanol - acetone (6:2:1, 20-hydroxyecdysone R_f 0.33). In each case visualisation under U.V. showed a major spot which had the same R_f as the standard and showed the colour reactions of 20-hydroxyecdysone with vanillin spray.

The U.V. spectrum of extract XXXIII (see Figure 49, page 135) however showed no $\lambda_{max.}$ at 244 nm.[61] The t.l.c. plates eluted with the solvent systems described above were therefore heated (130°) until the vanillin spray had completely charred the absorbed compounds. The results showed that 20-hydroxyecdysone no longer corresponded to the major spot observed for extract XXXIII (see Figure 75), and this was represented by material at R_f 0.13 in ethyl acetate - ethanol (8:2) and R_f 0.37

in chloroform - methanol - acetone (6:2:1).

FIGURE 75

Thin Layer Chromatograms of Extract XXXIII and Authentic 20-Hydroxyecdysone (S, standard) after Charring with Vanillin Spray



A Ethyl acetate - ethanol (8:2)

B Chloroform - methanol - acetone (6:2:1)

Preparative Thin Layer Chromatography of Extract XXXIII

The remaining extract XXXIII (33 mg., equivalent to approximately 2.90×10^4 nymphs) was subjected to p.t.l.c. (using two 20 x 20 cm. plates) eluting with ethyl acetate - ethanol (8:2). The band corresponding to the R_f (0.32) of 20-hydroxyecdysone was located under U.V. (see Figure 76), and the absorbed material was isolated from this band (extract XXXVI) and from the region R_f 0.00 to 0.30 (extract XXXVII) which included the material only visualised on charring with vanillin spray. The two new extracts were assayed for moulting activity and the results are given in Table 43 accompanying Figure 76.

FIGURE 76

P.t.l.c. of Extract XXXIII

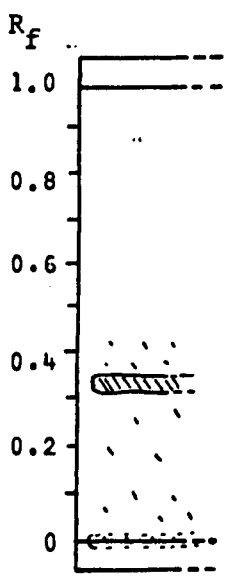


TABLE 43

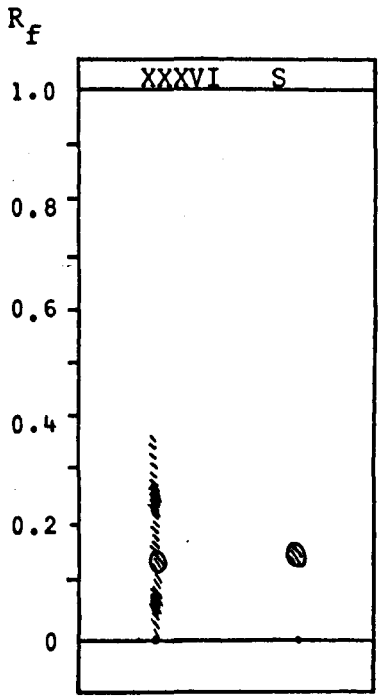
Cut	Extract	R_f range	Recovered mg.	Activity at dose: 100 µg.	10 µg.	1 µg.
	XXXVI	0.30 - 0.34	7.4	100%	89%	22%
	XXXVII	0.00 - 0.30	19.4	44%	36%	20%
	Control (all doses)				11%	

T.l.c. of extract XXXVI under the same conditions as those for p.t.l.c. followed by charring of the chromatogram with vanillin spray showed that it contained none of the material with a R_f of 0.13. The U.V. spectrum of extract XXXVI (see Figure 49, page 135) showed a shoulder at 244 nm. (95% EtOH) (estimated ε = 2000, $\log_{10}\varepsilon$ = 3.30, authentic 20-hydroxyecdysone, lit.[61] ε = 12400, $\log_{10}\varepsilon$ = 4.09) which indicated that it contained approximately 16% 20-hydroxyecdysone.

Portions of extract XXXVI were subjected to t.l.c. on silica gel PF_{254} plates and prepared sheets of aluminium oxide F_{254} (neutral type T, Merck, deactivated in the atmosphere over saturated aqueous sodium chloride solution) using the following eluting solvents: chloroform - methanol - acetone (6:2:1), acetonitrile - methanol (7:3) and ethyl acetate - methanol (8:2). A separation of components in the extract was observed on aluminium oxide sheet after eluting with chloroform - methanol - acetone (6:2:1) and visualising with vanillin spray followed by charring (see Figure 77, a reference sample of 20-hydroxyecdysone, R_f 0.16, was also chromatographed).

FIGURE 77

Thin Layer Chromatogram of Extract XXXVI and Authentic 20-Hydroxyecdysone (S, standard) on Aluminium Oxide Sheet after Charring with Vanillin Spray



Preparative Thin Layer Chromatography of Extract XXXVI

The remaining extract XXXVI (5.9 mg., equivalent to approximately 2.31×10^4 nymphs) was subjected to p.t.l.c. on a 20 x 20 cm. aluminium oxide F_{254} sheet (neutral type T, Merck, deactivated in the atmosphere over saturated aqueous sodium

chloride solution) eluting with chloroform - methanol - acetone (6:2:1). The band corresponding to the R_f (0.16) of 20-hydroxyecdysone was located under U.V. (see Figure 78), and the absorbed material was isolated from this band (extract XXXVIII) and the regions R_f 0.00 to 0.14 (extract XXXIX) and 0.18 to 0.65 (extract XL) on either side. The extracts XXXVIII to XL were assayed for moulting activity and the results are given in Table 44 accompanying Figure 78.

