A CHEMICAL INVESTIGATION OF THE DEVELOPMENT HORMONES PRESENT IN THE EGGS OF THE DESERT LOCUST (*Schistocerca gregaria*)

by

Santo Scalia

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Department of Chemistry
University of Keele

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Abstract

Ecdysteroids levels in the developing eggs of Schistocerca gregaria have been determined, at daily intervals, using HPLC with ultraviolet detection. Ecdysone, 2-deoxyecdysone and 20-hydroxy-ecdysone, in free and conjugated form, were identified, ecdysone being the main compound. Conjugated ecdysteroids predominated throughout embryogenesis; 19 $\mu g\ g^{-1}$ of conjugated ecdysone were found in day-12 eggs whereas the free compound was present at 2.2 $\mu g\ g^{-1}$. The same pattern of titre fluctuation was observed for both free and conjugated forms.

The levels of conjugates were found to be ten-fold higher than in a previous investigation by GC. It was demonstrated that HPLC and GC methods gave consistent results and the earlier error was traced to incomplete hydrolysis of the conjugates caused by enzyme inhibition.

The ecdysteroids assay in the separated embryo and yolk of S. gregaria eggs at different stages of embryonic development was begun. The results so far obtained indicated that ecdysteroids were located in the yolk immediately after oviposition. The major compound was ecdysone, present free at 40 ng/egg but, mainly, conjugated at 334 ng/egg. During late embryogenesis, ecdysone, which was lacking in the yolk, occurred in the embryo where its titre rose to 48.4 ng/egg and 228 ng/egg for the free and conjugated form respectively.

A new method was devised for the direct qualitative and quantitative analysis of the ionic conjugated ecdysteroids found in the eggs. The conjugates were separated and quantified by ion-paired

HPLC-UV on a reversed-phase column eluted with a buffered aqueous-methanol gradient containing tetrabutylammonium ions.

Experiments have been made on the effect of phosphate, ionic strength and a specific β-glucuronidase competitive inhibitor on the efficiency of the enzymic hydrolysis of the conjugates to provide more evidence on the chemical nature of these compounds. The results obtained eliminated the possibility of the conjugates being glucuronides.

The isolation of juvenile hormone from the eggs of S. gregaria was attempted, using HPLC as analytical method. The identification of the hormone was not accomplished with the amount of biological material extracted. Using an internal standard, it was shown that the level of juvenile hormone is S. gregaria eggs is below 47 ng g^{-1}.

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INTRODUCTION

The growth and differentiation of insects is influenced by the tough cuticle, or exoskeleton, which serves to support and contain their internal organs. As this cuticle is incapable of growth, in order to expand, the insect has to shed periodically its outer integument. This process, called moulting, involves the separation of the epidermal cells from the old cuticle (or apolysis), the secretion of a new cuticle, then the shedding of the old cuticle (or ecdysis), the expansion and the hardening of the new cuticle. Thus the insect, emerged from the egg as an immature larva or nymph, grows and acquires adulthood and sexual maturity through repeatedly moulting process. The form that the insect takes in the interval between moults is called an instar.

In the hemimetabola insects (e.g. locusts, grasshoppers, aphids and cockroaches) the various larval stages are fairly similar in appearance, differing mainly in size and showing gradually developing adult characteristics. However in the holometabola insects (e.g. beetles, moths, flies and ants) the development proceeds via a pupal stage in which the larval tissues are completely reorganized into the adult form.

The moulting process, which is essential for insect development, is controlled by two different hormones; the ecdysteroids, or moulting hormones, and the juvenile hormones. The former induce the synthetic activities necessary for moulting, whereas the latter influence the type of moult promoted by the ecdysteroids.

The Ecdysteroids

That a hormonal mechanism is responsible for initiating moulting in insects was first suggested by Kopéc in 1917[1]. However only in 1934 the classical studies of Fraenkel[2] and Wigglesworth[3] provided a convincing support for Kopéc's hypothesis and a demonstration of the presence of a circulating chemical factor which induces moulting. Following the work of several authors[4,5,6] performed with the methods of extirpation and reimplantation of presumed hormonal glands, it became clear that the moulting hormone is secreted by the prothoracic glands, or their equivalent (e.g. the brain-ring gland complex), at an appropriate time in response to a hormone released by the brain. This was convincingly demonstrated with simple ligation experiments by Williams[7].

While the biological studies were in progress, the purification of the insect moulting hormone was undertaken by Butenandt and Karlson, using the Calliphora bioassay[8] as a method for assaying the hormone. This work culminated in the isolation of the pure crystalline hormone, which was named ecdysone[9].

The complete structure of ecdysone was elucidated in 1965[10] by an X-ray diffraction technique called diffuse scattering; the assigned chemical structure was 2β,3β,14α,22R,25-pentahydroxy-5β-cholest--7-en-6-one (I, see Fig. 1).

Ecdysone (I)

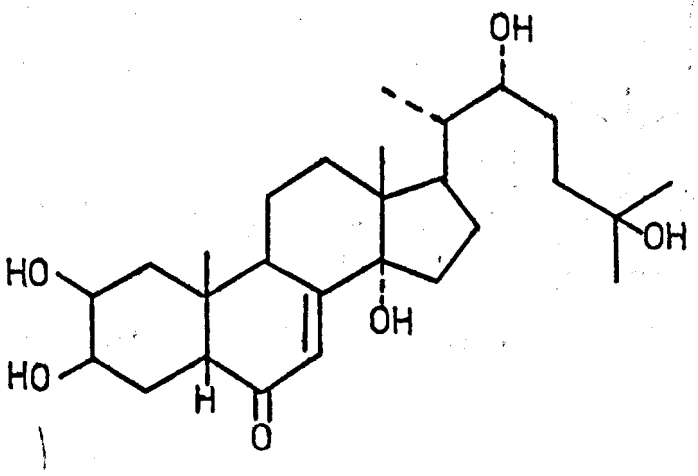
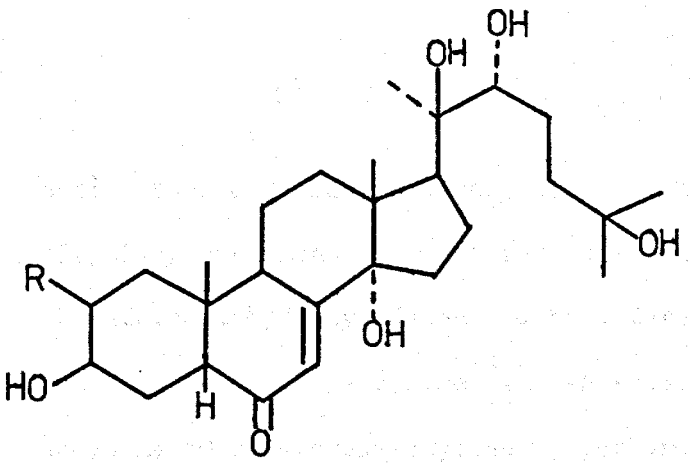


Figure 1

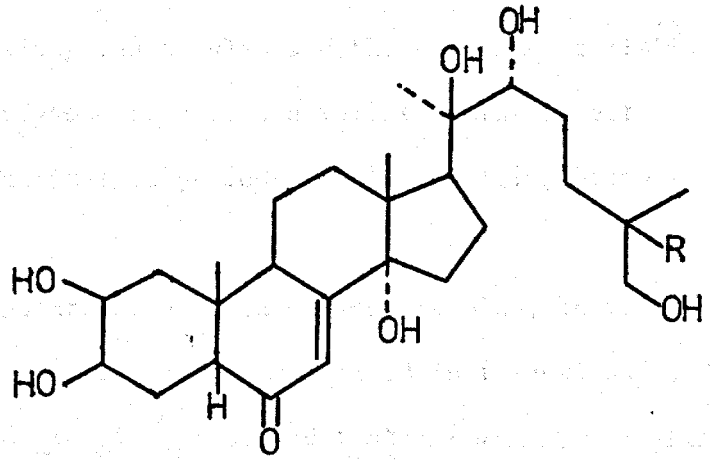
Since then a number of closely related steroids have been extracted from arthropods, purified and chemically characterized; e.g. 20-hydroxyecdysone (or β-ecdysone) (II), 20,26-dihydroxyecdysone (III)[12], 2-deoxy--20-hydroxyecdysone (IV)[13], 25-deoxy-20,26-dihydroxyecdysone (or inokosterone) (V)[14], 24-methyl-20-hydroxyecdysone (or makisterone A) (VI)[14], 25-deoxy-20-hydroxyecdysone (or ponasterone A) (VII)[15]. The structures of these compounds are given in Figure 2. The term ecdysteroids refers to the family of compounds structurally related to ecdysone, whereas moulting hormones refers to the compounds reacting physiologically like ecdysone.

Ecdysone and 20-hydroxyecdysone are the most abundant ecdysteroids. They have been found in all six insect orders studied and in the majority of arthropod species analysed so far[16]. 20-Hydroxyecdysone is more



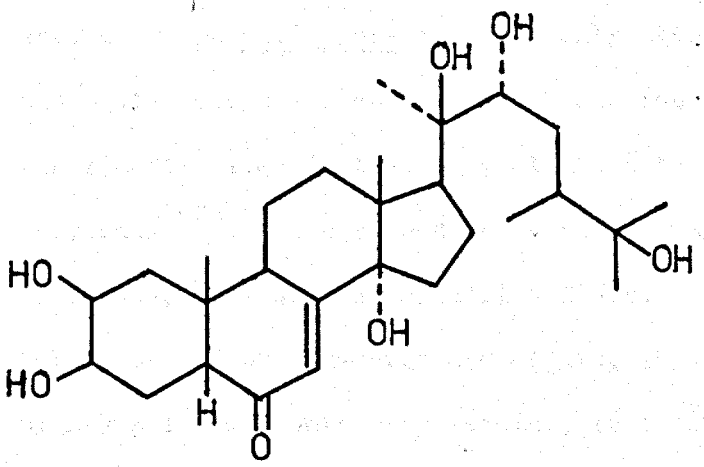
R = H, 2-Deoxy-20-hydroxy-ecdysone (IV)

R = OH, 20-Hydroxyecdysone (II)

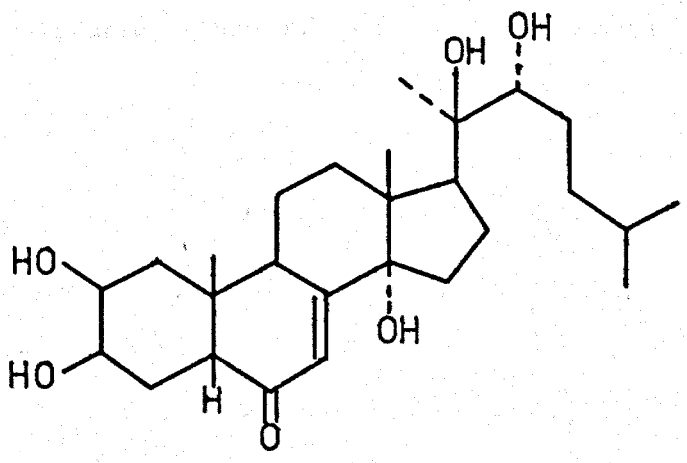


R = H, Inokosterone (V)

R = OH, 20,26-Dihydroxy-ecdysone (III)



Makisterone A (VI)



Ponasterone A (VII)

Figure 2

active than ecdysone in a variety of bioassays[17,18] and is the circulating hormone in most insect larvae[19,20] and crustaceans[22,23]. Therefore 20-hydroxyecdysone is considered the active form of the moulting hormone, ecdysone[16]. In insects, the conversion of ecdysone to 20-hydroxyecdysone is performed in peripheral tissue, e.g. the fat body, the malpighian tubules and the epidermal cells[19]. The ratio of 20-hydroxyecdysone to ecdysone varies during insect development[24-27], being maximal at periods of high moulting hormone titre[28,29].

