GAS CHROMATOGRAPHIC ANALYSIS OF ECDYSTEROIDS IN THE DESERT LOCUST *Schistocerca gregaria*

by

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

Abstract

The ecdysteroids present in 3rd, 4th, and 5th instar Schistocerca gregaria phase gregaria, and 5th instar Schistocerca gregaria, phase solitaria have been determined by gas chromatography with electron capture detection of their trimethylsilyl ethers. Both ecdysone and 20-hydroxyecdysone were detected with 20-hydroxyecdysone forming the major component. Titres of 20-hydroxyecdysone rose to a maximum of over 1 µg/insect during the 5th instar. Evidence of faecal excretion of ecdysteroids was sought, and small quantities of 20-hydroxyecdysone and polar conjugates of it were detected. These were not present in sufficient quantity to explain the observed fall in ecdysteroid levels in the insect. Both ecdysone and 20-hydroxyecdysone were present in the haemolymph of 5th instar insects (96 ng ml^{-1} and 432 ng ml^{-1} respectively).

Ecdysteroids in adult female insects were determined from the fifth-to-adult ecdysis to oviposition. The major ecdysteroids present were ecdysone and 20-hydroxyecdysone in the form of polar conjugates, the major component was ecdysone. These compounds were first detected a few days before oviposition and rose to 2-3 µg/insect at oviposition. These ecdysteroids could then be detected in the newly laid eggs.

Structural requirements for the detection of ecdysteroids using the electron capture detector were investigated. The tri-TMS ether of the model compound 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one was found to be approximately ten times more sensitive to electron capture than the 2β,3β-di-TMS 14α-hydroxy compound. More complex ecdysteroids with modifications to the side chain did not show this increase in electron capturing ability upon silylation of the 14α-hydroxyl group.

Chemical and enzymic procedures for cleaving polar ecdysteroid conjugates were investigated. Enzymic methods involving the use of Helix pomatia digestive juice were found to cleave ecdysteroid conjugates.

No success was seen using chemical methods involving heating in dioxan or pyridine, or incubating in tetrahydrofuran, or ethyl acetate containing perchloric acid.

The model compounds 3β-hydroxy-5α-cholestan-6-one, 3β-hydroxy--5α-cholest-7-en-6-one, and 3β,14α-dihydroxy-5α-cholest-7-en-6-one were synthesised from cholesterol. The syntheses of 3-dehydroecdysone and 3-dehydro-20-hydroxyecdysone were attempted without success. The solvent partition and gas chromatographic properties of the model compounds were determined.

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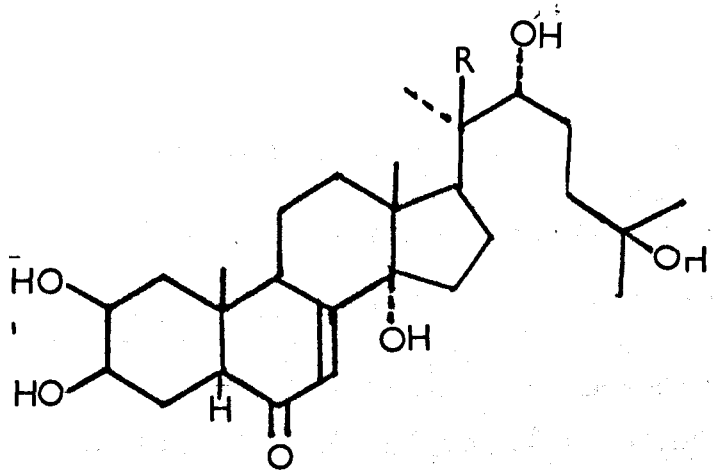
INTRODUCTION

The class Insecta is a subdivision of the phylum Arthropoda, which includes the Crustacea and Arachnida, creatures possessing a toughened jointed exoskeleton. Insects emerge from the egg as immature larvae still requiring a great deal of further growth and development before attaining adulthood and sexual maturity. The exoskeleton, whilst ideal for protection and as a support for internal organs, is in many ways incompatible with this growth and development, being essentially incapable of growth or modification. In order to overcome the constraints imposed upon them by the exoskeleton insects must periodically shed the hardened cuticle, and, in the period between this shedding and the hardening of the new cuticle rapid growth can occur.

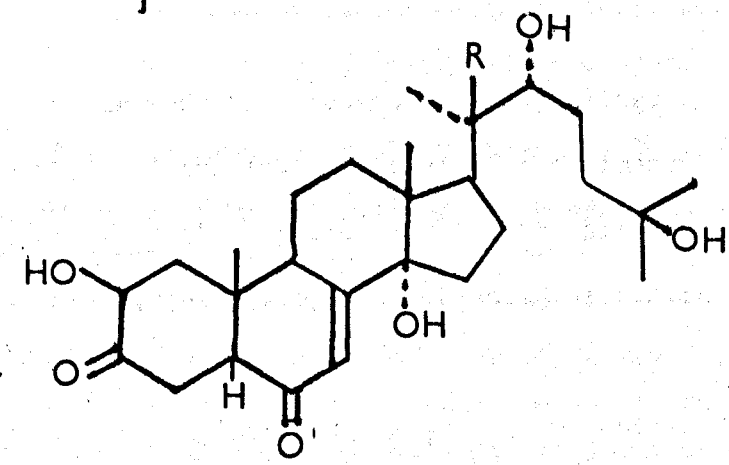
The term moulting covers the entire process of shedding the old cuticle, beginning with the secretion of the new cuticle, followed by apolysis (the separation of the old cuticle), then by ecdysis or loss of the old cuticle, and culminating in the hardening of the new cuticle some time later. As such the term moulting has been criticised for its lack of definition[1,2]. Frequently moult is used synonymously with ecdysis, as a consequence the hormones controlling the process have been called moulting hormones or ecdysteroids.

Two distinct types of development are seen amongst insects. The first is regular development of the larva towards the adult with each stage (instar) similar in appearance to the last, and the gradual development of adult characteristics. This type of development is characteristic of the Hemimetabola, which includes the more primative orders, Orthoptera (grasshoppers and locusts), Homoptera (aphids), and Dictyoptera (cockroaches). The moult cycle terminates in a larval to adult moult. An alternative form of development proceeds via a pupal stage with a fundamental rearrangement of tissues. This occurs in the Holometabola, represented



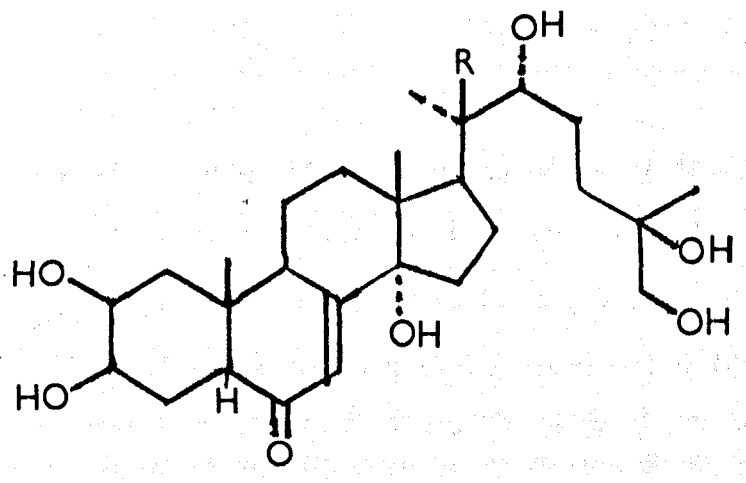
R = H, Ecdysone (I)

R = OH, 20 Hydroxyecdysone (II)



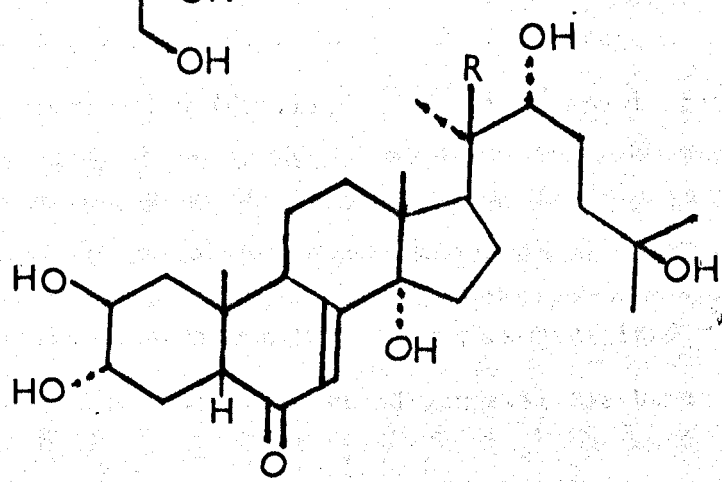
R = H, 3-Dehydroecdysone (III)

R = OH, 3-Dehydro-20-hydroxy--ecdysone (IV)



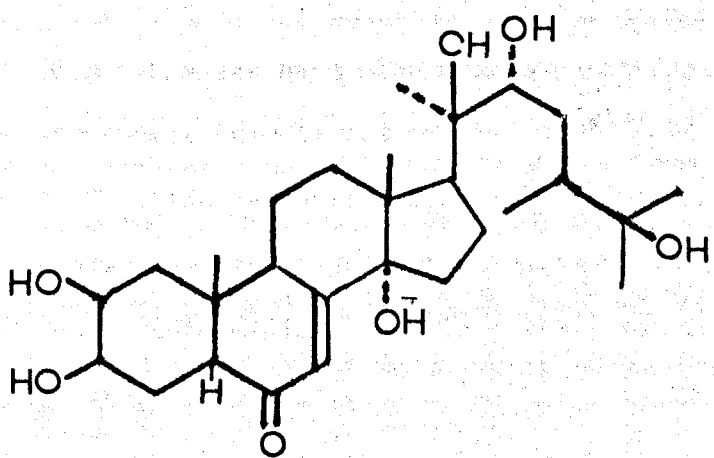
R = H, 26-Hydroxyecdysone (V)

R = OH, 20,26-Dihydroxyecdysone (VI)



R= H, 3-epi-Ecdysone (VII)

R = OH, 3-epi-20-Hydroxy--ecdysone (VIII)



MAKISTERONE A (IX)

Figure 1

by the higher insects, Coleoptera (beetles), Lepidoptera (butterflies and moths), Hymenoptera (bees, wasps and ants) and the Diptera (flies).

THE ECDYSTEROIDS

That moulting might be under endocrine control was postulated early in the twentieth century[3]; and the (by now) classic experiments of WIGGLESWORTH[4] on Rhodnius prolixus clearly demonstrated the presence of chemical substances in the blood, (haemolymph) which acted as hormones controlling moulting.

The first moulting hormone to be isolated, ecdysone, was obtained from the extraction of large quantities of the silkworm Bombyx mori[5]. The structure, determined by X-ray diffraction to be 2β,3β,14α,22R, 25-pentahydroxy-5β-cholest-7-en -6-one (1) was obtained in 1965, some eleven years after its original isolation[6,7]. Since that time a number of further ecdysteroids have been isolated from insects as well as compounds which appear to be metabolites of them. The structures of some of these compounds are given below (Fig.1). The origin and functions of these compounds is discussed later in the text.

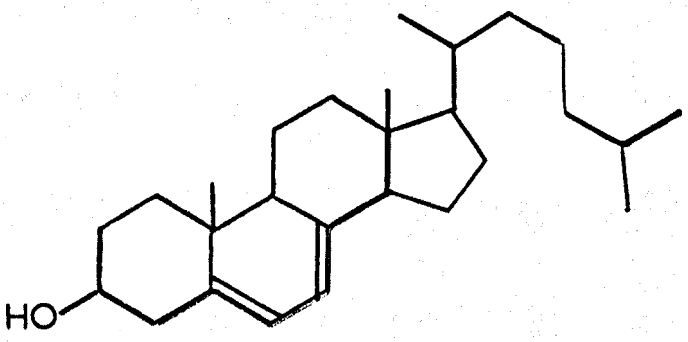
BIOSYNTHESIS OF ECDYSONE

The in vitro biosynthesis of ecdysone in the prothoracic gland (PTG), or its equivalent, (e.g. the brain-ring gland complex) has been demonstrated in a number of species. These include Calliphora erythrocephala[8], Bombyx mori[9], Tenebrio molitor[10] and Manduca sexta[11]. Other tissues have also been suggested as sites for ecdysteroid biosynthesis, namely the oenocytes[12,13] and the ovaries[14,15].

The metabolic pathway by which ecdysone is produced is under intensive study, and some aspects of it are becoming clear. A probable early intermediate on this pathway may result from the biosynthesis of 7-dehydrocholesterol (cholest-5,7-dien-3β-ol, X Fig.2), via cholesterol.

7-Dehydrocholesterol has been shown to be present in quantity in a number of insects, and its conversion to ecdysteroids has also been demonstrated[16,17].

Figure 2

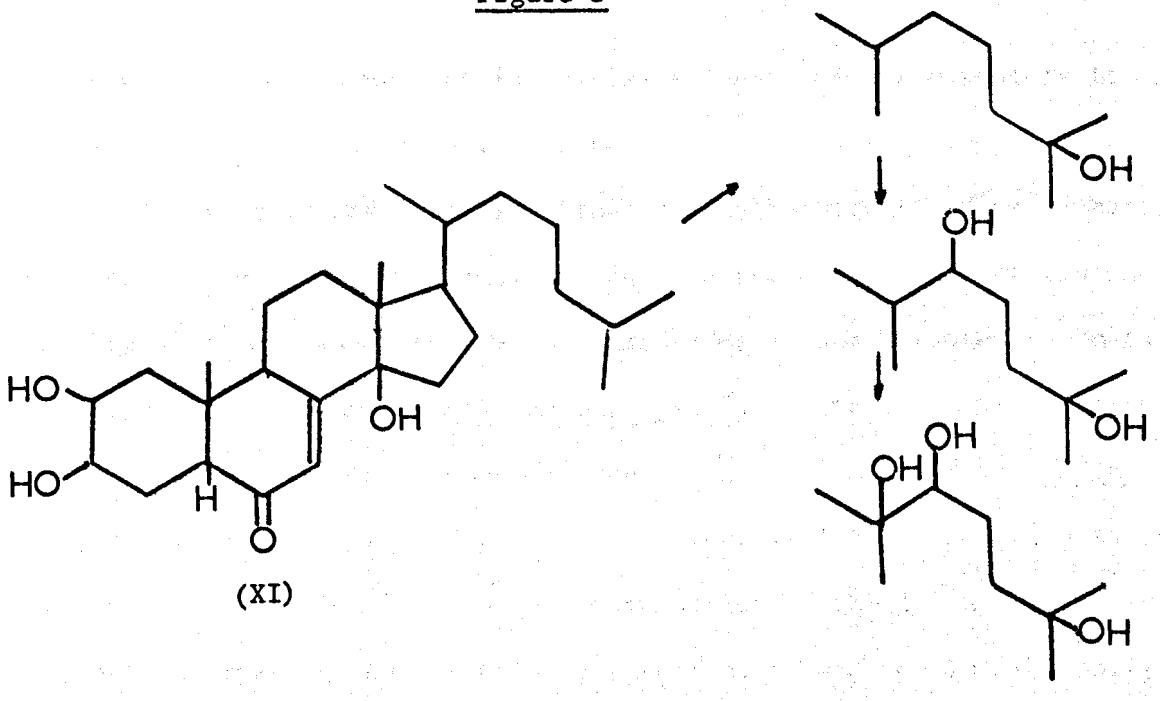


7-dehydrocholesterol (X)

The conversion of 7-dehydrocholesterol to ecdysteroids has been demonstrated in the desert locust, Schistocerca gregaria[27]. There is however simultaneous conversion of the radiolabelled 7-dehydrocholesterol to cholesterol. The authors suggest that there is an equilibrium between these two compounds, and that this is not a rate-determining step in ecdysone biosynthesis.

The strategy of challenging insects or tissues with possible intermediates in order to find out which compounds are involved in ecdysone biosynthesis has had some success, and has given valuable insight into the metabolism of the sterol nucleus and the order of hydroxylation of the side chain. However the exact sequence of this biosynthetic pathway remains unclear. In Manduca sexta the sterol 2β,3β,14α-trihydroxy--5β-cholest-7-en -6-one (X) is metabolised as shown in Figure 3. Interestingly this compound is not metabolised to ecdysteroids in either Sarcophaga or Gastrimargus but to a mixture of polar steroids[19].

Figure 3



(XI)

In Calliphora stygia 2β,3β,14α-trihydroxy-5β-cholest-7-en -6-one is also metabolised to moulting hormones, however whilst hydroxylation in Manduca begins at C-25, in Calliphora hydroxylation at C-22 precedes that at C-20 and C-25[16].

It seems that whilst hydroxylation of these ecdysteroid precursors takes place in a definite sequence the sequence may vary between species. It would appear that modification of the steroid nucleus must occur before hydroxylation of the side chain as 3β,25-dihydroxycholesterol and 3β,22-dihydroxycholesterol are not metabolised to ecdysone[20].

Whilst studies on possible precursors give useful information about the types of compound which may be involved in moulting hormone biosynthesis, the best evidence for a compound's participation on this pathway must come from its isolation. The presence of 3β-hydroxy-5α-cholestan-6-one has been demonstrated in the PTG of Bombyx mori and has been suggested as an intermediate[21]. In this case it is difficult to reconcile the involvement of both this and 7-dehydrocholesterol in the same biosynthetic pathway. This suggests that the early steps in ecdysone biosynthesis may also vary from species to species.

A much more complete biosynthetic route to ecdysone has been deduced from sterols present in ovaries and eggs of the migratory locust Locusta migratoria[22], shown in Figure 4.

It is still not clear if either 7-dehydrocholesterol or 3β-hydroxy--5α-cholest-6-one are intermediates in this sequence. One of the compounds, the ketodiol, 3β,14α-dihydroxy-5α-cholest-7-en-6-one has been shown to be converted to ecdysone in Calliphora stygia[23]. The authors considered the next hydroxylation to be at C-2 whereas in the scheme for Locusta (Figure 4) this occurs at C-25. The picture is further complicated by evidence that in some insects the introduction of the 14α-hydroxyl group may precede that of the 7-ene-6-one function. Evidence for the early introduction of the C-14 hydroxyl arises from the observation that incubation of compounds already containing the ene-one group leads to the production of 14α-deoxyecdysone in Manduca sexta[24]. It would appear therefore that there may be great variations in the biosynthetic pathway between species, not only in the hydroxylation of the side chain but also the formation of the ecdysteroid nucleus.

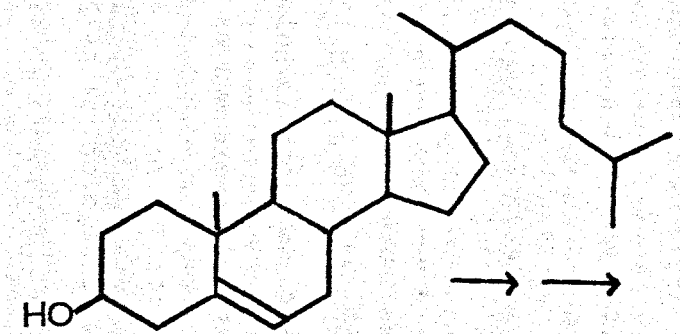
STRUCTURE AND ACTIVITY

The final step in ecdysone biosynthesis in Locusta ovaries, the C-2 hydroxylation[22] is interesting because it is the interaction of this hydroxyl group and the C19-methyl group which is responsible for the 5α to 5β isomerisation.[25] This results in cis-fusion of the A and B rings. This cis-fusion is an essential requirement for high biological activity.

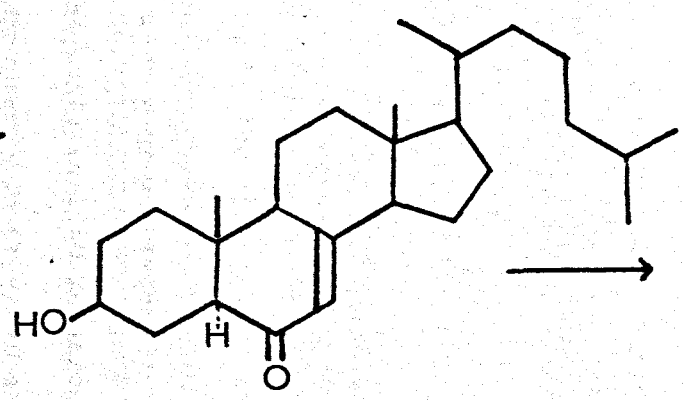
Other important structural requirements for biological activity are a 7-ene-6-one system, and a side chain with an R configuration at C-22 bearing an hydroxyl group[25]. For high activity an hydroxyl at C-3 is needed, however, 3-dehydroecdysteroids still retain some biological activity.

Figure 4

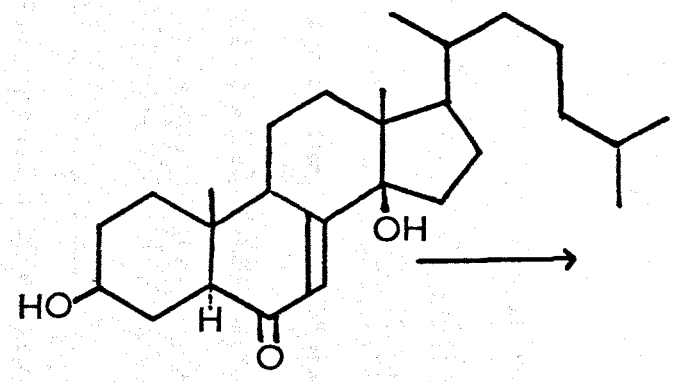
BIOSYNTHESIS OF ECDYSONE IN LOCUSTA MIGRATORIA



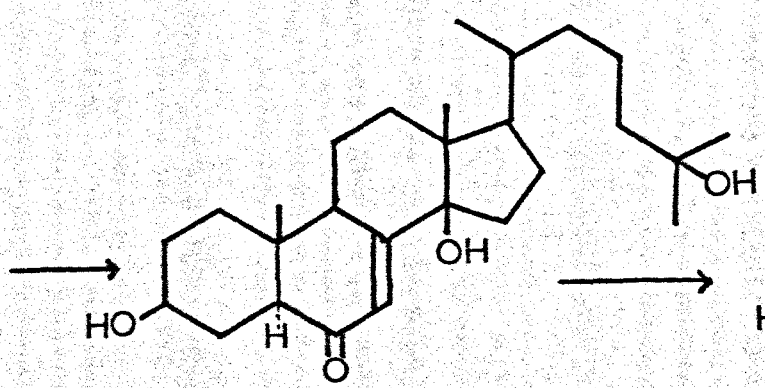
Cholesterol



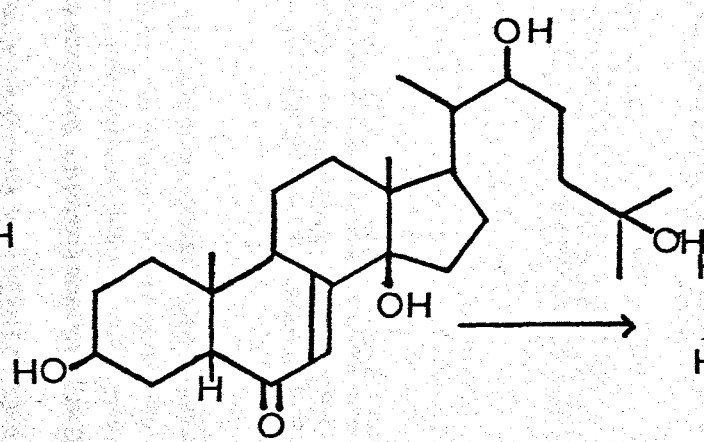
2β-Hydroxy-5α-cholest-7-en -6-one



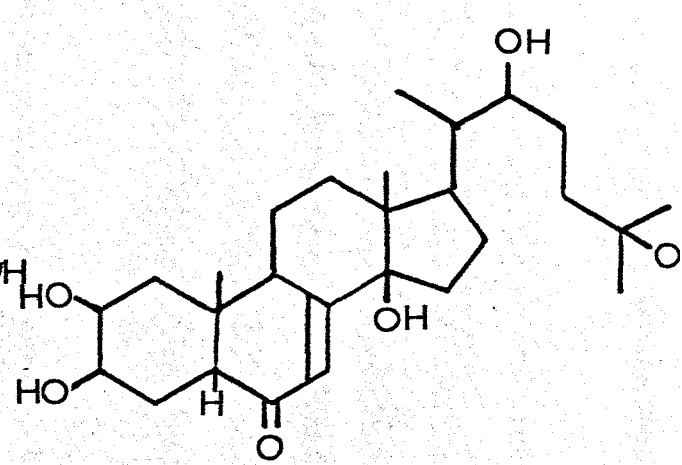
2β,14α-,Dihydroxy-5α-cholest-7-en-6-one



2β,14α,25Trihydroxy-5α-cholest-7- en -6-one



2-Deoxyecdysone



Ecdysone(1)

Whilst PTG's are able to synthesise ecdysone, in general they appear unable to further hydroxylate ecdysone to 20-hydroxyecdysone. This, because of its greater biological activity compared to ecdysone is regarded as the major moulting hormone in insects. The transformation of ecdysone to 20-hydroxyecdysone is believed to occur in a wide variety of other tissues, including malpighian tubules and fat body.

HYDROXYLATION OF ECDYSONE

Hydroxylation of ecdysone to 20-hydroxyecdysone appears to be performed by a mitochondrial or microsomal system involving cytochrome P450, NADPH, and oxygen[18,26].

It is becoming clear that the capacity of the C-20 hydroxylating system (as measured by the rate of conversion of injected ecdysone) varies during development, being maximal at periods of high moulting hormone titre[28]. This has led to the suggestion that the C-20 monooxygenase is induced by the rising hormone titre.

MODE OF ACTION OF MOULTING HORMONES

As early as 1960 an effect of ecdysone on chromosome puffing of giant chromosomes in Chironomus tentans was observed within 15-30 minutes of exposure to low doses of hormone (2×10^{-6} pg)[29]. The hypothesis was developed that ecdysteroids functioned at a transcriptional level activating specific genes, followed by mRNA production and synthesis of proteins required for moulting (e.g. DOPA decarboxylase[30]). Recently a general stimulation of RNA synthesis has been shown[31]. Effects on translation of preformed mRNA's have also been claimed[32]. In addition, effects on DNA biosynthesis have been observed[33].

A number of authors consider ecdysone to be a prohormone because of the greater biological activity of 20-hydroxyecdysone in systems incapable of C-20 hydroxylation[34-37]. In appears however that ecdysone

has biological activity in its own right, and a hormonal function for ecdysone in the moulting process should not be dismissed, as it may be that the prohormone concept is an oversimplification[38-40].

INACTIVATION AND EXCRETION OF THE ECDYSTEROIDS

It has been observed that moulting hormone titres can rise to very high levels before falling precipitously back to low levels, all in the space of a few hours. This poses the question, how is this rapid rise and fall brought about? Is the rise due entirely to *de novo* synthesis, or to the release of ecdysone synthesised in the early part of the instar and stored in an inactive form? Support for the concept of storage in an inactive form comes from studies on *Calliphora*[8] where inactive moulting hormone conjugates were detected in studies on ecdysone biosynthesis from cholesterol. Recently conjugated moulting hormones have been detected in large quantities in newly laid eggs of *S. gregaria*[41], *Bombyx mori*[42], and *L. migratoria*[43]. These may also represent a pool of inactive hormone for use by the developing embryo.

It has been suggested that ecdysteroid conjugates may be involved in the transport of ecdysones through the insect haemolymph[8]. Against this haemolymph proteins have been detected with high affinity for 20-hydroxyecdysone[44] but none for conjugates. In addition the proportions of conjugated ecdysteroid compound to free hormones found circulating in the haemolymph have been shown to be very low (< 10%)[45].

There would appear to be a number of possible routes for elimination of ecdysteroids (review[46]), what is not in doubt is that insects do possess a rapid and efficient method of reducing moulting hormone levels. Indeed the half life of injected moulting hormones in *Calliphora erythrocephala* is 3 hours[47] and in *Sarcophaga peregrina*, 1 hour[48]. Endogenous moulting hormone levels have also been observed to fall rapidly in a number of species including locusts, (e.g. *L. migratoria*[49], *S. gregaria*[50]).

Possibilities for the inactivation and excretion of the ecdysteroids include conjugation[49], excretion unchanged[51], further hydroxylation[52], side chain cleavage[53], changes in the conformation of the C-3 hydroxyl[54], the formation of 3-dehydro compounds,[55] or some combination of these.

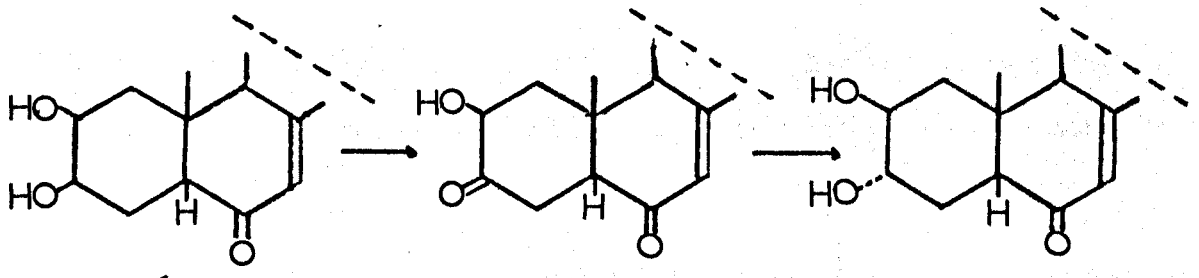
Quantities of unconjugated ecdysteroids were detected in the faeces of a number of insects[51], and more recently ecdysteroids and their metabolites have been observed in faecal extracts[49,62]. Conjugated moulting hormones were first detected in larvae and tissues of Calliphora erythrocephala incubated with radiolabelled 25 deoxyecdysone, and identified as glucosides[56]. In similar experiments on this and other insects a variety of inactive conjugates including sulphates[57], mixtures of sulphates[58], sulphates and glucuronides[59], and sulphates and phosphates[60] have been found. The occurrence of polar ecdysteroid conjugates in faeces coupled with their low level of biological activity suggests a function in inactivation and excretion.

3-dehydroecdysone and 3-dehydro-20-hydroxyecdysone, (III, IV, Fig.1), first reported in studies on Calliphora[55,61], have been observed in several studies on the fate of exogenous radiolabelled hormone. Indeed in 5 day old 5th instar L. migratoria 45% of the recovered radiolabelled hormone was identified as 3-dehydroecdysone[62]. However at the time of the endogenous hormone maximum (and thereafter) 3-dehydroecdysone production represented a very minor pathway, suggesting its formation was dependent upon the stage of development. Large quantities of radiolabel including 3-dehydroecdysone were recovered from the faeces of these insects. Indeed from experiments of this type ecdysone, 20-hydroxyecdysone, 3-dehydroecdysone, 3-dehydro-20-hydroxyecdysone and conjugates of all four compounds have been extracted from insects and faeces. 3-Dehydro compounds still retain a moderate amount of biological activity

(3-dehydro-20-hydroxyecdysone retains 25-30%[63] of the activity of 20-hydroxyecdysone) and whilst they may be involved in inactivation a hormonal function for 3-dehydroecdysteroids cannot be excluded.

The compound 3-epi-20-hydroxyecdysone, isolated from the meconium of Manduca sexta[54], and more recently from pupae of Sarcophaga peregrina[64] may be formed via 3-dehydro-20-hydroxyecdysone, and may represent a further step in its inactivation (Fig.5). The biological activity of 3-epi-20--hydroxyecdysone (VII) is 10 to 15% of that of ecdysone[54].

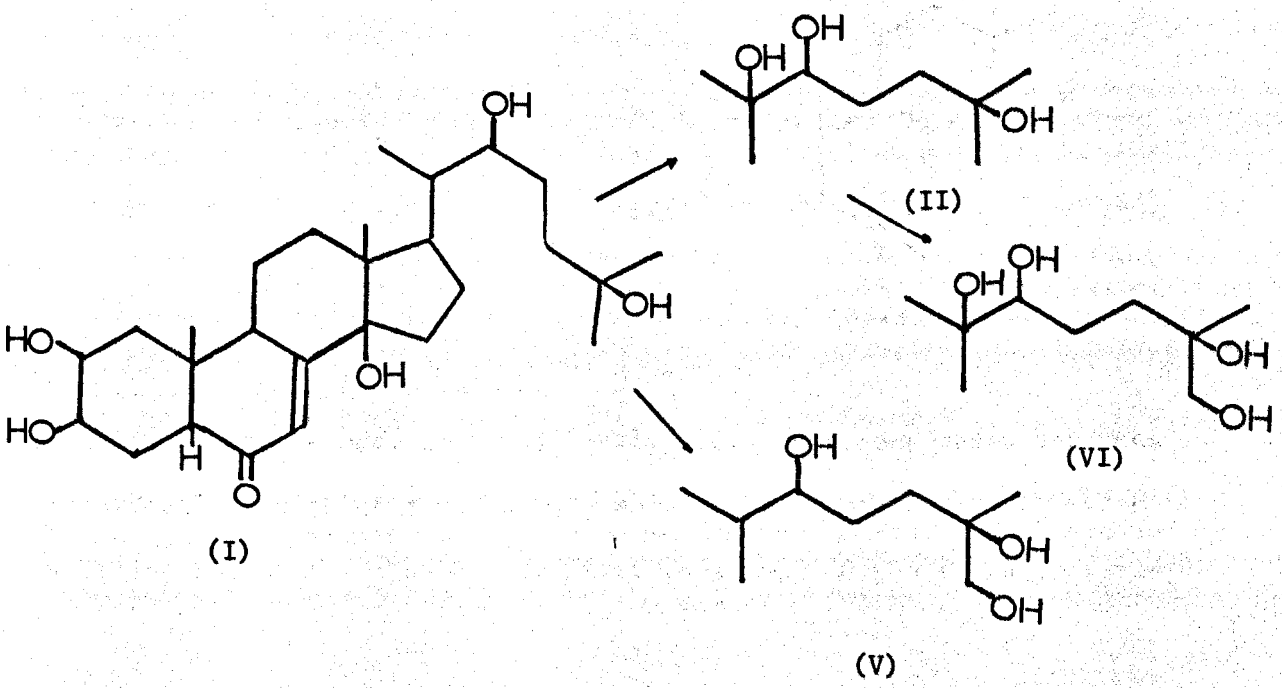
Figure 5



(I) (III) (VII)

Hydroxylation at C-26 has also been put forward as an inactivation process. Such compounds have been shown to occur in eggs[66] and pupae of Manduca sexta[52], and as a metabolite of radiolabelled ecdysone in Calliphora erythrocephala[65] (Fig.6). These C-26-hydroxyecdysteroids have 10% of the biological activity of the parent compound[52].