FIGURE 78

P.t.l.c. of Extract XXXVI

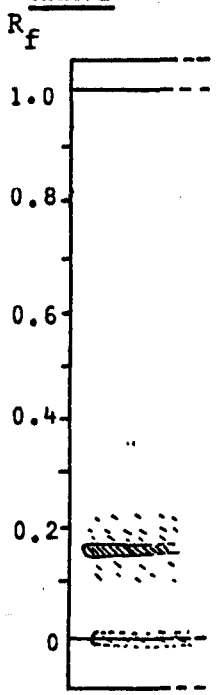


TABLE 44

Cut	Extract	R_f range	Recovered mg.	Activity at dose: 100 µg.	10 µg.	1 µg.
	XL	0.18 - 0.65	2.8	67%	66%	
	XXXVIII	0.14 - 0.18	1.2		100%	100%
	XXXIX	0.00 - 0.14	0.6		67%	50%
	Control (all doses)				17%	

Extract XXXVIII also showed 100% activity at a dose of 40 µg. and 75% activity at a dose of 0.1 µg.

T.l.c. of extract XXXVIII under the same conditions as those used for p.t.l.c. followed by visualisation with vanillin spray gave a single spot which had identical colour reactions and the same R_f (0.16) as an authentic sample of 20-hydroxyecdysone chromatographed on the same layer. The U.V. spectrum of extract XXXVIII (see Figure 49, page 135) exhibited a $\lambda_{max.}$ (95% EtOH) at 244 nm. (ε = 7900, $\log_{10}\varepsilon$ = 3.89) which indicated that it contained 64% 20-hydroxyecdysone.

Crystallisation of the remaining extract XXXVIII from ethyl acetate gave 20-hydroxyecdysone as white prisms m.p. 235 - 238° (decomp.) {lit.,[61] 241 - 242.5° (decomp.); mixed melting point with an authentic sample of 20-hydroxyecdysone 235 - 239° (decomp.)}.

DETERMINATION OF THE 20-HYDROXYECDYSONE CONTENT OF NYMPHS AT DAILY INTERVALS THROUGHOUT THE 5TH INSTAR BY GAS-LIQUID CHROMATOGRAPHY

Gregarious nymphs collected in the middle of the 4th instar were supplied by the Anti-Locust Research Centre, these were placed in cages at Keele, and reared on a diet of fresh grass. They were exposed to a daily photoperiod of 15 hours light and 9 hours dark. Cage lights gave the nymphs a temperature range of 29 to 40° during the light period, and the temperature dropped to a minimum of 26° during the dark period. The 5th instar lasted for approximately 12 days under these conditions.

Extraction of Nymphs

Groups of 8 to 10 nymphs were selected for extraction at daily intervals (day one represents 24 hours after the previous ecdysis) and were subjected to the following procedure.

The nymphs were anaesthetised with carbon dioxide and then homogenised with methanol (200 ml.) in a Waring blendor, this produced a methanol homogenate which had a similar consistency to that used for the large scale extraction of whole insects. The methanol homogenate was filtered as previously

described, and the filtrate evaporated to give an aqueous concentrate. This was partitioned between butanol (25 ml.) and water (35 ml.) and the phases were treated by the method described in the modified extraction of whole insects. The residue obtained after evaporation of the butanol was partitioned directly between light petroleum (14 ml.) and aqueous methanol (4:1, 28 ml.), and the two phases were also treated as described in the modified extraction procedure, but using aqueous methanol (4:1) instead of water. The aqueous methanol extract yielded a yellow-brown gum on evaporation. The weights of material obtained are given in Table 45.

TABLE 45

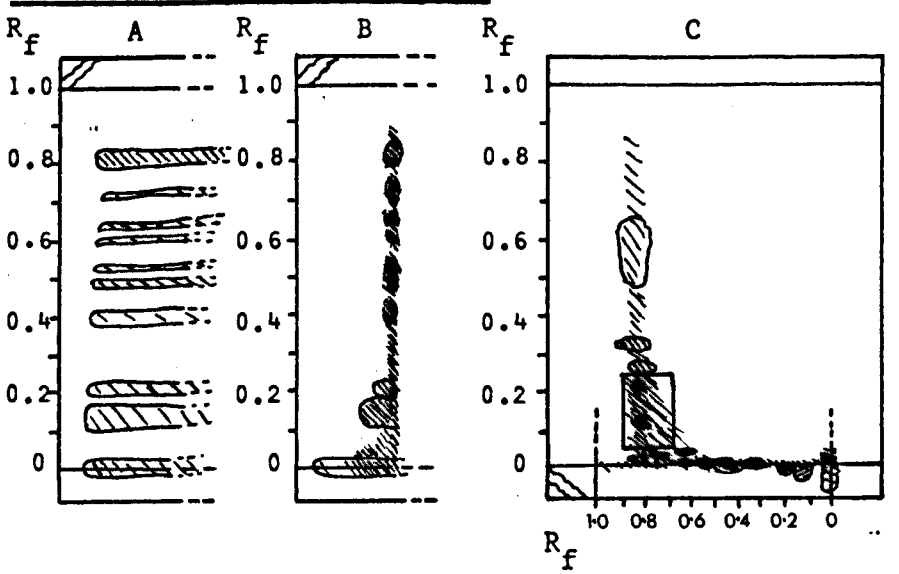
Instar	Day	Number of nymphs extracted	Butanol extract mg.	Aqueous methanol extract mg.
4th	8	10	166	6.9
	0	10	159	7.1
	1	9	146	3.8
	2	10	205	8.0
	3	10	240	8.0
	4	10	347	5.0
	5	8	320	8.3
5th	6	10	254	5.0
	7	10	460	7.0
	8	10	364	7.9
	9	10	286	8.0
	10	10	399	12.0
	11	10	445	8.0
	12	10	427	6.0
	0	10	411	5.6
	1	10	499	5.0
	2	10	520	3.9
Adults	3	10	586	4.0
	4	10	595	4.2
	14	10	643	10.2