The existence of polar derivatives of ecdysteroids, known as ecdysteroid conjugates, has also been demonstrated in insects and insect organs[16]. WILLIG et al.[30] observed that the brain-ring gland complex of Calliphora vicina contains polar compounds which were active in the Calliphora bioassay only after cleavage with α-glucosidase. Conjugated ecdysteroids have also been found in radiotracer experiments, i.e. in the study of the fate of injected tritiated ecdysone[31-33]. The structure of this group of ecdysteroid metabolites, in larvae, has not been established yet. From their electrophoretic behaviour and by enzymatic hydrolysis, it was concluded that they are highly polar or ionic ecdysteroids, tentatively identified as conjugated with glucose, sulphuric, glucuronic or phosphoric acid[16].

Microdetermination of Ecdysteroids

Since the concentration of ecdysteroids in insects is extremely small (10^{-9} to 10^{-6} g per g of tissue[34]), microdeterminations of the hormones in the biological material require efficient purification techniques and accurate and sensitive detection methods. Furthermore the study of the physiological mode of action of the hormones relies upon determination of levels of endogenous ecdysteroids at different stages of the insect development. Such correlative studies requiring a large number of analytical determinations, are made feasible only by assay methods which are rapid and efficient and which can be applied to routine analysis.

Several techniques are available for the assay of the ecdysteroids in insect tissues. Most of the earliest investigations on moulting hormones have been carried out by bioassay[35]. Although this method is fairly sensitive,[36] (5 to 50 ng) it requires laborious procedures and it lacks specificity in that it cannot distinguish between different chemical substances with the same physiological action[36]. For this reason the bioassay has been replaced by other analytical methods and it is seldom used, particularly in routine quantitative analysis.

A valuable technique for microdeterminations of ecdysteroids in crude extracts is radio-immuno-assay (RIA), which is based on the competition between labelled and unlabelled hormones binding to specific antibodies. RIA is widely used for ecdysteroid determinations because of its ease of operation, rapidity and sensitivity[34] (20 to 30 pg). Nevertheless, in common with bioassay, it cannot discriminate between different ecdysteroids because of the cross-reactivity of molecules of similar structure[37]. When more specific analyses are needed, RIA

must be preceded by purification and separation steps which lengthen the analysis time.

The specificity required for the assay of mixtures of ecdysteroids has been met by using chromatographic techniques which also allow quantitative determinations when coupled with suitable detectors.

Thin-layer chromatography (TLC) has been used extensively for the separation of ecdysteroids[36], because it is simple to use, gives good resolution and permits in situ quantification of the hormones under UV light due to the strong extinction coefficient of the 7-en-6-one chromophore present in ecdysteroids[34] (sensitivity,[38,39] 10^{-7} g). In addition to the relatively poor sensitivity, when compared with other techniques, TLC causes losses of small amounts of polar compounds, like ecdysteroids, due to their irreversible adsorption on activated silica surface[40]. Higher recoveries have been obtained by using reversed-phase TLC (RPTLC) with a non-polar surface bonded to silica[39]. However the latter have separation properties different from those of TLC.

Paper chromatography and conventional column chromatography are methods generally applicable for moulting hormones separations[41], although they both have inherent limitations with regard to speed and efficiency of separation and ease of quantification. Furthermore the recovery of ecdysteroids from silica or alumina columns has been found to be poor[40].

Gas chromatography (GC) has become a popular method for the assay of ecdysteroids because of its speed and high resolution[41]. Since ecdysteroids have low volatility and are thermally labile, they have to be converted to less polar derivatives to avoid decomposition and improve their chromatographic performance. The

derivatisation constitutes the main limitation of the GC method, since it lengthens the time per analysis, requires further purification steps and often causes low yield and production of mixtures of derivatives[36,41]. GC has been used first with a flame ionisation detector (FID) whose sensitivity (50 ng) is rather low for the concentration of moulting hormones in insects.[34] However the discovery[42] that ecdysteroids are sensitive to detection by the electron capture detector (ECD) enhanced the sensitivity of GC analysis to a pg level[43], permitting detection of physiological quantities of the hormones. Furthermore the use of GC-ECD produces an increase in detection selectivity since fewer compounds present in insect tissues will give an ECD response than a FID response[37]. GC coupled with mass fragmentographic detection (GC-MF) has also been used to measure ecdysteroid titres in several insect species[44]. In GC-MF analysis, the mass spectrometer serves as a highly specific detector for the GC, monitoring one or several fixed ions rather than scanning total spectra. Although this technique is not as sensitive as GC-ECD (GC-MF sensitivity is 0.1 ng) it enables simultaneous identification and quantitative determination of the compound analysed.[44]

High performance liquid chromatography (HPLC) was first introduced for the analysis of ecdysteroids by Hori[45]. With the improvement of equipment and packing materials it has become the most widely used technique. The use of microparticulate material (5-10 μm) as stationary phase has produced a dramatic increase in the resolving power of HPLC which has proved to be particularly useful for the assay of the moulting hormones in insect extracts containing several structurally similar ecdysteroids.[46] Two different types of stationary phase are available for HPLC; i.e. normal silica phase (NP) and

reversed-phase (RP), the latter system being more suitable for analysis of crude extracts since the very polar impurities associated with the ecdysteroids through the purification process are here eluted before the hormones and do not accumulate on the top of the column as happens with NP. One of the major advantages of HPLC for ecdysteroids analysis is that they can be directly injected into the column without prior formation of derivatives[27] as required for GC. Furthermore HPLC offers the advantage over TLC of a quantitative recovery of injected compounds[46] which can then be collected from the column and analysed by other techniques for conclusive identifications. As noted earlier, ecdysteroids strongly absorb UV light at 244 nm, this absorption band is convenient for detection purposes[36]. Consequently HPLC coupled with a UV detector has also been used for direct quantification of the hormones by recording the UV absorbance of the effluent from the column[27]. Although the detection limit[36] of HPLC-UV (10 ng) is inferior to GC-ECD or GC-MF, the speed and efficiency of separation and ease of quantification have made HPLC the method of choice for routine assay of moulting hormones in insect material, and particularly in insect ovaries, eggs and embryos, where the level of hormone is relatively high, and very large samples are therefore not required.

Microdetermination of Conjugated Ecdysteroids

The determination of the titre of the polar conjugates of ecdysteroids in insect tissues exhibits particular difficulties, in that the conventional methods used for the analysis of the free ecdysteroids are not suitable for these highly polar metabolites and, until very recently, their structures were unknown.

The conjugates are inactive in bioassays and also poorly immunoreactive[32,47]. Furthermore the RIA cannot discriminate between different compounds and it has produced inconsistent results in Locusta migratoria[48,49].

With regard to chromatographic analysis, the conjugated ecdysteroids are the most complex group of ecdysone metabolites. This is due to their ionic character as well as to the lack of reference substances. The conjugates are either strongly retained on NP columns or eluted rapidly in RP systems as broad peaks and poorly separated[49]. Individual ecdysteroid conjugates are also not resolved by TLC and ion-exchange chromatography (IEC) which are useful only for group separations of these compounds[50-52].

Quantitative analysis of these substances has been mainly performed on the free hormones released from the conjugates by enzymic hydrolysis with the digestive juice of the snail Helix pomatia[53-55]. The hydrolysis step not only adds to the time per analysis, but also it causes longer sample manipulation and the introduction of artifacts[56]. Moreover failure to achieve complete hydrolysis has been found to cause underestimation of the quantities of conjugates present in vivo[55].

Moulting Hormones Titres During Larval Development

The availability of more sensitive and specific methods for the detection of ecdysteroids, has made it possible to measure the fluctuation of the moulting hormone titre during insect development.

Several species have been investigated, data are available on Locusta migratoria (Orthoptera)[33], Manduca sexta (Lepidoptera)[57], Tenebrio molitor (Coleoptera)[58], Calliphora vicina (Diptera)[59], and Schistocerca gregaria (Orthoptera)[26]. Although the maximum concentration

of ecdysteroids detected varies widely from one animal to another[60], the investigations are generally in agreement in showing a sharp peak in hormone titre a few days before ecdysis, at the time of apolysis and initiation of the synthesis of the new cuticle. Furthermore the existence, during an instar, of a small peak, preceding the main hormone peak, has been reported by several authors[24,57,61]. Whereas the major peak is connected with the control of the secretion of the new cuticle, as previously stated, the physiological significance of the small peak has not yet been ascertained, being possibly related to renewed DNA synthesis and apolysis[29,62].

The presence of the two ecdysteroid peaks and the identification of 20-hydroxyecdysone as a major hormone have also been demonstrated in Schistocerca gregaria larvae[26].

Ecdysteroids in Insect Ovaries

As stated earlier, the ecdysteroids, synthesised in the prothoracic glands and further hydroxylated in the body, control the production of new cuticles during the moulting process in insects. In the light of this finding, it was assumed that the function of moulting hormones was limited to the post-embryonic development[63]. However this hypothesis had to be drastically revised by the discovery of moulting hormone activity in the ovaries of adult female insects[64-67], which do not moult and whose prothoracic glands are degenerated. Shortly after the first investigations on ovarian ecdysteroids based on unspecific and inaccurate bioassay and radioimmunoassay[47], the presence of these molecules in adult ovaries was conclusively demonstrated by GC and mass spectrometric analysis (GC-MS) in Locusta migratoria[68], Bombyx mori[69] and Macrotermes bellicosus[70]. These

studies have evinced that ovarian ecdysteroids are present mainly as high polarity compounds, assumed to be conjugated on the basis of enzymic hydrolysis by the digestive juice of the snail Helix pomatia[47,54,69,71,72]. Furthermore, in most of the adult female insects investigated, the ecdysteroids consisted essentially of conjugates of ecdysone and 2-deoxyecdysone[48,69,71,72] which predominate over 20-hydroxyecdysone, the major ecdysteroid present in the larval stage.

In Locusta migratoria ecdysteroids have been shown to be synthesised in the follicle cells surrounding the oöcyte and stored in the ooplasm of the terminal oöcytes[73]. Ovarian synthesis of ecdysteroids has also been demonstrated in the closely related locust, Schistocerca gregaria[74], in Galleria mellonella[75] and in Bombyx mori[76].

Several authors have proposed a requirement for ecdysteroids in the synthesis of vitellogenin, a precursor of the yolk protein[77,78]; nevertheless the major role of ovarian ecdysteroids is connected with the development of the embryo[47], since the bulk of these molecules is transferred to the eggs in many insect species[48,65,72,74].

Moulting Hormones in Insect Eggs

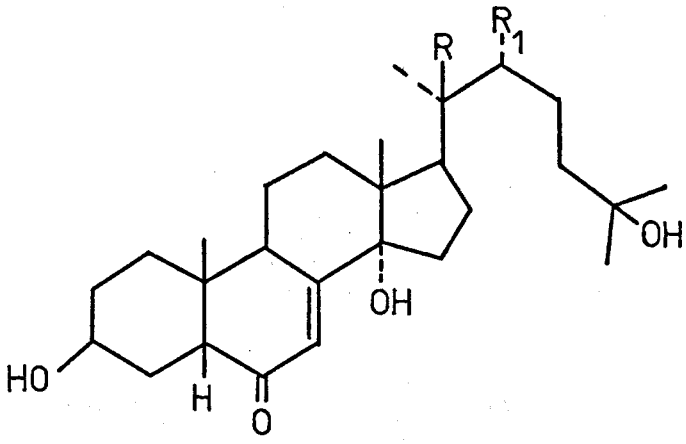
The first investigations on the presence of moulting hormones during embryonic development were carried out independently from studies on ecdysteroids in adult female insects. Reports on ecdysteroids in insect eggs have appeared since 1971[79]; detailed investigations have been performed on eggs of Manduca sexta[80], by Infrared (IR), MS, Nuclear Magnetic Resonance spectrometry (NMR); Bombyx mori[81] by HPLC, MS; Locusta migratoria[48] by GC-MS; Nauphoeta cinerea by GC-MS, and Schistocerca gregaria[53-55] by GC-ECD, HPLC, MS, NMR.