Figure 6

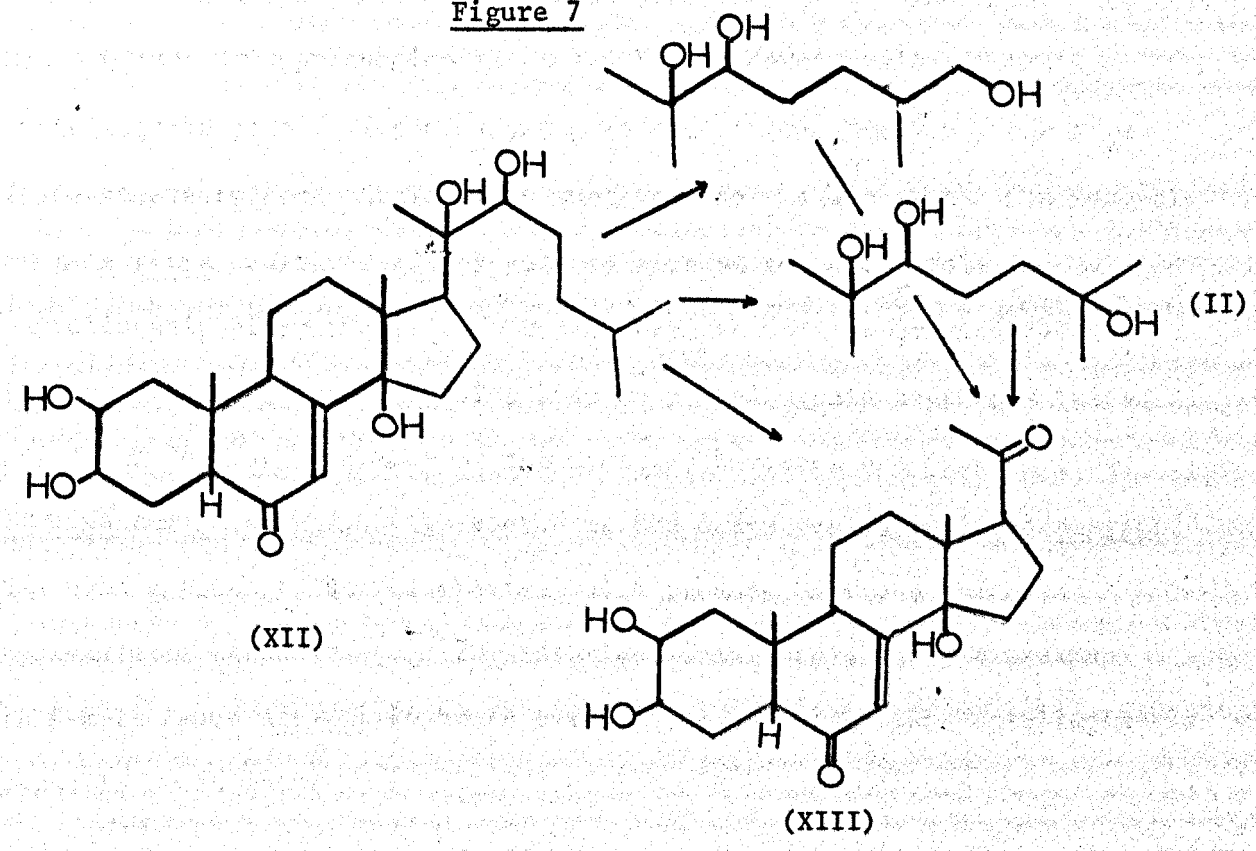


(I) (V)

In plants, enzymes capable of cleaving the side chain at C-20-22 and C-17-20 have been found[53] and evidence of similar enzymes in insects has been sought. The compound ponasterone A(2β,3β,14α,20,22-penta hydroxycholest-7-en -6-one) (XII) was metabolised to poststerone (2%) (XIII) in Bombyx mori, with concurrent production of 20-hydroxyecdysone and inokosterone[53] (Fig.7). Side chain scission has been inferred from the production of 4-hydroxy-4-methylpentonoic acid from 20-hydroxyecdysone in Calliphora stygia[67]. However this pathway accounted for only 0.3% of the exogenous hormone.

From this it is clear that which (if any) of the several pathways postulated for ecdysteroid elimination is of major importance is still unclear.

Figure 7



REPRODUCTIVE FUNCTIONS OF MOULTING HORMONES

One of the most exciting developments in recent years has been the identification of a number of reproductive functions performed by moulting hormones. These were first indicated 20 years ago in research

on adult female Bombyx mori, which appeared to contain quantities of an ecdysone like compound[68]. More recently ecdysteroids have been found in ovaries, eggs, or adult females of several species of insects including Leucophaea maderae[69], Bellicositermes bellicosus[70], Bombyx mori[42], Sarcophaga bullata[71], Aedes aegypti[72], and Manduca sexta[66,73]. Some of the functions of these moulting hormones are becoming clear and are considered below.

VITELLOGENESIS

A requirement for ecdysteroid in the production of vitellogenin has been shown for Sarcophaga bullata[74], Periplaneta americana[75] and Aedes aegypti[72,76]. The injection of > 50 ng of 20-hydroxyecdysone induced male Sarcophaga bullata to synthesise vitellogenin, which does not occur in male insects under normal circumstances. The authors suggest that vitellogenin production is caused by the derepression of the vittellogenin gene. The constant presence of 20-hydroxyecdysone seems to be necessary for vitellogenin synthesis in Aedes aegypti, its removal from in vitro cultures of fat body causing an immediate fall in vitellogenin biosynthesis[76]. There seems to be a programmed response to 20-hydroxyecdysone by the fat body as vitellogenin synthesis tails off after 30 hours, whether or not moulting hormones are present (both in vivo and in vitro). The source of the ecdysteroids in Aedes aegypti has been claimed to be the ovaries which produce ecdysone which is hydroxylated elsewhere[15]. 20-hydroxyecdysone levels reach a maximum in female Aedes aegypti 16 hours after a blood meal whilst vitellogenesis peaks 16 hours later.

DOPA DECARBOXYLASE PRODUCTION

The enzyme DOPA decarboxylase has been induced in female Aedes following an injection of 20-hydroxyecdysone[77]. This enzyme is involved in oocyte development. The evidence from these experiments

suggests that translational control of preformed mRNA's was involved. This enzyme is also involved in moulting in Calliphora erythrocephala where control of its synthesis appears to be via transcription[30].

OVARIAN DEVELOPMENT

Ecdysteroids have been implicated in the ovarian cycle of Folsomia candida[78] during late egg maturation and oocyte growth. Considerable quantities of ecdysone are synthesied by follicle cells of female Locusta migratoria towards the end of ovarian maturation[14]. The hormone also accumulates in the oocyte where it is transformed into inactive conjugates. These compounds are found in the eggs for several days after laying, the ratio of free to conjugated ecdysone decreasing progressively. A function in vittellogenesis is not thought likely as this is largely complete before moulting hormones appear. The fact that these moulting hormones are rapidly converted to inactive polor conjugates in the egg is felt by the authors to indicate that they are not used in embryonic development, and their function remains unclear. 20-hydroxy-ecdysone was not detected in any quantity in these insects and it would appear that the ovary, like the PTG, does not have the ability to further hydroxylate ecdysone.

MOULTING HORMONES IN EGGS AND EMBRYONIC DEVELOPMENT

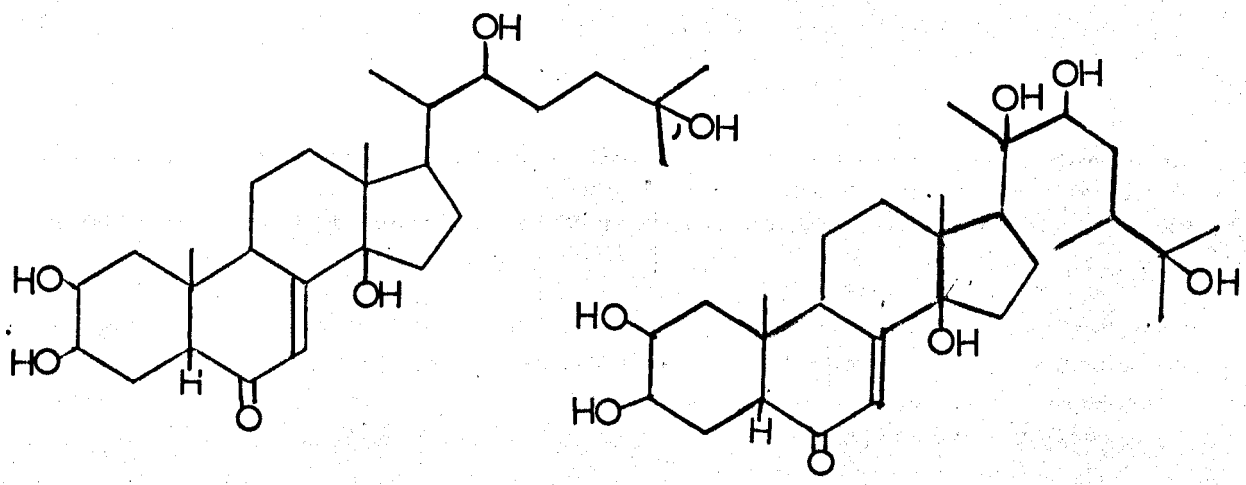
The eggs of Bombyx mori have been found to contain considerable amounts of ecdysteroids,in diapausing eggs this is mainly found as conjugated ecdysone[42] whereas in developing eggs free ecdysone is present[79]. This suggests that these conjugates are used as storage compounds until required in the form of free hormone by the developing embryo. 20-hydroxyecdysone was not found suggesting that the C-20 monoxygenase may be absent from embryos of Bombyx.

Ecdysone is however not the sole moulting hormone to have been isolated from insect eggs. The main ecdysteroid isolated from 24-44

and 48-68 hour old developing eggs of Manduca sexta was found to be 26-hydroxyecdysone[66,73]. Smaller quantities of ecdysone, 20-hydroxy-ecdysone, 20,26-dihydroxyecdysone along with small quantities of polar conjugates were also present. From this it would appear that whilst it is present, the C-20 hydroxylating system is of low activity.

1 Day old developing eggs of Oncopeltus fasciatus were found to contain only low levels of ecdysteroids. However after 4 days they were found to contain high levels of the C-28 ecdysteroid Makisterone A[80] (2β,3β,14α,20,22,25-hexahydroxy-24-methyl-5β-cholest-7-en -6-one (Fig.8)).

Figure 8



26-hydroxyecdysone (V) Makisterone A (IX)

This increase in the titre of Makisterone A is given as evidence of embryonic biosynthesis. It is also notable that in this case C-20 hydroxylation does occur.

The presence of ecdysteroids in ovaries and eggs of a large number of insects is well established, even if their function is not. It may be that they serve to activate embryonic genes in a general way, or they may serve to initiate the embryonic moult (or both). That they may serve to initiate the embryonic moult is supported by work on Melanoplus differentialis[81]. A puzzling feature is the apparent lack of a C-20 hydroxylating system in many eggs and ovaries. This results

in a complete reversal of the situation seen in postembryonic development, where 20-hydroxyecdysone is seen to be the predominant ecdysteroid.

THE RELEVANCE OF MOULTING HORMONE RESEARCH TO PEST CONTROL

The use of moulting hormones as pesticides is impractical for a number of reasons. Firstly as steroids they would be prohibitively expensive to synthesise on the large scale which would be required. Secondly the ability of moulting hormones to penetrate the insect cuticle is poor so that spraying may be impractical, and, thirdly, when fed to insects moulting hormones are not easily absorbed through the gut[82]. However the moulting hormone system is of such fundamental importance to insect (and crustacean) development that compounds which could interfere with it should be actively sought. Indeed some compounds have been found which interfere with moulting such as Dimilin[83], but these appear to interfere with chitin production[84], rather than moulting hormone levels[85]. Moulting hormone inhibitors remain in the future. Recent work in this laboratory has resulted in the development of an analytical technique for the determination of the ecdysteroid present in the desert locust[50]. The object of the research reported in this thesis was to use this technique to investigate the levels of moulting hormones and their metabolites during the last 3 larval instars, and from the larval-adult ecdysis to the point at which eggs were laid in the adult female.

DISCUSSION

Analysis of the Ecdysteroids and their Metabolites in the Desert Locust Schistocerca gregaria

Analytical Methods:

Much effort has gone into developing methods for determining ecdysteroids at the low levels found in insects and crustacea (1-3000 ng per individual), and at the start of this research a number of methods existed for accomplishing this. Bioassay techniques depended on the observation of apolysis,[86] puparium formation[87,88] or the breaking of diapause[89]. However such techniques are of limited sensitivity (10^{-7}g [90]), and lack specificity in that the same effects may occur with a wide range of ecdysteroids and other substances[90]. Radioimmunoassay is both sensitive (10^{-11}g [90]) and rapid, but may lack specificity due to the cross reaction of the antibody with a number of molecules of similar structure. Gas chromatographic methods when coupled to a suitable detector have the advantage of sensitivity (10^{-11}g [91]) and specificity in that they allow the separation and quantification of structurally similar ecdysteroids present in the same sample. Derivatisation of the hormones is required to stabilise the ecdysteroids to gas chromatography, and lack of proper control at this stage may lead to errors and artifacts. Thin layer chromatography, sensitive down to the microgram level (10^{-6}g [92]) using ultraviolet light to detect ecdysteroids was known, and this method has also had applications in detecting the products of the metabolism of radiolabelled hormones[65]. High performance liquid chromatographic methods of good resolution and sensitive down to the nanogram level (10^{-9}g) were under development[92].

Analysis of Ecdysteroids Present in The Desert Locust

In this study gas chromatography with the detection of the silylated hormones using an electron capture detector (ECD) was chosen because of its sensitivity, selectivity, and immediate availability. The method used was based on that of Morgan and Poole[50] and involves three solvent partition systems to remove most of the unwanted insect material, followed by silylation, thin layer chromatography (TLC) and gas chromatography (Fig. 9).

Solvent Partition Systems

1. Methanol Water : Light Petroleum

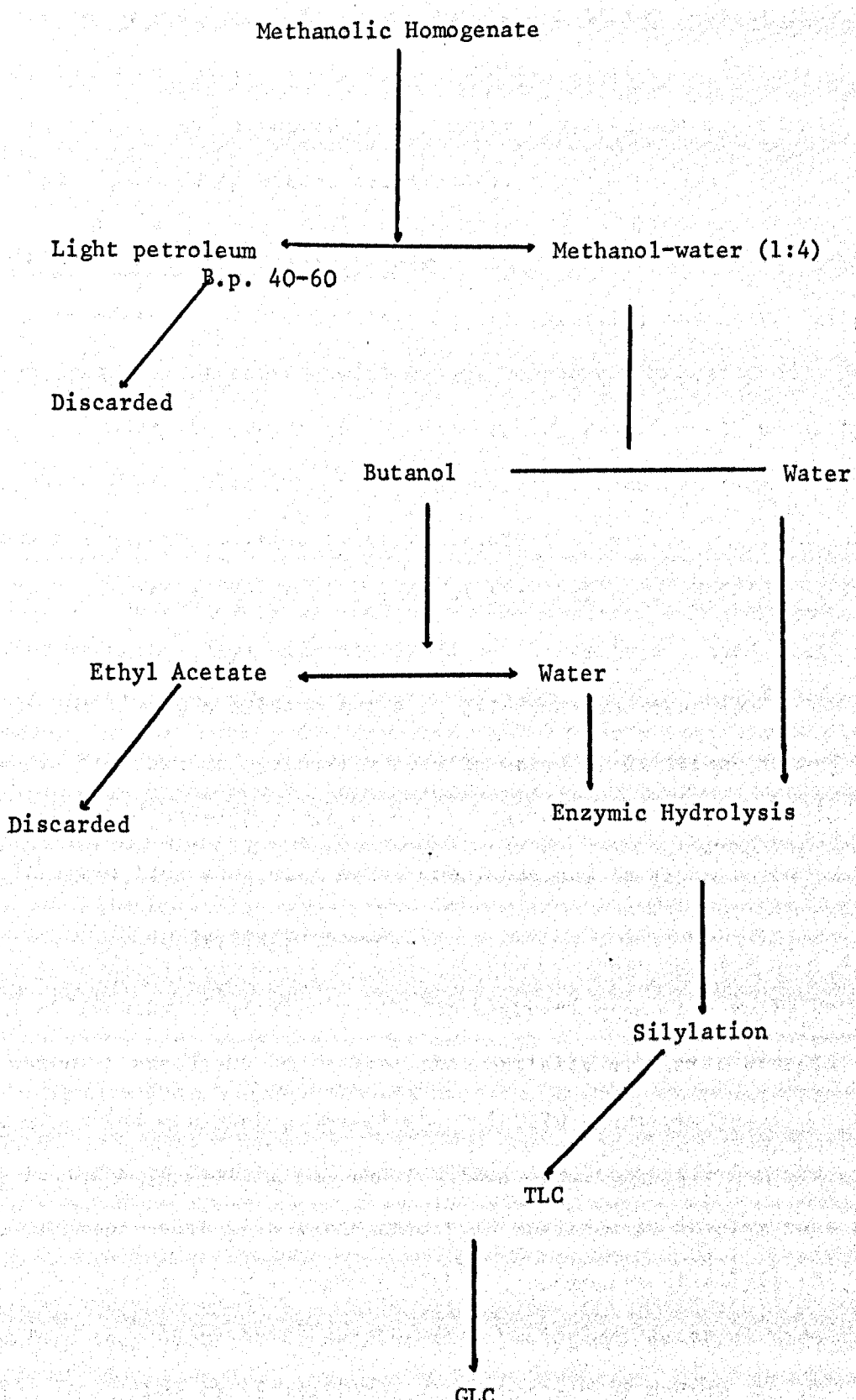
The solid residue obtained by the evaporation of the methanolic filtrate from the preliminary extraction of the biological material is first partitioned between aqueous methanol and light petroleum (b.p. 40-60). Lipids and non polar sterols, including ecdysteroid precursors such as cholesterol, 7-dehydrocholesterol, 3β-hydroxy-5α-cholestan-6-one, and 3β-hydroxy-5α-cholest-7-en-6-one are partitioned into the light petroleum phase. More polar substances including the ecdysteroids and their polar conjugates are partitioned into the aqueous methanol. To ensure the maximum recovery of ecdysteroids the number of backworkings of the light petroleum phase was increased from one in the Morgan and Poole method[50] to two, and the proportions of the two phases were also altered.

2. Butanol : Water

In the second partition ecdysteroids and compounds of similar polarity partition into the butanol phase of the butanol-water system. An examination of the aqueous phase using enzymes derived from the digestive juice of the snail Helix pomatia showed that polar ecdysteroid conjugates are partitioned into this phase. Indeed all the polar ecdysteroid conjugates observed in this study whether derived from faeces, eggs, or adult females were partitioned exclusively into this phase. This separation provided a convenient method for the determination of both free and conjugated ecdysteroids present in the same sample.

Extraction and Analysis of Ecdysteroids From Biological Material

Fig. 9



3. Water : Ethyl Acetate

As a further purification a partition between water and ethyl acetate gave ecdysteroids and compounds of similar polarity in the aqueous phase. The solid residue obtained on evaporation of the aqueous phase was transferred to a Reacti-vial as a methanolic solution. Examination of the ethyl acetate phase showed that neither ecdysteroids or their conjugates were present in detectable amounts.

Formation Of Silyl Ethers Of Ecdysteroids

Because of their high molecular weight, polarity, and instability at high temperatures the ecdysteroids cannot be gas chromatographed readily. To render the ecdysteroids more stable, reduce their polarity, and to increase their volatility it was necessary to convert some or all of the hydroxyl groups to trimethylsilyl ethers (TMS ethers). This was accomplished by heating the ecdysteroids as a solution in pyridine with an excess of trimethylsilylimidazole (TMSI). There is evidence that pyridine is not simply a solvent but also catalyses the reaction[92]. Silylation of the various hydroxyl groups occurred at different rates, depending upon their steric environments. The order of derivatisation has been inferred from work with model compounds and is given below[92].

$$2\beta, 3\beta, 22R, 25 > 20 \gg 14\alpha$$

The difficulty of silylating the 14α-hydroxyl group (up to 24 hours at 140°) makes it expedient to analyse the partially silylated steroid (tetrakis ether for ecdysone, pentakis ether for 20-hydroxyecdysone) which are formed more rapidly and under milder conditions (5½-6 hours at 100°). The presence of a large amount of moisture in samples causes hydrolysis of TMSI, and may interfere with the formation of silylated ecdysteroids; to prevent this, samples from biological material were dried under vacuum before silylation. The presence of sodium or

potassium acetate can lead to the generation of artifacts (discussed in detail later), as can the use of dirty Reacti-vials, or Reacti-vials contaminated with detergent or chromic acid.

Having formed the trimethylsilyl ethers it was necessary to purify the reaction mixture to some extent by removing excess silylating reagent, imidazole, and any polar biological material.

Thin Layer Chromatography of Silyl Ethers of The Ecdysteroids

The TLC clean-up could be omitted when pure ecdysteroids were silylated, providing that they were diluted before injection to protect the detector from contamination ($\sim$ 100 times dilution). However, when biological samples were to be determined the TLC procedure was essential to remove impurities which would otherwise effectively mask the response due to the ecdysteroids. TLC was performed on silica in a solvent system of toluene and ethyl acetate (7 : 3). The Rf values of some ecdysteroid silyl ethers are given in Table 9 p97 .

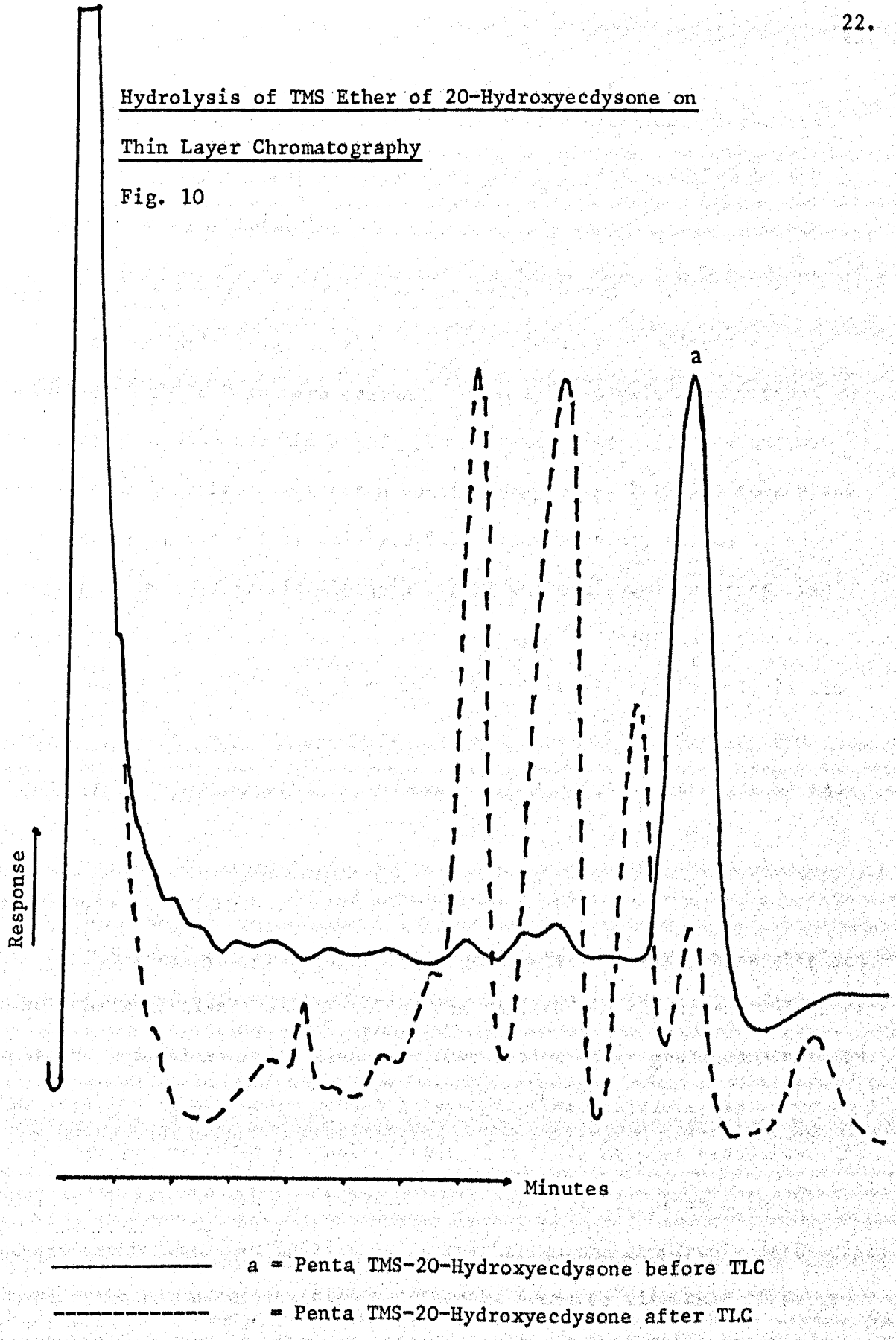
Recovery of Ecdysteroid TMS Ethers After TLC

Recovery of TMS ethers of ecdysteroids after TLC was effected by elution from the silica with diethyl ether. Recoveries of tetrakis TMS ecdysone and pentakis TMS 20-hydroxyecdysone were found to be quantitative over the range 10^{-4} to 10^{-9} g, losses became important when less than 2 ng were loaded on to the plate ($\sim$ 60% recovery). The most reliable method for determining percentage recovery was found to be as follows. The sample was divided into two portions, one portion was used for TLC, the other kept as a standard. After recovery from TLC both sample and standard were made up to the same volume, and their peak areas compared on gas chromatography. A lower value for the recovered sample compared to the standard was taken as evidence of losses on TLC.

Recoveries of hormone from the extraction and silylating procedures when determined for adult males spiked with hormone varied from 95%

Hydrolysis of TMS Ether of 20-Hydroxyecdysone on Thin Layer Chromatography

Fig. 10



(10^{-4} g hormone) to 85% (10^{-8} g hormone). The excellent recoveries from the TLC step suggests that most of these losses occurred in the extraction and partition steps.

Hydrolysis of TMS Ethers of the Ecdysteroids on TLC

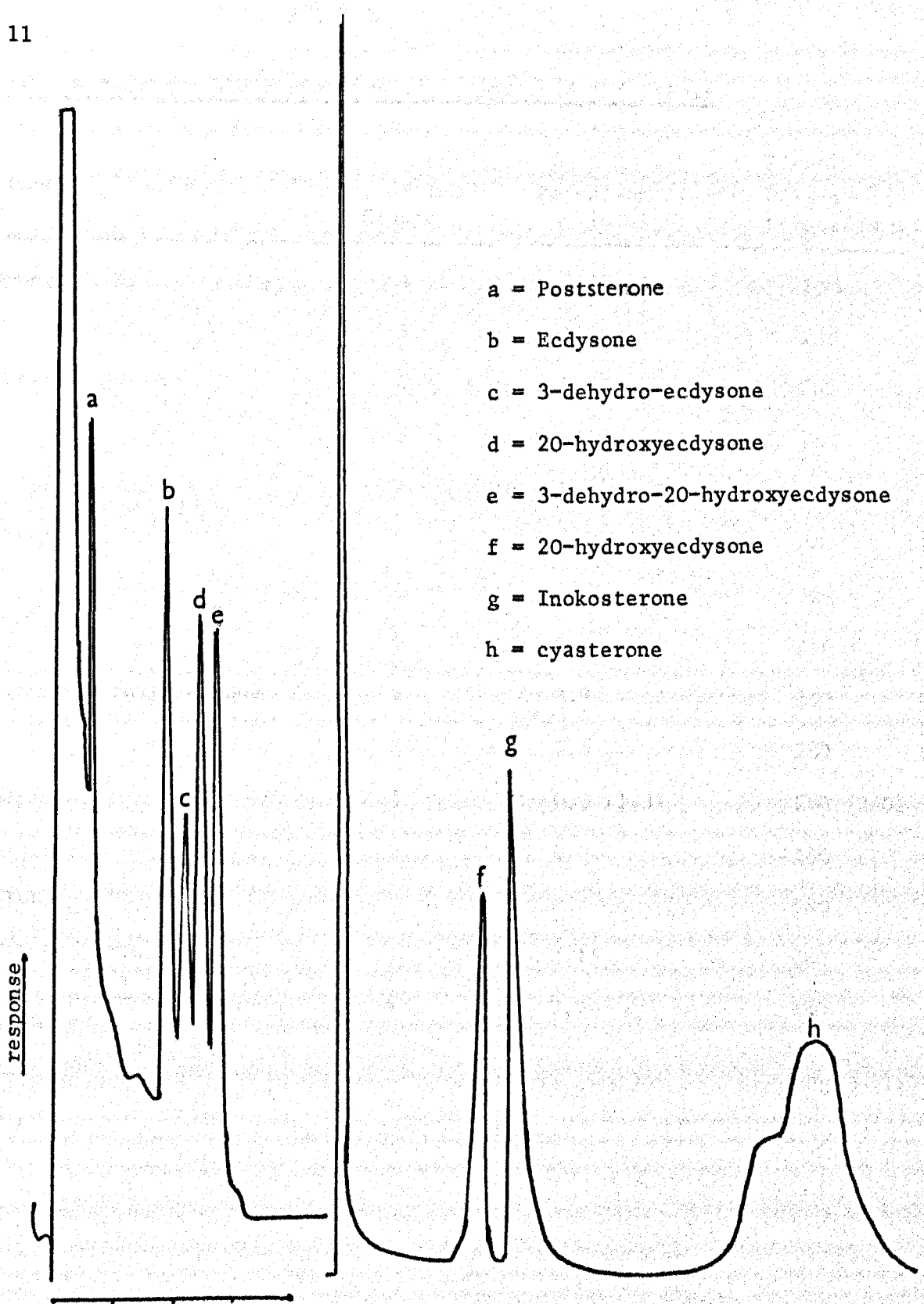
At an early stage in this study problems of hydrolysis of the ecdysteroid TMS ethers were encountered. These problems were found to be associated with the TLC step. Experiments with silylated hormone showed that hydrolysis was not a continuous process but only took place when the silylated ecdysteroid was in contact with dry silica. An example of this effect is given in Fig.10, where a sample of pentakis 20-hydroxyecdysone has been gas chromatographed before and after TLC. When samples were not allowed to dry out on the silica, hydrolysis was reduced to three or four percent. This problem seems to have been confined to just one batch of silica, and has not been observed in later batches.

Gas Chromatography

The ether was evaporated from the solution of TMS ethers obtained after their elution from the silica and replaced by a measured volume of toluene. Samples, in toluene were then subjected to gas chromatography. The identity of any ecdysteroids present in these extracts was determined by comparison of their retention times with those of pure standards. An additional test of the identity of the ecdysteroid(s) present in the sample can be obtained by silylating further to the completely derivatised form. The new retention time can then be compared with that of fully silylated standards. The retention times of ecdysteroids and related compounds are given in Table 1. A trace showing the relative gas chromatographic retention times of a number of ecdysteroids is shown in Fig.11.

Gas Chromatographic Properties of TMS Ethers of Ecdysteroids

Fig. 11



Conditions 280°, 5 ft 1% OV1 01 on CQ column, gas flow rate 50 ml min^{-1}, ECD

Table 1

Gas Chromatographic Properties of Ecdysteroid TMS Ethers

Ecdysteroid	No. of TMS Groups	Retention time (min)	Column temperature
Ecdysone	4	1.85	280
	5	1.65	280
20-Hydroxyecdysone	4	2.25	280
	5	2.45	280
	6	1.9	280
Inokosterone	4	2.55	280
	5	2.85	280
	6	2.2	280
2-Deoxy-20-hydroxyecdysone	4	3.9	280
	5	2.4	280
Poststerone	2	0.6 (1.3)	280 (260)
	3	0.9	260
Cyasterone			280
3-Dehydroecdysone	3	2.15	280
3-Dehydro-20-hydroxyecdysone	4	2.70	280

Detection of TMS Ethers of the Ecdysteroids

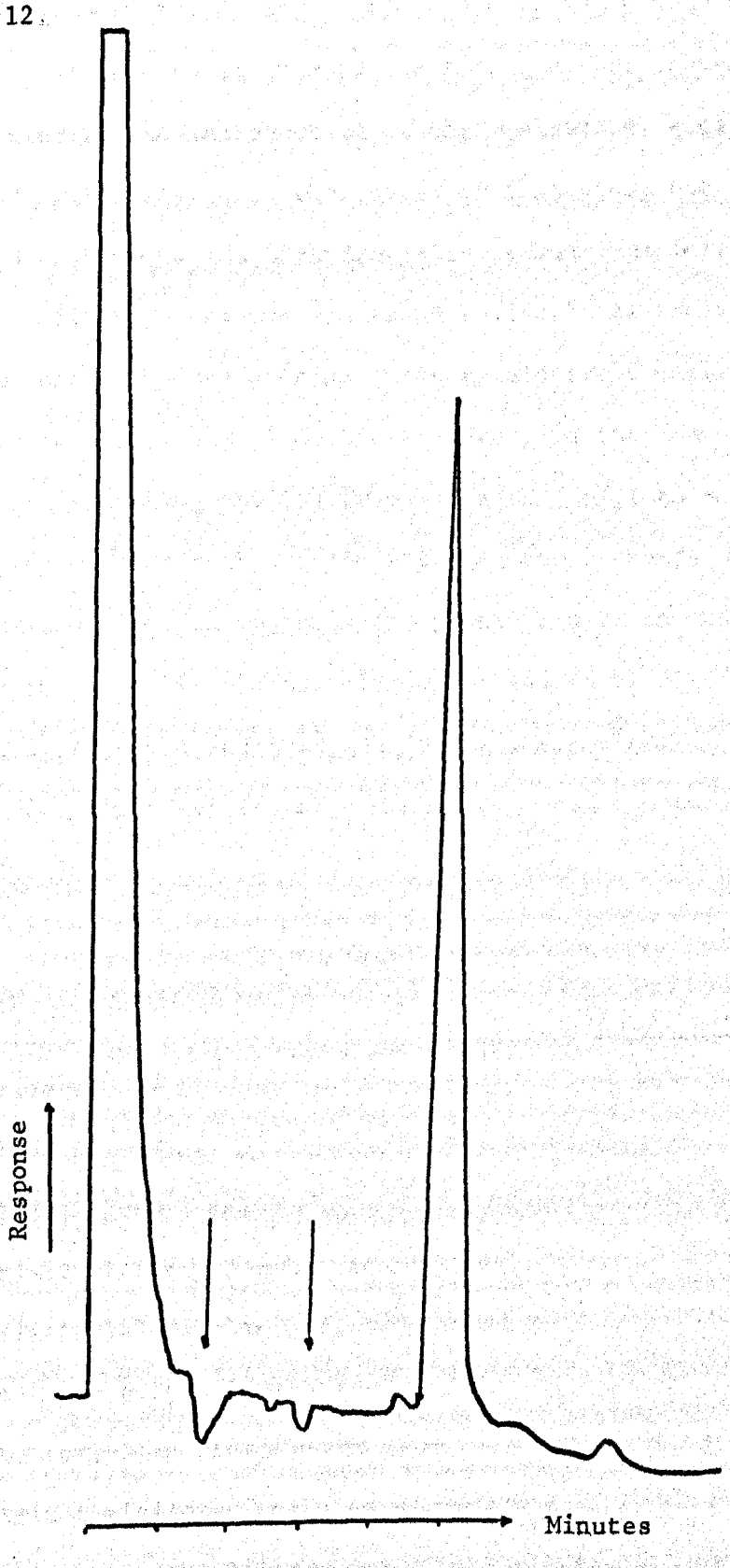
In this study the TMS ethers of the ecdysteroids were detected using either the flame ionisation detector (FID) or the electron capture detector (ECD). When samples derived from biological material, containing low levels of ecdysteroids were determined the electron capture detector was used exclusively. The ECD, unlike the FID, is a non-destructive detector using a Ni^{63} foil as a source of ionising radiation (β particles), to charge otherwise neutral molecules. In general the ECD is an insensitive detector to organic molecules, however where these are able to "capture" electrons the ECD is extremely sensitive and can detect down to 10^{-15} g of certain compounds[93]. Simply because it is non-destructive, relatively involatile compounds may condense onto the source and electrode. Over a period of time this build up of material may cause loss of sensitivity, and in extreme cases complete loss of response can occur. Consequently the response of the detector must be determined frequently, and fresh standard curves for the ecdysteriods must be prepared regularly. If contamination should become excessive the detector must be cleaned. The appearance of "negative peaks" as shown in Fig. 12, is indicative of detector contamination.

Structural Requirements for Electron Capture by the Ecdysteroids

In general steroids do not capture electrons and their determination using the ECD normally requires the introduction of an electron capturing substituent (e.g. the formation of a halomethylsilyl ether). Ikekawa *et al.* have described the formation of mixed trimethylsiloxyheptafluorobutyryl derivatives of the ecdysteroids which do indeed capture electrons[94]. However workers in this laboratory[91,92] and elsewhere[95] have shown that the ecdysteroids themselves will capture electrons strongly without the need to form halogenated derivatives. Work on model compounds[91] has shown that the electrophore involves the

"Negative" Peaks Due to Contamination of the Electron Capture Detector

Fig. 12.



Indicates position of "negative" peak

7-en-6-one-14α-hydroxyl function, with additional contributions from substituents on the A ring. Sterols with the 6 keto or 7-en-6-one groups but no substituents on the A ring do not capture electrons appreciably, the introduction of the 14α-hydroxyl greatly enhances electron capturing ability. In the course of this study the sterols 3β-hydroxy-5α-cholestan-6-one and 3β-hydroxy-5α-cholest-7-en-6-one have been synthesised and their ability to capture electrons tested. As found by Poole[92], the simple keto compound does not capture electrons well. However the introduction of the C-7 double bond to give the 7-en-6-one increases electron capturing ability from $\sim 6 \times 10^{-8}$ g/μl to $\sim 6 \times 10^{-10}$ g/μl. It is clear that both the 14α and 3β-hydroxyl groups are important in conferring electron capturing properties in these model compounds. Which of these groups is the most important source of electron capturing properties in the ecdysteroids is still not clear. A table of the electron capturing sensitivities of a number of model compounds is given below (Table 2).