Preparation of Nymph Extracts for Gas-Liquid Chromatography

In each case the extract from the aqueous methanol phase was treated with O-methyl hydroxylamine hydrochloride in pyridine followed by B.S.A. according to the standard procedure previously described. The residue obtained was then subjected to two dimensional p.t.l.c. using the method described for prothoracic gland extracts (see page 324). Some variation in the relative intensity and the number of the bands observed on p.t.l.c. occurred for different extracts, but a number of similar bands were observed for all the residues. The appearance of a typical plate under U.V. after each of the three stages, A to C, in the separation is shown in Figure 79.

FIGURE 79

Two Dimensional Preparative Thin Layer Chromatogram of a Typical Silylated Residue Originating from a Group of 5th Instar Nymphs (Day Three)



For each residue chromatographed as described above, the area of silica corresponding to the rectangle marked in Figure 79 (R_f region for the *syn*- and *anti*-isomers of tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) chromatographed under the same conditions) was scraped off and the absorbed material isolated using acetone and methanol. The material isolated was dissolved in acetone (3 µl.) and an aliquot (1 µl.) was injected onto a column of OV-210 on CQ using a sensitivity setting of 1×10^{-10} A (full scale deflection). Where peaks were observed which indicated that the original extract had contained 20-hydroxyecdysone, their areas were measured. The 20-hydroxyecdysone content of the extract was calculated from the calibration curve shown in Figure 32 (see page 93); the results are given in Table 46 and the variation in the 20--hydroxyecdysone titre per nymph throughout the 5th instar is plotted in Figure 53 (see page 143). The g.l.c. traces showed several common features and two typical traces (days 3 and 5 of the 5th instar) are shown in Figures 51 and 52 (see page 142). Under the conditions described above the two isomers of tetrakis--TMSi-ecdysone methoxime would have been located in two dimensional p.t.l.c. at the co-ordinates R_f (0.82, 0.20) and (0.82, 0.31). One of these would therefore have been eluted

for g.l.c. along with the 20-hydroxyecdysone derivatives. No peak corresponding to either of the ecdysone derivatives was however detected in g.l.c. for any of the extracts.

TABLE 46

Instar	Day	Number of nymphs extracted	Peak area sq. in.	20-Hydroxyecdysone in extract µg.	per nymph ng.
4th	8	10	0.60	1.04	104
	0	10	1.68	2.94	294
	1	9	0.99	1.73	192
	2	10	0.57	0.99	99
	3	10	0.98	1.71	171
	4	10	0.08	0.13	13
	5	8	0.16	0.29	36
5th	6	10	0.12	0.20	20
	7	10	0.25	0.44	44
	8	10	0.29	0.50	50
	9	10	0.23	0.39	39
	10	10	0.64	1.13	113
	11	10	0.79	1.37	137
	12	10	0.53	0.92	92
	0	10	1.55	2.70	270
	1	10	0.94	1.62	162
	2	10	0.92	1.59	159
Adults	3	10	0.12	0.20	20
	4	10	0.14	0.23	23
	14	10	0	0	0

Estimation of the Limit of Detection for the Procedure used to Determine 20-Hydroxyecdysone in Nymphs at Daily Intervals

Groups of 5 nymphs in the 14th day of adult development were extracted by the procedure used to determine the 20-hydroxyecdysone content of nymphs at daily intervals. At the start of each extraction a known amount of 20-hydroxyecdysone (in the range 0.2 to 10 µg.) dissolved in methanol was added to the methanol extract obtained for each group of nymphs. The extracts were prepared for g.l.c. as described for the extracts from nymphs taken at daily intervals. The results from g.l.c. showing the percentage recovery of 20-hydroxyecdysone are given in Table 47 and Figure 54 (see page 149).

TABLE 47

Weight of 20-hydroxyecdysone added µg.	Weight of 20-hydroxyecdysone determined from g.l.c. µg.	Percentage recovery
10.0	9.2	92%
1.00	0.67	67%
	0.77	77%
0.20	0	0%
	0	0%

INVESTIGATION OF PROTHORACIC GLANDS FOR MOULTING ACTIVITY

Freeze-dried extracts of prothoracic glands were prepared at the Anti-Locust Research Centre using the following procedure. Prothoracic glands were dissected from nymphs in the 8th to 11th day of the 5th instar and were homogenised with either water, aqueous ethanol (1:1) or ethanol. The homogenates were centrifuged and the resulting supernatants subjected to freeze-drying. Extracts prepared by the above procedure exhibited positive activity in the moulting hormone bioassay as shown in Figure 55 (see page 152).