On the basis of bioassay and RIA, egg ecdysteroids have been reported in other insect species such as Oncopeltus fasciatus[83], Leucophae maderae[84], Galleria mellonella[71], Drosophila melanogaster[85] and Calliphora vicina[59].

An important feature of egg ecdysteroids is their presence in a variety of molecular forms, in contrast with the simple composition of the ecdysteroids at post-embryonic stages. In additon to the commonly known ecdysone and 20-hydroxyecdysone, 2-deoxyecdysone (VIII), 3-epi-2-deoxyecdysone (IX) and ecdysone-3-acetate (X) have been identified in Schistocerca gregaria[55,86,87]; 2,22-bisdeoxy-20-hydroxy-ecdysone (XI) has been found in embryos of Bombyx mori[88]; 26-hydroxy-ecdysone and its 3-epi form (XII) have been detected in eggs of Manduca sexta[89] whereas 3-epi-ecdysone (XIII) has been discovered in Locusta migratoria embryos[90]. The structures of these compounds are given in Figure 3. The significance of the complex profile of naturally occurring ecdysteroids isolated from insect eggs is not understood; nevertheless it implies the presence of biosynthetic-metabolic pathways different from the ones occurring in post-embryonic development[74,89,91]. The latter observation is substantiated by the finding that 20-hydroxy-ecdysone is a minor component in the eggs, whereas it is the predominant ecdysteroid present in larval stages.

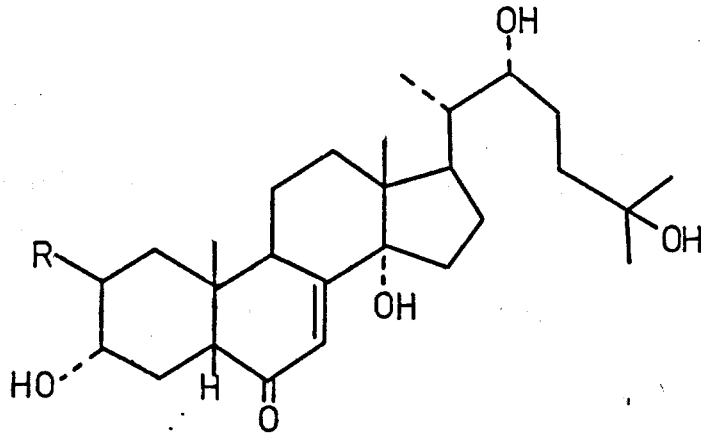
As in the ovaries, the ecdysteroids are present in the insect eggs not only in the free state[82,89] but also in an inactive, highly polar, conjugated form, which account for 80-90% of the total amount of the hormones during embryonic development of several species of insects[55,71,88,91].

In spite of the relatively large amount of these polar ecdysteroid derivatives, only at the end of this work have they been



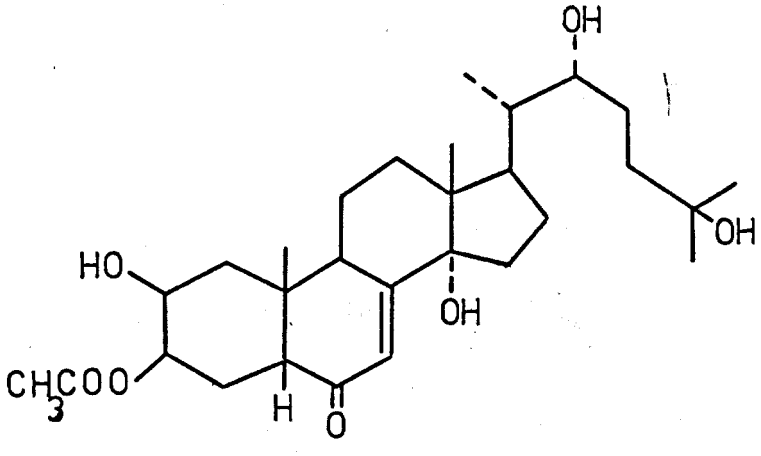
R = H, R_1 = OH, 2-Deoxyecdysone (VIII)

R = OH, R_1 = H, 2,22-Bisdeoxy-20--hydroxyecdysone (XI)

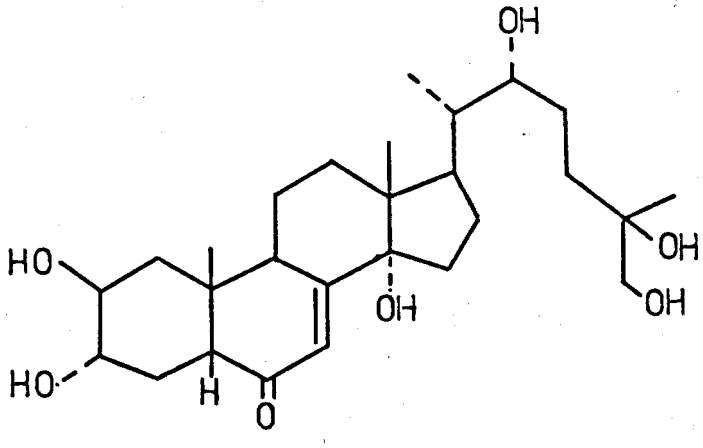


R = H, 3-Epi-2-deoxy-ecdysone (IX)

R = OH, 3-Epi-ecdysone (XIII)



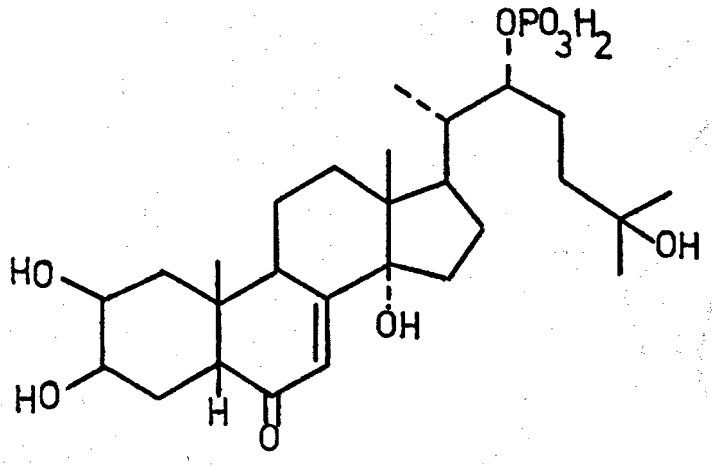
Ecdysone-3-acetate (X)



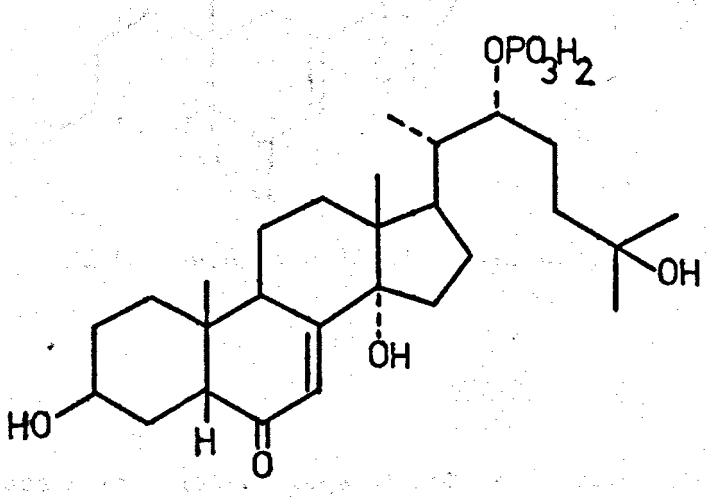
3-Epi-26-hydroxy-ecdysone (XII)

Figure 3

isolated in a pure form and chemically identified in newly laid eggs of Schistocerca gregaria by Fast Atom Bombardment MS and ^{1}H, ^{13}C and ^{31}P NMR spectroscopy[92]. The assigned structures of the two major conjugates are ecdysone-22-phosphate (XIV) and 2-deoxyecdysone-22--phosphate (XV) (see Figure 4).



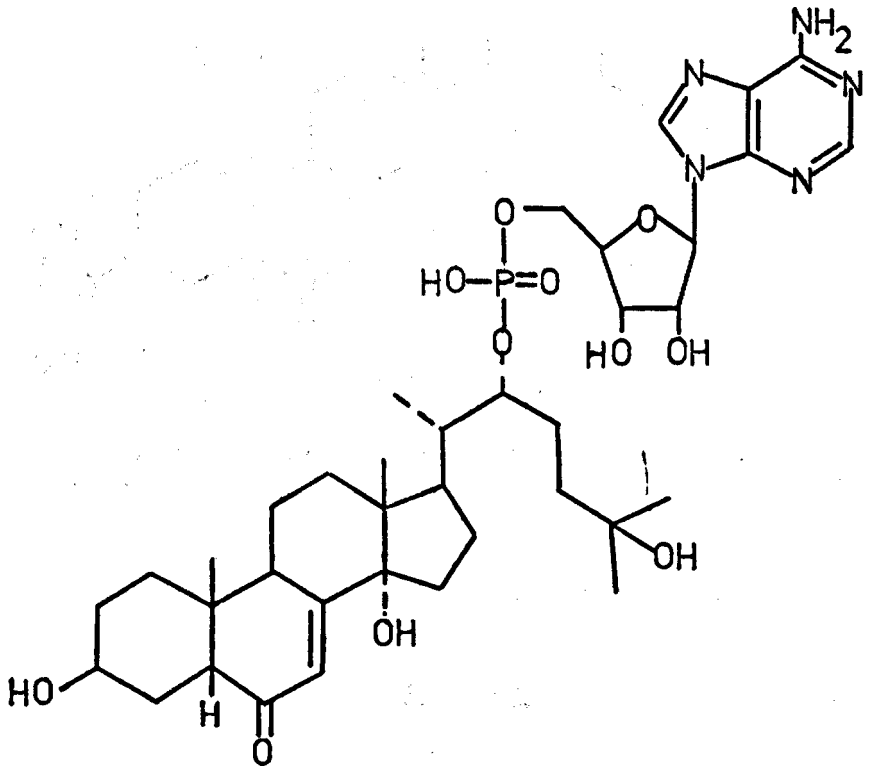
Ecdysone-22-phosphate (XIV)



2-Deoxyecdysone-22-phosphate (XV)

Figure 4

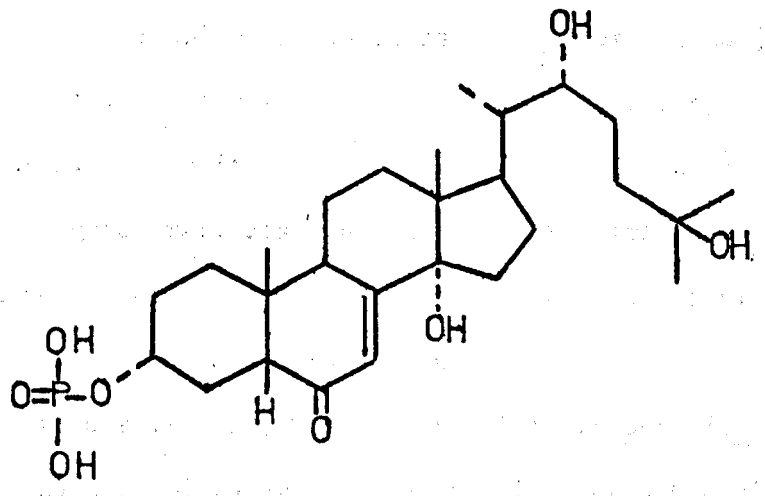
Shortly after, also in Locusta migratoria eggs the structure of the major conjugates was elucidated[90]. Using MS on a gold support in the Electron Impact (EI) and Chemical Ionization (CI) mode and ^{1}H, ^{31}P NMR the major conjugated ecdysteroid in newly laid eggs was identified as 22-adenosinemonophosphoric ester of 2-deoxyecdysone (XVI) (see below, Figure 5).



2-Deoxyecdysone-22-adenosinemonophosphate (XVI)

Figure 5

whereas in a later stage of embryonic development the major conjugate was shown to be 3-epi-2-deoxyecdysone-3-phosphate (XVII) (see Figure 6).