Table 2

Compound	Least detectable amount
Cholesterol	10^{-6} g*
5α-cholestan-6-one	10^{-8} g*
5α-cholest-7-en-6-one	10^{-8} g*
3β-trimethylsiloxy-5α-cholestan-6-one	10^{-8} g
3β-trimethylsiloxy-5α-cholest-7-en-6-one	10^{-10} g
5α-acetoxy-cholest-7-en-6-one	10^{-10} g*
14α-hydroxy-5α-cholest-7-en-6-one	10^{-11} g*
14α-trimethylsiloxy-5α-cholest-7-en-6-one	10^{-11} g*
2β,3β,14α-trimethylsiloxy-5α-cholest-7-en-6-one	10^{-12} g*

* From Poole[92].

The Effect of Formation of the 14α-TMS Ethers on Electron Capture Properties of the Ecdysteroids

Ecdysteroids with the 14α group present as the TMS ether have been reported to be approximately an order of magnitude more sensitive to electron capture detection than the corresponding 14α-hydroxy compounds.[90,92] This significant increase in sensitivity might become important where very low levels of ecdysteroids are to be determined. To test this observation mixtures of a number of partially and fully silylated ecdysteroids were prepared. The relative proportions of 14α-TMS ether and 14α-hydroxy compounds were determined using the FID, following which the ECD responses of the mixtures were determined. If the 14α-TMS ethers were an order of magnitude more sensitive to electron capture the relative peak heights on FID and ECD should be different. In the case of the model compound 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one this is indeed the case, the 14α-TMS ether of this compound being significantly more sensitive than the 14α-hydroxy compound. However in the case of more complex ecdysteroids where some modification has been made to the side chain the situation is quite different. The results of the determinations are summarised below (Table 3). It can be seen that for 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone the sensitivities of the partially and fully silylated compounds arevery close. However ecdysone, inokosterone and poststerone are approximately fifty to sixty percent less sensitive to electron capture when present in the fully silylated forms. Traces showing the FID and ECD responses of partially and fully silylated ecdysone, 20-hydroxyecdysone, and 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one are shown in Fig. 13. It is clear in the case of ecdysteroids with some modification to the side chain that there is no enhancement of the electron capturing properties of the fully silylated compounds.

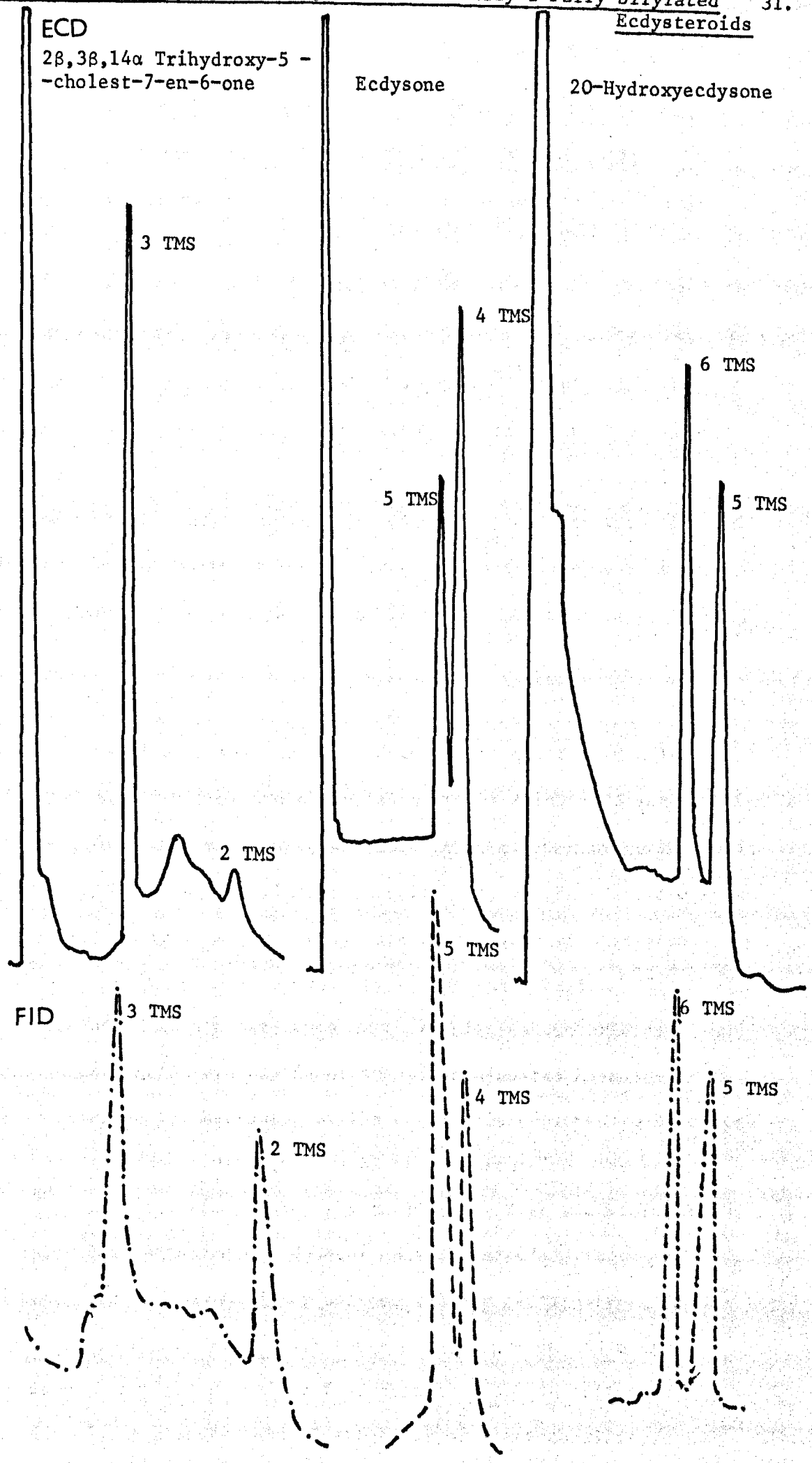
Table 3

Relative Sensitivities of Silyl Ethers to ECD

Compound	FID			ECD		
	14α OH*	14α TMS*	$\frac{14\alpha\ TMS}{14\alpha\ OH}$	14α OH*	14α TMS*	$\frac{14\alpha\ TMS}{14\alpha\ OH}$
Ecdysone	1.85	2.7	1.46	1.3	0.57	0.44
20-Hydroxyecdysone	1.26	1.5	1.2	1.80	1.96	1.08
2-Deoxy-20-hydroxyecdysone	2.01	2.93	1.39	0.5	0.76	1.52
Inokosterone	0.88	1.92	2.2	0.95	1.02	1.1
Poststerone	1.29	1.5	1.2	0.96	0.35	0.35
2β,3β,14α-Trihydroxy-5α--cholest-7- en -6-one	1.44	1.47	1.02	0.15	1.87	12.4

* Peak area in cm^3 derived from GC traces (see Fig.13)

Fig.13


ECD
2β,3β,14α Trihydroxy-5 - -cholest-7-en-6-one
Ecdysone
20-Hydroxyecdysone
3 TMS
2 TMS
4 TMS
5 TMS
5 TMS
6 TMS
5 TMS
FID
3 TMS
2 TMS
5 TMS
4 TMS
6 TMS
5 TMS

Given the difficulty of preparing the completely silylated compounds, and their similar electron capturing properties to the partially silylated compounds there would seem to be no advantages to using them in analysis except as a check on identity.

Limits of Detection of the Ecdysteroids

Pure ecdysteroids were detectable down to 10^{-11} g/µl using the electron capture detector. Biological samples contain a much greater level of impurities than pure standards which reduces the sensitivity of the method by both increasing the size of the solvent front and by increasing the "noise" on the traces. In practice this means that the limit of sensitivity of this method with biological material is 10^{-10} g/µl.

Ecdysteroid Conjugates

Derivatives of steroids have been detected in plants, mammals and insects and may be either non-polar, such as the fatty acid esters of cholesterol, or highly polar water-soluble esters of phosphoric or sulphuric acid, or ethers of glucose or glucuronic acid. These compounds are known under the general title of steroid conjugates. Conjugates have been recognised for over 50 years as a major route for the excretion of mammalian steroids. As a consequence of their clinical importance a number of methods for the hydrolysis of steroid conjugates have been developed, varying in the harshness of the conditions employed to liberate the steroid. More recently conjugates of the ecdysteroids have been detected[56-60], and successful hydrolysis to liberate free ecdysteroids has been achieved with enzymes. However enzymic methods have the limitation that the enzymes used may be specific for certain types of conjugate and so do not hydrolyse all the conjugate present in a sample. For this reason a chemical method is desirable that can be relied upon to hydrolyse conjugates regardless of structure or stereochemistry. A

number of chemical and enzymic procedures are considered below.

Hydrolysis of Sulphates by Heating at Neutral pH

Specific, non-acid hydrolysis of 3β-hydroxy-Δ5-sulphates has been reported[97], effected simply by heating the sample at neutral pH in buffer at 100° for 6 hours. The specificity of the reaction and the harsh conditions employed render this method unsuitable for the hydrolysis of ecdysteroid conjugates.

Oxidation of Glucuronides

The oxidative elimination of glucuronide conjugates is a technique of some clinical importance. The oxidation of the glucuronide by sodium periodate or bismuthate converts it to a formate ester, which may then be removed by mild acidic or basic hydrolysis[96]. However any vicinal diols in the steroid are simultaneously split. This has obvious disadvantages for the ecdysteroids which may have vicinal diols at C-2, C-3, and C-20, C-21. There is also controversy concerning the reproducibility of this method[96].

Solvolysis of Glucuronides and Sulphates

It has been shown that estrogen sulphates may be rapidly cleaved by standing in dioxan at room temperature[98] but hydrolysis did not take place in the presence of water, ethanol or other impurities. The addition of acid restores the ability of dioxan to hydrolyse sulphates even in the presence of impurities. The hydrolysis of conjugated steroids has now been reported in a number of acidified solvents, such as dioxan, tetrahydrofuran and ethyl acetate[96]. This method was initially limited to the hydrolysis of sulphates, however now both sulphates and glucuronides may be hydrolysed, either sequentially or together[99]. In the improved method sulphate or glucuronide conjugates

are hydrolysed in tetrahydrofuran (THF) or ethyl acetate, in the presence of small quantities of perchloric acid. A number of acid sensitive sterols were shown to be unharmed by the procedure, but others were degraded or rearranged. The (relatively) mild conditions used in this solvolytic procedure seem to make this one of the more suitable methods for achieving the hydrolysis of ecdysteroid conjugates chemically.

Enzymic Methods of Conjugate Hydrolysis

The mildest methods for hydrolysing steroid conjugates are those employing enzymes. One of the most common enzyme preparations for this, which has been used successfully in studies on ecdysteroid conjugates is obtained from the digestive juice of the snail Helix pomatia. This crude preparation contains two steroid sulphatases and a β glucuronidase. One of the sulphatases exhibits great specificity, hydrolysing 3βΔ5 and 3β-5α steroids exclusively, the other is thought to be a C-21 sulphatase[96].

Hydrolysis of Ecdysteroid Conjugates from the Desert Locust

Enzymic Procedures

The residues from the aqueous portions from the butanol:water and ethyl acetate:water partitions were taken up in acetate buffer. Half of each sample was then subjected to enzymic hydrolysis using the Helix pomatia enzymes, whilst the other was incubated without enzymes as a control. At the end of the overnight incubation both samples were extracted with butanol and their ecdysteroid content determined in the normal way. As a further control enzymic activity was determined both in buffer, and in portions of the buffered biological extracts, using artificial substrates. The results of experiments where conjugated ecdysteroids were sought in extracts obtained from biological material clearly demonstrated that conjugated ecdysteroids were present in the

aqueous portion derived from the butanol:water partition (from eggs, adult females, and faeces) and in no other of the fractions from the solvent partitions.

Conjugates of ecdysteroids and glucose have also been reported[56] and extracts of insects in the 5th larval instar were incubated with αD- and βD-glucosidases under similar conditions to those used for the Helix pomatia enzymes, but in phosphate buffer.

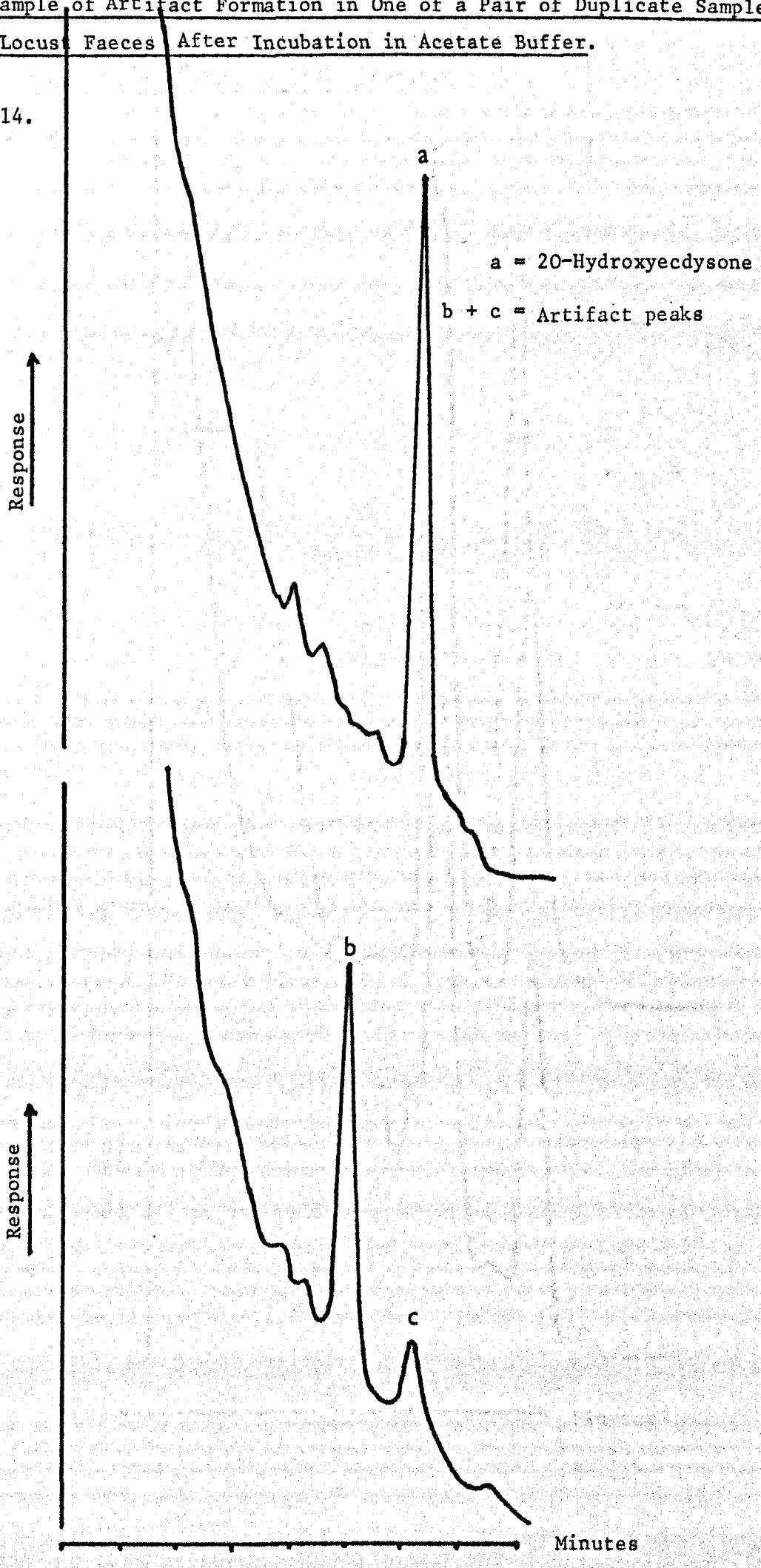
Artifact Formation

It soon became clear that there were problems associated with the enzymic method for the hydrolysis of ecdysteroid conjugates with the Helix pomatia enzymes. Duplicate samples gave different patterns of peaks on gas chromatography. This behaviour did not result from treatment with enzymes, but could result from simply incubating 20-hydroxyecdysone in acetate buffer. Examples of this effect are shown in Fig. 14, in one sample 20-hydroxyecdysone is present, in its duplicate, two peaks, neither of which has the same retention time as the pentakis TMS ether of 20-hydroxyecdysone are present. This result could be duplicated merely by adding a small quantity of sodium acetate to a silylating mixture containing 20-hydroxyecdysone. Thus it would seem that the carry-over of sodium acetate from the buffer into the Reacti-vial is responsible for the generation of this artifact Fig. 15.

These artifacts were formed in 80% of the ecdysteroid containing biological samples analysed. It was possible to quantify the 20-hydroxy-ecdysone present by summing the areas of the two peaks and comparing them with a standard curve of 20-hydroxyecdysone silylated in the presence of an acetate. However in the presence of acetate ecdysone is completely destroyed making its determination impossible. In addition to this, the effect of acetate on other metabolites of ecdysone and 20-hydroxy-

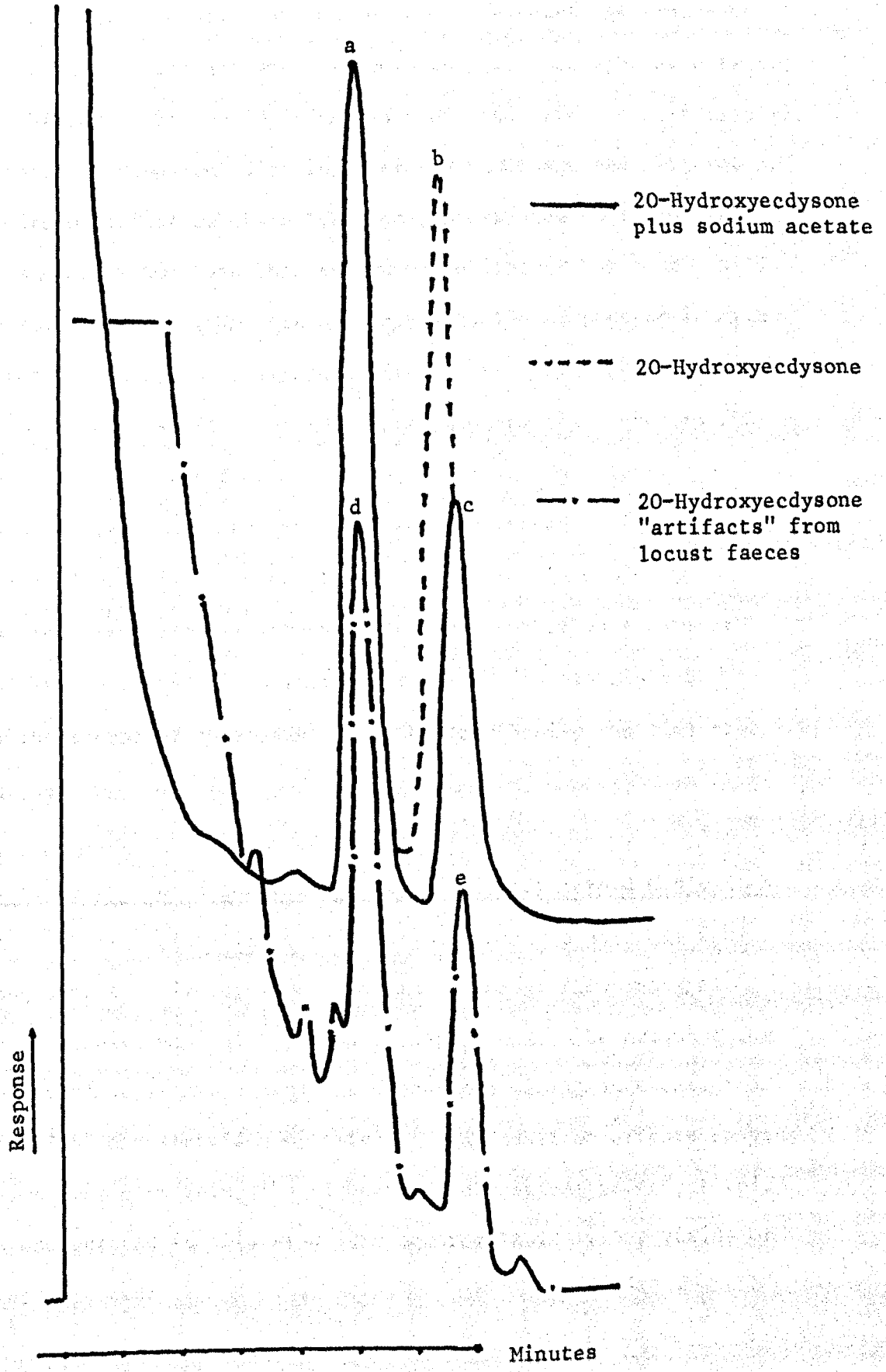
An Example of Artifact Formation in One of a Pair of Duplicate Samples From Locust Faeces After Incubation in Acetate Buffer.

Fig. 14.



Artifact Formation Due to Sodium acetate

Fig. 15



ecdysone (e.g. 3-dehydro compounds) which might have been present in faecal extracts was unknown. Because of the uncertainty generated by the use of acetate buffer alternatives were sought. Phosphate, citrate, and oxalate buffers were tested, of which phosphate gave the best results, sodium dihydrogen phosphate, disodium hydrogen phosphate and mixtures of both were without effect on the silylation process when added to the reaction mixture. Phosphate ions are known to inhibit one of the Helix pomatia enzymes (the 3βΔ5, 3β5α steroid sulphatase) when present in high concentration[96]. Control experiments with an artificial substrate showed that 100 mM phosphate buffer reduced sulphatase activity to 20% of its levels in acetate buffer. To compensate for this loss, the quantities of Helix pomatia enzymes used were increased five-fold. The yields of 20-hydroxyecdysone liberated from ecdysteroid conjugates present in biological samples were very similar whether phosphate or acetate buffers were used. No increase in yields was obtained by increasing the amount of enzymes used, or by lengthening the time of the incubation.

Efficiency of Enzymes Used for the Hydrolysis of Ecdysteroid Conjugates

The quantities of Helix pomatia enzymes added to extracts from various stages of the life cycle of the desert locust (800 units ml^{-1} sulphatase, 110 units ml^{-1} glucuronidase) were found to be adequate for the hydrolysis of ecdysteroid conjugates over the whole range detected (5-300 ng ml^{-1} of ecdysteroid conjugates). The addition of more enzymes to those solutions containing the highest concentrations of ecdysteroid conjugates encountered in this study did not result in higher yields of liberated ecdysteroids.

Contamination of Enzymes by Ecdysteroids and other Sources of Error

Ecdysteroids have been reported as contaminants of the Helix pomatia enzyme mixture (KARLSON, REES, personal communication). In this study contamination was found to vary between batches from zero to 1 ng/µl of ecdysteroids, depending upon the enzyme preparation. Suitable correction factors were applied to ecdysteroid yields.

Rarely spontaneous hydrolysis of conjugates in the control incubation was seen, and this could easily be mistaken for incomplete extraction of unconjugated ecdysteroids. Spontaneous hydrolysis was only observed when very high levels of ecdysteroid conjugates were present in samples, and never amounted to more than a few percent.

Attempted Hydrolysis of Ecdysteroid Conjugates by Chemical Methods

The enzymic methods used in this study appeared to be efficient at hydrolysing the ecdysteroid conjugates present in insects and faeces. However it was felt that a chemical technique would serve as a useful check to ensure that the enzymic method did hydrolyse all the conjugates present.

Attempted Solvolysis of Ecdysteroid Conjugates in Tetrahydrofuran and Ethyl Acetate

The first chemical hydrolysis attempted involved heating a sample of ecdysteroid conjugates in either THF or ethyl acetate in the presence of perchloric acid. This method has been used for the solvolysis of both sulphates and glucuronides[99]. After incubation at 50° for six hours the perchloric acid was removed by partitioning the reaction mixture between butanol and dilute sodium bicarbonate solution. The butanol was then removed by rotary evaporation and silylation and analysis was then performed in the usual way. When THF is used it

is important for reasons of safety to ensure that any peroxides present in the solvent are removed before the reaction is attempted. This was done simply by passing the THF through a short alumina column. When the stability of ecdysone and 20-hydroxyecdysone in the reaction mixture was determined it was found that complete destruction of the ecdysteroids occurred. Not surprisingly, ecdysteroid conjugates incubated under these conditions were also destroyed.

Attempted Solvolysis in Pyridine and Dioxan

The hydrolysis of steroid sulphates in both dioxan and pyridine has been reported[100]. To test its stability under the reaction conditions employed, 20-hydroxyecdysone was incubated in pyridine or dioxan for varying periods of time. At 100° approximately 70% of the 20-hydroxyecdysone present at the start of the reaction remained after one hour. These losses were considered to be low enough for the hydrolysis of ecdysteroid conjugates to be attempted. With pyridine as the solvent, the formation of the ecdysteroid TMS ethers could be carried out immediately the hydrolysis was stopped. The removal of dioxan proved difficult, but it was found that silylation could also be carried out in dioxan. Indeed, silylation of 20-hydroxyecdysone proceeds smoothly in dioxan without artifact formation. The time taken for the formation of the pentakis TMS ether of 20-hydroxyecdysone in dioxan is somewhat longer (∿ 10-12 hours) than for pyridine (5½-6 hours).

Samples of conjugates were either incubated at 100° for one hour or left at room temperature for a week. After this time samples were silylated and analysed for liberated ecdysteroids. No evidence for the hydrolysis of any ecdysteroid conjugates was obtained from the experiment. Further work is required to develop an effective, non-destructive chemical procedure for the hydrolysis of ecdysteroid conjugates.

Moulting Hormone Levels in the Desert Locust Schistocerca gregaria

The locusts have no king
Yet go they forth all of them by bands.
Proverbs 30.

Previous research in this laboratory demonstrated the presence of 20-hydroxyecdysone in 5th instar larvae of the desert locust[101]. The discovery that ecdysteroids captured electrons led to the development of a sensitive technique for the determination of these compounds in insects and crustacea[91,95]. Using this technique ecdysteroids in the 4th and 5th larval instars of the desert locust were identified and quantified[50]. In addition to ecdysone and 20-hydroxyecdysone a third, unidentified substance with gas chromatographic properties similar to those of an ecdysteroid was observed (referred to as compound C). The pattern of moulting hormones observed was similar for both 4th and 5th instars, a rapid rise in moulting hormone levels, reaching maximal titre, one or two days before ecdysis, followed by a rapid fall in titre to low levels at ecdysis.

These results, and the results from work on other insects led us to ask ourselves questions on a number of aspects of ecdysteroid metabolism in the desert locust.

Briefly these were as follows:-

Was the rapid rise in ecdysteroid titre observed during the 4th and 5th larval instars due to *de novo* synthesis or was this rise mediated by the release of hormone, synthesised during the first few days of the instar, and then stored in an inactive form as a polar conjugate?

Was the rapid fall in ecdysteroid levels preceding ecdysis due to the excretion of these compounds either unchanged or conjugated, or to their metabolism to other compounds (e.g. compound C)?

What is the nature of compound C?

Ecdysteroid Levels in 5th Instar Larvae of the Desert Locust

Ecdysteroid levels were determined for groups of ten 5th instar larvae of known ages from the 4th-5th larval ecdysis up to the larval--adult ecdysis. The results of these analyses are given in Table 4a, and Fig. 16. Initially ecdysteroids were found in detectable quantities on the fourth day of the instar. At this stage of development both ecdysone and 20-hydroxyecdysone are present in comparable amounts. Ecdysone levels then fall, whilst 20-hydroxyecdysone levels increase rapidly, reaching a maximum on the eighth day of the instar. After reaching a maximum on day eight, ecdysteroid titres then fall rapidly until at ecdysis only traces of 20-hydroxyecdysone remain.

Rate of Conversion of Ecdysone to 20-Hydroxyecdysone During the 5th Instar

The proportion of ecdysone and 20-hydroxyecdysone does not remain constant during the 5th instar. On day four the ratio of ecdysone to 20-hydroxyecdysone is approximately 1:2. At the time of the highest ecdysteroid titre (day 8) the ratio of ecdysone to 20-hydroxyecdysone has become 1:37. The variations in the relative proportions of ecdysone and 20-hydroxyecdysone presumably reflect changes in the ability of the C-20 monooxygenase to hydroxylate ecdysone. *In vivo* experiments using radiolabelled ecdysone have demonstrated that the capacity of the C-20 monooxygenase system in *Locusta migratoria* does indeed vary during the 5th instar[28]. *In vitro* experiments on fat body and malpighian tubules from the desert locust also show variations in C-20 hydroxylation dependant upon the age of the insects[18]. In both of these studies the rate of C-20 hydroxylation is low initially and increases to its highest levels at about the time of the highest titres of moulting hormones. From this study it would seem that the situation is as follows.

Table 4a

Ecdysteroids in 5th Instar S. gregaria, phase gregarious

Day of Instar	ng α/insect	ng β/insect	ng α/g	ng β/g	Wt. per insect g
1	-	-	-	-	0.67
2	-	-	-	-	0.75
3	-	-	-	-	1.00
4	9.25	16.4	8.56	15.2	1.08
5	-	39.9	-	30.2	1.32
6	-	85.8	-	52.9	1.62
7	9	302.8	5.29	177.6	1.7
8	29.5	1134	17.7	679	1.67
9	-	491.8	-	314.7	1.56
10	-	457.8	-	294.8	1.55
ecdysis	-	-	-	-	-

Results represent an average of 3 sample of ten insects each.

α = ecdysone β = 20-hydroxyecdysone

Table 4b

Ecdysteroids in 5th Instar S. gregaria phase solitarius

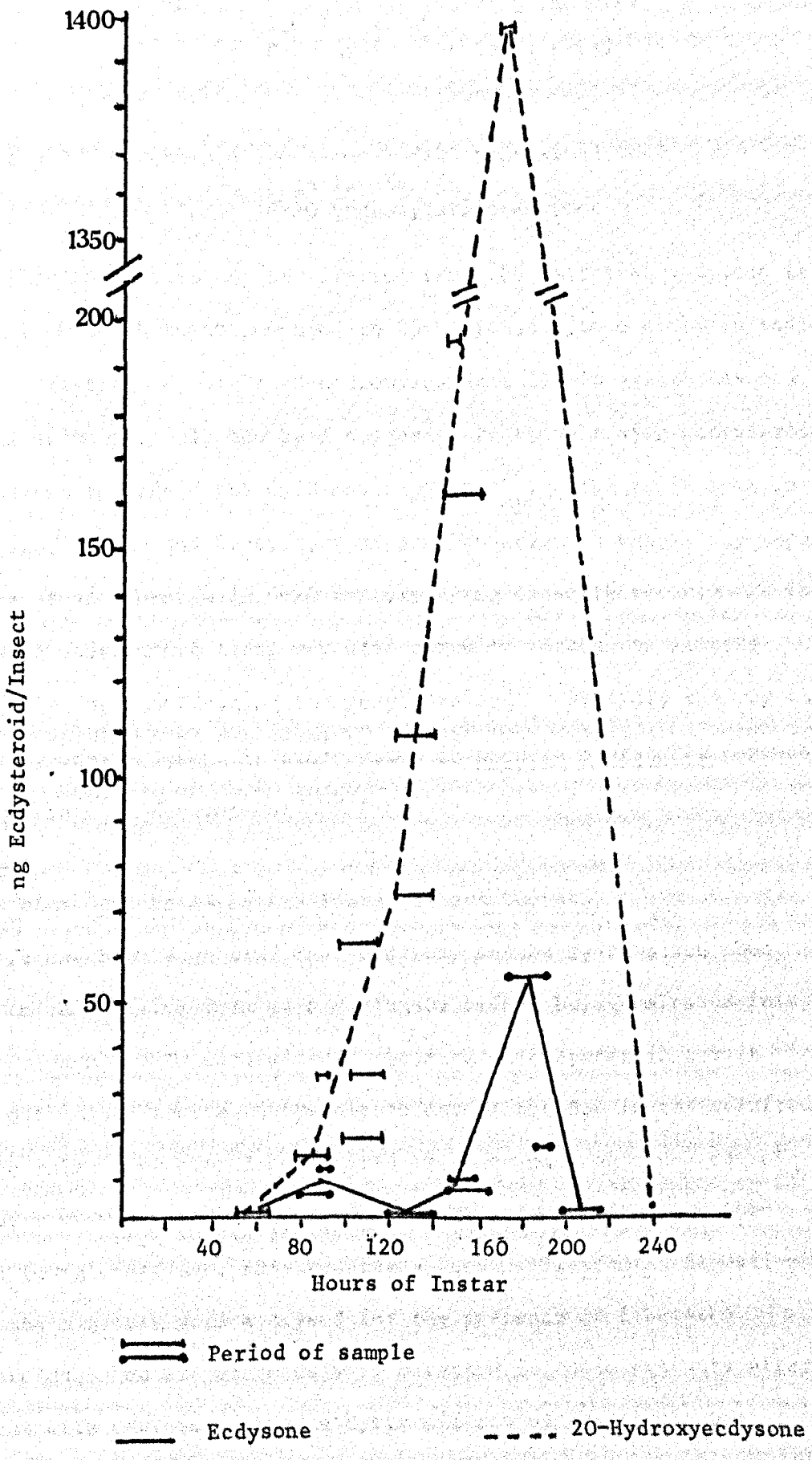
Day of Instar	ng α/insect	ng β/insect	ng α/g	ng β/g	Wt. per insect g
7	40	321.6	20.53	165	1.95
8	35	1201.8	17.93	615	1.95
9	3.3	536.5	1.92	318	1.69
10	-	10	-	5.82	1.72

Results represent the average of 3 samples of 1, 2 and 2 insects.

α = ecdysone β = 20-hydroxyecdysone

Ecdysteroids in 5th Instar S. gregaria phase gregaria

Fig. 16



Day 4

$$\text{Ecdysone} \xrightarrow[\text{low levels of C-20 hydroxylating enzymes?}]{\text{slow}} \text{20-hydroxyecdysone}$$

Day 8

$$\text{Ecdysone} \xrightarrow[\text{high levels of C-20 hydroxylating enzymes}]{\text{fast}} \text{20-hydroxyecdysone}$$

That this is so may be inferred from the fact that ecdysone is relatively more abundant compared to 20-hydroxyecdysone early in the instar, whilst on day eight 20-hydroxyecdysone levels greatly exceed those of ecdysone. It has been suggested that the rising ecdysteroid titre serves to induce the C-20 monooxygenase[28]. The variations in the ratios of ecdysone and 20-hydroxyecdysone observed in this study support the idea of an increase in C-20 hydroxylating capacity through the instar. The rising ecdysteroid titre may also serve to induce the enzymes responsible for inactivating the ecdysteroids. Certainly the way in which exogenous ecdysone is inactivated in Locusta migratoria depends upon the stage of development[40,62].

Storage of Ecdysteroids in 5th Instar Desert Locusts

It has been suggested that ecdysone may be synthesised then stored in an inactive form as a conjugate before being released into the haemolymph[8]. To determine if the rise in ecdysteroid levels observed from the fourth to the eighth day of the 5th instar resulted from the release of stored conjugates extracts made on each day of the instar were treated with enzymes. After incubation with the enzymes (αD-glucosidase, βD-glucosidase, and Helix pomatia digestive juice) the extracts were analysed for the presence of liberated ecdysteroids. No ecdysteroids were detected in these extracts after treatment with enzymes so that ecdysteroid conjugates to glucose,

glucuronic acid, or sulphate are not present in quantity at any stage of the 5th instar. It may be that conjugated ecdysteroids resistant to hydrolysis by the enzymes used in these experiments were present, in which case they would remain undetected. However in similar experiments on adult females (discussed later) considerable quantities of moulting hormones for which a function as storage compounds seems probable were liberated. Similar compounds present in quantity in 5th instar insects would have been easily detected by these experiments. This means that a completely different type of conjugate must be postulated if storage of ecdysteroids is important in the 5th instar.