General Purification Procedure

The following general procedure was used to examine extracts of 300 to 400 prothoracic glands for moulting activity. The freeze-dried extract (2-3 mg.) was partitioned between water (7 ml.) and butanol (5 ml.), and the aqueous phase was washed with butanol (3 ml. and 2 x 1 ml.). Similar weights of material were obtained in the aqueous and butanol extracts, regardless of which solvent was used in the homogenation. The aqueous extract gave, on evaporation, a residue (1-2 mg.) which was inactive when tested in the moulting hormone bioassay at doses of 200, 100 and 60 µg. per abdomen.

The butanol extract (0.7-1 mg.) was subjected to p.t.l.c. on a 5 x 20 cm. plate eluting with chloroform - 95% ethanol (4:1). Bands were detected by U.V. and areas of silica gel were scraped off and the absorbed material eluted with methanol directly into the sample tubes used to send the material for bioassay. The methanol was evaporated with a stream of nitrogen. Samples recovered from chromatograms were dissolved in water (400 µl.) and assayed at a dose of 20 µl. per abdomen, the solutions were diluted for lower doses. The results of homogenates prepared in water, aqueous ethanol (1:1), or ethanol follow.

(i) Prothoracic Glands Homogenised in Water

The freeze-dried extract (2.8 mg.) prepared from an aqueous homogenate of 400 prothoracic glands (each nymph has two glands) was subjected to the above procedure, and the bands detected had the appearance as shown in Figure 80. The areas of silica cut from the plate and the activities of the eluted material in the moulting hormone bioassay are given in Table 48 and in Figure 56 (see page 154).

FIGURE 80

P.t.l.c. of Glands
(Aqueous Extraction)

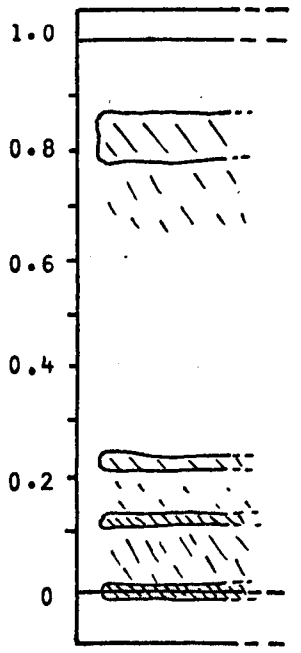


TABLE 48

Cut	Area	R_f range	Activity at dose: 20 µl.	5 x diluted
5		0.70 - 1.00	60%	17%
4		0.40 - 0.70	75%	60%
3		0.30 - 0.40	28%	22%
2		0.10 - 0.30	86%	83%
1		0.00 - 0.10	67%	78%
Control (both doses)				14%

(ii) Prothoracic Glands Homogenised in Aqueous Ethanol (1:1)

The freeze-dried extract (2.1 mg.) prepared from an aqueous ethanol (1:1) homogenate of 400 prothoracic glands was subjected to the general purification procedure. The bands detected had the appearance as shown in Figure 81. The areas of silica cut from the plate and the activities of the eluted

material in the moulting hormone bioassay are given in Table 49, and in Figure 57 (see page 154).

FIGURE 81

P.t.l.c. of Glands (aqueous Ethanol (1:1) Extraction)

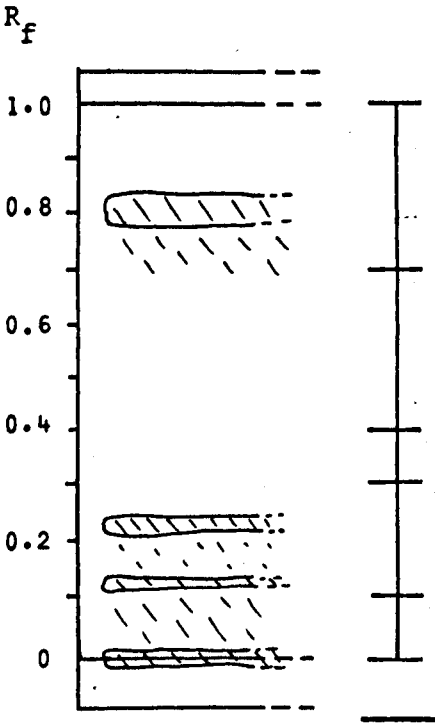


TABLE 49

Cut	Area	R_f range	Activity at dose: 20 µl.	5 x diluted
5		0.70 - 1.00	22%	44%
4		0.40 - 0.70	78%	86%
3		0.30 - 0.40	62%	37%
2		0.10 - 0.30	44%	38%
1		0.00 - 0.10	56%	86%
Control (both doses)				14%

(iii) Prothoracic Glands Homogenised in Ethanol

The freeze-dried extract (2.8 mg.) prepared from an ethanol homogenate of 300 prothoracic glands was subjected to

the procedure described above. The appearance of the bands is shown in Figure 82 and the results are given in Table 50, and in Figure 58 (see page 155).