3-Epi-2-deoxyecdysone-3-phosphate (XVII)

Figure 6

The structures of the conjugates from Schistocerca gregaria and Locusta migratoria eggs indicate variations in the type of conjugating group as well as in the position where this group is linked to the steroid nucleus. This may be explained by the two closely related species, Schistocerca gregaria and Locusta migratoria, having different biosynthetic pathways, but the real explanation must await further experiments.

However, when the present work was initiated, these highly polar derivatives of ecdysteroids were only tentatively identified as conjugates with glucose or with sulphuric, glucuronic and phosphoric acid on the

basis of their electrophoretic behaviour and by enzymic hydrolysis[32,50,55,72,94]. Since the enzyme preparations used were shown to be highly contaminated by other enzymes with hydrolytic activity[32], the evidence based on enzymatic cleavage of the conjugates was insufficient for an unequivocal structural assignment[16].

Following enzymatic hydrolysis of the conjugates, the nature of the ecdysteroids released can be determined by HPLC-UV, GC-ECD and GC-MS. Thus it has been shown that the ecdysteroidal moiety of the conjugates reflects the pattern of the free ecdysteroids, differences between the two classes of compounds being mainly quantitative[49,54,55,93].

A separated group of highly polar ecdysteroid derivatives, not split enzymically, has been reported in Schistocerca americana eggs[74], in Locusta migratoria larvae and in Pieris brassicae pupae[51]; however this group of hormones has not been further characterized.

Physiological Significance of Free Ecdysteroids in the Eggs

In spite of the existence of all the previously listed reports, the function of the moulting hormones in embryonic development remains unclear. It has been demonstrated for several years that insect embryos deposit up to three successive cuticles[94] and one possible function for ecdysteroids in eggs could be the control of embryo cuticulogenesis, as suggested by several authors[49,83,95,96]. However the hypothesis of a neuroendocrine control of the embryonic apolysis has been dismissed by other authors on the evidence that, under in vitro conditions, embryonic abdomens are capable of producing several cuticles long after having been severed from head and thorax[94,97,98].

In a study of the differentiation of the prothoracic glands in Schistocerca gregaria embryos[99] it was shown that the embryo, during

its development in the egg, forms two cuticles; the first one is considered as exclusively embryonic, the second one is that with which the insect hatches and begins its larval life. Moreover it was demonstrated by the same authors that, during embryonic development, an apolysis, similar to larval apolysis, occurs; nevertheless the embryonic apolysis were not under the control of embryonic prothoracic glands since embryos, severed from heads long before the prothoracic glands show any signs of activity, developed like normal embryos forming two cuticles.

Furthermore there is not sufficient information available on the compartmentalisation of the hormones in the egg system, i.e. whether the ecdysteroids detected in the whole egg are external or internal to the embryo. Although one report, based on RIA, exists on the ecdysteroids content of the egg and of the corresponding embryo in Locusta migratoria[49], the investigation was carried out on only one of the thirteen developmental stages. At stage six of the egg development (which occurs just after blastokinesis) it was shown that the hormone is essentially present in the embryo, although the latter represents only one tenth of the total egg weight.

In order to understand the functional relevance of moulting hormones in insect embryos, several investigations have been carried out by determining the fluctuation of ecdysteroid titres during the whole embryonic development and looking for any correlation between the varying amount of hormones and distinct stages of embryogenesis[53,55,82,83,93] From correlative studies in Locusta migratoria eggs, based on RIA, 4 distinct peaks of ecdysone concentration were observed, each coincident with the onset of a cuticle deposition[49]. From these data it was inferred that ecdysteroids act in controlling cuticulogenesis as they

do during postembryonic development. Correlations between moulting hormone peaks and cuticle deposition have also been reported from eggs of other insect species.[83,84,100]

With regard to the site of origin of ecdysteroids in embryo, the published results indicate that the hormones present in the early stages of embryogenesis, are of maternal origin[47,70-72,76]. However the remarkable changes in ecdysteroids levels during egg development and the rising hormone titre in the later phase of embryonic growth[49,55,82,83,101] could be produced either by interconversion of maternal ecdysteroids or de novo synthesis by the embryo.

Physiological Significance of Conjugated Ecdysteroids in the Eggs

As emphasised for the free form of the hormones, the physiological significance of the conjugated ecdysteroids in the eggs is not yet established. Conjugation diminishes the interaction with the steroid receptor and consequently can be considered to inactivate the hormone[16,102]. 26-Hydroxylation and 3-epimerization are also inactivating reactions which lower the hormonal activity[16]; thus they could represent alternative routes to inactivation. The latter assumption could explain the presence of 26-hydroxyecdysone and 3-epi-26-hydroxyecdysone as main hormones in Manduca sexta eggs, which are reported to contain only a small amount of conjugated ecdysteroids[80,89]. However the simultaneous presence of conjugation and 3-epimerization has been found to occur in later stages of embryonic development of Locusta migratoria eggs[90].

Moreover certain types of conjugates may be hydrolysed yielding free ecdysteroids with hormonal activity, as was first suggested by Willig[30]. The hypothesis that conjugates represent storage forms of

the steroid hormones has been proposed by several authors[91,93,103]. Lagueux et. al.[91] speculate that hydrolysis of conjugates could account for the production, in the embryo, of ecdysone peaks occurring before the differentiation of endocrine glands. Gande and Morgan[53] suggest that the conjugates are acting as inactive 'storage' compounds since the conjugated hormone peaks precede that of the free hormones. In the light of these observations, it is interesting that highly polar ecdysteroids, which are not susceptible to enzymic hydrolysis, have been detected at the end of embryogenesis in Schistocerca americana[74] and Locusta migratoria[90]. At that stage of the egg development, they probably act as inactivation products which cannot be reconverted to free hormones.

Another possible role of the conjugates could be the transport of the hormones, particularly in relation to the transfer of maternal ecdysteroids to the embryo. It has been shown that conjugated ecdysteroids are bound in the oöcyte to a large macromolecule, which shares several characteristics with vitellin, the major yolk protein[104]. These data could elucidate the mechanism by which the conjugated hormones, after their synthesis in the follicular cells are accumulated in the oöcyte where they persist during egg-laying[91,104].

So far there has been no evidence of the involvement of conjugates in the compartmentalisation of ecdysteroids in the egg system.

Juvenile Hormone

The classical experiments of parabiosis on Rhodnius prolixus by Wigglesworth[105] provided the first evidence in the insects of glands whose secretion permit the progressive differentiation from larva to

adult through the control of the type of moult induced by ecdysone. The glands responsible for this action were identified as the corpora allata, two paired organs situated in the insect's head and attached to the brain[105].

It was also shown that when the corpora allata are removed in early instars, moulting takes place as usual, but instead of another larva being produced, a diminutive adult emerges. However when the glands are removed in the last larval instar, normal moult to adult occurs[105-107]. Furthermore it was found that when corpora allata from earlier instars are implanted into the last larval instar, an extra larva stage is produced instead of an adult[108,109].

These experiments demonstrated that the corpora allata produce a hormone which acting in conjunction with ecdysone, guarantees the maintenance of larval characters in the growing insect until conditions favourable for maturation have been achieved[110,111]. Since this hormone induces the retention of juvenile characteristics, the name juvenile hormone (abbreviated JH) has been selected. Because of the role of the JH in maintaining the insect in an immature state, the gradual differentiation towards the adult form is attained by a progressive fall in hormone concentration in successive instars, until the last larval instar, when its absence allows adult formation[112].

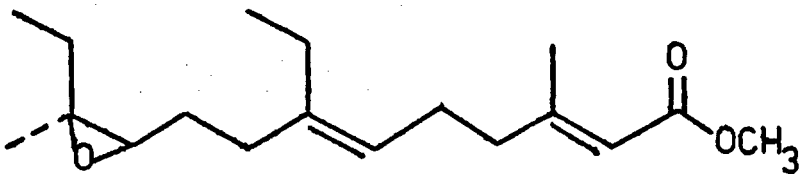
Although the activity of the corpora allata is turned off during the final moult, the glands are active again in the adult insect[113]. In view of this finding, it was demonstrated that JH promotes the synthesis of yolk proteins by the fat body and the uptake and incorporation of these proteins by the developing oöcytes[114-117]. Furthermore it was shown that the hormone controls the activity of the accessory sexual glands in female and male insects[118].

Because of the role of JH as metamorphic and reproductive regulator, it was not surprising that an unusually rich source of JH activity was discovered in 1956 in the abdomens of reproductive male Hyalophora cecropia moths[119]. The active compound contained in the extract was proved to be identical to the JH produced by the corpora allata, since the extracts applied locally to the cuticle of the last larval instar induced the epidermal cells to secrete another larval cuticle, whereas the surrounding cells produced a normal adult cuticle[120-123].

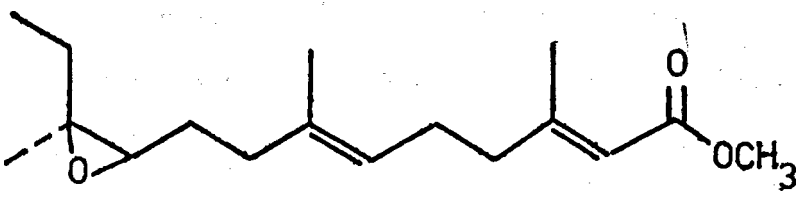
This feature prompted the development of a number of JH bioassays which all depend upon the presence of ecdysone to stimulate cuticle secretion and moulting[123,124]. The availability of sensitive and reproducible bioassays provided the prerequisite for the isolation and identification of endogenous JH from insect extracts.

Although highly purified JH extracts from Hyalophora cecropia were obtained since 1956, the characterisation of the hormone proved to be very difficult[125]. Only in 1967 the structure of the Hyalophora cecropia (Lepidoptera) JH was conclusively identified[126] as methyl (2E,6E,10-cis)-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate (JH1) (XVIII), using microchemical degradations or reactions, NMR and GLC-MS. Subsequently another juvenile hormone from the Cecropia moth was isolated and characterised[127]. It was shown to be a JH1 homologous, i.e. methyl(2E,6E,10-cis)-10,11-epoxy-3,7,11-trimethyl--2,6-tridecadienoate (JH2) (XIX). JH1 and JH2 constituted the first ethyl-branched homoterpenes occurring in animals[128]. They were shown to be optically active[129] and, in 1971, their absolute configuration was determined as 10R, 11S by stereoselective synthesis of precursors with known absolute configuration[130]. Later a third natural juvenile

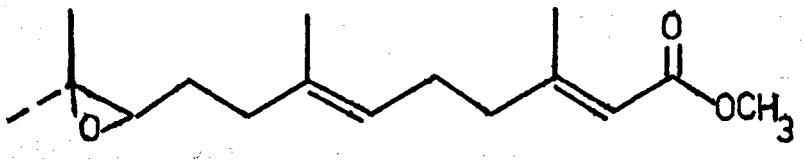
hormone was discovered[131], whose assigned chemical structure is methyl(2E,6E)-(10R)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate (JH3) (XX). This new terpene hormone was found in addition to JH2 from the culture medium of corpora allata of the moth Manduca sexta (Lepidoptera). The structures of JH1, JH2 and JH3 are given below (Figure 7).



JH1 (XVIII)



JH2 (XIX)



JH3 (XX)

Figure 7

Because of the difficulties of separating the lipophilic JHs from the masses of contaminating body lipids, the extraction and identification of the hormones could be achieved for several years

only on male saturnid moths[132], whose abdominal tissues contain large quantities of JH. The extraction and purification techniques adopted for the isolation of the Cecropia JH were not suitable for other insect orders in which the JH is present in much lower amounts[133]. In addition to the problem of the identification of JH in other insects, the requirement for physiological titre determinations of endogenous hormones, prompted the development of more sensitive and specific methods for the qualitative and quantitative analysis of JHs.