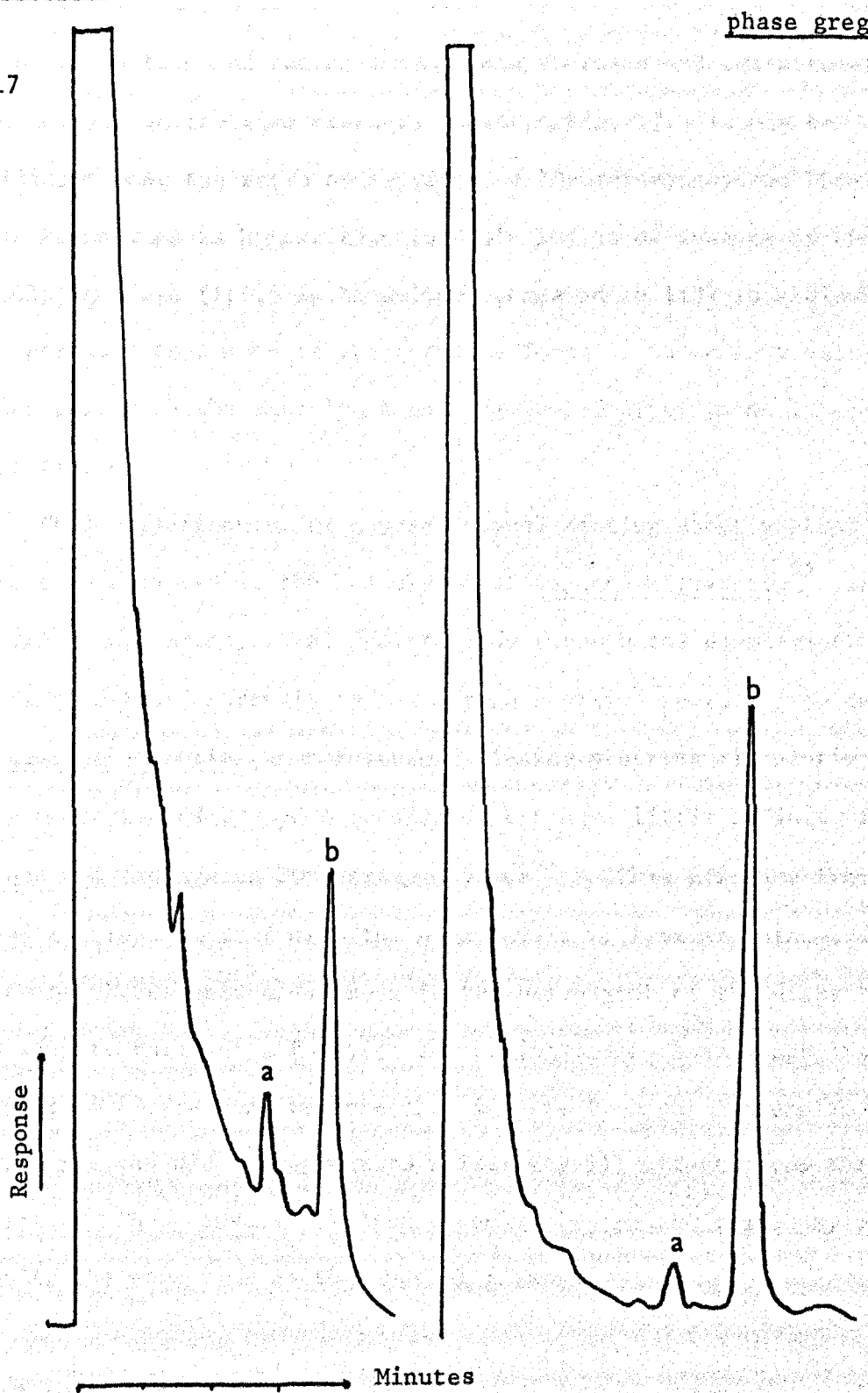
On the basis of these experiments, de novo synthesis of moulting hormones rather than the release of stored ecdysteroid conjugates would seem to be the most likely cause of the rapid rise in ecdysteroid titres observed during the 5th instar.

Transport of Ecdysteroids in the Haemolymph

It has been suggested that ecdysteroids may occur in the haemolymph bound to proteins[8,44] (either as free ecdysteroids or as their conjugates). Alternatively ecdysteroids may be found in the haemolymph simply as a solution of these hormones (either free or conjugated). The analysis of whole bodies for conjugated ecdysteroids performed in this study rules out their presence in large quantities. However if ecdysteroids were present in the haemolymph as conjugates, but in low concentration they might escape detection when the whole insect was analysed. To determine whether conjugated ecdysteroids were present in the haemolymph of 5th instar desert locusts, haemolymph was collected from insects with high ecdysteroid levels. For this study 2 ml of haemolymph was collected from a synchronous colony of approximately fifty insects, on day eight of the 5th instar. Analysis

Ecdysteroids in Haemolymph and Whole Bodies of 5th Instar S. gregaria, phase gregaria

Fig.17



Haemolymph, day 8

a = Ecdysone, 96 ng/ml

b = 20-Hydroxyecdysone, 432 ng/ml

Whole bodies, day 8

a = Ecdysone, 17.7 ng/insect

b = 20-Hydroxyecdysone 1134 ng/insect

of the haemolymph showed it to contain only ecdysone and 20-hydroxy-ecdysone. A trace of the haemolymph ecdysteroids and extracts of whole insects at the same stage is shown in Fig. 17. It may be significant that the ratio of ecdysone to 20-hydroxyecdysone observed in the haemolymph is higher than in whole bodies of insects of the same physiological age (1:4.5 in haemolymph compared to 1:37 in whole bodies). It is possible that much of the ecdysone found in whole body extracts is circulating in the haemolymph and the 20-hydroxyecdysone is accumulating in the tissues.

These experiments, of course, reveal nothing about whether proteins of the type observed in the haemolymph of Locusta migratoria[44] are involved in the transport of ecdysteroids through the haemolymph. Any ecdysteroids bound strongly to haemolymph proteins would not be detected. In Locusta migratoria the ecdysteroid binding proteins circulating in the haemolymph bind 20-hydroxyecdysone, but have little affinity for ecdysone and conjugated 20-hydroxyecdysone[44]. This evidence from a related species combined with the high levels of free ecdysteroids (and absence of their conjugates) seen in the haemolymph of the desert locust would seem to rule out a function for conjugates in haemolymph transport.

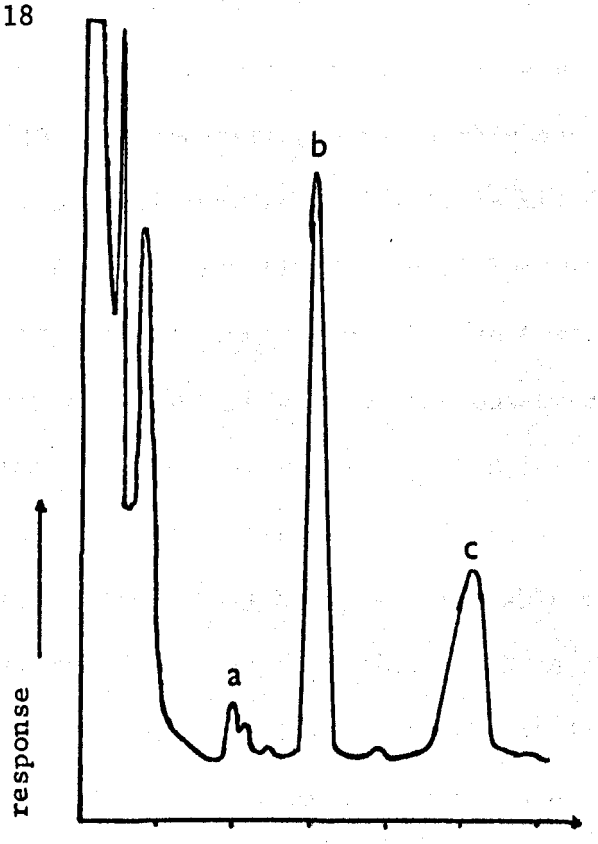
It is interesting to note that insects from which haemolymph had been removed did not appear to suffer any ill effects from this treatment. These insects moulted successfully on the tenth day of the instar at the same time as the control insects.

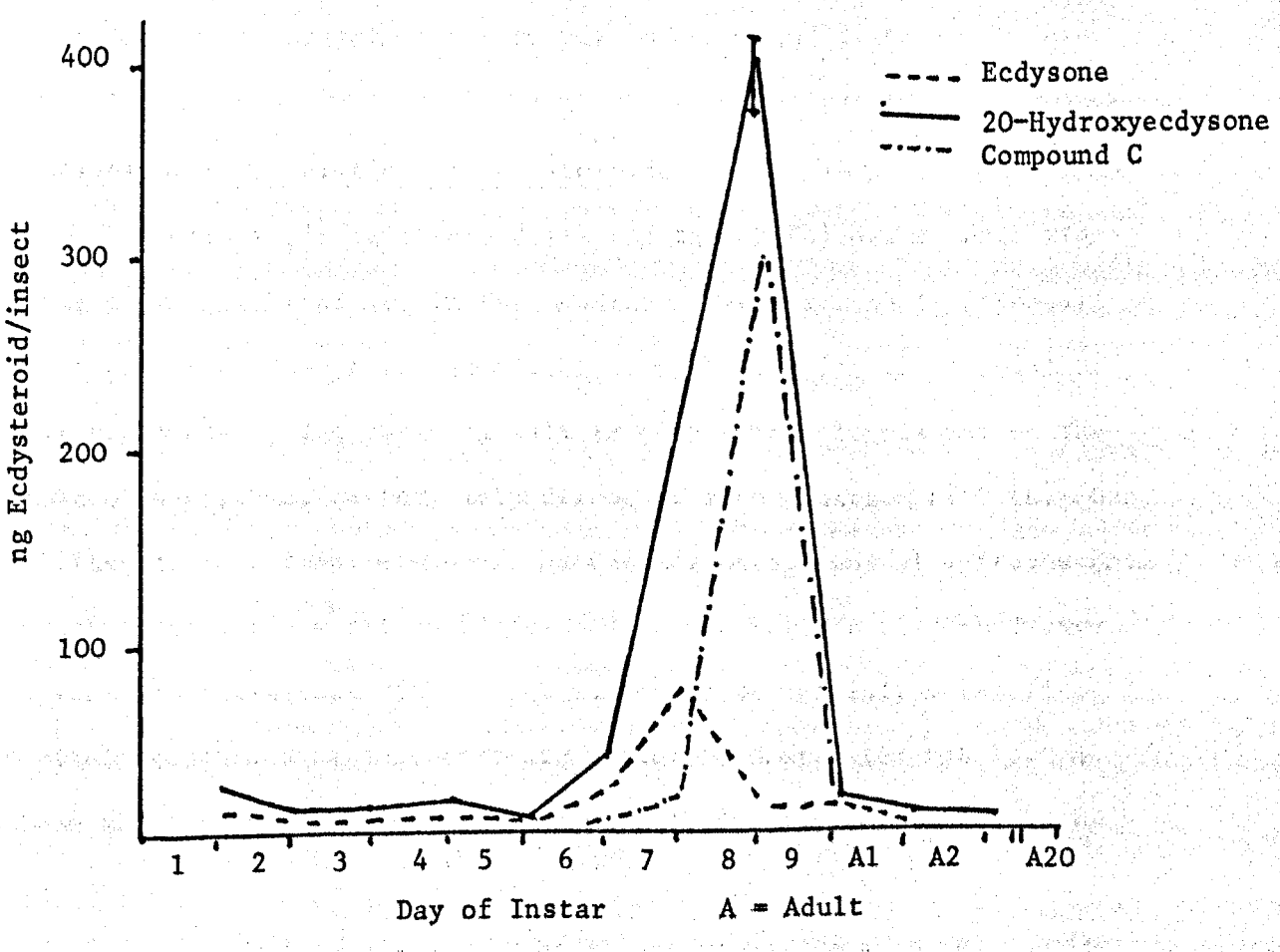
The Nature of Compound C

The earlier study on S. gregaria undertaken in this laboratory indicated the presence of a third, more polar, ecdysteroid (compound C) present in extracts of 4th and 5th instar insects[92]. This substance was found to be present in considerable quantities during the latter

"Compound C" In 5th Instar S. gregaria phase gregaria - From Poole[92]

Fig.18

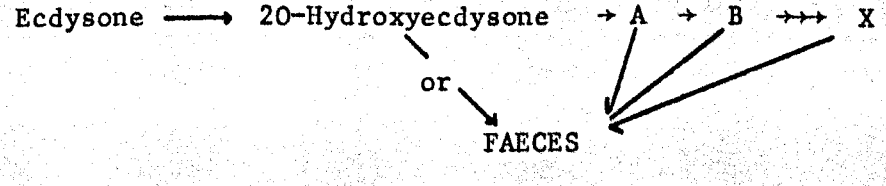




half of the instar. A gas chromatographic trace from the earlier work along with the results obtained for the 5th instar are reproduced in Fig. 18. A comparison between this and the earlier study reveals two important differences. Firstly in the present work no compounds of longer GC retention time thatn 20-hydroxyecdysone are present in any quantity at any time during the 5th instar. Secondly, at its maximum, the levels of 20-hydroxyecdysone observed in this study (∿ 1400 ng/insect) are much higher than those reported for the earlier work (400 ng/insect). It seems likely that these two facts are related, the results obtained in the earlier study being attributable to artifact formation. It is likely that artifact formation in the earlier study occurred during silylation, in a similar way to the artifact formation observed in this study in the presence of sodium acetate. No results comparable to those of POOLE[92] have been obtained anywhere in the present study, so it has been impossible to investigate the structure of compound C.

Inactivation and Excretion of Ecdysteroids

From the rapid fall in ecdysteroid titres following their high levels on day eight of the instar onwards it is clear that the desert locust possesses an active and efficient mechanism for the removal of these compounds. The observed fall in ecdysteroid levels may be the result of excretion, further metabolism, or a combination of both. Our inability to detect any compounds on gas chromatography of extracts from whole insects which might be identified as metabolites of 20-hydroxy-ecdysone indicates that either such compounds are rapidly metabolised to other substances or that they are rapidly excreted (or both). A scheme which might explain the observed fall in ecdysteroid levels is given below.



If faecal excretion of ecdysteroids is an important method for reducing the levels of ecdysteroids in the desert locust evidence of this might be obtained by examining the faeces. A number of studies on other insects have demonstrated the presence of ecdysteroids in faecal extracts [49,51,62].

Faeces were collected daily from synchronous colonies of 5th instar desert locusts, extracted in methanol, and analysed for the presence of ecdysteroids and their conjugates. The results of these experiments are shown in Fig.19 and Table 5. 20-Hydroxyecdysone is first detected in faecal extracts on the fifth day of the instar, which is the day after ecdysteroids are first detected in the insect. Conjugates of 20-hydroxyecdysone are detected in faecal extracts on the following day. From day six of the instar onwards free and conjugated 20-hydroxyecdysone are found in faecal extracts in approximately equal quantities. Amounts of excreted free and conjugated ecdysteroids in the faeces increase rapidly from day four reaching their maximum values on day eight. The highest values for ecdysteroids found in the faecal extracts are therefore present at the time of the highest ecdysteroid titres in the insect. After day eight the amount of 20-hydroxyecdysone present in the faeces declines steadily. However this is only true if the results are considered on the basis of hormone excreted per insect. If the results are considered simply on ng 20-hydroxyecdysone per gram of faeces, the quantity of hormone in the faeces appears to increase towards the end of the instar (Fig. 20). The quantity of faeces excreted per insect is shown in Fig. 21. The pattern of excretion of 20-hydroxy-ecdysone found in the faeces superimposed upon the amount of faeces excreted is shown in Fig. 22. There is no obvious correlation between the amount of faeces excreted and the quantity of 20-hydroxyecdysone detected in faecal extracts. The apparent increase in ecdysteroid levels

Table 5

20-Hydroxyecdysone Present in the Faeces of 5th Instar S. gregaria

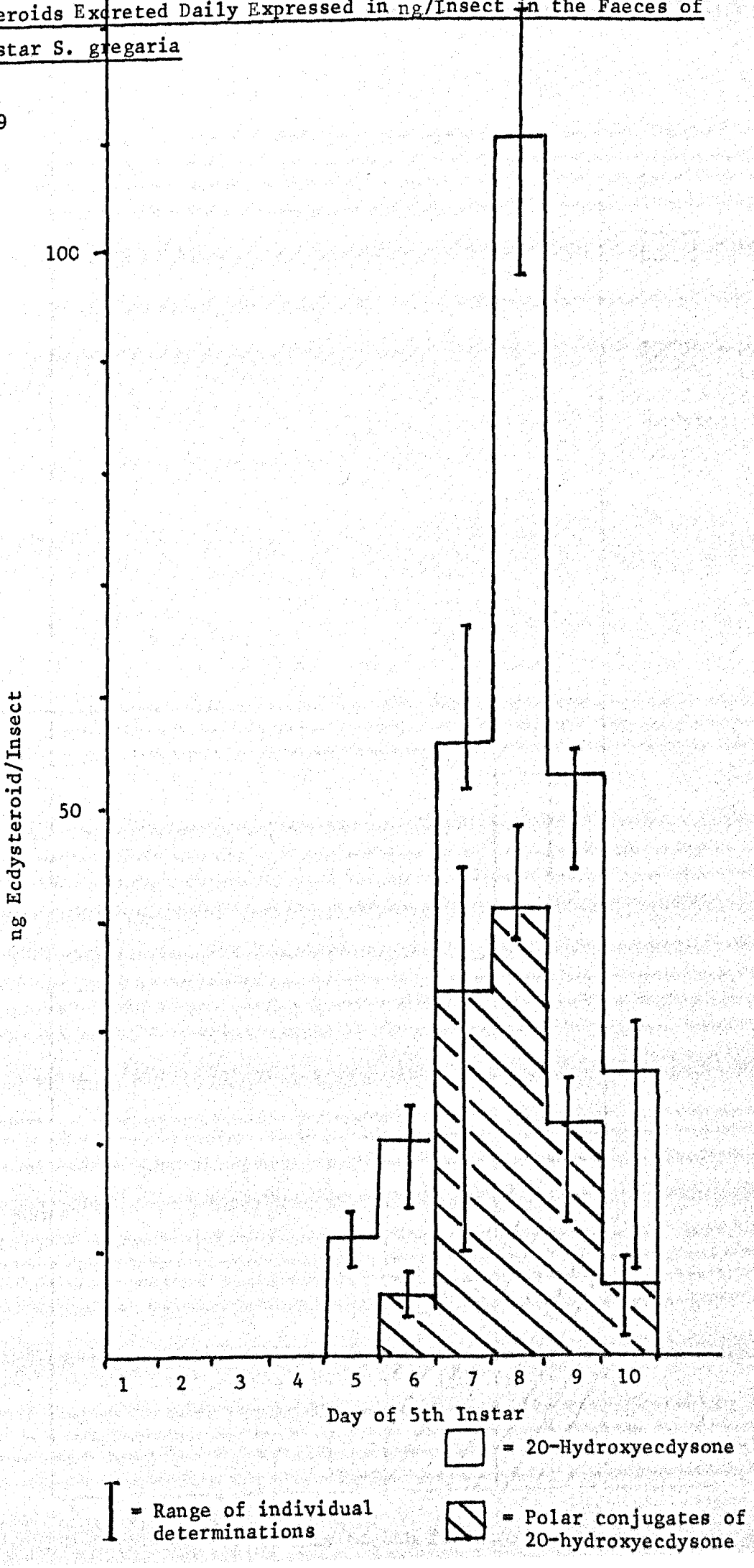
Day of Instar	Ng β+ conjugates in faeces/insect	% β conjugates	wt of faeces/insect mg	ng β/g faeces	% Daily excretion β
1	-	-	40	-	-
2	-	-	75	-	-
3	-	-	118	-	-
4	-	-	101	-	-
5	10.9	-	138	78	27.3
6	22.8	19.3	154	150	26.6
7	53.4	59.9	98	560	18.3
8	106.4	42.3	106	1003	9.3
9	52.5	41	38	1300	10.6
10	25.8	25.6	11	2340	5.6

Results represent the average of determinations on 3 colonies of 5th instar S. gregaria of approximately 50 insects each.

β = 20-Hydroxyecdysone.

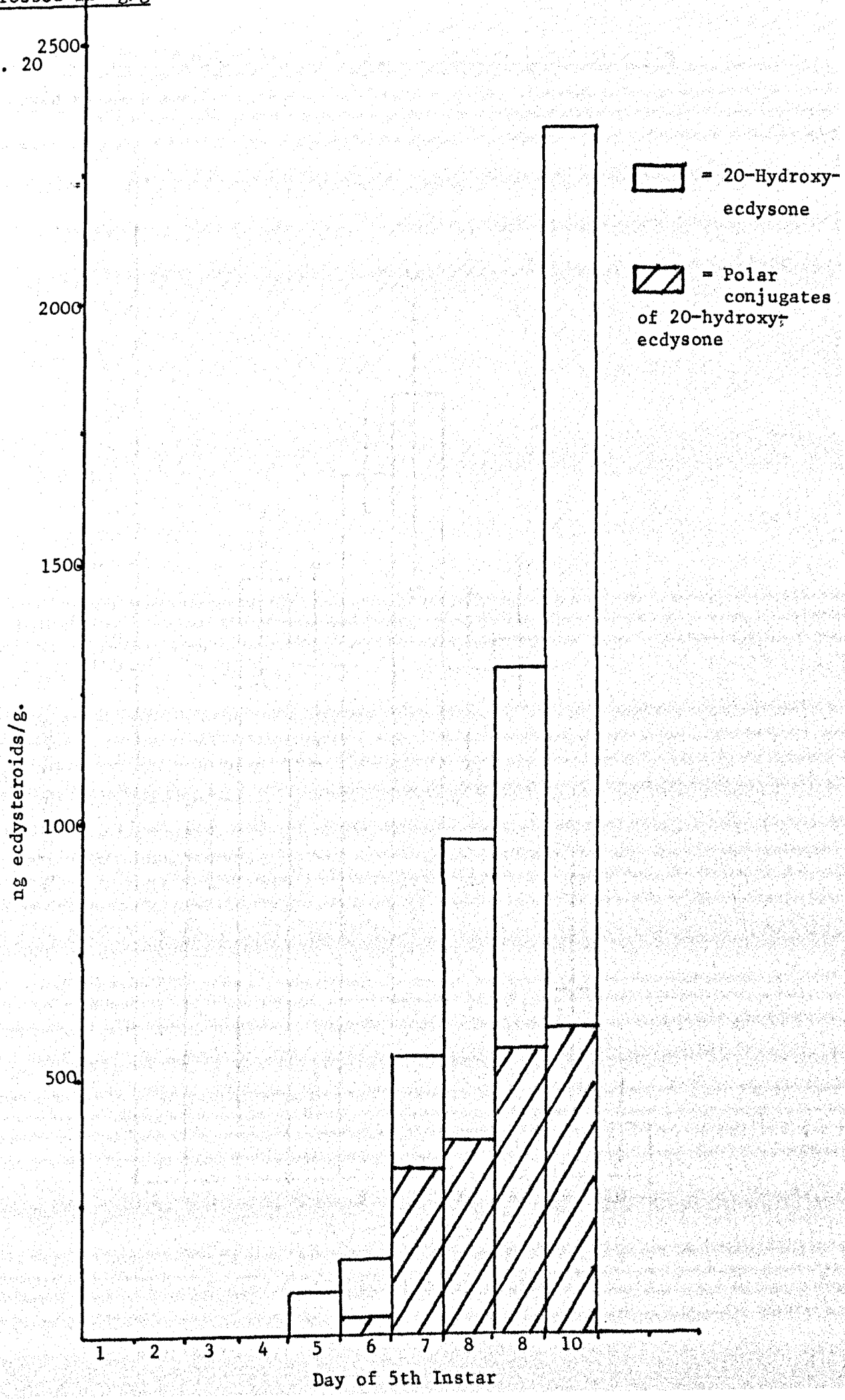
Ecdysteroids Excreted Daily Expressed in ng/Insect in the Faeces of 5th Instar S. gregaria

Fig. 19



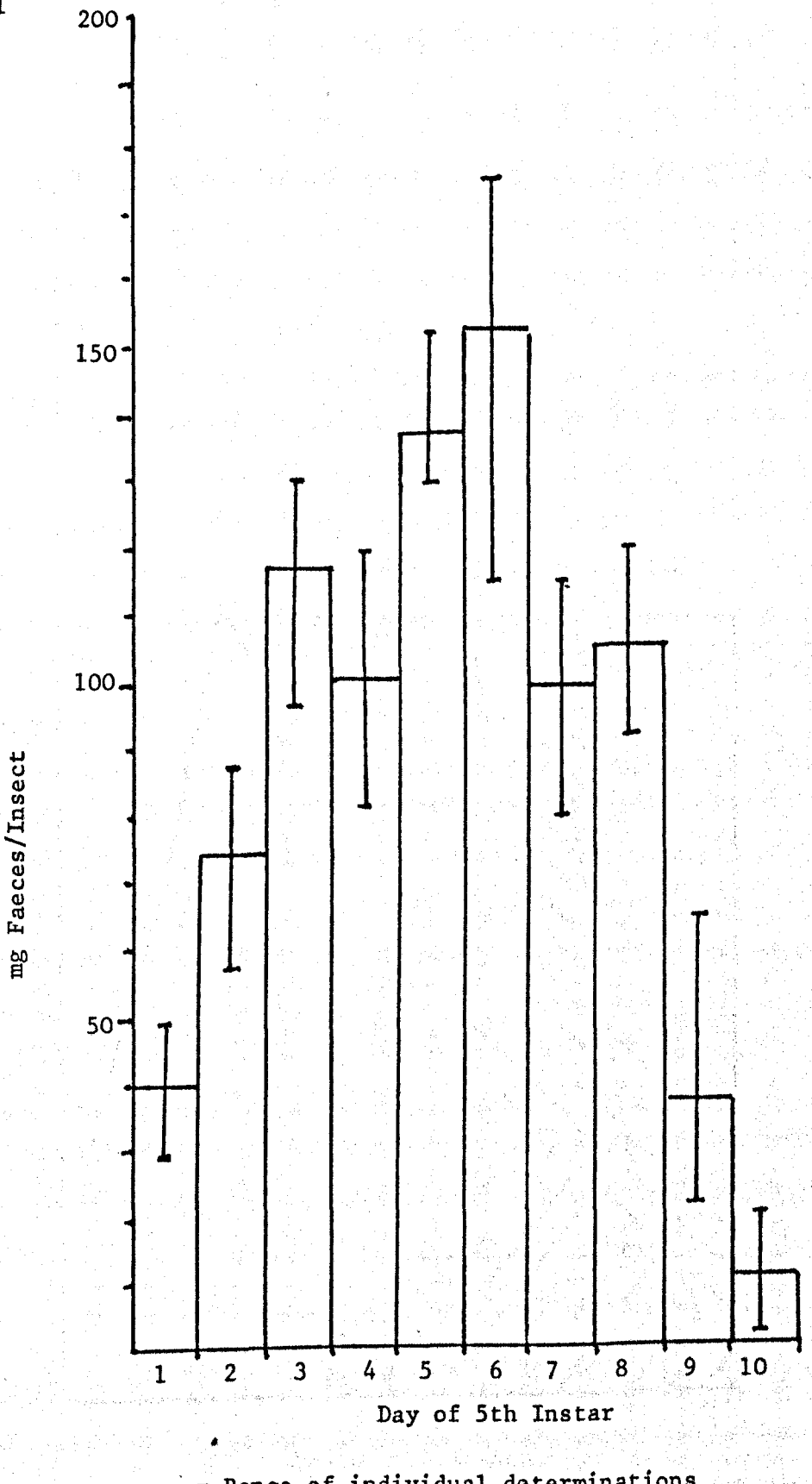
Ecdysteroids Excreted Daily in the Faeces of 5th Instar S. gregaria
Expressed in ng/g

Fig. 20



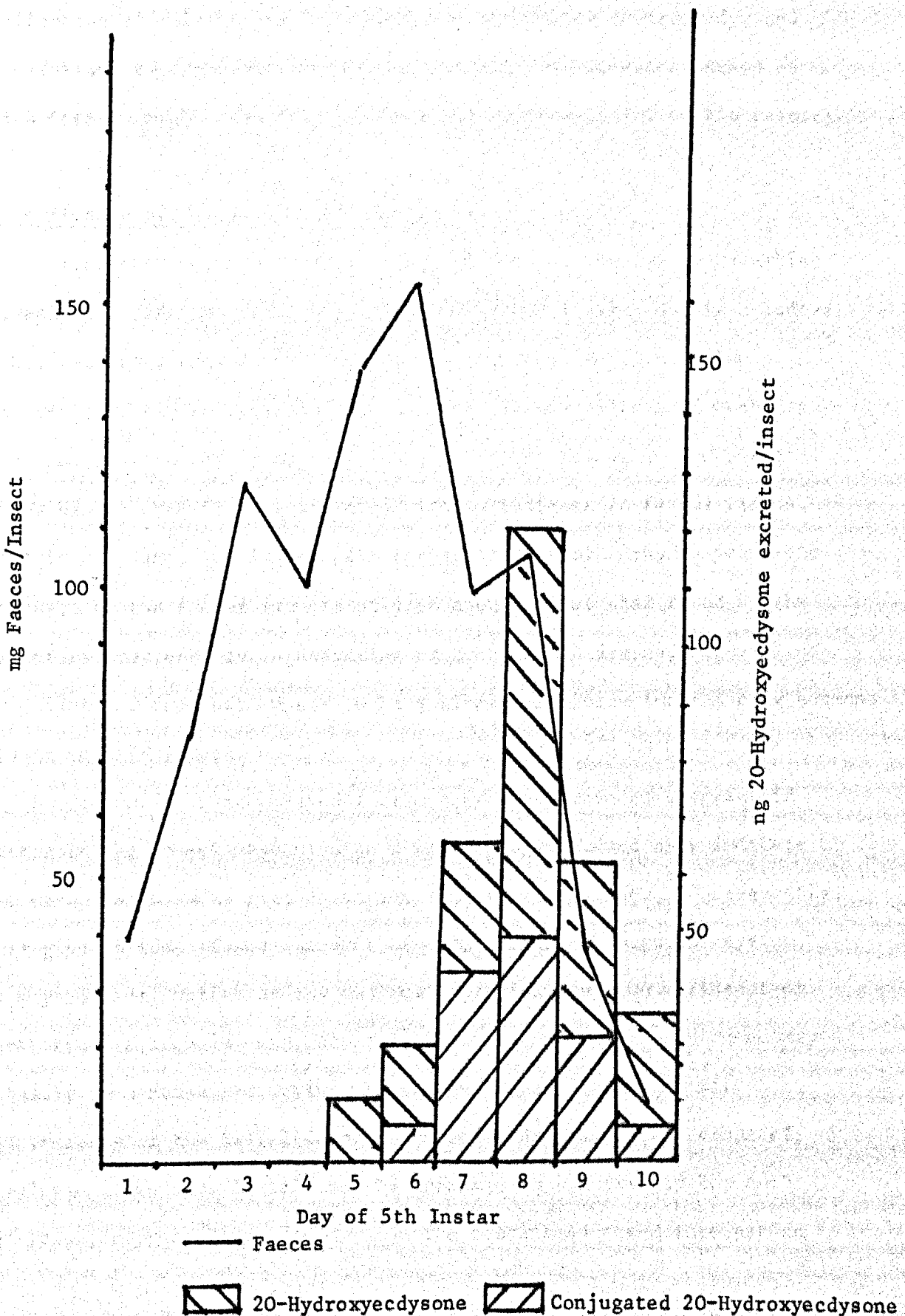
Quantities of Faeces Excreted Daily by S. gregaria, Phase gregaria expressed as mg/Insect

Fig. 21



Comparison of Excretion of Faeces and 20-Hydroxyecdysone Showing the Lack of any Correlation Between them.

Fig. 22



found in the faeces (exceeding the levels found in the insect) if quantities of hormone per gram of faeces is considered illustrates the need for care in experiments of this type. Failure to ensure the collection of all the faeces produced by the colony under study, and the failure to determine the average quantity of faeces produced by each insect, might result in an incorrect interpretation of the results.

Significance of Faecal 20-Hydroxyecdysone

The pattern of 20-hydroxyecdysone found in the faeces resembles closely the rise and fall of ecdysteroid levels in the insect. Indeed the levels of faecal 20-hydroxyecdysone mirror those seen in the insect. If the observed reduction in the insect titres of 20-hydroxyecdysone was the result of excretion, a drop in levels in the insect might be expected to be associated with an increase in faecal titres. This is not seen, and in any case the quantities of 20-hydroxyecdysone observed in the faeces amounts only to about 10% of that found in the insect for any one day. Excretion of 20-hydroxyecdysone, either free or conjugated does not appear to be a likely mechanism for reducing hormone levels in this insect.

It should be remembered that measurements made on ecdysteroid levels in the insect were made on a dynamic system, and only indicate the amount of hormone present at the moment of sampling. Consequently they give no idea of the turnover of ecdysteroids through the system. It may be that, whilst at its maximum approximately 1400 ng/insect of 20-hydroxyecdysone is seen, several times that quantity of hormone may actually be synthesised during the course of day eight. Until measurements of the half life of 20-hydroxyecdysone at this stage of development are made it will not be possible to say exactly how much 20-hydroxyecdysone is produced. On the other hand the determination

of the faecal ecdysteroids are made on a static system, so that the results represent the total amount of hormone excreted on any one day. With this in mind it can be seen that 10% is the maximum value which can be placed on faecal excretion of the ecdysteroidsas 20-hydroxyecdysone.

Possible Errors Resulting From Microbial Degradation of Faecal Ecdysteroids

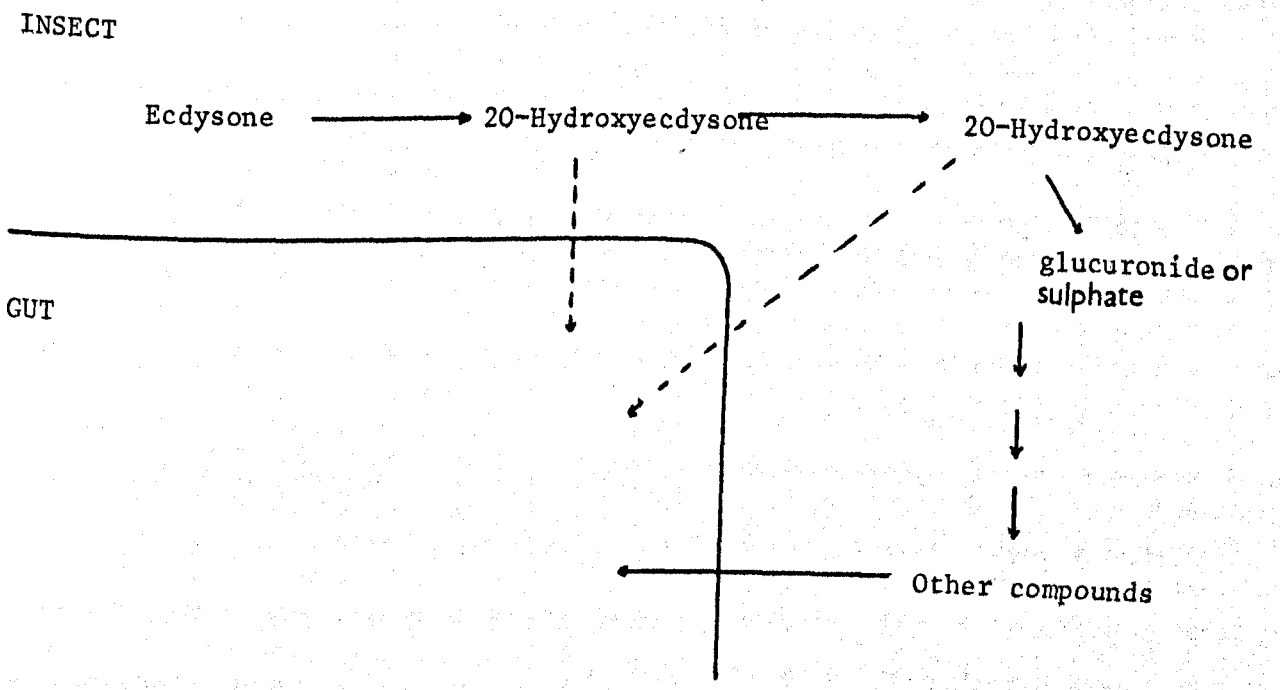
One possible reason for not observing larger quantities of 20--hydroxyecdysone excreted into the faeces may be degradation by bacteria present in the gut. If bacterial degradation of ecdysteroids does occur in the gut of the desert locust it would significantly distort the quantities detected. The insects used in this study were not reared aseptically so that bacterial degradation of excreted ecdysteroids is a possibility. In a similar study on Bombyx mori[103] degradation of ecdysteroids by gut bacteria was not found to be significant. However until suitable experiments are performed to rule out the possibility of degradation in the gut of the desert locust the conclusions on the fate of ecdysteroids from these excretion experiments must be treated with caution.