FIGURE 82

P.t.l.c. of Glands
(Ethanol Extraction)

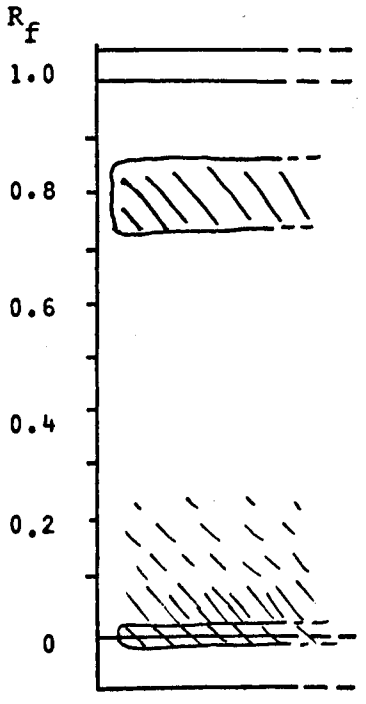


TABLE 50

Cut	Area	R_f range	Activity at dose: 20 µl.*	5 x diluted
5		0.70 - 1.00		20%
4		0.40 - 0.70		57%
3		0.30 - 0.40		28%
2		0.10 - 0.30		60%
1		0.00 - 0.10		50%
Control				25%

*Several abdomens died at this dose level and the results were unsatisfactory. This was not the fault of the extract.

INVESTIGATION OF PROTHORACIC GLANDS FOR 20-HYDROXYECDYSONE BY GAS-LIQUID CHROMATOGRAPHY

Freeze-dried extracts of prothoracic glands were prepared at the Anti-Locust Research Centre from aqueous homogenates as previously described. G.l.c. was carried out under the conditions described on page 311.

Preliminary Investigations

(i) The freeze-dried extract (1.2 mg.) prepared from 100 prothoracic glands removed from nymphs in the 8th to 11th day of the 5th instar was partitioned between butanol and water as previously described and the material from the butanol phase was partitioned directly between light petroleum (2.5 ml.) and aqueous methanol (7:3, 5 ml.), and the two phases were washed as described in the modified extraction of whole insects. The resulting aqueous methanol (7:3) and light petroleum phases were evaporated to yield hydrophilic and lipophilic extracts respectively.

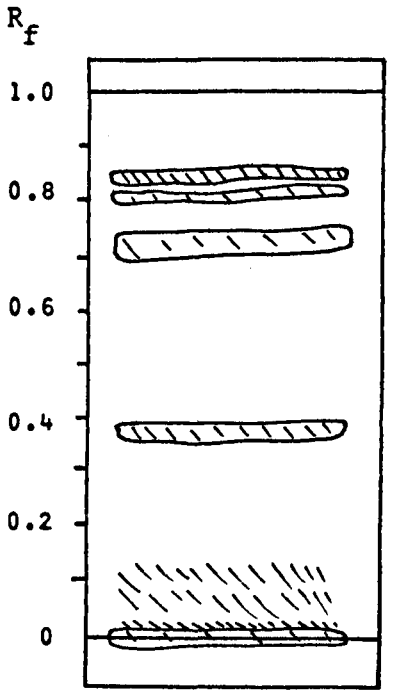
The hydrophilic extract was treated with O-methyl hydroxylamine hydrochloride in pyridine followed by B.S.A. according to the standard procedure previously described. A portion (10%) of the silylated residue was subjected directly

to g.l.c. This showed a complex pattern of peaks t_R less than four minutes and only traces of components having longer retention times were detected.

The remainder of the residue was subjected to p.t.l.c. on a 5 x 20 cm. plate eluting with chloroform - 95% ethanol (4:1). Bands detected by U.V. had the appearance as shown in Figure 83.

FIGURE 83

Preparative Thin Layer Chromatogram of the Silylated Hydrophilic Extract from 100 Prothoracic Glands



The material from the three bands in the R_f region 0.70 - 0.84 was isolated using acetone and methanol, g.l.c. showed essentially the same result as that for the crude extract above. No derivatives of 20-hydroxyecdysone or ecdysone were detected.

The lipophilic extract was also treated with O-methyl hydroxylamine hydrochloride in pyridine followed by B.S.A. as described for the hydrophilic extract, to give a silylated residue. This residue was run on t.l.c. eluting with chloroform - 95% ethanol (4:1), visualisation under U.V. showed an elongated spot in the region R_f 0.70 to 0.85.

(ii) The freeze-dried extract (1.4 mg.) prepared from a further 100 prothoracic glands removed from nymphs in the 8th to 11th day of the 5th instar was treated as described above to give a residue from the butanol phase. This was treated with O-methyl hydroxylamine hydrochloride in pyridine followed by B.S.A. as previously described.

The silylated residue from the lipophilic extract isolated in (i) showed components in the R_f region 0.70 to 0.85 when run on t.l.c. eluting with chloroform - 95% ethanol (4:1), and when tetrakis-TMSi-20-hydroxyecdysone methoxime (*20a*) was chromatographed under the same conditions it gave a spot R_f 0.82. Another solvent system was therefore required to chromatograph the silylated residue which contained the hydrophilic and lipophilic materials as a mixture. The silylated lipophilic

residue from (i) and a reference sample of tetrakis-TMSi-20--hydroxyecdysone methoxime (*20a*) were subjected to t.l.c. The eluting solvents used and the results obtained after visualising under U.V. are summarised below.