Analysis of Juvenile Hormones

Various techniques have been used, up to now, for assaying these hormones. Bioassays are very sensitive[121] (5 pg of JHs), yet they cannot discriminate between the known JHs and are liable to interference from other substances[134]. Furthermore they exhibit a different sensitivity to the three hormones[135], causing innaccurate qualitative and quantitative analysis[136]. A second type of assay available, namely the RIA, uses either native JH[137] or the corresponding diol produced on opening the epoxide ring[138]. The RIA method has the advantages of rapidity and sensitivity[137] (50 pg of JH), but it is not selective because of the presence in vivo of many cross-reacting substances[139]. If more specificity is needed, TLC and/or HPLC purification are also required with a consequent lengthening of the time per analysis. Moreover RIA suffers from lower precision compared to the physico-chemical procedures[139]. Because of the scattering of results produced it is not really suitable for physiological titre determinations.

HPLC is widely used in the purification of JHs containing insect extracts, because it offers a high separation efficiency. HPLC can

also be used for quantitative analysis when coupled with an appropriate detector. The more commonly available UV detector is not sensitive enough for the low concentration of JH in the biological samples[140]. However a new method based on HPLC coupled with MS has been recently developed[140]. Although the sensitivity achieved (10 pg) is satisfactory for the assay of JH in insect material, several technical parameters need to be further optimized for repetitive analyses.

The main technique for the analysis of JH has proved to be GLC which affords very efficient separations of these volatile hormones[133]. For the quantitative analysis several detectors can be coupled with the GC. The sensitivity of the FI detector (several nanograms) limits its use for the assay of JH in insect material[141,144]. However the sensitivity can be greatly enhanced by the ECD detector[133,142,143] (few picograms) which requires derivatization of the epoxide ring, to introduce "electrophoric" functionality into the JHs molecule. Unfortunately the derivatization step involves longer sample manipulation, further purification procedures and low recoveries due to formation of several derivatives and incomplete derivatization reactions[143-145]. JH determination by GC-MF of underivatized JHs is a very selective method, the possibility of interference from other natural compounds being minimal[134]. By the use of CI instead of EI the sensitivity of this technique has been improved to a picogram level.[144,146,147]

Juvenile Hormones in Larva and Adult Insect

With the improvements in specificity and sensitivity of the JHs assay systems, further aspects of the biochemistry of the hormone have been investigated.

Using radioactive dilution method and GC-MS analysis the first determination of endogenous JH in insect orders other than Lepidoptera

was accomplished[148]. Later Lanzrein et. al[146], using GLC-MF analysis in the CI mode, first demonstrated the presence of the three known JHs in the same insect. Furthermore it was found that JH1 and JH2 are mainly present in the larval stages, whereas the haemolymph of the adult female contained mostly JH3. From this evidence, the authors suggested that different JHs have different modes of action; JH3 being a gonadotropic hormone, whereas JH1 and JH2 are morphogenetic hormones.

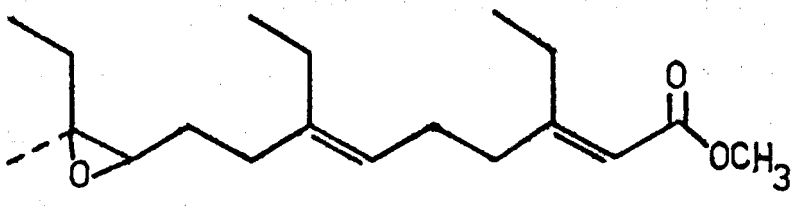
Since the advances in the methods for assaying the JHs in insect material at picomolar levels, several investigations have been carried out on the titre determinations of the hormones in the larva and adult insect[133,134]. When all the data available are compared, the larval values are found to range between 0.1 and 10 ng/g of insect tissue. These values are much lower than the corresponding ecdysteroids values (in S. gregaria[149] by a factor of 100). Since JH acts in conjunction with ecdysone in larva development, the remarkable difference in their titre may suggest the hypothesis of a function of ecdysteroids other than moulting hormones.

Juvenile Hormone in Insect Eggs

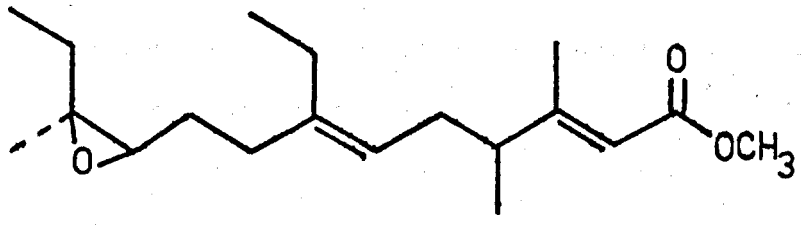
Using traditional bioassay techniques, JH-active substances have been found in eggs of insects since 1961[82,150,151]. However the data, presented in those investigations, were not a conclusive demonstration of the presence of JH during embryonic development, because of the possibility of interference by other natural substances that elicit a "JH response" in the bioassay[133].

The first chemical identification of any JHs in insect eggs has been reported quite recently by Bergot et al. in Manduca sexta[152]. These authors demonstrated the presence of JH1 and of a new JH which

was identified as methyl(2E,6E,10-cis)-10,11-epoxy-3,7-diethyl-11--methyl-2,6-tridecadienoate (JHO) (XXI). The JHO structure was assigned by comparison of the GC-MS data of the unknown and of its d_3-methoxyhydrin and tetrahydro derivatives with those of synthetic JHO and its d_3-methoxyhydrin and tetrahydro derivatives. However, the absolute configuration of this hormone has not been determined yet. More recently another new JH has been found in Manduca sexta eggs[153]. It has been shown to be methyl(2E,6E,10-cis)-10,11-epoxy-7-ethyl-3,4,-11-trimethyl-2,6-tridecadienoate (iso-JHO) (XXII) with the same structural analysis used for the identification of JHO. The structures of JHO and iso-JHO are shown below (Figure 8). In addition to JHO and iso-JHO, JH1, JH2 and JH3 have also been identified in insect eggs[153] indicating the presence of more complex structural forms of JHs in the eggs than in the larval or adult stages. It is interesting to emphasise that an analogous situation is observed for the pattern of the ecdysteroids in embryonic and post-embryonic development.



JHO (XXI)



iso-JHO (XXII)

Figure 8

Although the presence of JHs in insect eggs has been established, the role of the hormone in the developing embryo remains speculative. To shed some light on the physiological significance of JH during embryogenesis, investigations of the fluctuation of JH titres in developing eggs have been undertaken. Although too few representative species have been systematically examined to draw definitive conclusions, a common pattern is already emerging from these correlative studies. Quantitative analysis carried out using bioassays[82,83,154] or GC-MF of the JH-d_3 methoxyhydrin[153] have shown the appearance of a JH peak during late embryogenesis, when the ecdysteroid titres also reach a peak[82,83,153]. This finding suggests an interaction between JH and ecdysteroids during embryonic development, similar to that in the larval moult. In the eggs, as well as in the larvae, the quantity of JH present is much smaller than the corresponding levels of ecdysteroids[89,153,154]; this may imply that the latter could control physiological processes other than moult. In the light of this comparison between JH and moulting hormones during embryogenesis, it should be also emphasised that the presence of conjugated JH has never been detected, whereas the ecdysteroids are found mainly as conjugates in several species of insect eggs.

The site of origin of JH in the embryonic development is also conjectural. Because of the appearance of JH during late embryogenesis, i.e. when corpora allata differentiated, it has been suggested that JH is produced by the embryonic endocrine glands[82,151,154].

Relevance of Ecdysteroids and Juvenile Hormones for Insect Control

The problems of persistence in the environment and lack of selectivity, caused by present methods of insect control, have stimulated different approaches to the development of new pesticides. One of these is to use analogues or antagonists of insect growth regulators, such as JH and ecdysteroids, to control pest populations. These new substances should overcome one of the problems connected with the conventional insecticides, because they are specific to insects and not toxic for mammals[113,155]. Furthermore the insect pest would not be able to develop resistance to hormones that are necessary for their normal development[155].

Most insects die soon after they have been exposed to large quantities of ecdysone. Such pathological response represents the first example of hyperhormonism in invertebrates[155,156]. Despite this effect of exogenous ecdysone on insects, its use as a pesticide is impractical because the hormone penetration through the cuticle is very poor and its large scale synthesis prohibitively expensive[157]. Nevertheless the discovery of compounds which act as anti-ecdysone or as ecdysone inhibitors[158], could be of interest for the development of hormone-based insecticides. Furthermore a better understanding of the function of ecdysone, in the insect eggs, could provide the possibility of interfering with the moulting hormones induced process also at the embryonic stage.

In contrast to ecdysteroids, JH is already used as an agent of insect control[159]. It has been shown in various insect orders (Lepidoptera, Hemiptera, Coleoptera and Orthoptera) that last-stage larva or pupa exposed to JH moult into monsters which are unable to mature and die in a short time[159]. However several insects became

insensitive to JH a few days after the last larval moult[155]; therefore the practical application of JH as an insecticide is limited, in the larval stages, by its timely and accurate application. On the other hand it has been demonstrated that JH, applied to adult female, or young embryos, prevents the embryonic or post-embryonic development[160,161]. Also in Schistocerca gregaria the application of JH has been reported to block embryogenesis[162]. In the light of this evidence the most direct and potentially useful application of JH, for pest control, is in the disruption of embryo development.

DISCUSSION

Aim of the Investigation

The purpose of the research reported in this thesis, was to shed some light on the physiological significance of moulting hormones and juvenile hormones during embryogenesis in Schistocerca gregaria (S. gregaria); a better understanding of the role of these hormones in the eggs being of fundamental importance for the control of insect pest growth.

A prerequisite for any study on site of production, mode of action and eventual fate of physiologically active substances is their chemical identification and quantification at distinct and characteristic stages of the developmental process. Bearing this in mind, we have approached the problem of the role of ecdysteroids and JHs during embryogenesis in S. gregaria by determining the hormones titre in the developing eggs. Such a study, requiring numerous hormone assays is feasible only if a rapid purification and quantification procedure is available. Therefore, one of the aims of this work was to develop a suitable method for the analysis of many samples.

Another aspect of the presence of moulting hormones during embryogenesis which has not been fully investigated is their compartmentalisation within the egg. Since the determination of the location of the ecdysteroids is essential for any speculation on their mechanism of action, we have also begun the measurement of the levels of moulting hormones external and internal to the embryo.

Furthermore, when the present research was undertaken, the exact nature of the conjugating group in the highly polar ecdysteroid

conjugates was not established. Since it is important to know the chemical structure of the hormones present, in addition to their titre, the identification of the conjugate moiety of ecdysteroids has been attempted by indirect approaches which allowed us to draw structural inferences.

Assay of the Ecdysteroids in the Eggs of Schistocerca Gregaria

Although a previous investigation on ecdysteroid titre in S. gregaria eggs was carried out by GC with ECD after silylation of the ecdysteroids[53], in this study HPLC with UV detection was chosen for the direct separation and quantification of the ecdysteroids. Although HPLC-UV is less sensitive and selective than GC-ECD, the former technique offers several advantages in that the sample may be directly analysed avoiding manipulation losses, destruction by heat and contamination from reagents necessary for the formation of derivatives for GC and also involving shorter time per analysis. The HPLC method used, based on that of Scalia and Morgan[55], involves two solvent partition systems to remove most of the unwanted egg material, followed by a further purification of the extract with Sep-Pak C_{18} cartridges and, finally, by the HPLC analysis (Fig. 9). In Figure 10 the flow diagrams of the sample preparation for the GC-ECD and HPLC-UV methods are compared. The latter requiring fewer purification steps is clearly more suitable for routine analysis of extracts of S. gregaria eggs, particularly because the relatively high levels of hormone present do not need the greater sensitivity of the GC-ECD method.