Function of 20-Hydroxyecdysone Conjugates

The 20-hydroxyecdysone found in the faeces of the desert locust is almost equally divided between the free and conjugated forms, a result similar to that found after the excretion of radiolabelled ecdysone by Locusta migratoria[40]. If these substances are not present in the faeces in sufficient quantity to account for the fall in ecdysteroid titres in the insect the function of the conjugates is unclear. It is known however that conjugated ecdysteroids are not biologically active[56]. This lack of biological activity certainly suggests that conjugates are somehow involved in inactivation of the ecdysteroids, however the small amounts

present in our extracts indicates that this is not the whole story. A possible function of the conjugation of ecdysteroids is to rapidly inactivate the hormone before it is metabolised to some other compound(s), which may or may not then be excreted.

Fig. 23.



Conjugation might also serve to activate the ecdysteroid to the enzyme system responsible for its further metabolism. One of the organs active in the metabolism of ecdysteroids is the malpighian tubule[27,58], the normal organ of excretion in insects[104]. It may be that the 20-hydroxyecdysone and conjugates of it found in the gut are not excreted but are present because of some unspecific leakage from the malpighian tubules during the course of ecdysteroid metabolsm and degradation.

No other peaks were seen on gas chromatography of faecal extracts which might represent large quantities of ecdysteroid metabolites. If however side scission is part of the inactivation process such compounds

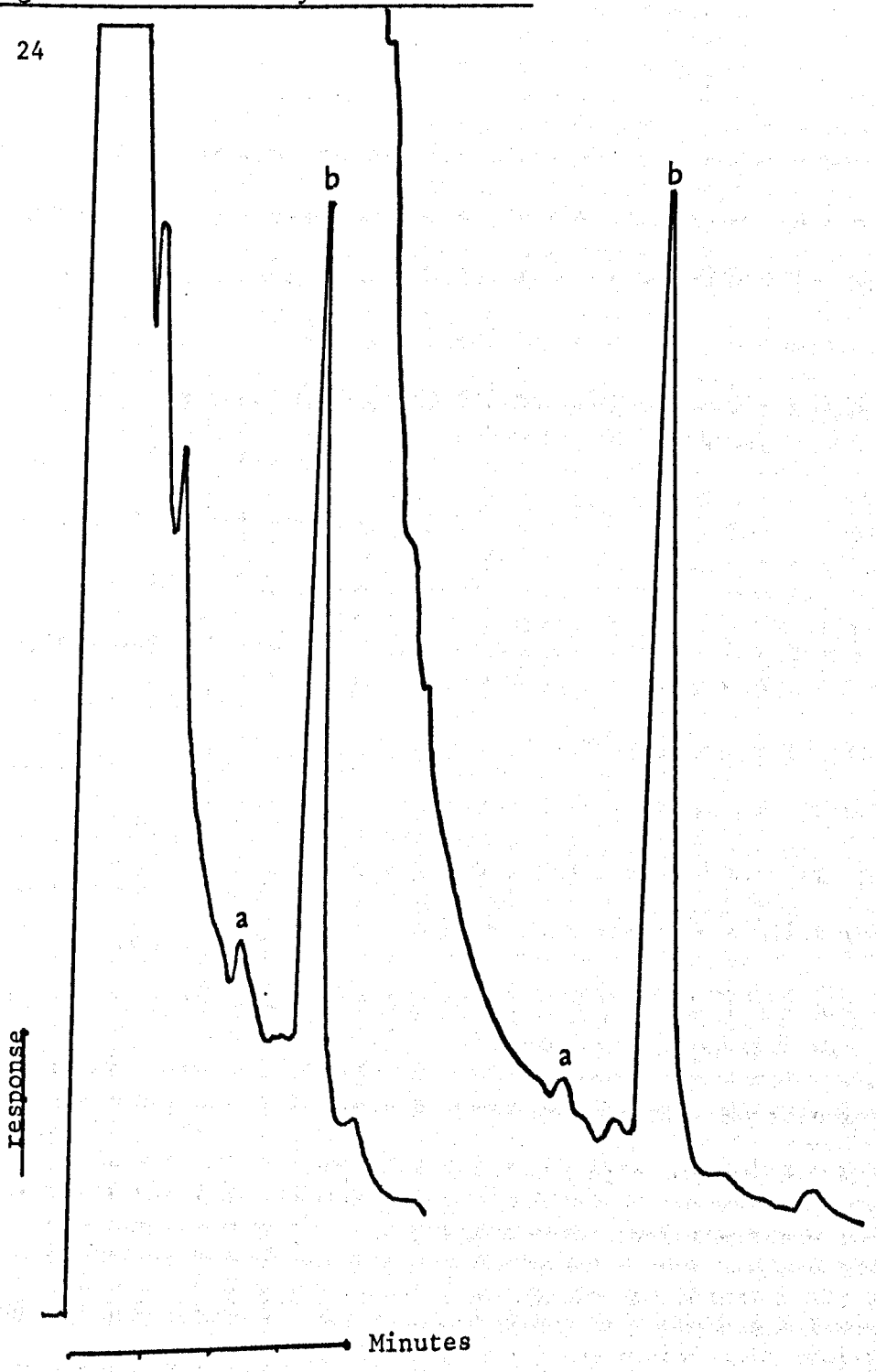
would be lost in the solvent front. Experiments with poststerone (Fig.7, structure XIII) have shown that its polarity is such that it will be partitioned into the butanol phase of the butanol water partition. It forms a silyl ether which is ECD sensitive, but because of its lower molecular weight, it would be lost in the solvent front in any analysis of the ecdysteroids. Insufficient time prevented a systematic examination of faeces for poststerone. If the inactivation process involved the loss of the 7-ene-6-one group from the ecdysteroid molecule sensitivity to electron capture would also be lost. Such compounds, lacking the 7-ene-6-one group would escape detection in this assay.

Metabolism of the Ecdysteroids in 5th Instar S. gregaria

Evidence from these experiments and results from other laboratories suggests that ecdysteroids may be metabolised in the following way. Ecdysone synthesised in the prothoracic gland is released as ecdysone into the haemolymph. It is then transported to organs such as the fat body[103] gut and malpighian tubules[27,58] where it is hydroxylated to 20-hydroxyecdysone. 20-Hydroxyecdysone in turn is released into the haemolymph where it travels to its target organs (either free, or bound to proteins[44]). From the target organs the hormone goes to those tissues (fat body[103] and malpighian tubules[58]) responsible for inactivation and excretion of the ecdysteroids, where it is modified to destroy its biological activity. Ecdysteroids may then be excreted or further degraded. The results of this study for excretion, either conjugated or unchanged suggest that this is not a major pathway for removing ecdysteroid from the desert locust. Evidence for degradation as opposed to excretion (conjugated or unchanged) comes from recent experiments on Sarcophaga. These showed that the percentage of radiolabelled ecdysone which could be recovered from pupae decreased from 98% at zero time to 50% after 3 hours. The recovered label was in the

Gas Chromatograph of Ecdysteroids Present in S. gregaria and L. migratoria at the Ecdysteroid Maximum.

Fig. 24



L. migratoria

S. gregaria

a = Ecdysone

b = 20-Hydroxyecdysone

form of 20-hydroxyecdysone and its conjugates. No radioactivity could be detected in any of the other fractions isolated from the pupae. The inability of the investigators to account for the lost label was taken by them as evidence of the degradation of the exogenous ecdysteroids.

Ecdysteroids in Extracts of Locusta migratoria

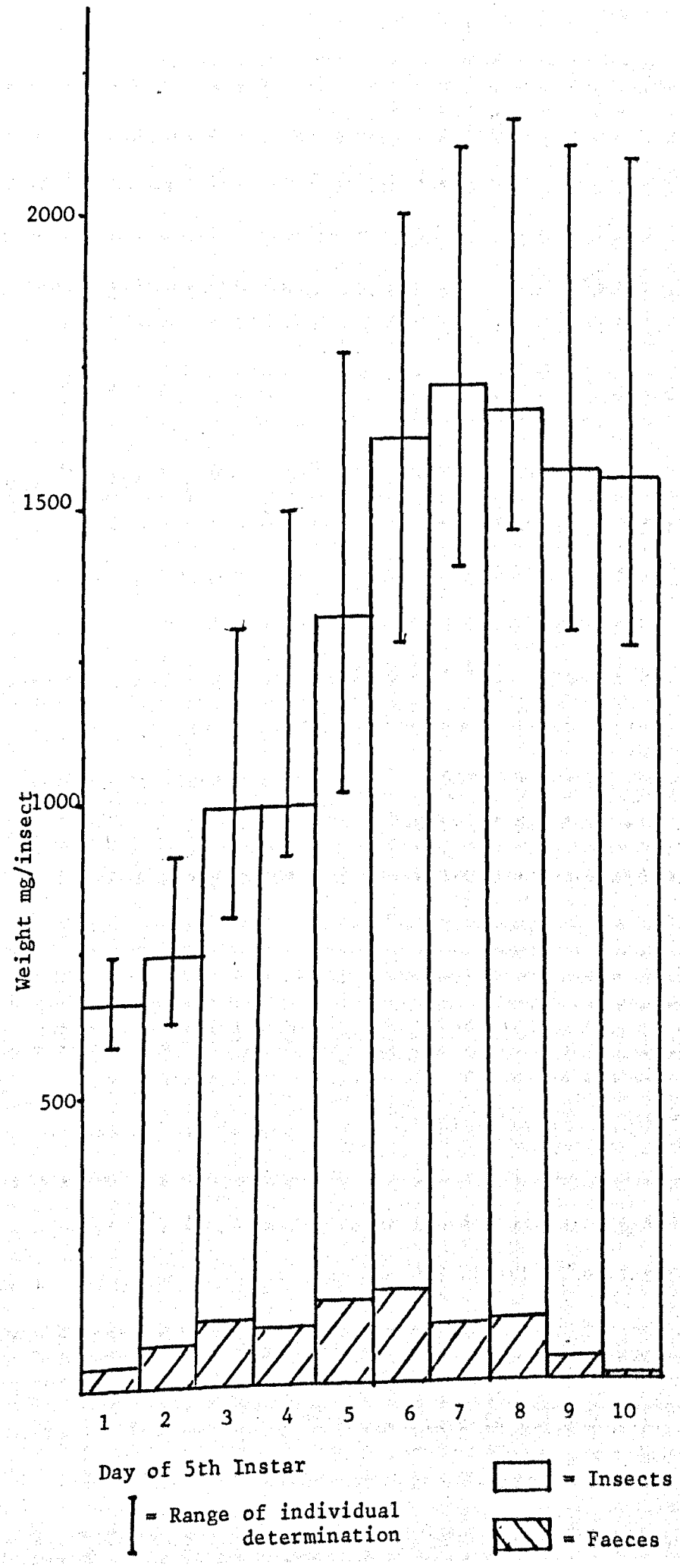
To see if the results obtained in this study of the desert locust were specific for this insect or more generally applicable, ecdysteroids in the related species Locusta migratoria were examined. Analysis of the ecdysteroids present in similar samples of both species of locust made under the same conditions in the same laboratory should allow a direct comparison to be made between them. Analysis of extracts of 5th instar Locusta migratoria made at about the time of the hormone maximum (∿ day eight of a ten to twelve day instar) revealed that the type and proportion of ecdysteroids found in the migratory locust was very similar to those found in the desert locust. A gas chromatogram from one of these extracts is given in Fig. 24. Both ecdysone and 20-hydroxyecdysone are present with 20-hydroxyecdysone forming the major component. This is in agreement with a similar study on the migratory locust performed by KOOLMAN et al.[49]. No other ecdysteroid-like compounds are present, and the trace is indistinguishable from one obtained from the desert locust. It may therefore be assumed that the metabolism of ecdysteroids in Schistocerca gregaria and Locusta migratoria is very similar. Results obtained from one species should be applicable to the other.

The Increase in Weight of the Desert Locust During the 5th Instar

The increase in weight of the desert locust during the 5th instar is not regular, as is demonstrated by the results shown in Fig. 25. There is a rapid increase in weight during the first three days of the

Weight of 5th Instar S. gregaria, phase gregaria, Insects and Faeces

Fig. 25



instar and then a pause. That this pause is due to a decrease in feeding is reflected by the slightly reduced quantity of faeces produced on day four (the quantity of faeces produced on a standard diet being directly related to the amount of food consumed). After this pause weight is put on rapidly until the sixth and seventh days of the instar. From the weight of faeces recovered, the largest quantities of food are consumed on day six. Following the seventh day of the instar weight falls slightly and by the tenth day of the instar (as judged by the weight of faeces collected) feeding has almost ceased. These results are similar to those of Davey[105] for the desert locust.

The reasons for the discontinuous increase in the weight of these 5th instar insects is not clear. It is tempting to speculate that the increase in weight observed on days five and six is the result of increased feeding triggered by the rising moulting hormone titre. The reduced feeding on day ten is probably a consequence of the approach of ecdysis. It would be interesting to observe the effect of the removal of the PTG on the rate of growth, and eventual weight attained of 5th instar desert locusts. In this way an indication of the effects of ecdysteroids on feeding might be obtained.

Ecdysteroids in 5th Instar Solitary Phase Desert Locusts

Locusts can exist in two extreme forms, these are the solitary and the gregarious phases[106]. These phases exhibit distinct morpholigical and behavioural differences.

It is known that phase (or caste) determination in insects is under endocrine control, and juvenile hormone is known to affect this process[121]. The possibility that ecdysteroids might have a function in the determination of phase in the desert locust arises from the following observations. According to the views of ELLIS and CARLISLE insects in the solitary phase

have (relatively) larger prothoracic glands than insects in the gregarious phase[108]. Partial ablation of the gland (reducing it in size to approximately that of a gregarious insect) resulted in insects which were much more gregarious in character than shown in operated controls[109]. As the prothoracic gland is generally accepted as a site of ecdysone biosynthesis the increased size of the gland in the solitary phase insect might be expected to result in higher levels of ecdysteroids in the solitary insect (compared to the gregarious insect). We therefore undertook a comparison of ecdysteroid levels between the solitary and gregarious insects.

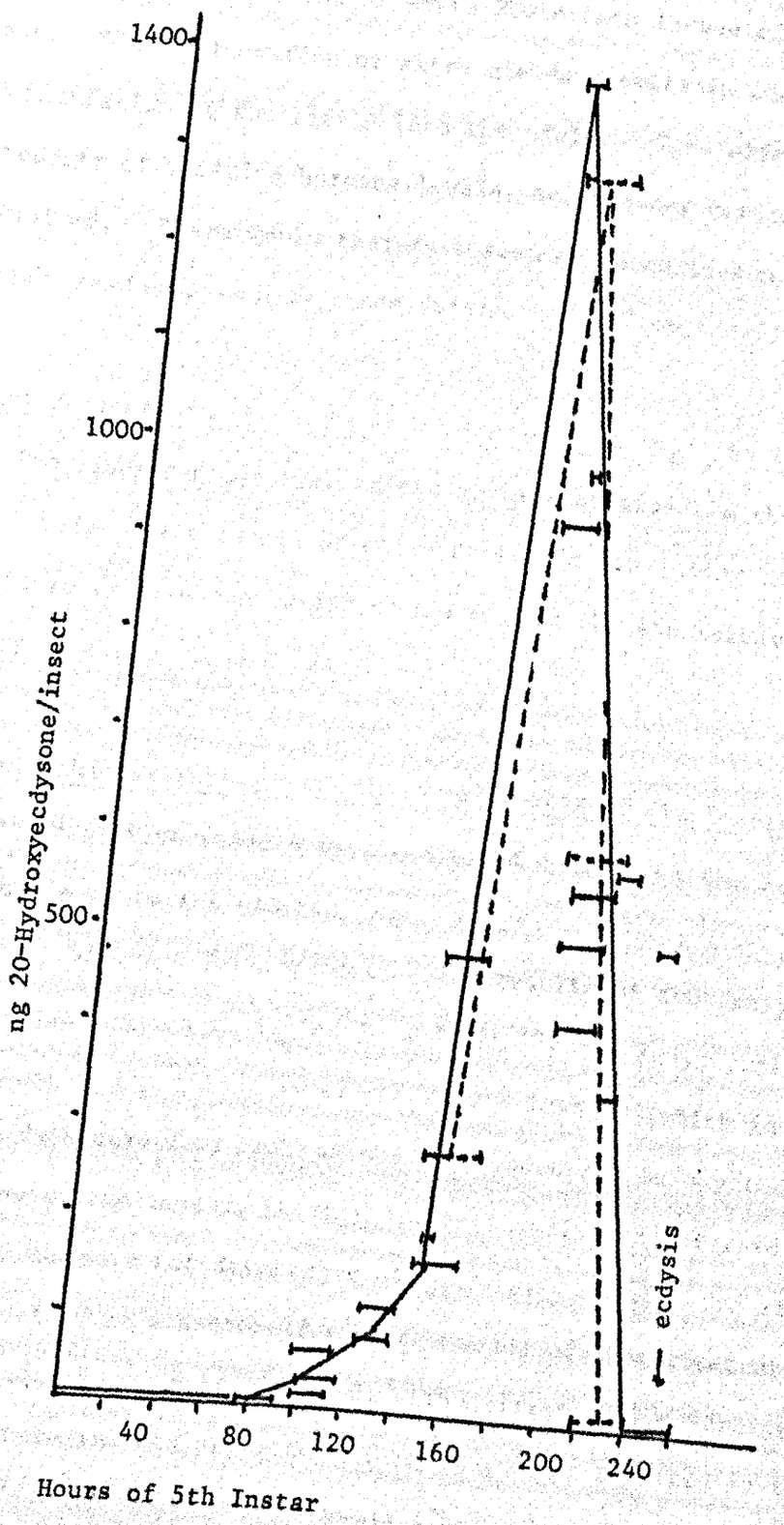
Pattern and Titre of Ecdysteroids in Solitary 5th Instar Desert Locusts

Rearing large numbers of insects in the solitary phase is difficult because of the space required, for this reason ecdysteroid levels were determined only for the last few days of the 5th instar. Determinations were made on individual insects (three per day). The results of these analyses are given in Table 4b. The major moulting hormone observed in the solitary insect is 20-hydroxyecdysone, with ecdysone present in smaller quantities. Fortunately the length of the 5th instar in these insects was the same as that of the gregarious insect, which greatly simplifies comparison. The coincidence between the phases of both the time of production and levels of ecdysteroids is notable. Levels of 20-hydroxyecdysone in solitary and gregarious insects are compared in Fig. 26. From the similarity between the types, pattern and levels of moulting hormones it would seem that ecdysteroids do not have a function in phase determination in this insect.

This being the case the results of Carlisle and Ellis[108-109] are perhaps best interpreted in the light of some additional endocrine

Levels of 20-Hydroxyecdysone in Solitary and Gregarious 5th Instar S. gregaria

Fig. 26



function of the PTG. Recently Joly et al.[109] have demonstrated the effect of the implantation of extra PTG's into larvae of the migratory locust. The implantation of extra glands results in the more rapid differentiation of the larvae into the adult. This effect is approximately independent of moulting hormone levels, and another hormone is thought to be involved. There would therefore seem to be no reason to exclude hormonal functions such as phase determination from the PTG.

Moulting Hormones in the 3rd and 4th Larval Instarsof the Desert Locust

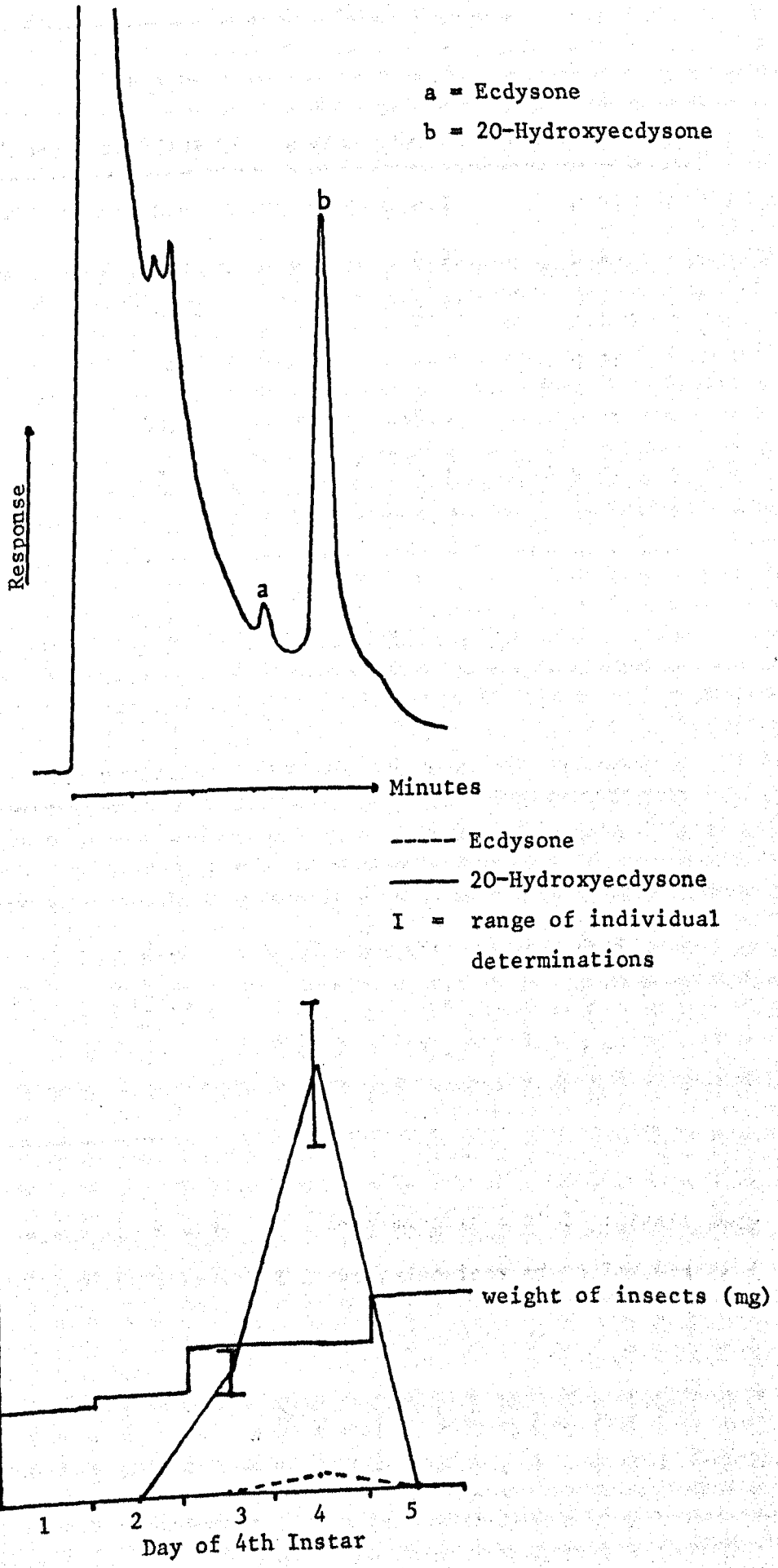
The discrepancies between the results obtained in this and the earlier study of the levels of ecdysteroids in 5th instar insects[90-92] led us to re-examine the levels of hormone in the 4th instar, and extend the study to cover the 3rd instar.

Ecdysteroid Titres During the 4th Larval Instar of the Desert Locust

Groups of five insects were collected daily from the 3rd-4th larval ecdysis up to the 4th-5th larval ecdysis. Two groups of five insects were collected for each day. The results of the analyses of these samples are given in Table 7 and Fig. 27. Ecdysteroids were first detected on the third day of the fourth instar, (which in this case was of five days duration) and maximum titre occurred on day four. By the fifth day of the instar, shortly before ecdysis, ecdysteroids had again fallen and were not detectable at ecdysis. As with the studies on the 5th instar, no ecdysteroid like compounds of longer retention time than 20-hydroxyecdysone were observed. The titres of 20-hydroxyecdysone observed in these insects are much higher than those reported in the earlier study[90,92]. It therefore seems likely that the results reported in the earlier study of the 4th instar suffered from the same artifact formation that appears to have occurred during their study of the 5th instar.

Ecdysteroids in 4th Instar S. gregaria, phase gregaria.

Fig. 27



Ecdysteroids in 3rd Instar S. gregaria, phase gregaria

Table 6

Day of Instar	ng α/insect	ng β/insect	ng α/g	ng β/g	wt per insect g
1	-	0.6	-	4.2	0.14
2	-	1.4	-	8.75	0.16
3	-	0.25	-	1.56	0.165
4	-	-	-	-	0.24
5	1.8	33.5	6.4	119.6	0.28
6	-	7	-	25.9	0.27
ecdysis	-	-	-	-	-

Ecdysteroids in 4th Instar S. gregaria, phase gregaria

Table 7

Day of Instar	ng α/insect	ng β/insect	ng α/g	ng β/g	wt per insect g
1	-	-	-	-	0.28
2	-	-	-	-	0.31
3	-	39.8	-	86.5	0.46
4	4.5	133.3	12	289.1	0.46
5	-	10	-	48.2	0.6
ecdysis	-	-	-	-	-

Table 6 - Results represent the average of 2 determinations of 10 insects each

Table 7 - Results represent the average of 2 determinations of 5 insects each

α = Ecdysone

β = 20-Hydroxyecdysone

20-Hydroxyecdysone is the major ecdysteroid found in 4th instar insects, with smaller quantities of ecdysone also present. A gas chromatographic trace from day four of the 4th instar is shown in Fig. 27. Quantities of ecdysteroids both per insect and per gram are lower than those seen during the 5th instar.

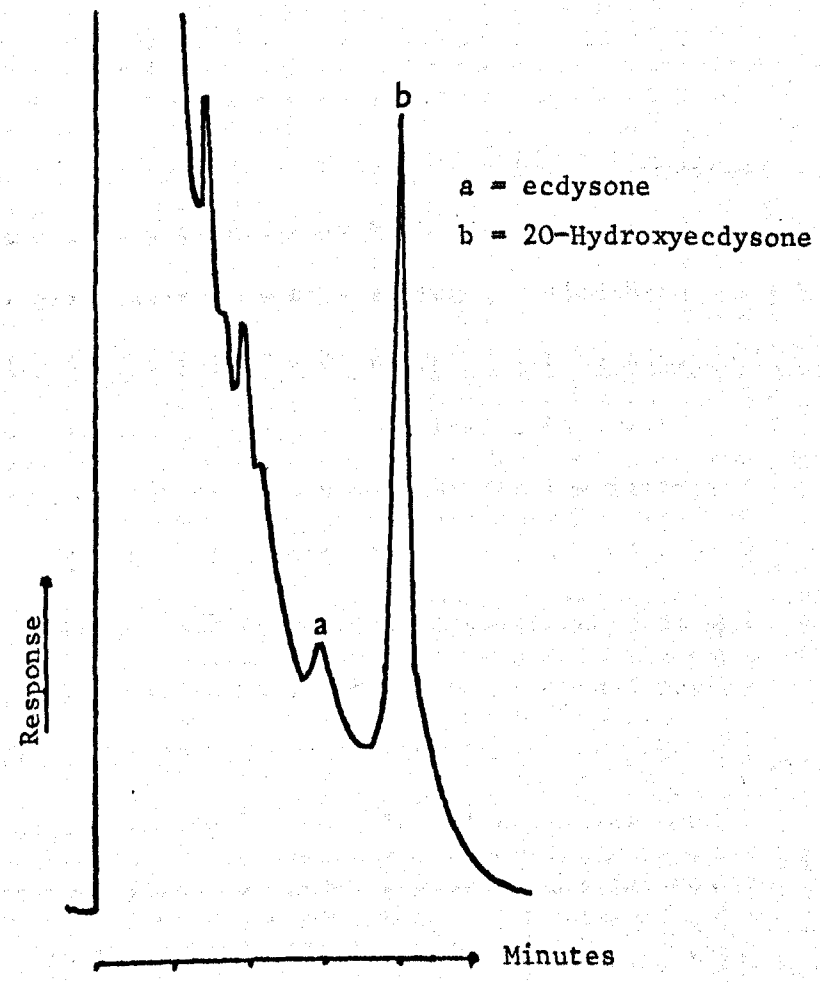
Extracts of the 4th instar insects were treated with Helix pomatia digestive enzymes and examined for the presence of conjugated ecdysteroids, however none were detected.

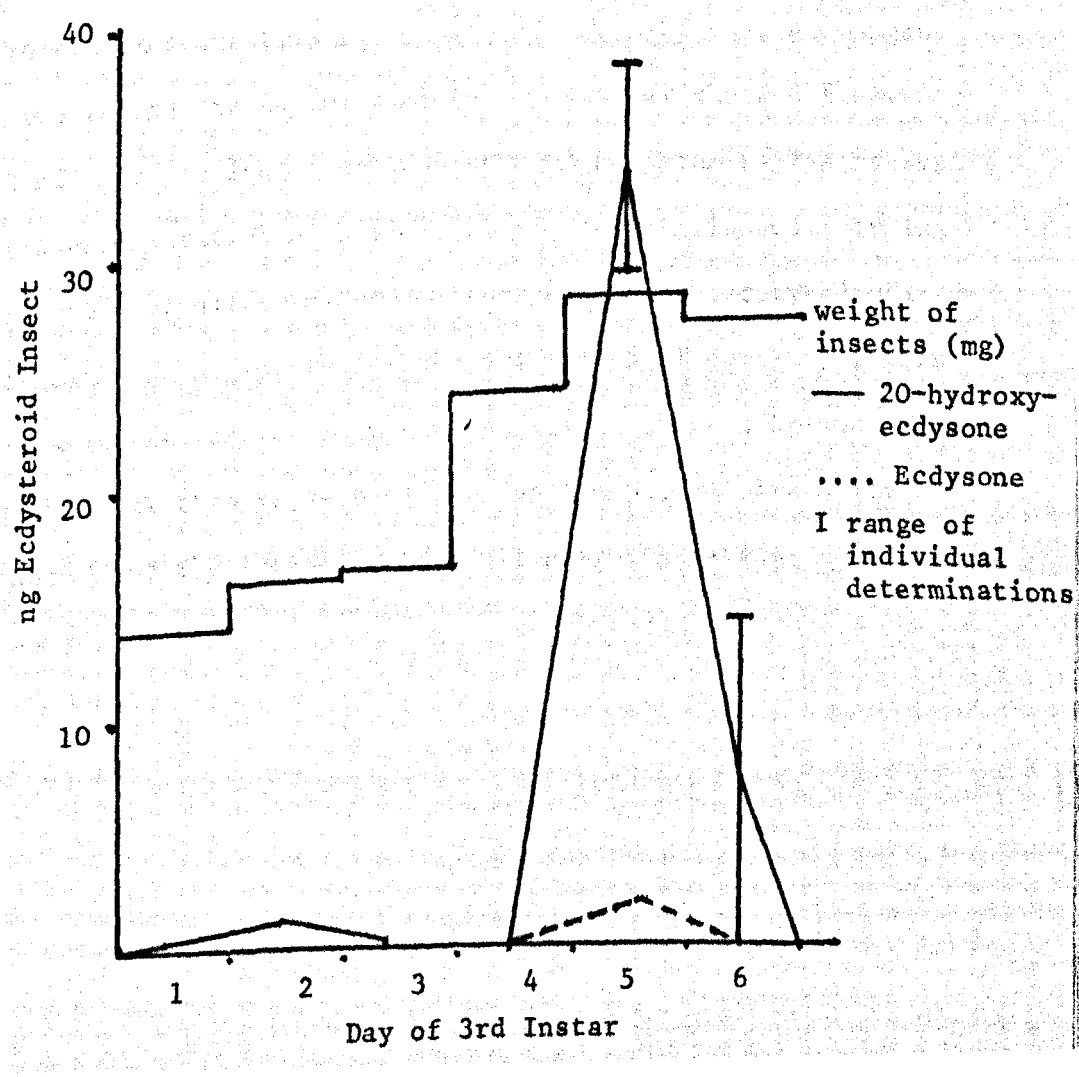
Ecdysteroid Titres During the Third Larval Instar of the Desert Locust

Ecdysteroid levels were determined for each day of the 3rd instar from the 2nd-3rd larval ecdysis to the 3rd-4th larval ecdysis. Groups of ten insects per day were collected thoughout the six days of the 3rd instar. Two groups of ten insects were determined for each day. Small quantities of ecdysteroids were detected during the first few days of the instar, with ecdysteroid titres increasing to a maximum on the fifth day. From their maximum levels on the fifth day ecdysteroid titres fell rapidly during the sixth day to below the limits of detection at ecdysis. The results of the analysis are shown in Fig. 28 and Table 7. The 3rd instar is similar to the 4th and 5th instars in that the major ecdysteroid present is 20-hydroxyecdysone with smaller quantities of ecdysone. A gas chromatographic trace from the fifth day of the instar is shown in Fig. 28. Levels of ecdysteroids per insect and per gram are lower than those observed in the 4th instar. No conjugated ecdysteroids were detected in extracts of 3rd instar larvae after treatment with Helix pomatia enzymes. No ecdysteroid-like compounds other than ecdysone or 20-hydroxyecdysone were detected.