Eluting solvents	Silylated lipophilic residue R_f	Tetrakis-TMSi-20--hydroxyecdysone methoxime (*20a*) R_f
Light petroleum	0.00	0.00
Light petroleum-ether (4:1)	0.00 - 0.08 0.33 - 0.42 0.65 - 0.70	0.10 0.21
Light petroleum-ether--EtOH (78:20:2)*	" "	"

*Same result as light petroleum-ether (4:1)

The silylated residue containing both the hydrophilic and lipophilic materials was subjected to p.t.l.c. on a 5 x 20 cm. plate eluting with light petroleum - ether (4:1). The silica gel within the R_f region 0.08 - 0.25 was scraped off the plate and the absorbed material isolated using acetone and methanol. The g.l.c. of this material showed a complex pattern of peaks t_R less than seven minutes and only traces of components having longer

retention times were detected. Peaks corresponding to derivatives of ecdysone and 20-hydroxyecdysone could not be identified.

Investigation of Prothoracic Glands at Daily Intervals Throughout the 5th Instar by Gas-Liquid Chromatography

Freeze-dried extracts of 100 prothoracic glands were prepared at daily intervals throughout the 5th instar,* and these were individually subjected to the following procedure. Each extract was treated with O-methyl hydroxylamine hydrochloride in pyridine followed by B.S.A. as previously described in the standard procedure, and the silylated residue was subjected to two dimensional p.t.l.c. as follows.

Stage A The material was applied as a narrow band (5 cm. in length) to the lower left hand corner of a 20 x 20 cm. p.t.l.c. plate. This was eluted with chloroform - 95% ethanol (4:1) followed by drying with a stream of nitrogen.

Stage B The plate was then eluted twice with chloroform - 95% ethanol (1:1) at right angles to the initial

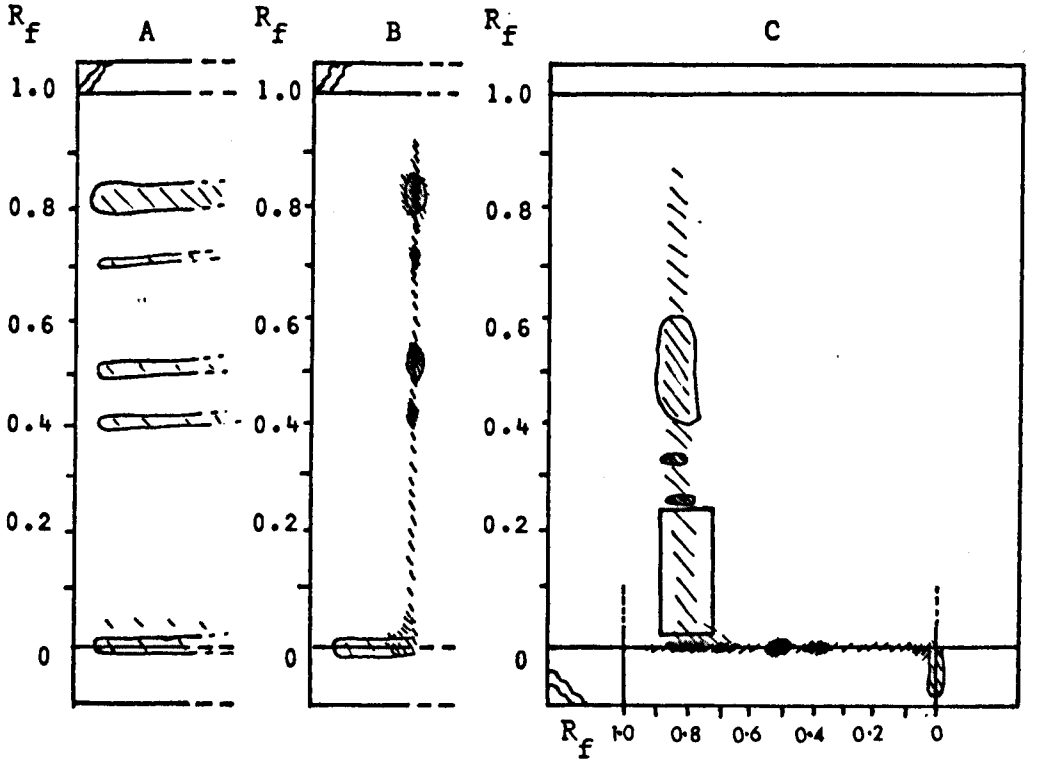
*Under the conditions used to rear nymphs at the Anti-Locust Research Centre the instar lasted 10 to 11 days. The insects were reared in a room maintained at 26°. Room lighting was provided for 12 hours a day and for eight of these additional cage lights gave locusts a range of temperatures from 26 to 40°.

direction of elution, just sufficiently to close up the bands into a line of spots, after each elution the plate was dried with a stream of nitrogen.

Stage C The spots were then eluted with light petroleum - ether (4:1) again at right angles to the initial direction of elution, and the plate was dried with a stream of nitrogen. The appearance of the p.t.l.c. plates under U.V. were similar for the extracts of glands from each day of the instar, and the appearance of a typical plate at each of the three stages, A to C, of the separation is shown in Figure 84.