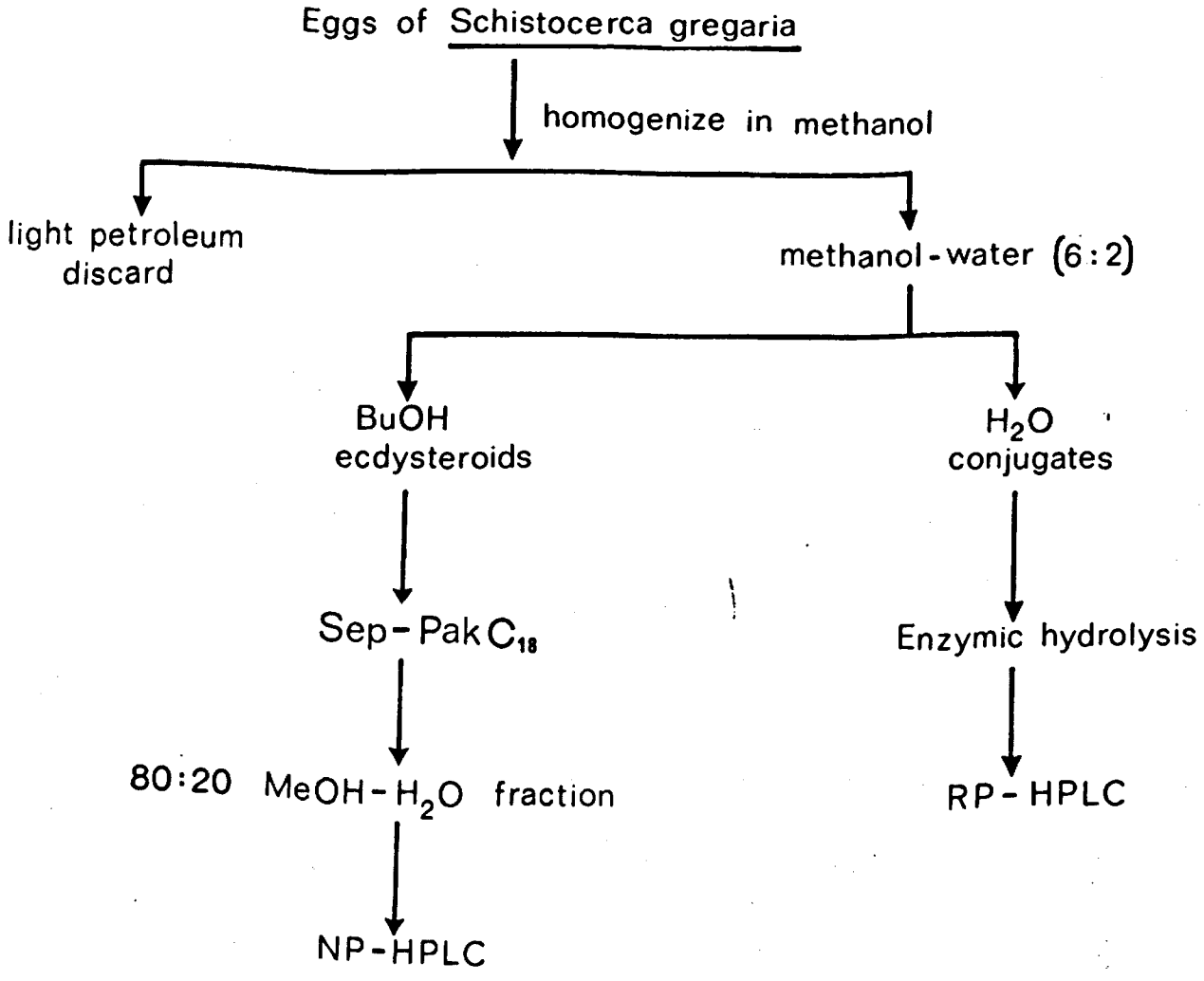


Figure 9. Extraction and purification procedure for the HPLC analysis of ecdysteroids from S. gregaria eggs.

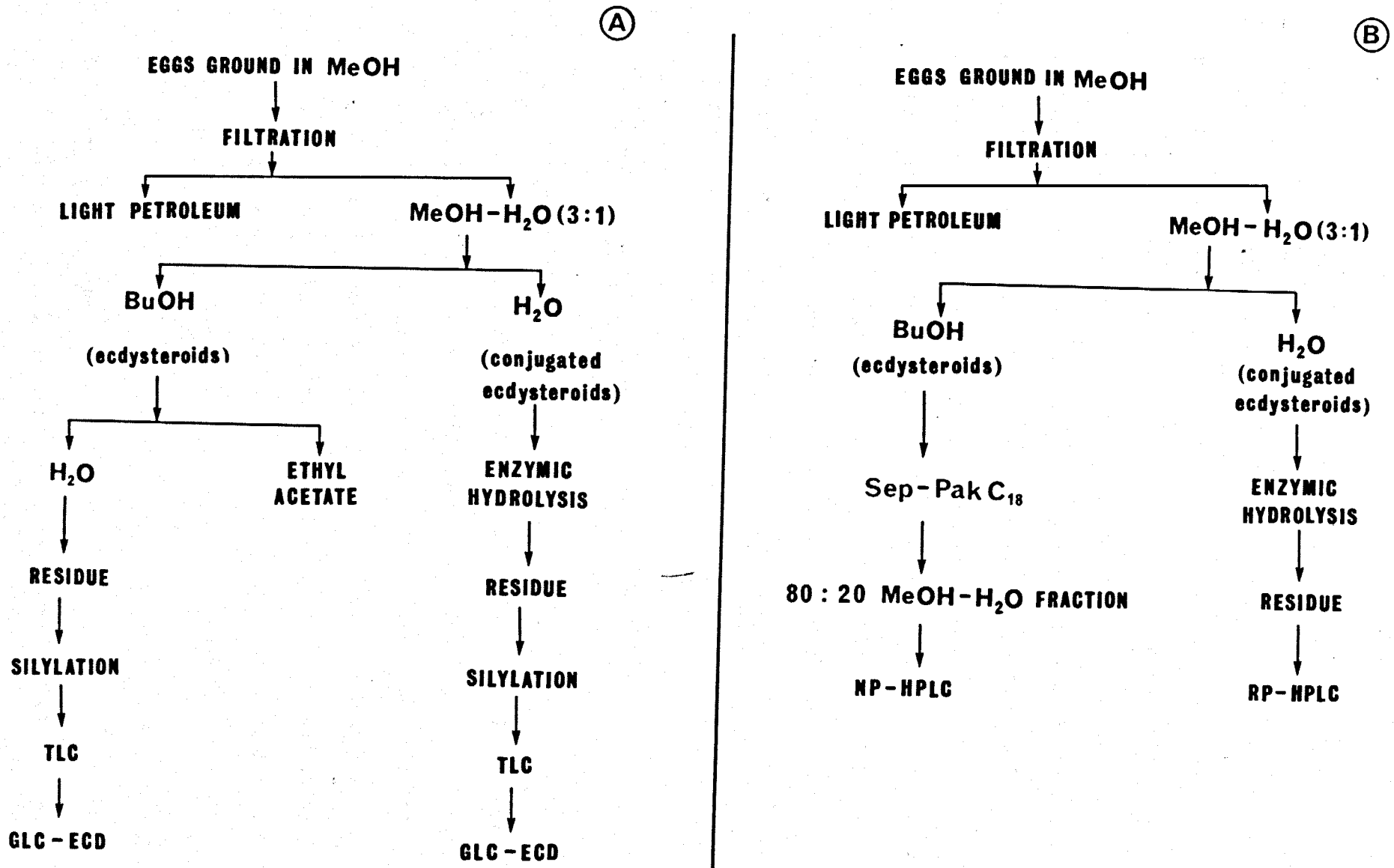


Figure 10. Analysis scheme for the GC-ECD (A) and HPLC-UV (B) methods.

Preliminary Extraction Procedure

The extraction was performed by grinding the eggs in methanol in a glazed mortar, at room temperature. However, in the course of the work, it has been found that ecdysteroid recoveries were greater when a glass mortar was used instead of the glazed one. The efficiency of recovery was determined by adding to the biological sample a known amount of 2-deoxy-20-hydroxyecdysone (5×10^{-7} g per sample) as internal standard. Using a glazed mortar we obtained recovery values of 70 - 75%, whereas more than 85% recovery was attained with a glass mortar. The irreversible adsorption of ecdysteroids on the porous surface of a much-used glazed mortar accounted for the losses of hormones. These losses are particularly important at the low levels of ecdysteroids present in our samples.

Solvent Partition Systems

1. Methanol-Water:Light Petroleum.

This step, involving the removal of lipids from the biological extract, is carried out by partitioning it between light petroleum (b.p. 40-60) and methanol-water (3:1, V/V). Lipids and non-polar sterols, including ecdysteroid precursors such as cholesterol, 7-dehydrocholesterol, 3β-hydroxy-5α-cholestan-6-one are partitioned into the light petroleum phase[168]. More polar substances, including the ecdysteroids and their polar conjugates are quantitatively partitioned into the aqueous methanol.

2. n-Butanol:Water

The second partition corresponds to the removal of very polar substances. During this partition the free ecdysteroids are found

in the butanol phase, whereas the highly polar conjugates are in the water layer. The conjugated ecdysteroids are partitioned quantitatively into the water phase provided that careful attention is paid to phase separation and back-washing of fractions to be discarded[93,163]. The quantitative transfer of the conjugated ecdysteroids into the aqueous layer has been demonstrated by checking the butanol phase for conjugates. It was found that only 2% of ecdysone, 2.5% of 20-hydroxyecdysone and none of 2-deoxyecdysone conjugates were present in the butanol phase. The losses of conjugates in the butanol layer being not significant, the partition step provided a simple and effective method of separating the free and conjugated ecdysteroids.

A chromatographic procedure was also explored for the separation of free and conjugated ecdysteroids. Briefly, the egg extract from the aqueous methanol-light petroleum partition, was dissolved in 5 ml of 20% (V/V) methanol in water, injected into a Sep-Pak C_{18} cartridge and eluted successively with 4 ml of 40% methanol in water and 7 ml of 80% methanol in water. The three fractions obtained were then separately analysed for free and conjugated ecdysteroids, as described later. Since the 40% methanol in water fraction was found to contain conjugated 2-deoxyecdysone and free 20-hydroxyecdysone, the chromatographic method was discarded.

Hydrolysis of Conjugates

In this study the term conjugated hormones refers to the highly polar ecdysteroids which are hydrolysed by the digestive juice of the snail Helix pomatia to release the free compounds.

Dinan and Rees[74] have shown, by injecting labelled cholesterol into adult ovaries of S. americana gregaria, that highly polar labelled ecdysteroid derivatives were produced in the eggs. These derivatives were not split enzymically and have not been further studied. Too little is known about these compounds to include them in the present study; furthermore they are not detectable by the method of hydrolysis and chromatography, and it is not known whether they are normal metabolites of endogenous hormone.

Because of their ionic character[11,32,50,55], the conjugates form a class of compounds which is difficult to analyse directly by chromatography. For this reason, when this investigation was undertaken, the only method available for the assay of these substances consisted in hydrolysing them to release the free hormones which were then chromatographed[49,54,71,93]. Successful hydrolysis of conjugates has been achieved with enzymes, usually from the digestive tract of the snail Helix pomatia, the digestive juice of the snails being particularly rich in hydrolases, especially arylsulphatase, steroidsulphatase, β-glucuronidase and phosphatase[32,55,164,165]. However, enzymic methods have the limitation that artifacts can be introduced[56] and the enzyme used may be specific for certain types of conjugates and, consequently, not able to hydrolyse all the conjugates present in the sample. Therefore a chemical method was desirable that can be relied upon to split conjugates regardless of structure or stereochemistry. Several attempts have been made in this direction by others. Treatment with strong mineral acids or strong bases at elevated temperature has been shown to be unsatisfactory, since it leads

to the destruction of α-diketones and α-hydroxyketones[167,168]. Milder conditions have been attained by solvolysis of conjugates in dioxan, tetrahydrofuran or ethyl acetate in the presence of small quantities of perchloric acid at room temperature[168]. Although a number of acid-sensitive sterols were found to be unharmed by the procedure, others were degraded or rearranged[168]. Also the use of ion exchange resin as catalyst for the solvolysis of conjugates has been reported[164]. However, the method did not effect any improvements of the enzymic hydrolysis procedure, therefore it was abandoned[164].