Ecdysteroids in 3rd Instar S. gregaria, phase gregaria

Fig. 28



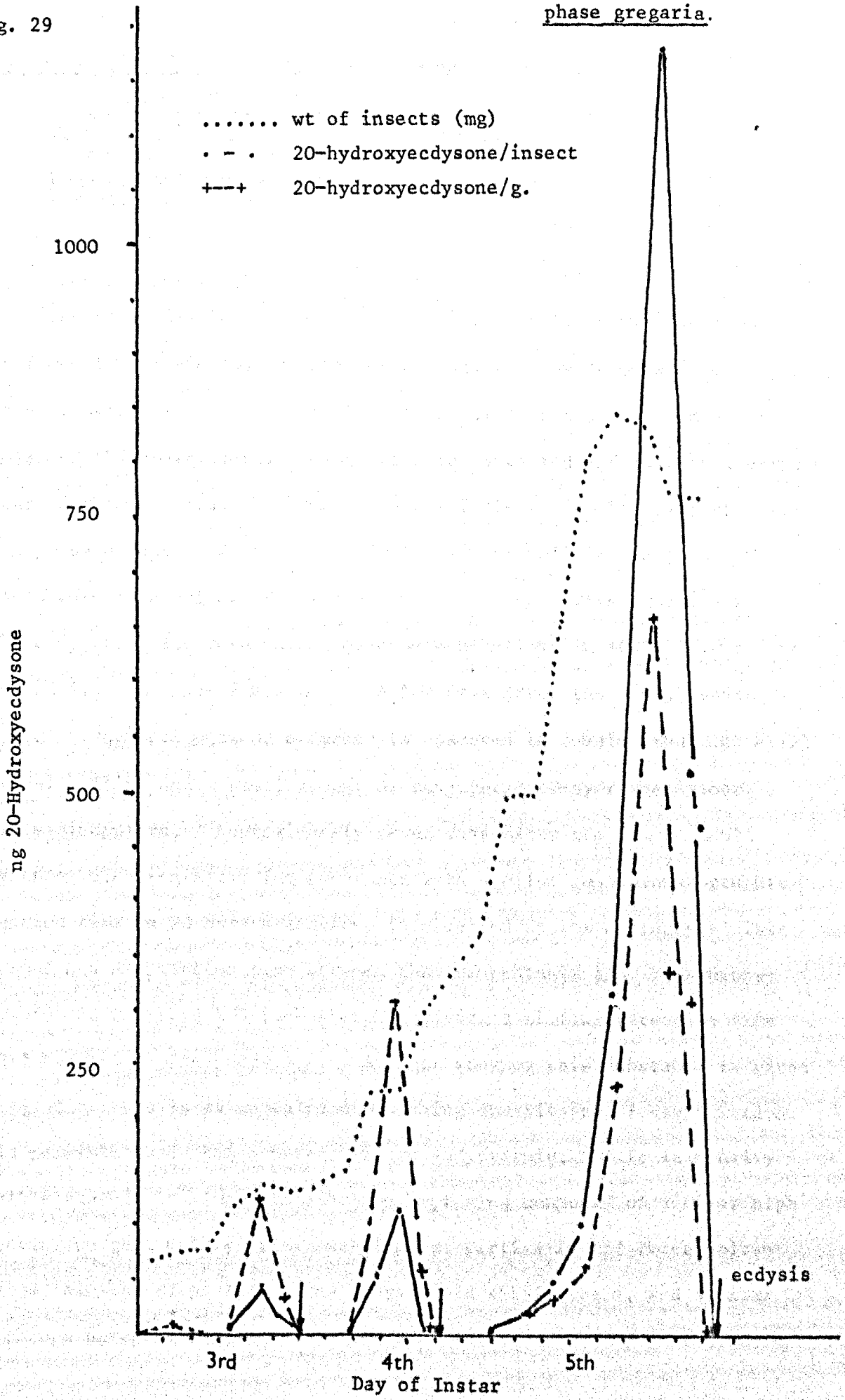


Comparison of Ecdysteroids During the 3rd, 4th and 5th Larval Instars of the Desert Locust

During all three larval instars examined in this study the major moulting hormone observed was 20-hydroxyecdysone with lower levels of ecdysone. No other ecdysteroids were seen at any stage. All three instars are basically similar in the patterns of ecdysteroids seen, with ecdysteroids appearing midway through the instar and rising to their maximum levels approximately four fifths of the way through the instar. In all three instars ecdysteroids have fallen to low levels at ecdysis. The levels of 20-hydroxyecdysone for all three instars (both per gram and per insect) are shown in Fig. 29. From this it can be seen that during the course of development titres of 20-hydroxyecdysone (both per gram and per insect) increase progressively. In the 3rd and 4th instars, levels per insect are about one third of the titres per gram (1:3.3 for the 3rd and 1:2.7 for the 4th instar). In the 5th instar this situation is reversed and titres per insect exceed titres per gram (1:0.6). Of course changes in volume have occurred and it is not easy to say whether these differences in the ratios of hormone per insect against hormone per gram represent real differences in the concentration of hormone at the target tissues. It is tempting to speculate that different concentrations of 20-hydroxyecdysone at target tissues might be necessary for the activation of particular sets of genes. Changes in the levels of hormone at target tissues between the 3rd and 5th instars might then be responsible for the progressive activation of the genes required for the formation of adult characteristics.

Levels of 20-hydroxyecdysone in 3rd, 4th and 5th Instar S. gregaria phase gregaria.

Fig. 29



Ecdysteroids in Adult Females of the Desert Locust

We are the army of Allah
We produce 99 eggs
If the hundredth were complete
We would consume the whole world
and all that is in it

Mohammeden proverb

The adult females and eggs of a number of species of insect have been found to contain considerable quantities of ecdysteroids (see introduction). In order to see if ecdysteroids were present in adult females of the desert locust, insects were extracted and their ecdysteroid content determined from the final, adult ecdysis up to the point at which the eggs were laid. Groups or individuals were collected daily. Male insects were also collected for the first three days after the 5th to adult ecdysis. The results of these determinations on adult insects are shown in Fig. 30 and Table 8. A few days after the final, adult ecdysis a transient peak of ecdysone is observed in female (but not male) insects. This, and a small amount of conjugated 20-hydroxyecdysone rapidly disappears. Approximately seven days after the 5th to adult ecdysis an electron capturing substance with similar gas chromatographic retention time to 20-hydroxyecdysone is observed in the extracts. This compound has a retention time between that of pentakis TMS, 20-hydroxy-ecdysone and pentakis TMS inokosterone, giving a similar retention time to 3-dehydro-20-hydroxyecdysone. A trace showing this substance is given in Fig. 31. It is detected in diminishing quantity until day thirteen. It is possible that this substance is an ecdysteroid, but it is equally possible that it is simply an electron capturing compound of similar high molecular weight, with similar partition properties in all three solvent systems, similar Rf on TLC to the ecdysteroid silyl ethers, and similar gas chromatographic properties. It is at least noteworthy that nowhere

Ecdysone In Adult Female S. gregaria

Fig.30

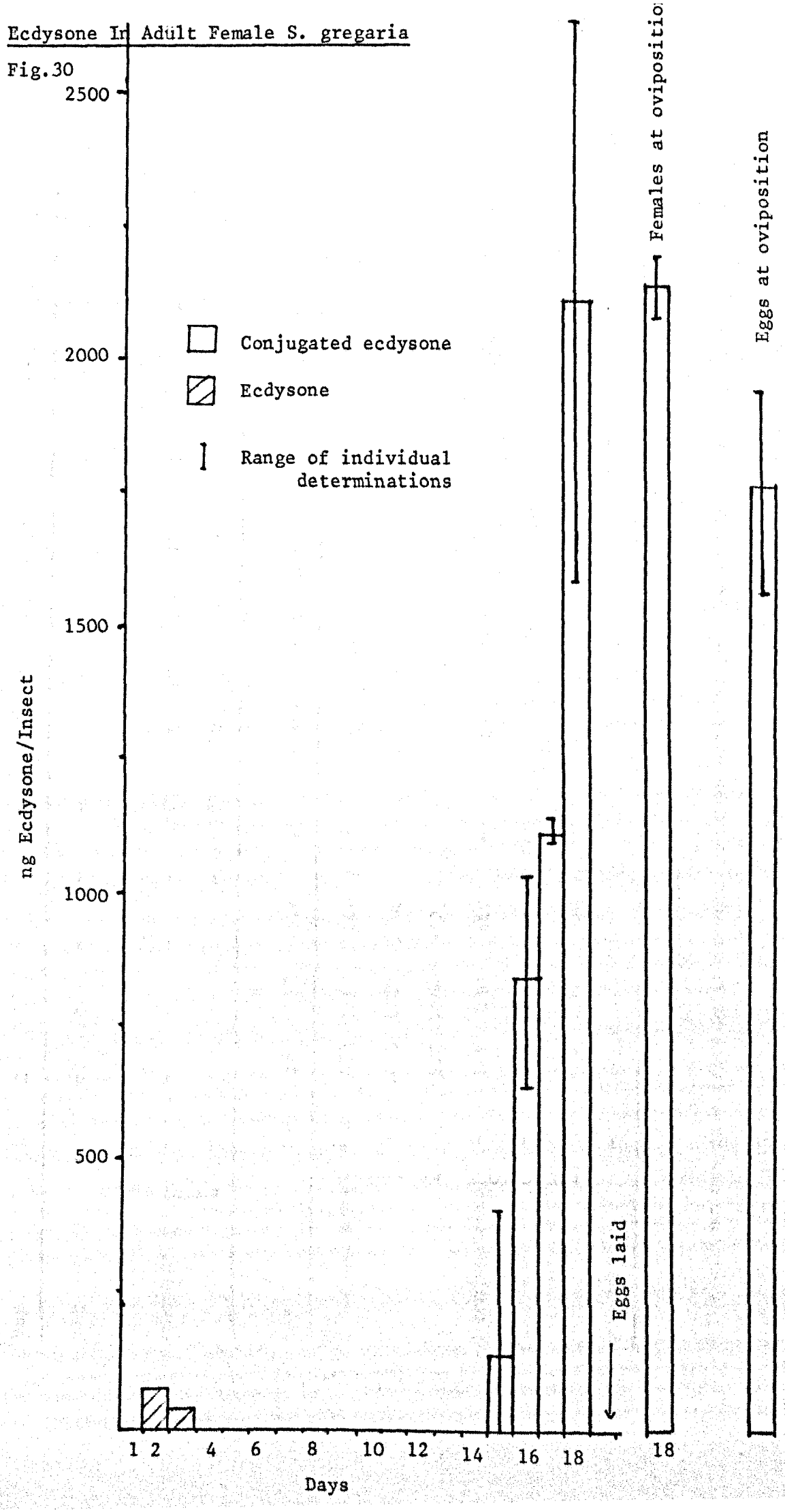


Table 8

Ecdysteroids in Female Adult Schistocerca gregaria

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
α ng	-	80	28.9	-	-	-	-	-	-	-	-	-	-	-
β conjugates ng	-	-	56.3	-	-	-	-	-	-	-	-	-	-	-
unknown* ng	-	-	-	-	-	243	266	105	150	192	90	160	365	-
	-	-	-	-	-	175	30	90	-	220	-	100	-	-

Day	15	16	17	18	19	Females at oviposition	Eggs at oviposition
α conjugates ng	418	653	1160	2666	-	2210	1960
	-	1050	1120	1600	-	2100	1580
	-	870	1108	2123	-	-	-
β conjugates ng	-	196	330	460	-	368	392
	-	225	210	224	-	300	354.8
	-	205	211	350	-	-	-
unknown ng	-	-	-	-	-	-	-

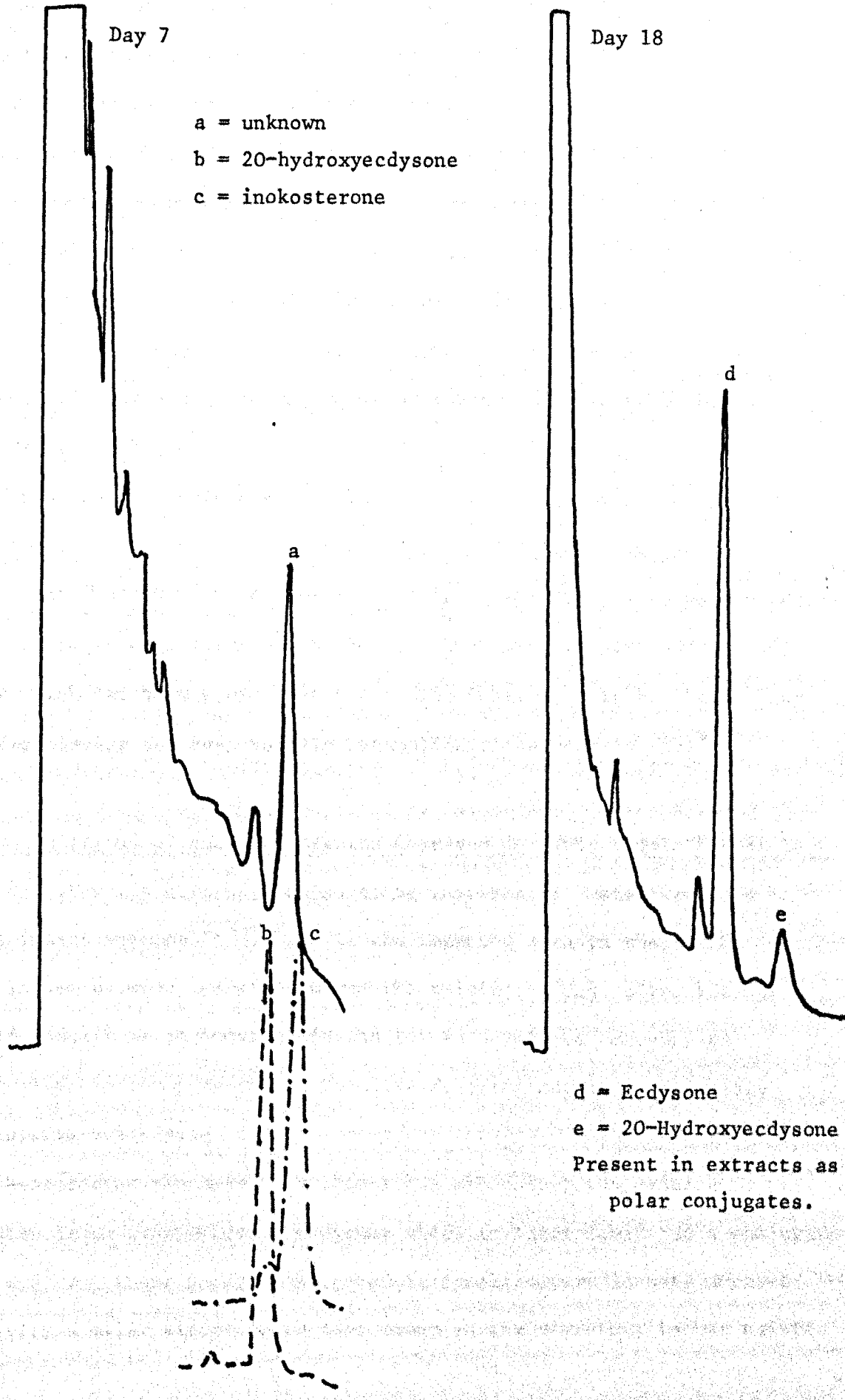
α = ecdysone β = 20-hydroxyecdysone

* The unknown was quantified as if it were an ecdysteroid giving the same response per ng as ecdysone or 20-hydroxyecdysone.

Analyses performed were as follows: Days 1-3, 1 determination of 3 insects. Days 4-14 2 determinations of 3 insects ea.
Days 15-19 3 determinations of 3 individual insects.
Females at oviposition, 2 determinations of 2 individual insects
Eggs at oviposition, 2 determinations of 2 egg pods.

Ecdysteroids in Adult Female S. gregaria

Fig. 31



else in this study have any non-ecdysteroidal peaks been found to gas chromatograph in the ecdysteroid silyl ether region. The production of an artifact during silylation of either ecdysone or 20-hydroxyecdysone was ruled out by the addition of these compounds to extracts. On analysis these extracts contained only ecdysone and 20-hydroxyecdysone, with no artifact formation having occurred. Further investigation of larger quantities of insect material would be necessary to identify this substance, and no time was available to pursue this study.

Conjugated ecdysone is first observed in extracts on day fifteen, conjugated 20-hydroxyecdysone is detected shortly afterwards. Rapid biosynthesis of conjugated ecdysone follows until by the eighteenth day the insects contain impressive quantities of these conjugated ecdysteroids ($2\text{-}3 \times 10^3$ ng). When insects which had just laid their eggs were examined for ecdysteroids none were detected. The newly laid eggs however were found to contain large quantities of conjugated ecdysteroids, equal to that which had been present in the female insects. It has been shown that after laying the eggs rapidly metabolise these ecdysteroids[41].

Possible Functions of the Ecdysteroids Observed in Adult Female Desert Locusts

Ecdysteroids have been shown to be involved in vitellogenesis in several insect species[72,74-76]. If the compound seen in the female desert locust between the seventh and the thirteenth days after the 5th to adult ecdysis is an ecdysteroid and not an unrelated compound of accidentally similar properties, then its function may be to stimulate vitellogenin synthesis.

Research on the migratory locust has shown that the ovaries synthesise large quantities of ecdysone which is later found as a conjugate in the egg[14]. These experiments on adult female desert locusts suggest that a very similar situation to that found in the migratory locust exists.

It would seem that the most likely function of these conjugated ecdysteroids is as storage compounds, forming a pool of inactive hormone on which the embryo may draw as required. It has been suggested that because they are found as inactive conjugates these ecdysteroids do not have a function in embryonic development.[14] Given the very active metabolism and probable biosynthesis of ecdysteroid seen in the eggs of the desert locust[41] this view seems unlikely.

A curious feature of the ecdysteroids found in these insects is the very high ratio of ecdysone to 20-hydroxyecdysone. In the juvenile stages of the desert locust 20-hydroxyecdysone is the predominant ecdysteroid, but this is completely reversed in the adult females and eggs. It has been suggested that ovaries and embryos may be unable to hydroxylate ecdysone to 20-hydroxyecdysone[42,66,73], but the presence of some conjugates of 20-hydroxyecdysone in our extracts shows that a capacity for effecting this transformation does exist. Whether the high proportion of ecdysone observed means that ecdysone has a special function in embryonic development not seen at later stages is not clear. Also it is quite possible that having sequestered the ecdysone from the stored hormone provided by its parent the embryo hydroxylates it to 20-hydroxyecdysone to produce local high concentrations of 20-hydroxyecdysone at target tissues. A gas chromatograph showing the ecdysone and 20-hydroxyecdysone found in adult females at the time of oviposition is shown in Fig.31.

Synthesis of Model Steroids

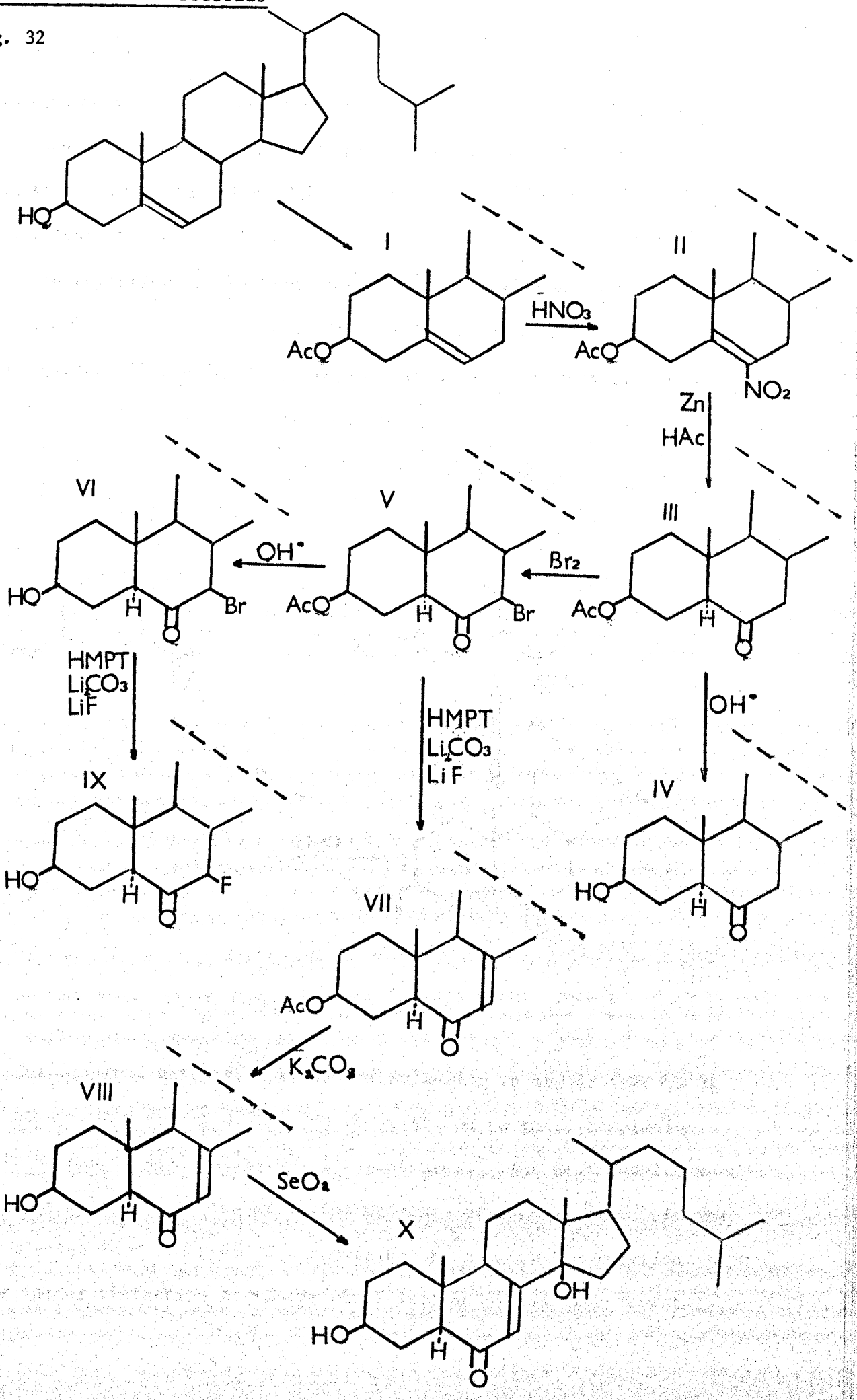
One of the limitations of gas chromatography is that unknown compounds must be identified by some other technique, such as linked mass spectrometry, or comparison of retention times with known compounds. By itself gas chromatography is therefore limited to the identification of compounds for which standards may either be purchased or synthesised. Recently a number of compounds have been suggested as intermediates in the biosynthesis of ecdysone following their isolation from biological tissues[21,22]. In order to develop suitable analytical methods to enable some of these compounds to be sought in extracts of the desert locust, 3β-hydroxy-5α-cholestan-6--one(IV) was synthesised by a known route[110] from cholesterol. 3β-Hydroxy--5α-cholest-7-en-6-one(VIII) and 3β,14α-dihydroxy-5α-cholest-7-en-6-one (X), which have previously been synthesised from 7-dehydrocholesterol[111], were synthesised by a new route from 3β-hydroxy-5α-cholestan-6-one. The main advantage of this synthesis is that the required compounds are synthesised in good yield from cholesterol, available in a pure form at low cost in comparison with 7-dehydrocholesterol.

Synthesis of 3β-Hydroxy-5α-cholestan-6-one(IV, Fig. 32)

3β-Hydroxy-5α-cholestan-6-one has been identified in extracts of the PTG of *Bombyx mori*[21] and suggested as one of the intermediates in the synthesis of ecdysteroids. This compound was synthesised from cholesterol in the following manner[110]. The cholesterol was first acetylated to cholesterol acetate (I) which was then nitrated to 6-nitrocholesteryl acetate (II) in high yield. The large quantities of saturated saline solution required for removing the excess fuming nitric acid makes the purification of the product lengthy. The attempted use of sodium carbonate solution to effect the neutralisation of the excess acid led to the darkening of the solution and the production of a brown gum.

Synthesis of Model Steroids

Fig. 32



When this darkening occurred the yield of 6-nitro-cholesteryl acetate was significantly reduced. Washing with dilute sodium bicarbonate to remove most of the excess acid, followed by washing to neutrality with saturated saline gave good yields of product and reduced the length of time taken for purification.

The formation of the keto compound, 3β-hydroxy-5α-cholestan-6-one (IV) was effected by refluxing 6-nitro-cholesteryl acetate in acetic acid in the presence of zinc dust. The suggested reaction pathway which proceeds through an imine is given below[112].

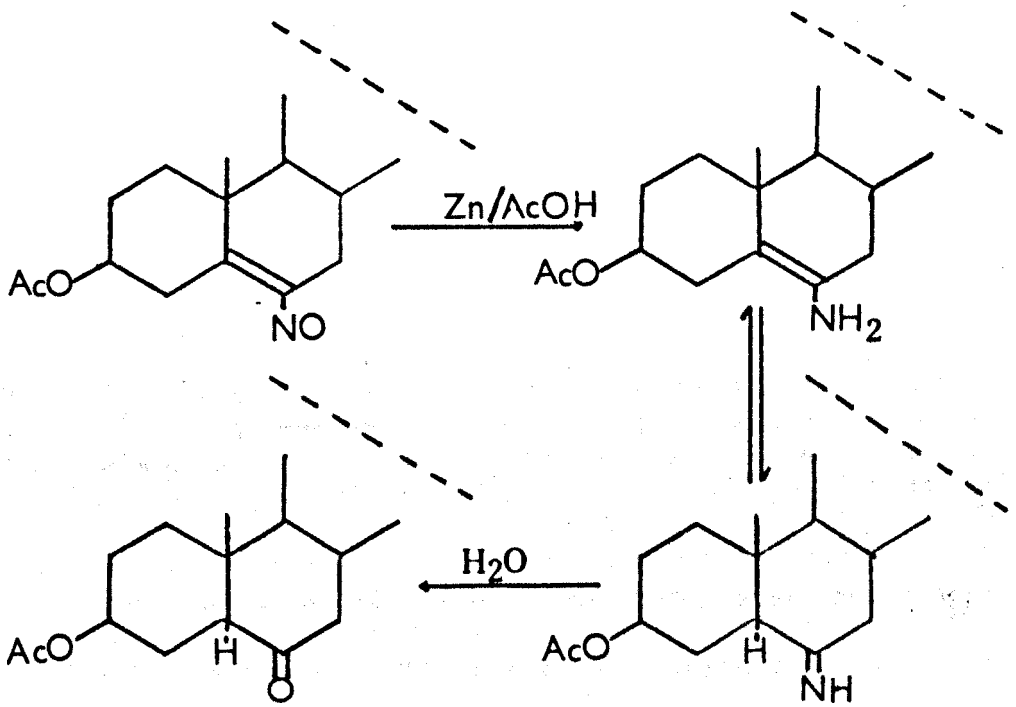
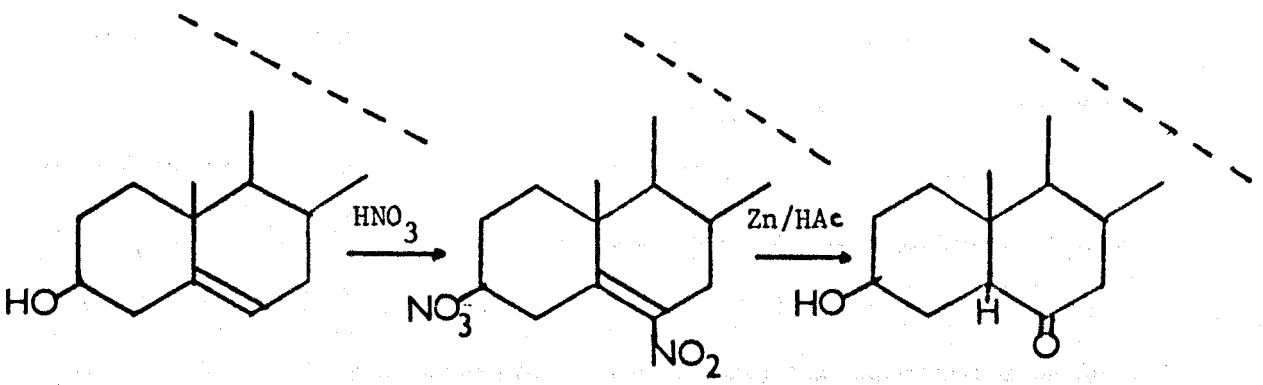


Fig. 33

The original authors[110] suggested refluxing in acetic acid for 3½ hours, however the time required for the reaction to go to completion seems to depend on the quality of zinc dust used. The best results were obtained by refluxing overnight in the presence of excess zinc. It was also suggested that the product was obtained by pouring the reaction mixture (after filtration to remove zinc) into an excess of cold water,

and filtering off the precipitated 3β-acetoxy-5α-cholestan-6-one. This was found to be an inefficient method for isolating the product. Higher yields of product were obtained by diluting the reaction mixture with water followed by extraction with dichloromethane. 3β-Hydroxy-5α-cholestan-6-one(IV) was obtained in high yield by alkaline hydrolysis. 3β-Acetoxy-5α-cholestan-6-one was dissolved in a mixture of methanol, water and THF (80:20:50) containing sodium hydroxide. The THF was required to dissolve the ketoacetate. An alternative route for the preparation of 3β-hydroxy-5α-cholestan-6-one from cholesterol without prior acetylation of cholesterol has been reported[113]. This method was attempted on a small scale.

Fig.34



The product from the nitration of cholesterol proved difficult to purify and this method was not investigated further.

Synthesis of 3β-Hydroxy-5α-Cholest-7-en-6-one(VIII, Fig. 32)

3β-Hydroxy-5α-cholest-7-en-6-one(VIII) is obtained by the dehydrobromination of 3β-acetoxy-7α-bromo-cholestan-6-one(V) followed by mild ester hydrolysis to yield the required 3β-hydroxy- compound (VIII).

Bromination of 3β-acetoxy-5α-cholestan-6-one(III) to give 3β-acetoxy-7α-bromo-5α-cholestan-6-one(V) was performed by refluxing an ethereal solution of the keto acetate with bromine[112]. Bromination may occur in either the C-5 or C-7 positions, however formation of the 5α-bromo

steroid is under kinetic control, whilst formation of the 7α-bromo compound is under thermodynamic control[112]. By refluxing the reaction mixture thermodynamic control of bromination was ensured so that the C-7α-bromo steroid was the major product of the bromination of 3β-acetoxy--5α-cholestan-6-one. However this is not the case with all 6-keto steroids. Previous work in this laboratory[92] on the bromination of the model compound 5α-cholestan-6-one under conditions where thermodynamic control was operating produced a mixture of 5α-bromo, 7α-bromo and 5α,7α dibromo steroids. It is notable that the only differences between 3β-acetoxy--5α-cholestan-6-one(III) which may be brominated selectively at C-7 in high yield and 5α-cholestan-6-one is the possession by the former of a substituent at C-3. Differences in the chemical reactivity of these steroids are also seen in the dehydrobromination step which generates the 7-en-6-one group. Dehydrobromination of 7α-bromo-5α-cholestan-6-one using lithium carbonate in dimethyl formamide has been shown to proceed smoothly and quickly[92]. Under the same conditions (refluxing under nitrogen for 1 hour) no dehydrobromination of 3β-aceoxy-7α-bromo-5α--cholestan-6-one(V) was observed. Increasing the reaction time by refluxing overnight was also ineffective, as was refluxing in collidine for 70 hours. However when dehydrobromination using a mixture of lithium fluoride, lithium carbonate, hexamethylphosphorictriamide and powdered soda glass[114] was attempted moderate yields of 3β-acetoxy-5α-cholest-7--ene-6-one(VII) were obtained. The reaction may be conveniently monitored by gas chromatography, and best yields were obtained by refluxing the reaction mixture overnight.

Formation of 3β-hydroxy-5α-cholest-7-en-6-one(VIII) was attempted by two routes. These were firstly, dehydrobromination of the 3β-acetoxy bromoketone(V), followed by mild ester hydrolysis, and secondly formation of the 3β-hydroxybromoketone(VI), followed by dehydrobromination to the

required product. In the first method mild ester hydrolysis (effected by refluxing in methanolic potassium carbonate solution) is necessary to protect the en -one function, which is unstable to strong acid and alkali. The product, 3β-hydroxy-5α-cholest-7- en -6-one(VIII) crystallises out of the reaction mixture during the hydrolysis, and may be collected on cooling by filtration. This crude material was then redissolved in butanol, and partitioned between butanol and water to remove any remaining traces of potassium carbonate. Failure to ensure that the product is free of potassium carbonate before drying is attempted results in the formation of a high melting point solid. In this fashion good yields of 3β-hydroxy-5α-cholest-7- en -6-one(VIII) compound were obtained. Dehydrobromination of 3β-hydroxy-7α-bromo-5α-cholestan-6-one (VI) on the other hand did not result in the formation of the 7-ene-6-one compound. Instead a halogen exchange reaction between the lithium fluoride present in the reaction mixture and the bromine in the steroid took place. This resulted in the formation of 3β-hydroxy-7β-fluoro--5α-cholestan-6-one(IX).

Synthesis of 3β,14α-Dihydroxy-5α-Cholest-7- en -6-one(X Fig.32)

The 14α-hydroxyl group may be introduced by selective allylic oxidation with selenium dioxide in dioxan[111]. When this was performed on 3β-hydroxy-5α-cholest-7- en -6-one(VIII) the product formed was a red gum. After repeated recrystallisation a colourless crystaline solid was obtained. The small amount of material obtained precluded rigorous purification and characterisation. However mass spectra of this compound show an increase in molecular weight of 16, corresponding to the addition of an hydroxyl group to the starting material. The ultraviolet spectrum of the product shows a strong absorbance at 240 nm indicating that the 7- en -6-one function is intact. Given the selectivity of the selenium

dioxide oxidation it seems likely that the major product of the reaction was 3β,14α-dihydroxy-5α-cholest-7-en-6-one(X). Further evidence in support of this conclusion is obtained from the gas chromatographic properties of silyl ethers of this compound. Firstly the retention time of the partially silylated product is greater than that of the silylated starting material, which would be expected from the increase in molecular weight and polarity of the product. The production of a 14α-TMS ether of 3β,14α-dihydroxy-5α-cholest-7-en-6-one should require similar conditions to those required for the silylation of the 14α position in ecdysone and 20-hydroxyecdysone. This is exactly what is observed when the compound obtained from the selenium dioxide oxidation was subjected to prolonged heating in an excess of TMSI (140°, 24 hr.). The fully silylated compound had a much shorter retention than the partially silylated steroid, (Fig. 35) and this is what would be anticipated by analogy to partially and fully silylated ecdysteroids for 3β,14α-dihydroxy--5α-cholest-7-en-6-one.

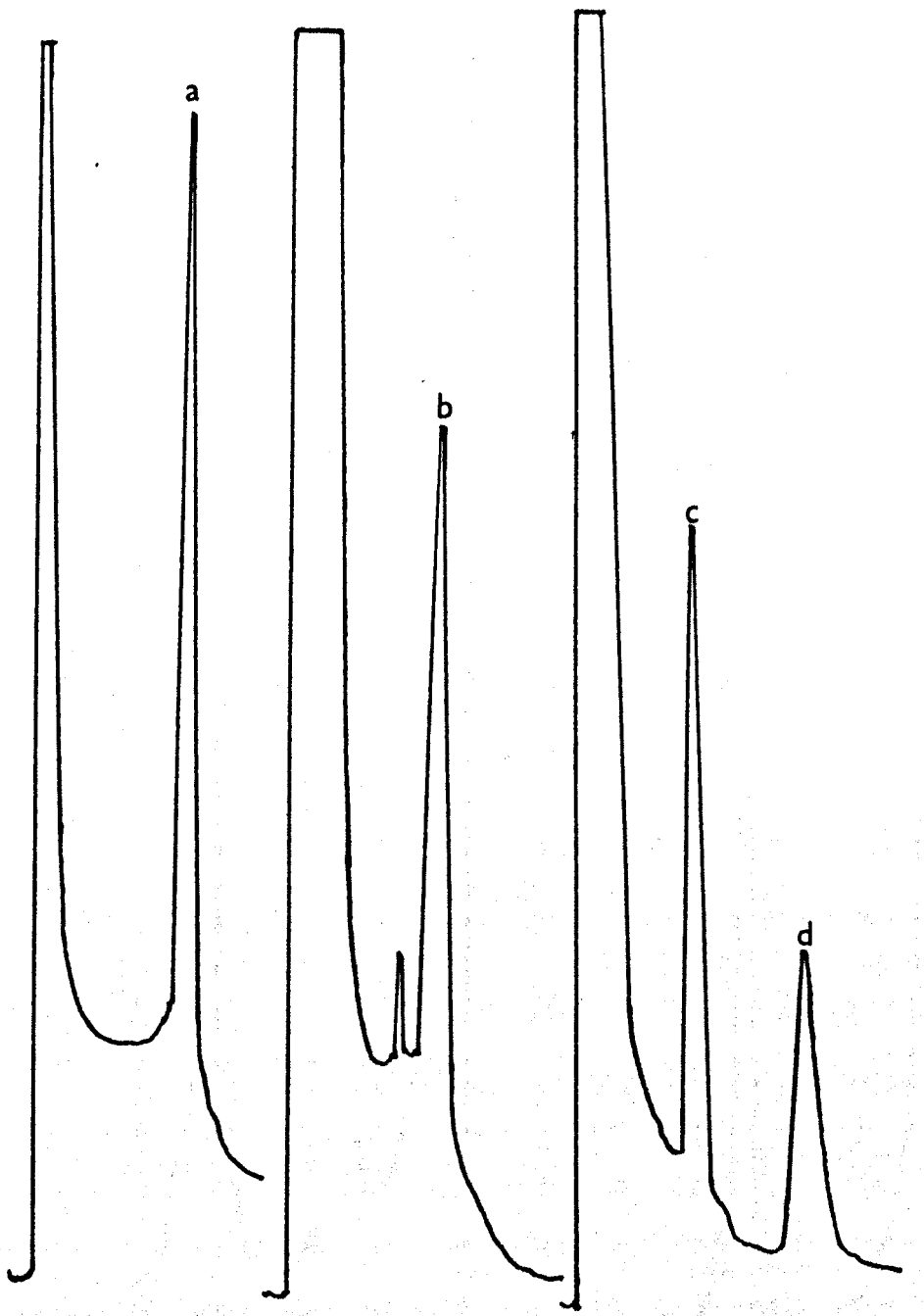
A notable feature of the synthetic route used to obtain these model compounds is that at no stage is any purification procedure other than recrystallisation required.