FIGURE 84

Two Dimensional Preparative Thin Layer Chromatogram of a Typical Silylated Residue Originating from 100 Prothoracic Glands (Day Three)



For each residue chromatographed as described above, the area of silica corresponding to the rectangle marked in Figure 84 (R_f region for the *syn*- and *anti*-isomers of tetrakis--TMSi-20-hydroxyecdysone methoxime (*20a*) chromatographed under the same conditions) was scraped off and the absorbed material isolated using acetone and methanol. The material isolated was dissolved in acetone (3 µl.) and an aliquot (1 µl.) was injected onto a column of OV-210(3%)on CQ using a sensitivity setting of 1×10^{-10} A for full scale deflection.

The gland extracts from each day in the 5th instar all gave similar g.l.c. results when treated under the conditions described above. In each case a complex pattern of peaks was observed t_R less than 10 minutes, and only traces of components having longer retention times were detected. Two typical g.l.c. traces (days three and five of the 5th instar) are shown in Figures59 and 60 (see page 164). Peaks corresponding to derivatives of ecdysone and 20-hydroxyecdysone could not be identified.

INVESTIGATION OF BLOOD FROM 5TH INSTAR NYMPHS FOR MOULTING ACTIVITY

Freeze-dried blood supernatant was prepared at the Anti-Locust Research Centre by removing blood from nymphs using a Hamilton syringe and a number 30 needle, centrifuging to remove cells and suspended material, and subjecting the cell free supernatant to freeze-drying.

General Purification Procedure

The freeze-dried portions of supernatant from groups of 40 nymphs were examined for substances with moulting activity by partitioning the material between water and butanol, and then partitioning the butanol extract between light petroleum and aqueous methanol (4:1) as described in previous procedures.

The aqueous methanol (4:1) extracts were subjected to p.t.l.c. on 5 x 20 cm. plates eluting with chloroform - 95% ethanol (4:1). Bands were detected by U.V. and areas of silica gel were scraped off and the absorbed material eluted with methanol directly into the sample tubes used to send the material for bioassay. The methanol was evaporated with a stream of nitrogen. Samples recovered from chromatograms were dissolved in water (400 µl.) and assayed at a dose of

20 µl. per abdomen. The results for blood supernatants prepared from 5th instar nymphs follow.

(i) Early 5th Instar Blood

The results for freeze-dried blood supernatant (310 mg.) prepared from 40 nymphs in days one to five of the 5th instar are summarised below, and in Figure 85 and in the accompanying Table 51.

Solvent partition	Extract	Weight mg.	Activity at 50 µg. dose
Water-butanol	Aqueous	281	
	Butanol	21	23%
Light petroleum--aqueous methanol (4:1)	Light petroleum	16	
	Aqueous methanol (4:1)	3.5	
	Control		16%

FIGURE 85

P.t.l.c. of Blood Extract (Early 5ths)

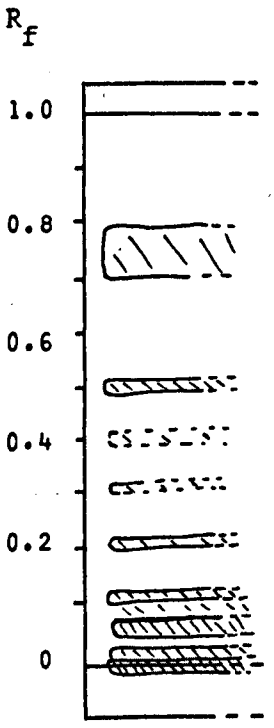


TABLE 51

Cut	Area	R_f range	Activity at dose: 20 µl.
5		0.70 - 1.00	13%
4		0.40 - 0.70	13%
3		0.30 - 0.40	14%
2		0.10 - 0.30	25%
1		0.00 - 0.10	10%
Control			16%

(ii) Late 5th Instar Blood

The freeze-dried supernatant (245 mg.) prepared from 40 nymphs in days 6 to 11 of the 5th instar was subjected to the procedure previously described and the results are summarised

below, and in Figure 86 and the accompaying Table 52.

Solvent partition	Extract	Weight mg.	Activity at 50 µg. dose
Water-butanol	Aqueous	221	
	Butanol	19	25%
Light petroleum--aqueous methanol (4:1)	Light petroleum	14	
	Aqueous methanol (4:1)	3.5	
	Control		12%

FIGURE 86

P.t.l.c. of Blood Extract (Late 5ths)

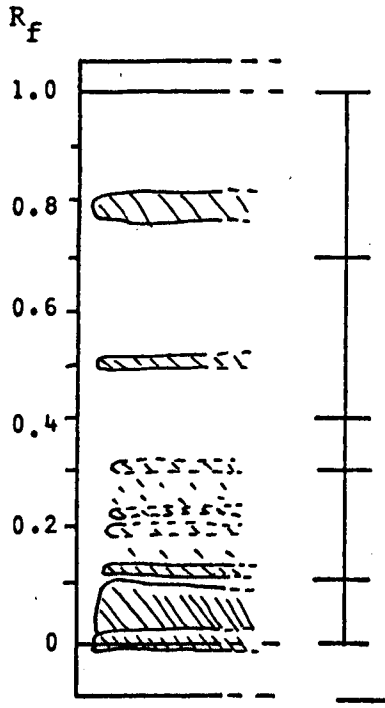


TABLE 52

Cut	Area	R_f range	Activity at dose: 20 µl.
	5	0.70 - 1.00	22%
	4	0.40 - 0.70	10%
	3	0.30 - 0.40	14%
	2	0.10 - 0.30	29%
	1	0.00 - 0.10	11%
	Control		12%

The variation of moulting activity with R_f for the early and late 5th instar blood samples is plotted in Figure 61 (see page 171).