In this work the hydrolysis of the polar conjugated ecdysteroids was accomplished in acetate buffer by the mixture of enzymes contained in the Helix pomatia digestive juice. The amount of enzyme used was shown to give complete hydrolysis of the conjugates, increasing enzyme caused no incremental change in ecdysteroids released. A blank determination made with buffer and enzyme was checked for the presence of ecdysteroids. With our detection method no ecdysteroids were found in the batches of enzyme used in this study. Furthermore no solvolysis of conjugates was observed in the absence of enzyme.

Purification of the Ecdysteroids Released by Enzymic Hydrolysis

Two different methods have been utilised in order to purify the hormones freed by enzymatic hydrolysis from the impurities present in the incubation medium. The first one, based on that of Gande and Morgan[53], involves the extraction of the hydrolysis mixture with n-butanol and the analysis of the material from the butanol phase by RP-HPLC, as described later.

However, during the course of the investigation, another method was developed which consists of injecting the incubation medium, placed in a glass syringe, through a Sep-Pak C_{18} cartridge, and subsequently eluting it, as reported in the experimental section. The fraction from the cartridge containing the ecdysteroids, was then chromatographed by RP-HPLC[55]. Ecdysteroid recoveries from the Sep-Pak C_{18} were shown to be quantitative in the range of levels of hormones (i.e. 0.1 - 10 µg) present in the egg sample (2-3 g) used for the extraction.

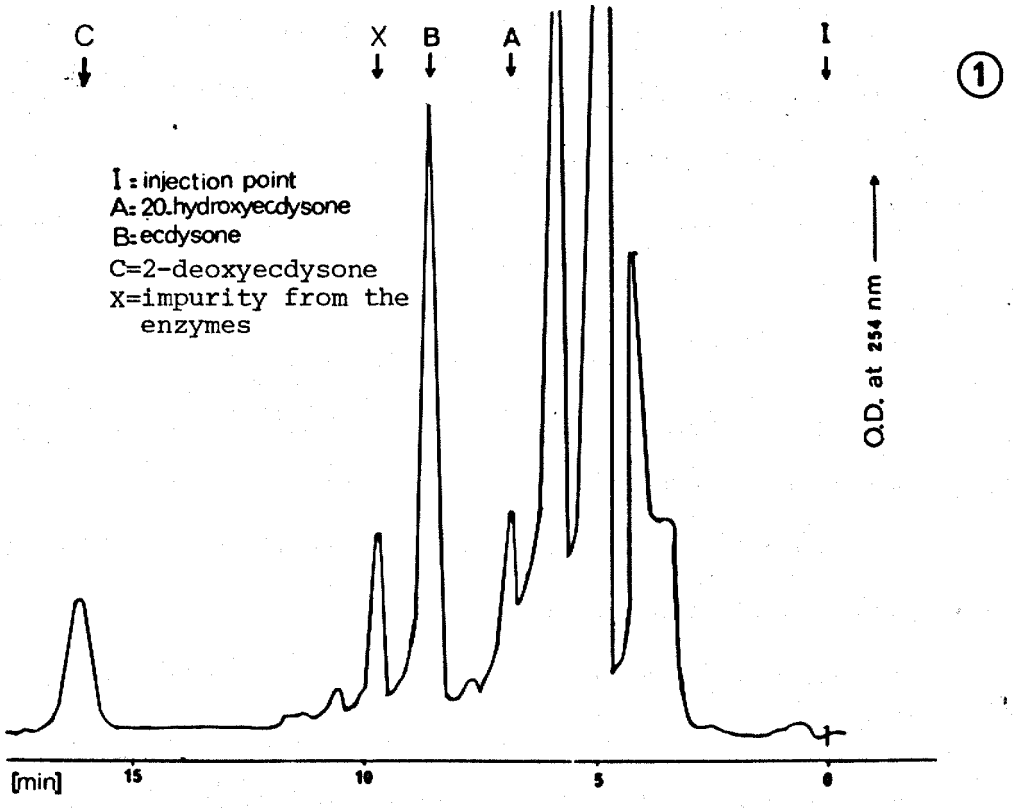
The RP-HPLC traces of an extract of hydrolysed ecdysteroid conjugates purified by partition and by Sep-Pak C_{18} are shown in Figure 11. The comparison of the two traces indicates that the cartridge achieves a more efficient purification since it produces a smaller initial peak. For this reason the chromatographic method was adopted throughout the investigation.

Purification of the Free Ecdysteroids

It was necessary to carry out a more extensive purification of the free ecdysteroids fraction than of the conjugates, because of the large amount of impurities still present after the solvent partition and which interfere with the HPLC analysis. This was not necessary with the conjugate fraction because: (a) the amount of ecdysteroids present was approximately ten fold greater than in the free form, and (b) the enzymic hydrolysis and the subsequent chromatography on a Sep-Pak C_{18} acted as a purification step.

In order to achieve a rigorous purification of the free ecdysteroids fraction, different techniques have been assessed.

The classical TLC method for preparation of biological



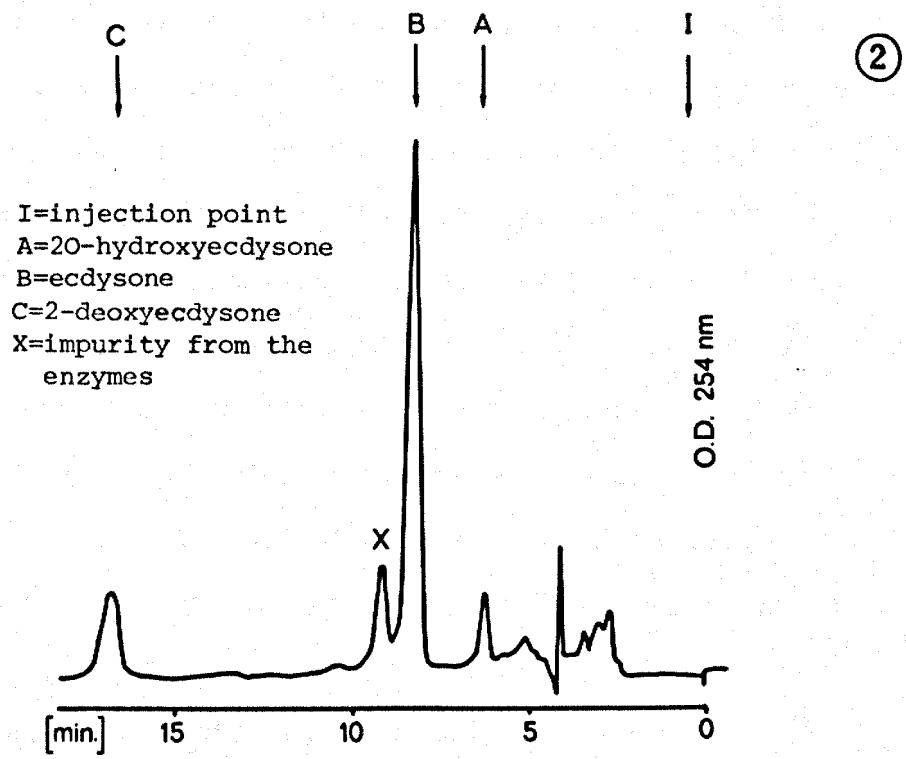


Figure 11. Typical RP-HPLC trace of an extract of hydrolysed ecdysteroid conjugates purified by partition (1) and by Sep-Pak C_{18} (2). Operating conditions: 25 cm x 5 mm I.D. column of Hypersil ODS eluted with methanol-water (60:40, V/V; 0.8 ml min^{-1}).

samples is not suitable for ecdysteroid-containing extracts since the polar moulting hormones are not recovered efficiently from silica material, due to irreversible adsorption[40]. This loss through adsorption can become particularly significant when small quantities (ng range) of compound are available[41], as in our case.

RP-TLC Plates for Purification of the Free Ecdysteroids

RP-TLC plates should be more suitable than normal TLC for the quantitative recovery of ecdysteroids, since the non-polar surface bonded to silica should reduce the irreversible adsorption loss. We have, therefore, explored their usefulness by comparing the recovery of small samples of pure ecdysone, in the range 10^{-6}-10^{-7} grams, from various types of RP-TLC plates with that of NP-TLC plates. The results are given in Table 1.

While recoveries varied from 50 to 60% on NP-TLC, all the RP-TLC plates examined showed much higher recoveries, up to 90%[39]. Recoveries were slightly better in all cases for the larger amounts of ecdysone. The longer the carbon chain chemically bonded to the silica surface, the better the recovery, but the difference is small. The recovery from plates coated with C_{18} groups in the laboratory was improved by capping the unreacted surface silanol groups with dimethyldichlorosilane or trimethylchlorosilane. Both Merck and Macherey, Nagel & Co. plates are available with or without a fluorescent indicator added, and we have examined the effect of the indicator on recovery. No difference was found in recovery of ecdysone for the same manufacturer's plates with or without fluorescer, but the indicator plates contain elutable components absorbing UV light, which gave a number of peaks when the sample

Table 1

Recovery (%) of ecdysone from RP-TLC plates compared with NP-TLC plates.

Type of TLC plates	Amount applied (g) 1.10^{-6}	5.10^{-7}	1.10^{-7}
NP-TLC	56	56	51
Merck C_2	84	84	79
Merck C_8	82	79	80
Merck C_{18}	87	84	82
Macherey, Nagel & Co. 100% C_{18}	88	83	75
Whatman C_{18}	76	76	71
Laboratory-made, not capped	82	69	*
capped DMCS	87	83	*
capped TMCS	91	80	*

Results were single determinations for laboratory-made plates, others are the average of two determinations.

* Not tested.

is analysed later by HPLC.

From the results reported, RP-TLC appears to be clearly preferable to NP-TLC for the purification of the moulting hormones. However, one factor limiting the use of RP-TLC plates is their hydrophobic nature, which restricts the choice of solvent in which the sample can be applied, as well as the choice of mobile phase. Furthermore, RP-TLC plates suffer from the disadvantage that only small amounts of biological extract can be chromatographed; therefore several plates (from 10 to 20) have to be used to handle the sample size typical of this stage of the purification procedure. For these reasons, the RP-TLC plates were discarded for the purpose of clean-up of biological extracts from S. gregaria eggs.

RP-Medium Pressure Liquid Chromatographic Purification of Free Ecdysteroids

More recently, in a number of purification procedures for ecdysteroids from insect extracts, TLC has been replaced by preparative column chromatography[27,44,54,169-171]. The latter technique has also been examined in this study. Because of the poor recovery of ecdysteroids from activated silica surface, a large particle size reversed-phase support was used here instead of the more common silicic acid. The purification was carried out on a large-diameter preparative column packed with 30-40 μm particles of C_{18} Magnusil H. The ecdysteroids containing sample was injected in a large volume (4 ml) and the hormones eluted with a step gradient of methanol in water. The method offers noticeable advantages in that it allows large amounts of sample to be chromatographed, it gives quantitative recoveries (> 90%), and it also produces an efficient purification.

The fraction containing ecdysteroids from RP-medium pressure liquid chromatography (briefly RP-MPLC) can then be directly assayed by NP-HPLC (see Fig. 12).

Sep-Pak C_{18} for the Purification of the Free Ecdysteroids

Another method has been devised for the purification of the free ecdysteroids. It involves the use of a Sep-Pak C_{18} cartridge. The ecdysteroids fraction from the eggs, obtained in a water phase, after the butanol-water partition, was injected through the cartridge and successively eluted as described in the experimental section. The fraction of the eluate, containing the ecdysteroids, was then used for direct NP-HPLC analysis. Also in this case the recovery was shown to be quantitative. A typical NP-HPLC trace of an extract of free ecdysteroids purified by Sep-Pak C_{18} is shown in Figure 13. This procedure is not more efficient than the RP-MPLC one, but it is simpler and faster, i.e. more suitable for assay of a large series of samples. For this reason it was preferred to the RP-MPLC method in the titre determination of egg ecdysteroids.