Gas Chromatography of Model Compounds

Gas chromatographs of all three model compounds using the FID are given in Fig. 35. As detailed in Table 1 3β-hydroxy-5α-cholestan-6-one does not readily capture electrons, whilst 3β-hydroxy-5α-cholest-7-though more sensitive to electron capture detection does not capture electrons as strongly as the ecdysteroids. However both steroids readily form pentafluorophenyldimethylsilyl ethers ("Flophemesyl ethers"), and Poole[92] has shown that such "Flophemsyl" ethers can be detected by electron capture in the nanogram range. A gas chromatograph of the "Flophemsyl"

Gas Chromatographic Properties of the TMS Ethers of the Model Compounds

Fig. 35



a. TMS ether of 3β-hydroxy-5α-cholestan-6-one

b. TMS ether of 3β-hydroxy-5α-cholest-7-en-6-one

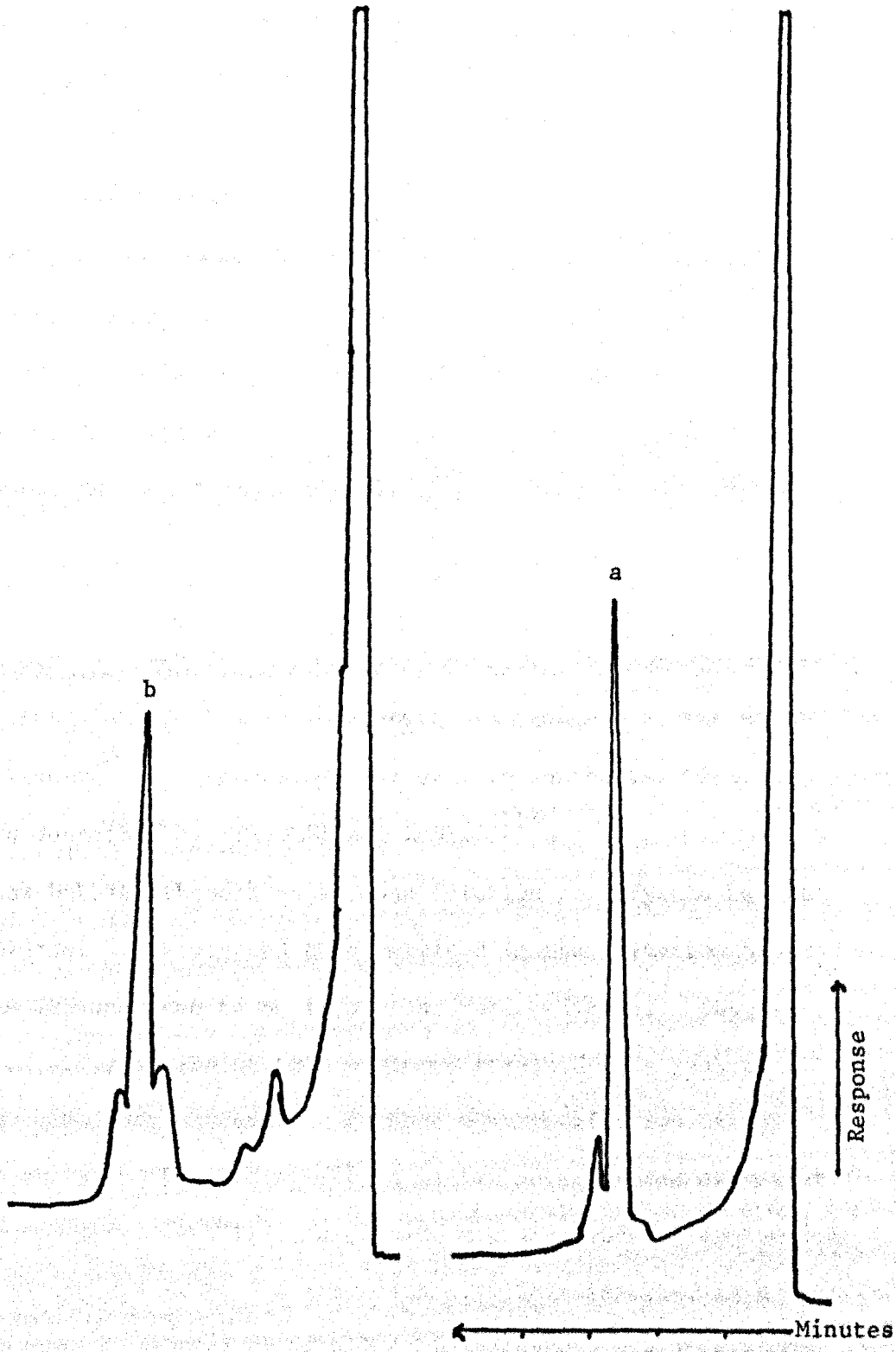
c. Completely silylated form of 3β,14α-dihydroxy-5α-cholest-7-en-6-one

d. Partially silylated form of 3β,14α-dihydroxy-5α-cholest-7-en-6-one

Conditions: 5 ft OV101 on CQ column, temp 266°, gas flow rate 50 ml min^{-1}
E C D.

Flophemsyl Derivatives of Model Compounds

Fig. 36



a. Flophemsyl ether of 3β-hydroxy-5α-cholestan-6-one

b. Flophemsyl ether of 3β-hydroxy-5α-cholest-7-en-6-one

Conditions. 5 ft 1% OV101 on CQ column, temp. 292°, gas flow rate 50 ml min^{-1}. FID

ethers of both 3β-hydroxy-5α-cholestan-6-one and 3β-hydroxy-5α-cholest--7-en-6-one obtained using the FID is given in Fig. 36. The electron capturing properties of these "Flophemsyl" ethers were not determined.

Solvent Partition Properties of Model Compounds

All three model compounds (IV, VIII, X) were found to partition into the light petroleum phase of the aqueous methanol-light petroleum partition (Fig. 9). Therefore if present in the desert locust these compounds would not have appeared in extracts containing ecdysone and 20-hydroxyecdysone, but would have been discarded during the extraction procedure.

Attempted Synthesis of 3-Dehydroecdysone and 3-Dehydro-20-hydroxyecdysone

3-Dehydroecdysteroids have been reported as products of the metabolism of ecdysone in insects[55,61]. An attempt was made to synthesise these compounds using the mathod described by SPINDLER and KARLSON[115].

Oxygen was bubbled through an aqueous solution of ecdysone in the presence of a platinum catalyst and this resulted in the formation of a product which on TLC appeared to be less polar than ecdysone. Gas chromatography of silyl ethers of this compound resulted in a peak of longer retention time than ecdysone. Further characterisation of this compound was prevented by its decomposition whilst being stored overnight as a methanolic solution (at 0^{o}). An attempt was also made to synthesise 3-dehydro-20-hydroxyecdysone by the same method. This resulted in a mixture of compounds. Samples of both 3-dehydroecdysone and 3-dehydro--20-hydroxyecdysone were obtained from another laboratory and no further work on these syntheses was attempted.

Both compounds have similar solvent partition properties to ecdysone and 20-hydroxyecdysone and appear to be stable in methanol for several weeks (at room temperature).

Suggestions for Further Work

In this study the end products of ecdysone biosynthesis, ecdysone and 20-hydroxyecdysone have been measured in the desert locust from the 2nd-3rd larval ecdysis to the point at which the female adult laid her eggs. However nothing has been revealed by this work about the biosynthetic intermediates involved in the production of these hormones. The synthesis of a number of possible intermediates (3β-hydroxy-5α--cholestan-6-one, 3β-hydroxy-5α-cholest-7-en-6-one, and 2β,14α-dihydroxy--5α-cholest-7-en-6-one) should allow analytical methods for their determination in the desert locust to be developed. It should then be possible to determine whether these compounds are found in the desert locust at times when ecdysones are being synthesised. However in order to show that these compounds are biosynthetic intermediates for ecdysteroids in the desert locust it will probably be necessary to use radiolabelled steroids. With radiolabelled steroids it should (if they are on the biosynthetic route to ecdysone in the desert locust), be possible to demonstrate their conversion to radiolabelled ecdysteroids. Using this approach 3β-hydroxy-5α-cholestan-6-one has been implicated in ecdysone biosynthesis in the PTG of *Bombyx mori*[21] and the metabolism of 3β-hydroxy and 3β,14α-dihydroxy-5α-cholest-7-en-6-one (identified in eggs of the migratory locust[22]) has been studied in isolated PTG's of *Manduca sexta*[117].

Metabolites of 20-hydroxyecdysone which might have been inactivation products were sought both in the insect and in faeces, however apart from a few percent found as polar conjugates no clues as to the inactivation and/or excretion of ecdysteroids were found. The injection of several hundred nanograms of radiolabelled 20-hydroxyecdysone into 5th instar insects late in the instar (days eight and nine) followed by extraction a few hours afterwards might help to resolve this issue. It would enable

the solvent partition properties of the metabolites to be determined, and therefore assist in the development of analytical procedures to help to identify these compounds.

The nature of the polar conjugates detected in this and other studies has been suggested as either sulphates or glucuronides on the basis of their hydrolysis with enzymes. However an unambiguous identification would be useful. Because of the comparatively low levels of conjugates found in faeces this would seem to be an unpromising source of material on which to develop procedures for the isolation of large quantities of conjugates. Eggs on the other hand contain large quantities of polar conjugates so that it should be possible to isolate enough to determine their nature. Using similar methods it might then be possible to identify the conjugates present in the faeces.

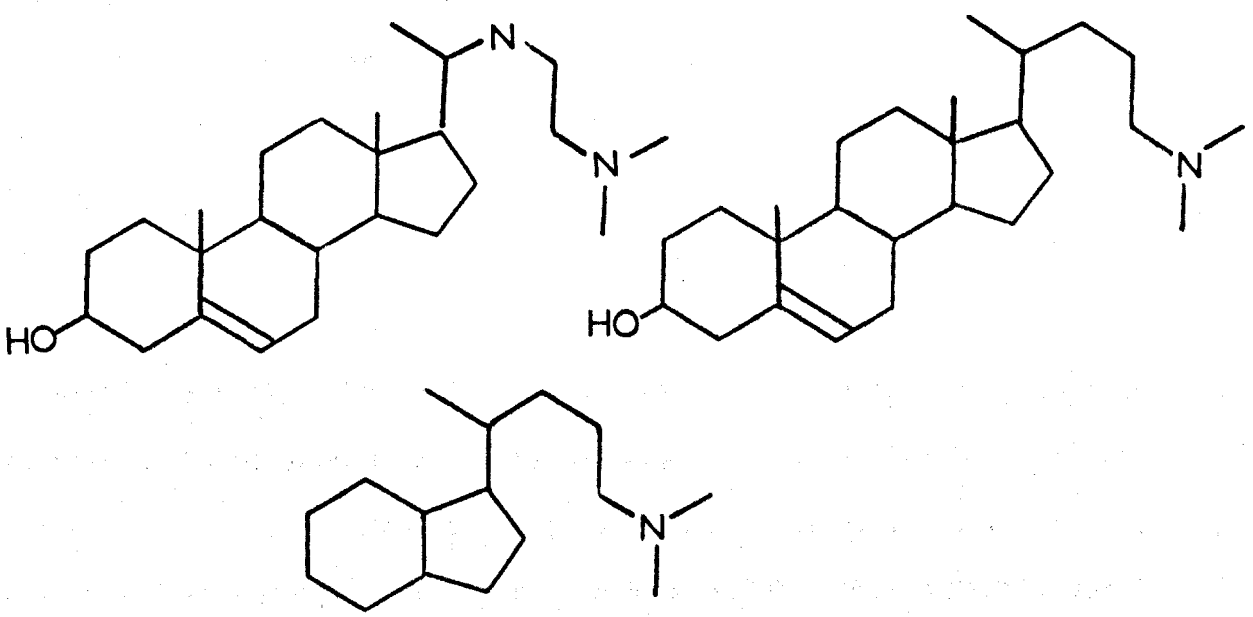
Polar ecdysteroid conjugates have been detected in the eggs of a number of species of insect (see introduction), it might therefore be worthwhile analysing eggs from crustacea to see if similar compounds are present.

Because of the apparent involvement of ecdysteroids in reproduction an investigation into whether there is sexual dimorphism during larval development with regard to the ecdysteroids should be undertaken. This study was performed upon mixed populations of both sexes and would not have revealed any differences which might exist between them.

This study has demonstrated the power of gas chromatography coupled with a sensitive and selective detector for the determination of both the type and quantity of ecdysteroids present in a variety of biological materials (haemolymph, faeces and insects). This has been done for the 3rd, 4th, and 5th instars and for adult females. A concurrent study in this laboratory[116] has determined levels of hormones in eggs and 1st and 2nd instar desert locusts. We now know how ecdysone and 20-hydroxy-

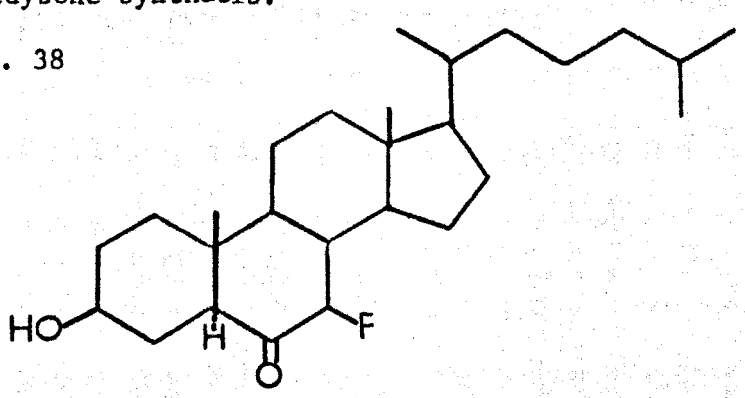
ecdysone and their polar conjugates vary through the life cycle of the desert locust and which compounds are present at particular stages. With this information it should now be possible to select suitable stages of the life cycle and use these to test the effectiveness of possible moulting hormone inhibitors on ecdysteroid biosynthesis and metabolism. Recent work in this area[118,119] has shown that certain azosteroids and simple amines can affect moulting hormone biosynthesis. Some of these compounds are illustrated in Fig. 37.

Fig. 37



The compound 3β-hydroxy-7β-fluoro-5α-cholestan-6-one (Fig. synthesised in this study (and compounds similar to it) might, because of its strategically placed fluorine, be expected to have inhibitory effects on ecdysone synthesis.

Fig. 38



With information derived from studies of this type on the structure and activity of these compounds it may be possible to develop non-steroid inhibitors of the ecdysones possessing the characteristics required for pesticides.

When testing these potential inhibitors for biological activity it would be valuable to be able to measure the levels of some or all of the intermediates on the pathway to ecdysone. This would give an idea of the point at which the inhibitor was exerting its effect and allow related compounds to be compared. Not all the steroids on the pathway to ecdysone may be equally sensitive to analysis using the ECD. Whilst they can be made sensitive to electron capture using "Flophemsyl" reagents this may not prove to be a satisfactory solution. However all ecdysteroids and most of the suggested biosynthetic intermediates so far postulated contain a C-6 keto group. This should allow a fluorescent derivative to be formed using Dansylhydrazine[120], which it should be possible to analyse by HPLC. This would provide selectivity in that good resolution of each of the intermediates should be possible and with the fluorescent Dansyl group the method should be sensitive down to very low levels (picograms). It might also be possible, with an HPLC system to use a shorter clean up procedure.

By using gas chromatography, the desert locust has been persuaded to reveal some of the secrets of its moulting hormones. In the future, development of other analytical techniques in combination with gas chromatography may enable us to obtain a complete understanding of the biosynthesis and excretion of these compounds.

Experimental Methods

Preliminary Procedures

Pure ("Spectroscopic") methanol was prepared by distillation of methanol from magnesium methoxide.

"ECD grade" toluene for gas chromatography was prepared from sulphur free toluene in the following way. Tolune (1ℓ) was shaken with concentrated sulphuric acid (2 x 100 ml, cooled below 30^{o}). The toluene was then shaken with an equal volume of water followed by an equal volume of 5% sodium bicarbonate solution. The toluene was dried over calcium sulphate, distilled from phosphorous pentoxide and stored over molecular sieves.

All other solvents were laboratory grade; where dry solvents are used (e.g. ether and pyridine) drying was effected using sodium wire or molecular sieves as appropriate. Water used for partitions and washing apparatus was distilled.

Instrumentation

Gas chromatography was performed on a Pye Series 104 gas chromatograph with FID and ^{63}Ni ECD detectors.

Infrared spectra were obtained using a Perkin-Elmer 257 infrared spectrophotometer as solutions in chloroform.

Ultraviolet spectra were obtained using a Perkin-Elmer 402 ultraviolet-visible spectrophotometer as solutions in ethanol, using cells of 1 cm path length.

Mass spectra were obtained using a Hitachi-Perkin-Elmer RMU-6 mass spectrometer with direct insertion of the compound on a probe.

NMR spectra were obtained on a Jeol FX100 100 MHz NMR spectrometer as solutions in deuterochloroform.

Cleaning of Glassware and Reacti-vials

Glassware was cleaned by soaking in a bath of caustic detergent overnight, followed by extensive washing in water, then acetone. Reacti-vials were cleaned by soaking in chromic acid overnight. Acid was removed by washing with saturated sodium bicarbonate solution, and repeated washing with distilled water. Reacti-vials were then rinsed with acetone and dried at 140° for at least 30 min. Material not removed by soaking in chromic acid was removed by abraiding it with bleaching powder ("Briz"), after which the Reacti-vial was washed in water, then soaked in chromic acid as above.

Preparation of Thin Layer Chromatography Plates

Silica gel (Kieselgel PF_{254} E. Merck Darmstadt) was washed in excess methanol (reagent grade) with gentle warming followed by filtration and a second wash in methanol. The methanol was decanted and the silica dried and sieved in a fine mesh sieve. Slurries for plate preparation were made up in distilled water. Thin layers were prepared by slurrying the silica gel in water onto glass plates (20 x 20 cms) to a thickness of 0.6 mm. The glass plates had been prepared by washing in detergent, followed by several washes in distilled water, with a final wash in acetone. After the silica had been slurried onto the plates they were left to dry at room temperature. Plates were activated before use by heating at 100° for 1 hour, after which they were allowed to cool in a dry box (over dessicating silica gel), where they were stored until required.

Preparation of Trimethylsilylimidazole (TMSI)

Imidazole (13.6 g) was heated under reflux for 2 hours with hexamethyldisilazane (24.2 g) in the presence of concentrated sulphuric acid (2 drops). The mixture then fractionally distilled under vacuum

to give trimethylsilylimidazole as a colourless liquid. This was then stored in 1 ml glass vials sealed under nitrogen until required. B.p. 90° at 12 mm Hg. Average yield, 85%

Silylation of Pure Ecdysteroids

The method used was essentially that of MORGAN and POOLE[90]. Samples of ecdysteroids (∿ 0.1 mg) in dry pyridine (200 µl) were placed in 1 cm^3 Reacti-vials (Pierce & Warriner, Chester, U.K.). To this solution was added TMSI (100 µl) and the mixture heated for 5½ hours at 100°. This produced a partially silylated ecdysteroid in which the 14α-hydroxyl group was not converted to a TMS ether. To silylate the 14α--hydroxyl group required more vigorous conditions, such as heating at 140° for up to 24 hours. The TMS ethers produced in this way were then either diluted with ECD grade toluene, or subjected to thin layer chromatography, followed by gas chromatography as described below. Hormone derivatives prepared by this method are stable in excess TMSI in the fridge for several weeks.

Silylation of Model Compounds

The method used for the silylation of model compounds was similar to that used for the ecdysteroids. The sterol (∿ 0.1 mg) in pyridine (200 µl) was reacted with TMSI (100 µl) at 100° for 10 min in a 1 cm^3 Reacti-vial. The reaction mixture was then diluted with toluene and a sample removed with a microlitre syringe for gas chromatography.

Formation of Flophemsyl Derivatives of Model Compounds

The sterol (∿ 0.1 mg) in pyridine (100 µl) was reacted with pentafluourophenyldimethylsilylamine at 100° for 10 min in a 1 cm^3 Reacti-vial. The reaction mixture was then diluted with toluene and subjected to gas chromatography.

Thin Layer Chromatography of the TMS Ethers of Pure Ecdysteroids

The products of the silylation reactions described above were diluted with toluene (200 µl) and spread in a line at the origin of a thin layer plate (20 x20 cm) using a Pasteur pipette. The Reacti-vial was then washed with toluene (2 x 300 µl) and the washings also spread at the origin. Solvent was evaporated from the silica with a hair dryer between applications. The plates were then developed in toluene--ethyl acetate (7:3). Under these conditions ecdysteroids have the following R_f values (Table 9).

Table 9

R_f Values of TMS Ethers of the Ecdysteroids on Silica*

Ecdysteroid	No. of TMS Groups	R_f
Ecdysone	5	0.69
20-hydroxyecdysone	4	0.54
	5	0.67
	6	0.75

* From Poole[92]

After development, the plate was dried with the aid of a hair dryer. Ecdysteroids were visualised under UV fluorescence and the appropriate R_f band scraped off. If ecdysteroids were below the level of detectability by UV fluorescence then the R_f band 0.5-1 was taken.

Recovery of the TMS Ethers of the Ecdysteroids after TLC

The silica obtained by scraping the appropriate R_f band from the plate was packed into a glass column (∿ 100 x 11 mm), constricted at one end, and with a glass wool plug to retain the silica. The silylated

ecdysteroids were then eluted from the column using dry ether (15 ml). The eluent was collected in a centrifuge tube. Ether remaining on the column was eluted by blowing it out with a stream of nitrogen (5 psi).

The ether was then evaporated from the centrifuge tube using a stream of nitrogen and gentle heat from a hair dryer. When only a small volume of ether remained (∿ 50 µl) toluene was added (500 µl). Evaporation was continued until the volume was reduced to 100 µl, (Measured with a syringe), Samples (1 µℓ) were then taken for gas chromatographic analysis.

Conditions for Gas Chromatography of Silylated Ecdysteroids

Analysis of the TMS ethers of the ecdysteroids was effected using gas liquid chromatography with either the FID or ECD to detect the silylated hormone. Conditions for analysis by flame ionisation were, nitrogen flow rate 60 ml min^{-1}, oven temperature 280°. For the ECD the detector oven temperature was 300°, detector pulse space 50 µ sec, pulse width 0.75 µ sec, pulse height 47-60V, with a nitrogen carrier gas flow rate of 50 ml min^{-1}. When the column carrier gas was switched off a flow of purge gas of 15 ml min^{-1} of nitrogen was maintained through the detector. The ECD was not used for one hour after the purge gas had been switched off to allow conditions to stabilise. With both FID and ECD, direct on column injection was used with an 11 cm needle. The column was a 5 ft glass column containing 1.5% OV 101 on Gas Chrom Q.

Typical retention times for a range of ecdysteroid TMS ethers are given in Table 1 (measured from the solvent front).

The quantity of hormone present in a sample was determined by comparison of the peak area of the sample against a calibration graph (Fig. 39).

Preparation of Calibration Graphs Using Pure Hormones

A known quantity of ecdysteroid was converted to its trimethylsilyl ether as described above. The reaction was monitored by taking samples (∿ 1 µl), diluting with toluene, followed by gas chromatography. When the desired silyl ether had been formed a quantity of the reaction mixture (5 µl) was diluted in toluene (500 µl) and then serially diluted. Samples from these serial dilutions were then injected (in triplicate). From the peak areas obtained a standard curve was prepared.

Because of progressive contamination of the ECD with use its sensitivity declines. Standard curves were therefore prepared regularly, and the sensitivity of the detector was determined before and after each set of determinations on biological samples. A typical standard curve for ecdysone and 20-hydroxyecdysone is given in Fig. 39.

Maintenance of Column

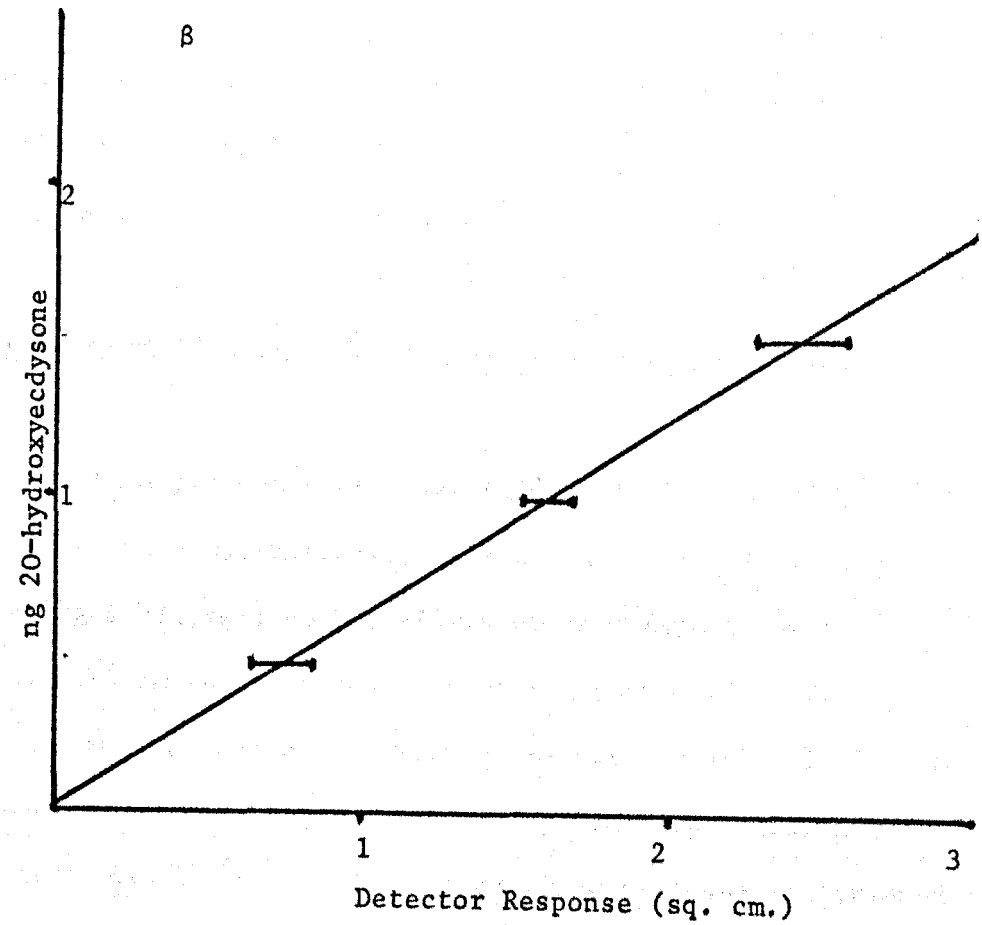
Because of the on-column injection technique used in this study the first few centimetres of column packing became contaminated with involatile material which reduced the performance of the column and led to degradation of the TMS ethers. Therefore this first few centimetres of column packing was regularly replaced (∿ every 300 injections) with fresh material. The column was then conditioned overnight at 300^{o} before being used for analysis.

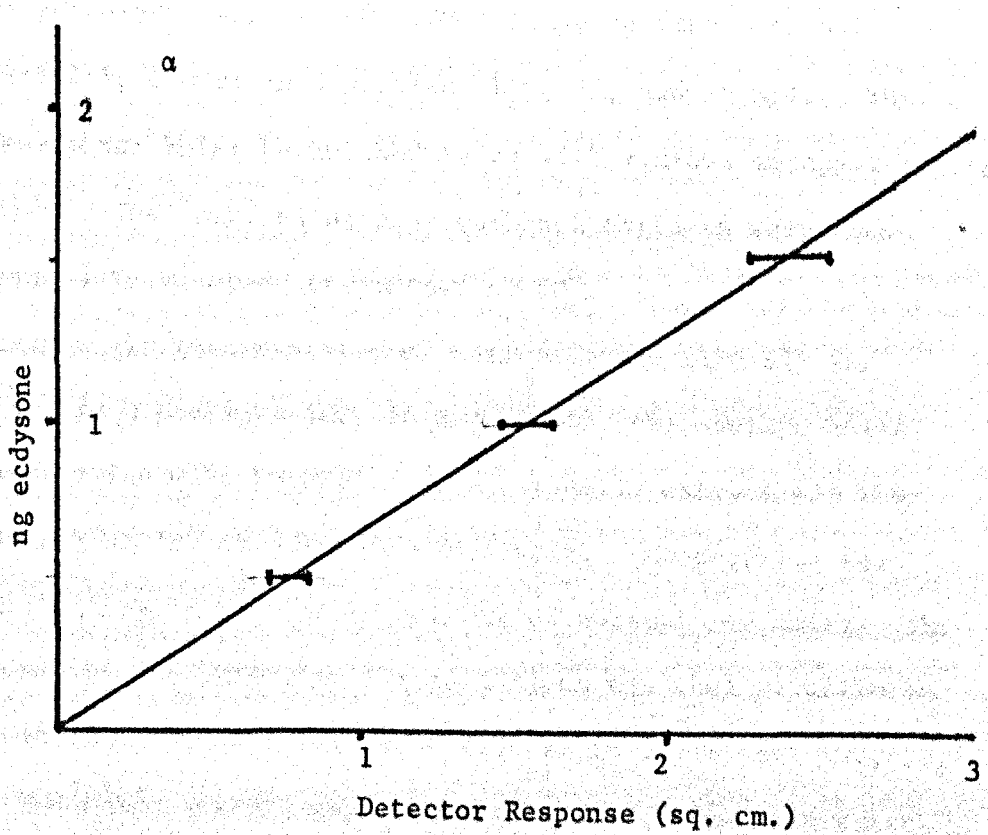
Maintenance of Electron Capture Detector

Because it is a non-destructive detector the ECD is prone to contamination with involatile materials eluting from the column. When it became necessary the detector was cleared in the following way. The detector was removed from the gas chromatograph and washed sequentially in benzene (75 ml) and hexane (75 ml). The waste solvents were then

Standard Curves for the Response of the Electron Capture Detector to TMS Ecdysone and 20-Hydroxyecdysone

Fig. 39





diluted with more solvent (750 ml) and disposed of as waste solvent. The detector was then remounted in the detector oven, purged with nitrogen, and heated at 350° for 12 hours. Mild contamination could sometimes be removed by "baking out" the detector at 350° overnight.

Extraction of Ecdysteroids and Ecdysteroid Conjugates from Biological Material

Insects

The extraction procedure used was similar to that of Morgan and Poole[92], and is shown diagramatically in Fig. 9. Insects were ground in methanol using a blender, either singly or in groups of up to ten insects. The mixture was filtered through a sintered glass funnel (porosity 3) and the residue washed with an excess of methanol (3 x 200 ml).

The combined methanolic filtrates were then evaporated in vacuo on a rotary evaporator at 50°. The residue from this was partitioned between light petroleum (b.p. 40-60, 100 ml) and aqueous methanol (water:methanol 4:1, 250 ml in 3 portions 100, 75, and 75 ml). The light petroleum phase being backwashed twice with aqueous methanol before being discarded. The combined aqueous methanol extracts were then reduced to dryness in a rotary evaporator at 50°.

The residue was then partitioned between water (100 ml) and butanol (250 ml, in 3 portions 100, 75 and 75 ml), the aqueous phase being backwashed twice with butanol. The butanolic extract was then reduced to dryness at 50° on a rotary evaporator. Evaporation was assisted by distilling an azeotropic mixture of butanol and water. The butanol residue from the butanol-water partitions was then partitioned between ethyl acetate (100 ml) and water (250 ml in 3 portions 100, 75, and 75 ml). The ethyl acetate phase was backwashed twice with water and discarded. The combined aqueous phases were then reduced to dryness by rotary evaporation as an azeotropic mixture with butanol. The residue

from the evaporation of the aqueous phase from this final extract was then taken up in "spectroscopic" methanol (15 ml). This was transferred to a centrifuge tube and the volume reduced (2 ml) under a stream of nitrogen, with gentle warming from a hair dryer. The resulting solution was then transferred to a Reacti-vial (1 cm^3), evaporated to dryness, and dried under vacuum over boiling acetone for 1 hour.

The aqueous phase from the butanol-water partition was usually evaporated to dryness by rotary evaporation (as an azeotrope with butanol). It was then taken up in phosphate or citrate buffer and analysed for conjugated ecdysteroids as described later.

Faeces

Faecal pellets were collected daily from 5th instar nymphs of S. gregaria, (phase gregaria), and separated from grass, bran and other contaminating material using tweezers. Removal of contaminating material by sieving, or by blowing it away using compressed air was attempted but was not found to be effective. The pellets were then placed in a mortar, moistened with methanol, and ground with a pestle. The resulting fibrous mass was then stirred in excess methanol overnight (100 ml methanol g^{-1} faeces). The fibrous material was then removed by filtration on a sintered glass funnel (porosity 3), and the filter cake was then washed with excess methanol (1 ℓ g^{-1} faeces). The combined methanolic filtrates were reduced to dryness by rotary evaporation at 50°. Subsequent partitions were identical to those used for insects.

The filter cake was also extracted with water to extract any very polar material. This aqueous extract was reduced to dryness by rotary evaporation as an azeotrope with butanol at 50°. The extract was then examined for conjugated ecdysteroids. None were detected.

Haemolymph

Haemolymph collected from 5th instar larvae of S. gregaria (phase gregaria) at the time of the endogenous moulting hormone maximum was mixed with an excess of methanol (100 ml methanol/ml haemolymph). The extract was left overnight at 0°, and filtered. The solid residue was washed with methanol (2 x 250 ml) and the combined filtrates evaporated to dryness at 50° on a rotary evaporator. The solid residue from this stage was then partitioned between aqueous methanol and petroleum ether (as described above), reduced to dryness, and partitioned between butanol and water (as described above). Both aqueous and butanolic phases were evaporated to dryness and examined for the presence of free and conjugated ecdysteroids as described elsewhere.

Extraction and Hydrolysis of Ecdysteroid Conjugates

The aqueous phase from the butanol water partition (Fig. 8) of insect homogenates, haemolymph or faeces was evaporated to dryness by azeotropic distillation at 50° on a rotary evaporator. The residue from this step was then dissolved in either phosphate or acetate buffer (10 ml, 100 mM). This was then divided into two portions (2 x 5 ml), one of which was subjected to enzymic hydrolysis (see below), the other incubated without enzyme under the same conditions, as a control. The same procedure was used to examine samples derived from the aqueous phase of the water-ethyl acetate partition. After incubation in a thermostatted waterbath at 37° overnight, samples were made up to 100 ml with distilled water and extracted with butanol (250 ml in 3 portions, 100, 75, and 75 ml). The butanolic phase was then evaporated to dryness in the normal way, and the residue analysed for ecdysteroids liberated by the enzymes.