INVESTIGATION OF BLOOD FROM 5TH INSTAR DESERT LOCUSTS FOR 20-HYDROXYECDYSONE BY GAS-LIQUID CHROMATOGRAPHY

Freeze-dried blood supernatant was prepared at the Anti-Locust Research Centre as previously described. The blood cells and suspended material removed by centrifugation were extracted with aqueous ethanol (1:1), centrifuged, and the supernatant subjected to freeze-drying.

Purification of Extracts

The freeze-dried materials from the centrifuged blood and blood cells were prepared from two groups of locusts, A and B, each containing 25 nymphs in day 10 of the 5th instar. These materials were partitioned between butanol and water as described for the groups of nymphs taken at daily intervals throughout the 5th instar. The weights of material obtained are given in Table 53.

TABLE 53

Group	Freeze-dried material	Extract	Weight mg.
A	Blood supernatant	Aqueous	395
		Butanol	17
	Cell extract	Aqueous	17
		Butanol	2.2
B	Blood supernatant	Aqueous	363
		Butanol	18
	Cell extract	Aqueous	15
		Butanol	2

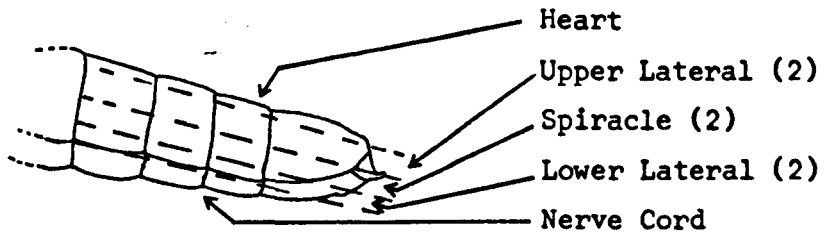
Preparation of Extracts for Gas-Liquid Chromatography

Each of the butanol extracts described above was treated with O-methyl hydroxylamine hydrochloride in pyridine followed by B.S.A., and the silylated residues obtained were subjected to two dimensional p.t.l.c. as previously described.

In each case the absorbed material was isolated from the area of silica gel corresponding to the R_f region for tetrakis--TMSi-20-hydroxyecdysone methoxime (*20a*). The materials were each dissolved in acetone (3 µl.) and an aliquot (1 µl.) was injected onto a column of OV-210 (3%) on CQ using a sensitivity setting of 1×10^{-10} A for full scale deflection. All the materials gave a complex pattern of peaks t_R less than nine minutes and only traces of components having longer retention times were detected. No peaks indicating the presence of ecdysone or 20-hydroxyecdysone, in the original extract, were identified. Similar results were obtained when the above procedure was repeated for the freeze-dried blood supernatants.

INVESTIGATION OF OENOCYTE BEARING TISSUE FROM 5TH INSTAR NYMPHS FOR 20-HYDROXYECDYSONE BY GAS-LIQUID CHROMATOGRAPHY

Freeze-dried extracts of oenocyte bearing fat body and epidermal tissue were prepared at the Anti-Locust Research Centre using the following procedure. For the epidermal tissue, strips were removed from the abdomens of 20 nymphs in the 10th day of the 5th instar as illustrated below.



The strips of tissue were homogenised with aqueous ethanol (1:1); the homogenates were centrifuged and the resulting supernatants freeze-dried. The fat bodies were removed from the same nymphs and were treated as for the tissue strips to give a freeze-dried extract.

Initial Purification Procedure

The freeze-dried materials were extracted by the procedure described for the aqueous concentrates originating from the groups of nymphs taken at daily intervals throughout

the 5th instar, to give the aqueous methanol (4:1) extracts from the second solvent partition. The weights of material obtained are given in Table 54.

TABLE 54

Tissue* (epidermis)	Material from 1st partition, mg.		Material from 2nd partition, mg.	
	Aqueous	Butanol	Light petroleum	Aqueous methanol (4:1)
Fat body	30	60	54	4.9
Heart	8	21	19	1.5
Upper lateral	42	40	38	2.6
Spiracle	64	58	54	3.3
Lower lateral	6	9	7	1.0
Nerve cord	39	54	51	1.5

*As the freeze-dried extracts were slightly sticky and not completely dry their weights are not given, but may be estimated from the sum of the weights isolated after the first partition.

Preparation of Tissue Extracts for Gas-Liquid Chromatography

Each of the aqueous methanol (4:1) extracts described above was subjected to an identical procedure to that described for blood extracts, and g.l.c. was carried out under the same conditions. All the tissue samples described above gave a complex pattern of peaks t_R less than six minutes, and only traces of components having longer retention times were detected. Peaks indicating the presence of 20-hydroxyecdysone in the original extracts could not be identified. Similar results were obtained when the above procedure was repeated for spiracle and lower lateral tissue extracts.

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