The conditions of the enzymic hydrolysis were as follows:- Solutions were made of the extracts in buffer at the appropriate pH (for

Helix pomatia enzymes pH 5.3, α-D-glucosidase pH 5, and β-D-glucosidase pH 6). The pH of the solution was then checked with a pH meter, and if necessary adjusted using dilute hydrochloric acid or sodium hydroxyide solution. The quantities of enzyme used routinely were as follows

Enzyme	Activity	Source
β-glucuronidase	110 Fishman units ml^{-1}	Koch Light, England
Aryl sulphatase	800 Ray units ml^{-1}	Koch Light, England
α-D-glucosidase	2.2 units ml^{-1}	Sigma, England
β-D-glucosidase	1 unit ml^{-1}	Sigma, England

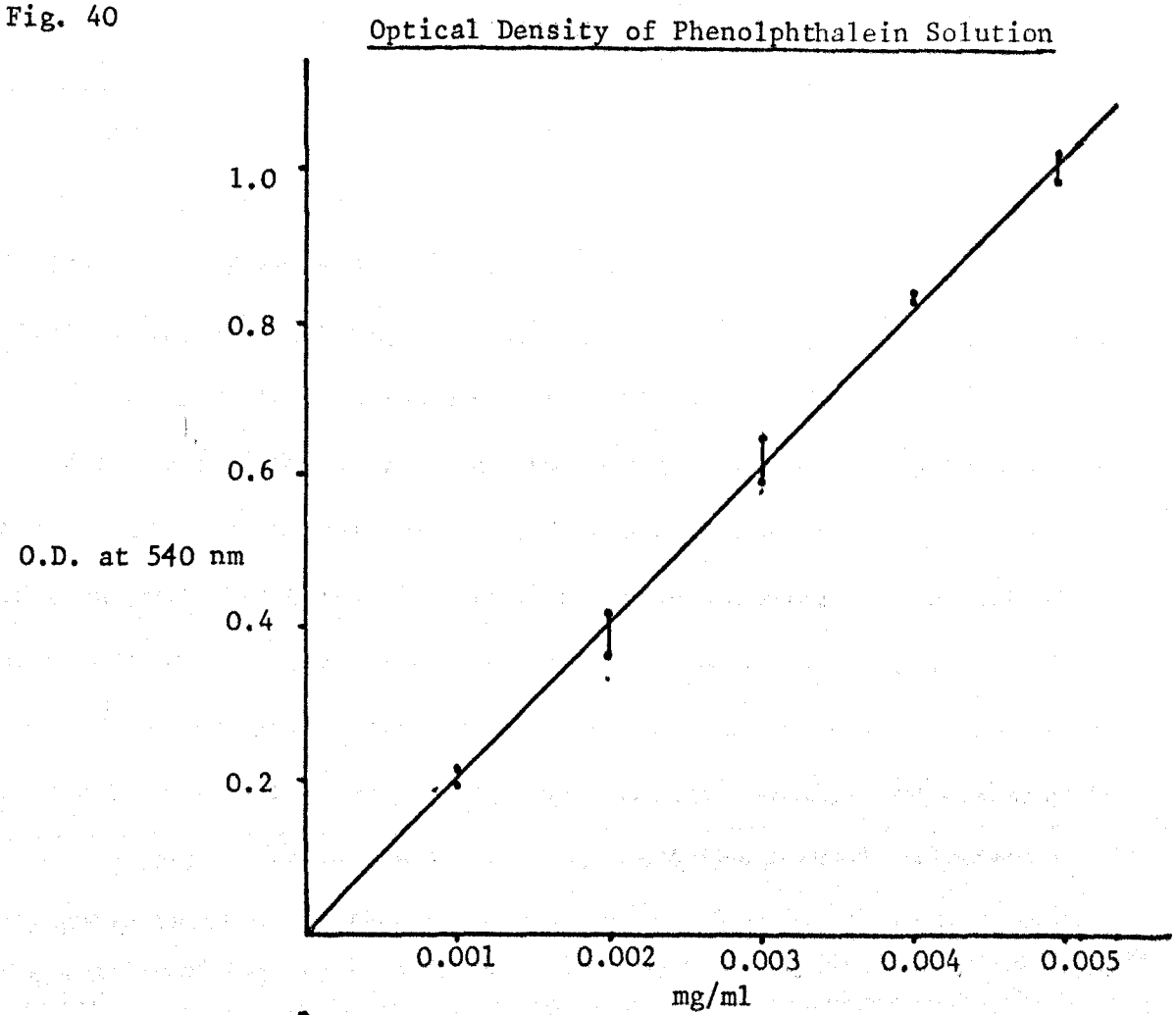
Determination of Enzymic Activity

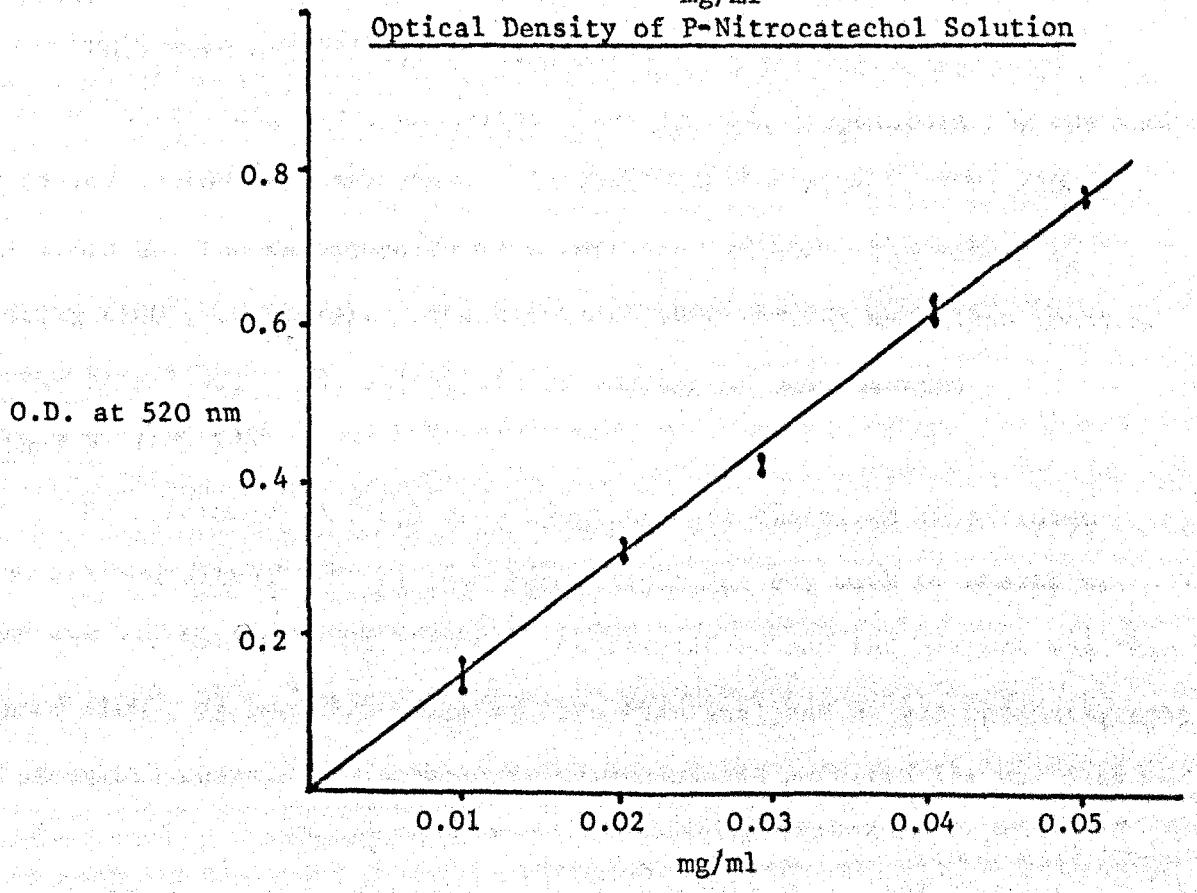
The activity of the aryl sulphatase was conveniently checked using a solution of p-nitrocatechol sulphate (0.1 ml, 1 mg ml^{-1}) in acetate buffer (0.8 ml, 100 mM, pH 5.3) at 37°, to which was added a diluted sample of enzyme (in 0.1 ml). The mixture was then incubated for five minutes, the reaction was stopped by the addition of dilute sodium hydroxide solution (0.1 m, 2 ml), and the optical density determined immediately in a spectrophotometer (520 nm). The quantity of p-nitrocatechol liberated was then estimated by reference to a calibration graph prepared using p-nitrocatechol (Fig. 40).

The activity of the β-glucuronidase was conveniently determined using a solution of phenolphthalien glucuronide (0.5 ml of 1 mg ml^{-1}) in acetate buffer (0.4 ml, 100 mM, pH 5.3) at 37°, to which was added a diluted sample of enzyme mixture (in 0.1 ml). The mixture was incubated for five minutes, and the reaction stopped using dilute sodium hydroxide solution (0.5 m, 5 ml). Liberated phenolphthalien was determined by measuring the optical density of the resulting solution at 540 nm, and comparing this with a calibration graph prepared using pure phenolphthalien (Fig. 40).

Standard Curves for Estimating Enzymic Activity

Fig. 40





Attempted Solvolysis of Ecdysteroid Conjugates

Method 1

This method involved incubating samples in tetrahydrofuran or ethyl acetate towhich perchloric acid had been added. THF was prepared by distillation, dried over molecular sieves, and then passed down a short column of aluminia (Fluka, grade 1) to remove peroxides. Pure ecdysteroids (0.1 mg) or ecdysteroid conjugates (derived from eggs or faeces of S. gregaria) were dissolved in THF (5 ml), and the solution was made 0.01M with perchloric acid. The mixture was then heated on a waterbath at 50° for six hours, and then neutralized with sodium bicarbonate solution (0.1 m, 100 ml). The resulting solution was extracted with butanol (3 x 250 ml). The combined butanol phases were then backwashed once with water, followed by rotary evaporation, and the residue was analysed for ecdysteroids in the normal way. These conditions for the solvolysis of ecdysteroid conjugates resulted in the degradation of the hormone in both solvents.

Method 2

Pure hormone (0.1 mg) or conjugates were dissolved in dioxan (200 µl, distilled, dry), and either left at room temperature for one week or heated at 100° for one hour. To this reaction mixture TMSI (100 µl) was added and the ecdysteroids were converted to their TMS ethers by heating (100°, 10 hours). The silylated ecdysteroids were then analysed in the normal way. No solvolysis of conjugates was observed.

Method 3

Pure hormone (0.1 mg) or conjugates were dissolved in pyridine (200 µl) and either left at room temperature for one week or heated at 100° for one hour. TMSI (100 µl) was then added and the mixture was then heated (100°, 5½ hours). Samples were then analysed by gas chromatography as described above. No solvolysis of conjugated ecdysteroids was observed.

Silylation of Ecdysteroids from Biological Material

After having gone through the partition and enzymic hydrolysis steps the sample was taken up in pure methanol ("spectroscopic", 15 ml) and transferred to a Reacti-vial as described earlier. This resulted in a brown tarry residue, which was dried under vacuum (54°, 1 hour) and then dissolved in pyridine (200 µl) and stored overnight (0°). To this solution TMSI (100 µl) was added, and the sample was then heated in an oven to convert the ecdysteroids to their silyl ethers (100°, 5½ hours). The reaction mixture was then streaked onto a TLC plate as described earlier.

Insect Breeding

Schistocerca gregaria were reared under crowded conditions (phase gregaria) either at Keele or at the Centre for Overseas Pest Research, London. Solitary insects (phase solitaria) were reared individually in the Department of Biology, University of York. Both phases were reared at 34° (day), 28° (night) with a photoperiod of 12 hours light and 12 hours darkness. Illumination was provided by a 40 watt bulb in the cages. Insects were fed on grass and bran twice daily, old grass and faeces were removed daily. Both solitary and gregarious insects were reared from the same genetic stock. Under these conditions instar length for gregarious insects was as follows:

1st instar - 4 days, 2nd instar - 4 days, 3rd instar - 6 days,
4th instar - 5 days, 5th instar - 10 days.

Females laid eggs within two to three weeks after the larval-adult ecdysis. The presence of a mature male was found to speed up the rate of maturation of both male and female insects. Eggs were laid into dampened sand contained in aluminium tubes 1½" in diameter, 5" in length. The sand was sterilised by heating for several days at 100°.

Eggs were incubated in the cages under the same conditions as those described for insects. Eggs generally hatched two to three weeks after laying.

Locusta migratoria were reared at Keele under the same conditions as those used for Schistocerca gregaria.

The cages in which the insects were kept were of wood or metal construction, and were cleaned regularly to prevent the spread of disease. A solution of phenol in water, or 'TCP' was used to sterilise the cages.

Collection of Samples

Insects were taken at suitable intervals, either individually or in groups, anaesthetised with nitrogen, weighed, and homogenised in methanol using a blender. Samples were then stored at 0^{o} until analysed.

Faeces were collected daily from cages containing approximately fifty 5th instar insects, separated manually from grass and bran, weighed, and stored at 0^{o} until analysed.

Haemolymph was collected from a synchronous colony of fifty 5th instar Schistocerca gregaria. A hypodermic needle was used to pierce the cuticle at the joint between the abdomen and the rear leg. The insects were held upside down between the thumb and forefinger, with the leg held away from the body with the other hand. Haemolymph was collected using a Pasteur pipette, transferred to a graduated centrifuge tube. When the volume had been determined it was mixed with an excess of methanol ($10\ ml\ ml^{-1}$), and stored at 0^{o} until analysed.

Synthesis of Model Compounds (Fig. 32)

Cholesteryl Acetate (I)

Cholesterol (80 g) was dissolved in pyridine (400 ml) and acetic anhydride (360 ml) and the mixture stirred overnight at room temperature.

The reaction mixture was diluted with water (2ℓ), cooled, and extracted with ether (4ℓ). The ether layers were backwashed with sodium carbonate solution, water, and then dried over magnesium sulphate. The ether was removed by rotary evaporation and the residue of yellow crystals was then recrystallised from absolute ethanol to yield cholesteryl acetate (72g, 77%) as colourless crystals m.p. 114° (lit. 116°)[122]

6-Nitrocholesteryl Acetate (II)

Cholesteryl acetate (1, 70g) in ether (1ℓ) in a round-bottomed flask, fitted with a mechanical stirrer, condenser, thermometer and dropping funnel, was cooled in an ice/salt bath. To the solution was added fuming nitric acid (600 ml) dropwise over 3 hr. The temperature was kept between 5 and 10° during the addition. Stirring was continued for a further hour after the addition of the acid was complete, after which the mixture was transferred to a 5ℓ separating funnel and washed to neutrality with dilute sodium carbonate solution (0.1 m, 2 x 5ℓ) followed by saturated salt solution (15ℓ). The aqueous phases were backwashed with ether and discarded. The combined ether layers were reduced to dryness by rotary evaporation to yield an oil. On titration with methanol the oil yielded a crystaline solid which on recrystallisation from methanol gave 6-nitrocholesterol as a white solid. (43g 56%) m.p. 102-3° (lit. 101-2°)[110]

3β-Acetoxy-5α-cholestan-6-one (III)

To 6-nitrocholesteryl acetate (11, 40g) in glacial acetic acid (800 ml) contained in a 2ℓ round-bottomed flask fitted with a mechanical stirrer and condenser, water (80 ml) was added. To the resulting solution zinc dust (80g) was added in small quantities over 1 hour. The whole was then refluxed overnight. The reaction mixture was filtered

whilst hot, and the filter cake washed with glacial acetic acid (2 x 100 ml). The solution was then diluted with water (2ℓ) and extracted with dichloromethane (4 x 1ℓ). The dichloromethane was removed by rotary evaporation and the crude product was recrystallised from methanol to yield 3β-acetoxy-5α-cholestan-6-one as colourless crystals. (24g, 54%) m.p. 128-9 (lit. 127-129)[110] ν_{max} 3000, 1710 cm^{-1} (Found C, 79.15; H, 11.48 requires C, 78.43; H, 11.04).

3β-Hydroxy-5α-cholestan-6-one (IV)

3β-acetoxy-5α-cholestan-6-one (III, 0.8g) in a mixture of methanol, water, and THF (150 ml, 80:20:50) containing sodium hydroxide (1g) was stirred at room temperature overnight. Methanol and THF were removed by rotary evaporation, water added (200 ml) and the whole extracted with dichloromethane (3 x 250 ml). The organic extract was dried over magnesium sulphate and the solvent removed by rotary evaporation. The product was recrystallised from methanol to give 3β-hydroxy-5α-cholestan-6-one as colourless needles (0.6g, 83%) m.p. 142-143 (lit. 142 144°)[110] ν_{max} 3000, 1710 cm^{-1}. (Found C, 80.5; H, 11.50, requires C, 80.54; H, 11.52).

3β-Acetoxy-7α-bromo-5α-cholestan-6-one (V)

Bromine (8g) in glacial acetic acid (100 ml) was added dropwise to a refluxing solution of 3β-acetoxy-5α-cholestan-6-one (III, 20g) in ether (300 ml) and acetic acid (60 ml) contained in a three necked round-bottomed flask fitted with dropping funnel, condenser and thermometer. The resulting mixture was refluxed overnight. Ether was removed by rotary evaporation at room temperature. Water (6 ml) was added to the reaction mixture, and the resulting precipitate redissolved by heating over a water bath (100°). The solution was then allowed to cool, crude

3β-acetoxy-7α-bromo-5α-cholestan-6-one crystalising out as a yellow solid. The crude material was recrystallised three times from aqueous acetic acid (5% water) to give 3β-acetoxy-7α-bromo-5α-cholestan-6-one as pale buff crystals.

(17.7g, 77%), m.p. 145° (lit. 144-5°)[113] ν_{max} 2950, 1710 cm^{-1}

(Found: C, 66.81%; H, 9.29%; requires C, 66.5%; H, 9%)

3β-Hydroxy-7α-bromo-5α-cholestan-6-one (VI)

The acetate (V, 5g) in a mixture of water, methanol and THF (20:80:50, 100 ml) containing sodium hydroxide (4g) was stirred overnight. The removal of THF and methanol, by rotary evaporation, produced a white precipitate. The mixture was extracted with butanol (3 x 250 ml). The organic layers were then washed with water until neutral. The butanol was then removed by rotary evaporation as an azeotrope with water. The crude product was recrystallised from ethanol to give 3β-hydroxy-7α-bromo--5α-cholestan-6-one as colourless crystals.

(2.2g 66½), m.p. 166-168 ν_{max} 2950, 1710 cm^{-1}

(Found: C, 59%: H, 8.3%, requires C, 63.6%; H, 9.5%)

3β-Acetoxy-5α-cholest-7-en-6-one (VII)

The bromoketone (V, 20g) in hexamethylphosphorictriamide (HMPT, redistilled anhydrous, 200 ml) was mixed with lithium carbonate (13.4g), lithium fluoride (5.2g) and powdered soda glass (4g). The reaction mixture was stirred under nitrogen at 120° for 10 hours. The mixture was then cooled, poured into water (1ℓ) and left to stand. The yellow brown gum obtained from this was collected by filtration, and purified by recrystalisation to give 3β-acetoxy-5α-cholest-7-en-6-one as white crystals.

(60 g, 36%) m.p. 151-152.5° (lit. 150-152°)

ν_{max} 2960, 1725, 1665 cm^{-1}. U.V. max 246 nm (ethanol)

E 7305 (Found: C, 78.53%; H, 10.61%, requires C, 78.45%; H, 10.53%)

3β-Hydroxy-5α-cholest-7-en-6-one (VIII)

To 3β-acetoxy-5α-cholest-7-en-6-one (VII, 1g) in refluxing methanol (200 ml) a solution of potassium carbonate in aqueous methanol (400 mg, 100 ml methanol, 100 ml water) was added dropwise. The mixture was refluxed for 2 hours, then reduced in volume to 100 ml by evaporation, and allowed to cool. Upon cooling the product crystallised out of solution and was collected by filtration. The crystals were then dissolved in butanol (200 ml) and backwashed twice with water (2 x 100 ml). The aqueous layers were pooled and backwashed with butanol (100 ml). The combined butanolic extracts were then reduced to dryness by rotary evaporation, and the product recrystallised from absolute ethanol to give 3β-hydroxy-5α-cholest-7-en-6-one as colourless crystals.

(0.8g, 88%), m.p. 190-192° (lit. 192-3°)[123]

ν_{max} 2950, 1660 cm^{-1} U.V. max 247 nm (ethanol) E. 12017

(Found: C, 80.79%; H, 11.29%, requires C, 81%; H, 11%)

3β-Hydroxy-7β-fluoro-5α-cholestan-6-one (IX)

3β-Hydroxy-7α-bromo-5α-cholestan-6-one (VI, 2g) was dissolved in HMPT (30 ml) to which was added lithium carbonate (1.4g), lithium fluoride (0.52g) and powdered soda glass (400 mg). The reaction mixture was heated overnight at 100° under nitrogen. The mixture was then cooled, filtered and poured into an excess of cold water (500 ml). The resultant precipitate was collected by filtration and the product recrystallised from methanol to give 3β-hydroxy-7β-fluoro-5α-cholestan--6-one as colourless crystals.

(1.5g, 92%) m.p. 168-170° ν_{max} 2900, 1710 cm^{-1}

Found: C, 71.53%; H, 11.12%, requires C, 70%; H, 10.5%)

3β,14α-Dihydroxy-5α-cholest-7-en-6-one (X)

3β-Hydroxy-5α-cholest-7-en-6-one (VIII, 400 mg) in dioxan (15 ml) was heated at 85° for 1 hour with selenium dioxide (400 mg, resublimed). The mixture was then poured into water (200 ml) and extracted with butanol (3 x 250 ml). The butanolic extract was reduced to dryness by rotary evaporation to give a pink gum. The product was recrystallised twice from absolute ethanol to give 3β,14α-dihydroxy-5α-cholest-7-en-6-one as colourless crystals.

(200 mg 48%) m.p. 190-200° with decomp. (lit 231-234°)[111]

U.V. max 244 nm (ethanol) ε 9583

3-Dehydroecdysone and 3-Dehydro-20-hydroxyecdysone

Samples of pure hormones (1 mg) were dissolved in distilled water (1 ml). To this solution was added a portion of platinum catalyst (1 mg) prepared by the reduction of platinum dioxide by hydrogen. The platinum dioxide was reduced in acetic acid at room temperature overnight whilst stirred under hydrogen at atmospheric pressure. The catalyst was washed extensively before use with distilled water (5 x 50 ml). Oxygen was bubbled through the reaction mixture of ecdysteroid and catalyst, which was stirred using a magnetic stirrer. The reaction was followed using TLC (dichloromethane:methanol 80:20) and gas chromatography. With ecdysone one major product was seen on TLC after an hour, at which point the reaction was stopped. The reaction was stopped by diluting the reaction mixture with water (100 ml) and then extracting with butanol (3 x 100 ml). Butanol was removed by rotary evaporation. This product was less polar than ecdysone on TLC but of longer gas chromatographic

retention time. After an hour the reaction mixture containing 20-hydroxyecdysone produced three spots on TLC and the reaction was stopped. These compounds decomposed on storage in methanol overnight (0^{o}) preventing further characterisation. A comparison of the gas chromatographic retention times of these compounds and those of 3-dehydroecdysone and 3-dehydro-20-hydroxyecdysone obtained from another laboratory showed no close correspondence.

References

1. P.M. Jenkin, and H.E. Hinton, Nature, 1966, 211, 871.
2. P.M. Jenkin, Ann. Endocr., 1966, 27, 331.
3. S. Kopec, Biol. Bull., 1922, 42, 323.
4. V.B. Wigglesworth, J. Exp. Biol., 1940, 17, 180.
5. A. Butenandt, and P. Karlson, Z. Naturf. 1954, 96, 389.
6. R. Huber, and W. Hoppe, Chem. Ber., 1965, 98, 2403.
7. P. Karlson, H. Hoffmiester, H. Hummel, P. Hocks, and G. Spiteller, Chem. Ber., 1965, 98, 2394.
8. A. Willig, H.H. Rees, and T.W. Goodwin, J. Insect Physiol., 1971, 17, 2317.
9. H. Chino, S. Sakurai, T. Ohtaki, N. Ikekawa, H. Mizazaki, M. Ishibashi, and H. Abuki, Science, 1974, 183, 529.
10. F. Romer, H. Emmerich, and S. Nowcock, J. Insect Physiol., 1974, 20, 1975.
11. D.S. King, W.E. Bollenbacher, D.W. Borst, W.V. Vedeckis, J.D. O'Connor, P.I. Ittycheriah, and L.I. Gilbert, Proc. Nat. Acad. Sci., U.S.A., 1974, 71, 793.
12. F. Romer, Experientia, 1973, 29, 1417.
13. G. Studinger, and A. Willig, J. Insect Physiol., 1975, 21, 1793.
14. M. Lagueux, M. Hirn, and J.A. Hoffmann, J. Insect Physiol., 1977, 23, 109.
15. H.H. Hagedorn, J.D. O'Connor, M.S. Fuchs, B. Sage, D.A. Schlaeger, and M.K. Bohm, Proc. Nat. Acad. Sci., U.S.A., 197 , 72, 3255.
16. W.E. Robbins, J.N. Kaplanis, J.A. Svoboda, and M.J. Thompson, Ann. Rev. Entomol., 1971, 16, 53.
17. M.J. Thompson, J.N. Kaplanis, W.E. Robbins, and J.A. Svoboda, Adv. Lipid. Res., 1973, 11, 219.

18. P. Johnson, and H.H. Rees, Biochem. J., 1977, 168, 513.

19. D.S. King, and J.B. Siddall, in W.J. Burdette (Editor), "Invertebrate Endrocrinology and Hormonal Heterophylly", Springer-Verlag, New York, 1974, p147.

20. M.J. Thompson, J.N. Kaplanis, W.E. Robbins, S.R. Dutky, and H.N. Nigg, Steroids, 1974, 24, 359.

21. S. Sakurai, N. Ikekawa, T. Ohtaki, and H. Chino, Science, 1977, 198, 627.

22. C. Hetru, M. Lagueux, L. Bang, and J.A. Hoffmann, Life Sci., 1978, 22, 2141.

23. M.N. Galbraith, D.H.S. Horn, E.J. Middleton, J.A. Thomson , and J.W. Wilkie, J. Insect Physiol., 1975, 21, 23.

24. L.I. Gilbert, W. Goodman, and W.E. Bollenbacher, Int. Rev. Biochem., 1977, 14, 1.

25. K. Slama, M. Romanuk, and F. Sorm, "Insect Hormones and Bioanalogues", Springer-Verlag, New York, 1974, p.303.

26. R. Feyereisen, C.R. Acad. Sci. Paris, Ser. D., 1977, 284, 1931.

27. P. Johnson, and H.H. Rees, J. Insect Physiol., 1977, 23, 1387.

28. R. Feyereisen, and J.A. Hoffmann, J. Insect Physiol., 1977, 23, 1175.

29. A. Clever, and P. Karlson, Exptl. Cell. Res., 1960, 20, 623.

30. E. Shaaya, and C.E. Sekeris, F.E.B.S. Lett., 1971, 16, 333.

31. K. Scheller, and P. Karlson, J. Insect Physiol., 1977, 23, 285.

32. N. Fong, and M.S. Fuchs, Mol. and Cellular Endocr., 1976, 4, 341.

33. F. Bulliere, and D. Bulliere, J. Insect Physiol., 1977, 34, 1475.

34. D.S. King, Gen. Comp. Endocr. Supp., 1972, 3, 221.

35. L.I. Gilbert, and D.S. King, in M. Rockstein "The Physiology of Insecta", Academic Press, New York, Vol. 1., 1973, p.250.

36. D.S. King, W.E. Bollenbacher, D.W. Borst, W.V. Vedeckis, J.D. O'Connor, P.I. Ittycheriah, and L.I. Gilbert, Proc. Nat. Acad. Sci., U.S.A., 1974, 71, 793.

37. W.E. Bollenbacher, W.V. Vedeckis, L.I. Gilbert, and J.D. O'Connor, Dev. Biol., 1975, 44, 46.

38. R. Oberlander, J. Insect Physiol., 1972, 18, 223.

39. U. Clever, I. Clever, I. Storbeck, and N.L. Young, Dev. Biol., 1973, 31, 47.

40. P. Karlson, J. Koolman, and J.A. Hoffmann, Am. Zool., 1975, 15 supp 3, 49.

41. A.R. Gande, and E.D. Morgan, J. Insect Physiol., 1979, 24, in press.

42. T. Mizuno and E. Ohnishi, Dev. Growth Diff., 1975, 17, 219.

43. M. Lagueuex, M. Hirn, and J.A. Hoffmann, J. Insect Physiol., 1977, 23, 109.

44. R. Feyereisen, Experientia, 1977, 33, 1111.

45. R. Feyereisen, M. Lagueux, and J.A. Hoffmann, Gen. Comp. Endocr., 1976, 24, 319.

46. E.D. Morgan, and C.F. Poole, Comp. Biochem. Physiol., 1977, 57b, 99.

47. R. Shaaya, Z. Naturf., 1969, 24b, 718.

48. T. Ohtaki, and C.M. Williams, Biol. Bull., 1970, 138, 326.

49. J. Koolman, J.A. Hoffmann, and M. Dreyer, Experientia, 1975, 31, 247.

50. E.D. Morgan, and C.F. Poole, J. Insect Physiol., 1976, 22, 885.

51. H. Hoffmeister, C. Rufer, and H. Ammon, Z. Naturf., 1965, 20b, 130.

52. M.J. Thompson, J.N. Kaplanis, W.E. Robbins, and R.T. Yamamoto, Chem. Commun., 1967, 650.

53. H. Hikino, Y. Ohizumi, and T. Takemoto, Chem. Commun., 1971, 1036.

54. J.A. Thomson, D.H.S. Horn, M.N. Galbraith, and E.J. Middleton, in W.J. Burdette (Editor) "Invertebrate Endocrinology and Hormonal Heterophylly", Springer-Verlag, New York, 1974, p153.

55. P. Karlson, H. Bugany, H. Dopp, and G.A. Hoyer, Hoppe-Seylers Z. Physiol. Chem., 1970, 353, 1610.

56. H. Heinrich, and H. Hoffmeister, Z. Naturf., 1970, 25b, 358.

57. J. Koolman, J.A. Hoffmann, and P. Karlson, Hoppe-Seylers Z. Physiol. Chem., 1973, 354, 1.

58. G.M. Price and G.B. Russell, Biochem. Soc. Trans., 1975, 3, 75.

59. P. Karlson, and J. Koolman, Insect Biochem., 1973, 3, 409.

60. A. Sannasi, and P. Karlson, Zool. Jb. (Physiol.), 1974, 78, 378.

61. J. Koolman, Hoppe-Seylers Z. Physiol. Chem., 1978, 359, 1315.

62. J.A. Hoffmann, J. Koolman, P. Karlson, and P. Joly, Gen. Comp. Endocr., 1974, 22, 90.

63. G. Richards, J. Insect Physiol., 1978, 24, 329.

64. A.E. Moribayashi, and T. Ohtaki, J. Insect Physiol., 1978, 24, 279.

65. D.R. Greenwood, and G.B. Russell, Experientia, 1977, 34, 667.

66. J.N. Kaplanis, W.E. Robbins, M.J. Thompson, and S.R. Dutky, Science, 1973, 180, 307.

67. D.N. Galbraith, D.H.S. Horn, E.J. Middleton, J.A. Thomson., J.B. Siddall, and W. Hafferl, Chem. Commun., 1969, 1134.

68. P. Karlson, and D. Stamm, Hoppe-Seylers Z. Physiol. Chem., 1956, 306, 109.

69. D.S. King, and E.P. Marks, Life Sci., 1974, 15, 147.

70. C. Bordereau, M. Hirn, and M. De Reggi, C.R. Acad. Sci., Paris, Ser. D., 1976, 282, 885.

71. R. Huybrechts, and A. De Loof, J. Insect Physiol., 1977, 23, 1354.

72. A.M. Fallon, H.H. Hagedorn, G.R. Wyatt, and H. Laufer, J. Insect Physiol., 1974, 20, 1815.

73. J.N. Kaplanis, W.E. Robbins, M.J. Thompson, and S.R. Dutky, Steroids, 1976, 37, 675.

74. W.J. Bell, and G.R. Sams, J. Insect Physiol., 1975, 21, 173.

75. W. Fong, and M.S. Fuchs, J. Insect Physiol., 1976, 22, 1493.

76. M.K. Bohm, M. Behan, and H.H. Hagedorn, Physiol. Entomol., 3, 17.1978

77. W.F. Fong, and M.S. Fuchs, Insect Biochem., 1976, 6, 123.

78. C. Palevody, J.P. Delbeque, and J. Delachambre, C.R. Acad. Sci. Paris, Ser. D., 1977, 285, 1323.

79. E. Ohnishi, T. Mizuno, N. Ikekawa, N. Awata, and S. Sakurai, J. Insect Physiol., 1977, 23, 317.

80. J.N. Kaplanis, S.R. Dutky, W.E. Robbins, M.J. Thompson, E.L. Lindquist, D.H.S. Horn, and M.N. Galbraith, Science, 1975, 190, 681.

81. R.K. Boohar, and D.H. Bucklin, Am. Soc. Zool., 1977, 4, 496.

82. P.E. Ellis, E.D. Morgan, and A.P. Woodbridge, Pest Articles News Summaries, 1970, 16, 434.

83. J.L. Marx, Science, 1978, 197, 1170.

84. L. Clarke, G.H.R. Temple, and J.F.V. Vincent, J. Insect Physiol., 1977, 23, 241.

85. M.P. O'Neill, G.M. Holman, and J.E. Wright, J. Insect Physiol., 1977, 23, 1243.

86. E.D. Morgan, A.P. Woodbridge, and P.E. Ellis, Acrida, 1975, 4, 69.

87. G.Fraenkel, Proc. Roy. Soc. B., 1935, 118, 1.

88. P. Karlson, Vitamins and Hormones, 1956, 14, 227.

89. C.M. Williams, Biol. Bull., 1968, 134, 344.

90. E.D. Morgan, and C.F. Poole, Adv. Insect Physiol., 1976, 12, 17.

91. C.F. Poole, E.D. Morgan, and P.M. Bebbington, J. Chromatog., 1975, 104, 172.

92. C.F. Poole, Ph.D. Thesis, Univ. of Keele, 1975.

93. P.M. Burkinshaw, E.D. Morgan, and C.F. Poole, J. Chromatog., 1977, 132, 548.

94. N. Ikekawa, F. Hattori, J. Rubio-Lightbourn, H. Miyazaki, M. Ishibashi, and C. Mori, J. Chromatog. Sci., 1972, 10, 223.

95. D.W. Borst, and J.D. O'Connor, Steroids, 1974, 24, 637.

96. H.L. Bradlow in S. Bernstein and S. Solomon (Editors),"Chemical and Biological Aspects of Steroid Conjugation", Springer-Verlag New York, 1970, p130.

97. E. Dingmanse, H. Borcharot, and E. Laquer, Biochem J., 1937, 31, 500.

98. G.A. Grant and D. Beal, Recent Prog. Hormone Res., 1950, 5, 307.

99. G.M. Jacobsohn and S. Lieberman, J. Biol. Chem., 1962, 237, 1469.

100. J. McKenna and J.K. Norymberski, J. Chem. Soc., 1957, 3889.

101. E.D. Morgan, A.P. Woodbridge, and P.E. Ellis, J. Insect Physiol., 1975, 21, 979.

102. H. Chino, L.I. Gilbert, J.B. Siddall, and W. Hafferl, J. Insect Physiol., 1970, 16, 2033.

103. M. Hikino, Y. Ohizumi, and T. Takemoto, J. Insect Physiol., 1975, 21, 1953.

104. O.W. Richards and R.G. Davies in"Imm's General Textbook of Entomology", Chapman and Hall, London, 10th Ed., 1977, p248.

105. P.M. Davey, Bull. Ent. Res., 1954, 45, 539.

106. B. Uvarov, "Grasshoppers and Locusts", Cambridge University Press, Vol. 1, 1966, p332.

107. D.B. Carlisle and P.E. Ellis, 4^{e} Un. Int. Etúde Insectes Soc. Pavia. 1962, 219.

108. P.E. Ellis and D.B. Carlisle, Nature, 1961, 190, 368.

109. L. Joly, P. Joly, and M. Lagueux, C.R. Acad. Sci. Paris, Ser. D., 1977, 285, 543.

110. C.E. Anagnostopoulou and L.F. Fieser, J. Amer. Chem. Soc., 1954, 76, 532.

111. M.N. Galbraith, D.H.S. Horn, and E.J. Middleton, Aust. J. Chem., 1974, 27, 1087.

112. R.M. Dodson and B. Riegel, J. Org. Chem., 1948, 13, 424.

113. I.M. Heilbron, H. Jackson, E.R.H. Jones, and F.S. Spring, J. Chem. Soc., 1938, 102.

114. R. Hanna, Tet. Letters, 1968, 17, 2105.

115. K.D. Spindler, J. Koolman, F. Mosora, and H. Emmerich, J. Insect Physiol., 1977, 23, 441.

116. A.R. Gande, Ph.D. Thesis, Univ. Keele, 1979.

117. W.E. Bollenbacher, M.N. Galbraith, L.I. Gilbert and D.H.S. Horn, Steroids, 1977, 29, 47.

118. W.E. Robbins, M.J. Thompson, J.A. Svoboda, T.J. Shortino, C.F. Cohen, S.R. Dutky, and O.J. Duncan, Lipids, 1975, 10, 353.

119. J.A. Svoboda, M.J. Thompson, W.E. Robbins, and J.N. Kaplanis, Lipids, 1978, 13, 742.

120. R. Chayen, R. Dvir, S. Gould, and A. Harell, Anal. Biochem., 1971, 42, 283.

121. K.K. Nayar, "Elements of Insect Endocrinology", Prentice Hall, 1973, p31.

122. L.F. Fieser and M. Fieser, "Steroids", Reinhold Publishing Corp., New York, 1959, p.28.

123. C. Djerassi, J.C. Knight and H. Brockmain, Chem. Ber., 1964, 97, 3118.