Detection of Ecdysteroids

Due to the presence of an unsaturated ketone function, the ecdysteroids present a strong absorbance in UV light[34] (λ243 nm, $\varepsilon \simeq 12{,}000$). This absorbance allows the direct detection of the hormones by UV monitoring of the effluent from the HPLC column.

The quantification of the ecdysteroids present in each biological sample was performed on the HPLC trace by comparison of the peak areas with calibration graphs prepared with authentic hormones. The standard curves for ecdysone, 2-deoxyecdysone and

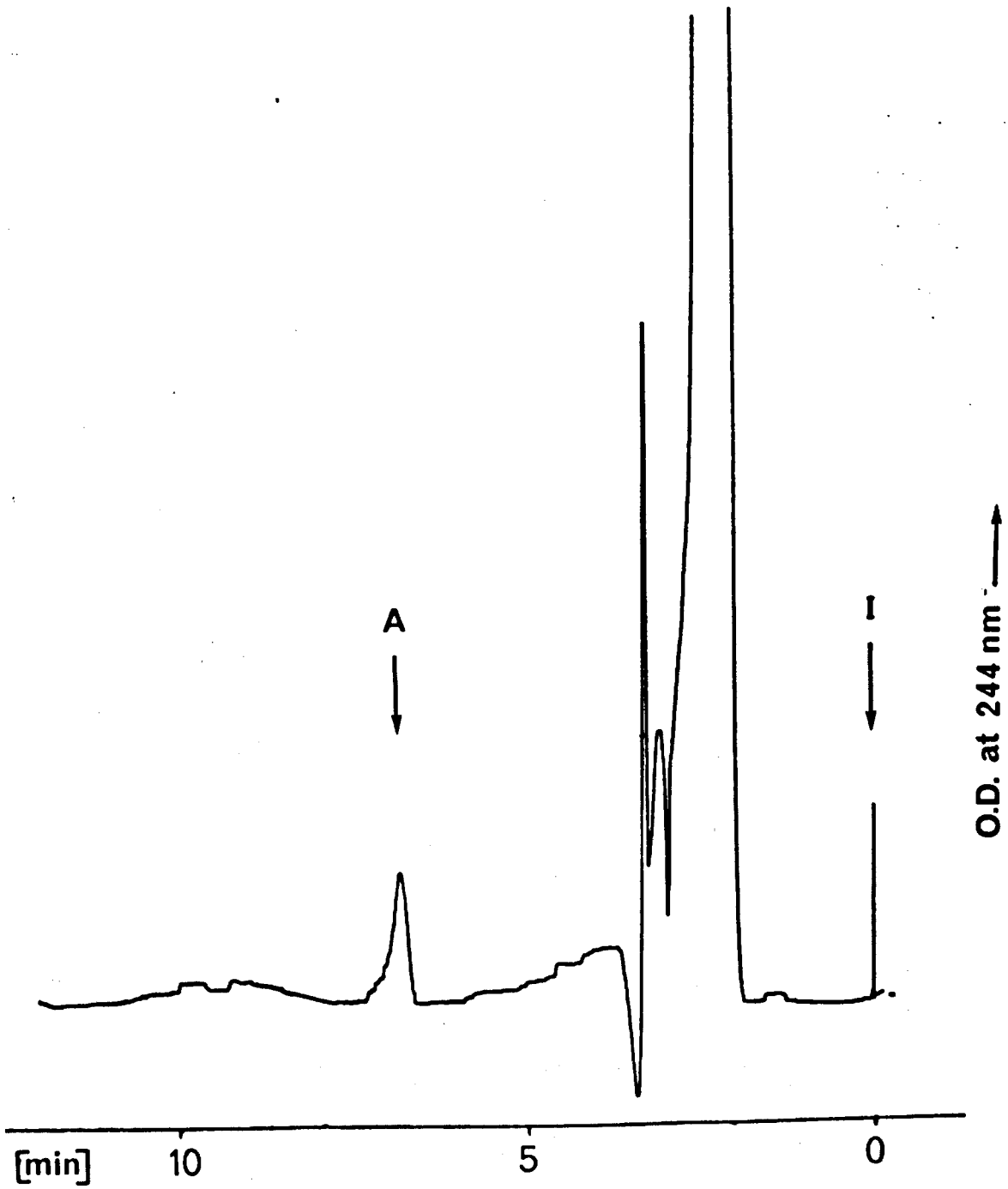


Figure 12. Typical NP-HPLC trace of an extract of free ecdysteroids from S. gregaria eggs, purified by RP-MPLC.

Operating conditions: 25 cm x 5 mm I.D. column of Hypersil eluted, under isocratic conditions, with methylene chloride-isopropanol-water (125:30:2, v/v; 1.5 ml min^{-1})

Peak A = ecdysone. 'I' indicates the injection point.

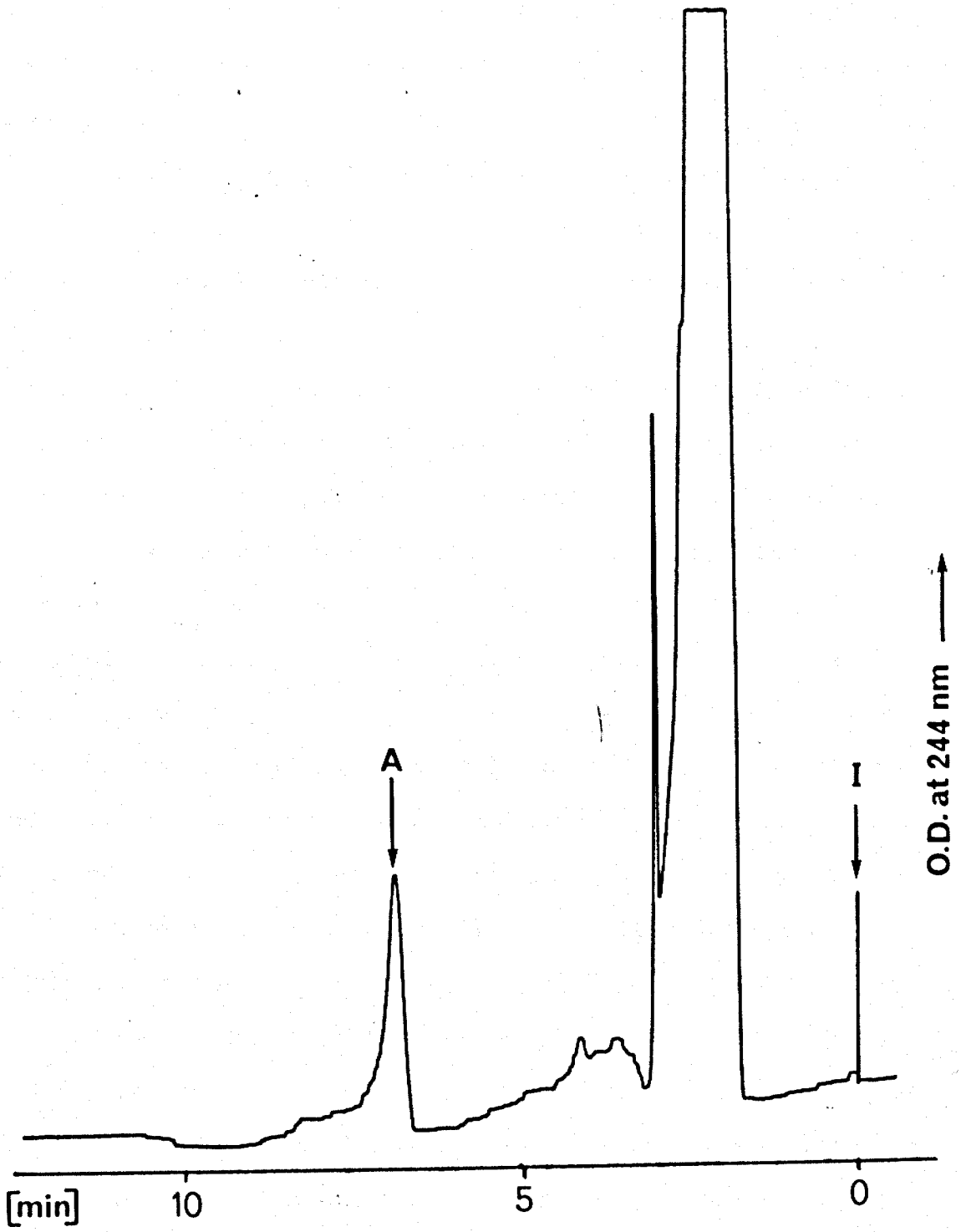


Figure 13. Typical NP-HPLC trace of an extract of free ecdysteroids, from S. gregaria eggs, purified by Sep-Pak C_{18}.

All conditions as in Fig. 12.

Peak A = ecdysone. 'I' indicates the injection point.

20-hydroxyecdysone are reported in the Experimental Methods section. The response of the UV detector was found to be linear in the range investigated (i.e. 10-400 ng).

The sensitivity is 10 ng of sample injected, but since only one tenth of a 50 µl solution could be conveniently injected onto the HPLC column, the limit of detection is 50 ng per gram tissue when 2 g of biological material are used.

The choice of the mobile phase for the HPLC-UV analysis is restricted by the necessity of it being completely transparent at the detector wavelength used. Because of the impurities present, the ordinary commercial products are not suitable for UV detection, particularly for trace analysis at high sensitivity. Therefore the eluents for HPLC are purified to improve their transparency at the operational wavelength.

The formation of gas microbubbles in the sample flow cell of the detector causes excessive baseline drift. In order to avoid it, dissolved gases are eliminated from the eluent by thoroughly purging it with helium, which has an extremely low solubility in virtually all liquids.

Moreover, because of the poor specificity of the UV detection, adequate sample purification procedures and highly efficient HPLC separations must be adopted in order to separate the ecdysteroids from any UV absorbing impurities present in the egg extracts which would interfere with the detection of the hormones.

Identification of Ecdysteroids

In spite of the greater resolution provided by the HPLC technique, a single chromatographic system is not sufficient for positive

identification of the ecdysteroids. Therefore accurate determinations require the analysis on chromatographic systems with different selectivities. For this purpose non-destructive detection techniques, such as the UV method used in our procedure, have to be employed to allow the collection of the material eluted from the column and its further analysis by other chromatographic methods.

Identification of the individual hormones was thus based on the observation that the biological sample co-chromatographed with authentic compound on RP-HPLC, NP-HPLC and silica gel TLC. Furthermore when the eluate corresponding to biological sample peaks was collected from the HPLC and silylated, the trimethylsilyl ether derivatives obtained were found to co-chromatograph with silylated authentic compounds on silica TLC and GC with ECD.

Several attempts at identification of the hormones and their TMS derivatives by mass spectrometry were unsuccessful because the quantity of compound isolated from the egg extract was below the detection limit of the instrument.

Reversed-Phase Ion Pair HPLC Analysis of Conjugates

As previously emphasized, when the present study was undertaken the assay of the highly polar ecdysteroid conjugates was carried out indirectly and laboriously by collecting a polar fraction containing the conjugates by solvent partition[55] or chromatography[54], then hydrolysing the conjugates overnight with the crude enzyme preparation of the snail Helix pomatia, collecting the released ecdysteroids and determining them directly by HPLC with UV detection[54,55], or GC-ECD after derivatisation[41]. The hydrolysis step is generally too time-consuming for routine analysis. Further-

more it causes longer sample manipulation and represents a further source of possible errors, as shown in a previous investigation on the levels of conjugates in the eggs of S. gregaria[55]. In order to overcome the difficulties connected with the enzymic hydrolysis procedure, work was undertaken to develop a direct and rapid method for the separation and quantification of the conjugated ecdysteroids in S. gregaria eggs.

Because of their ionic character, the conjugates are difficult to analyse directly by liquid chromatography. Only group separations of these compounds have been achieved by chromatographic systems such as silicic acid[54], reversed-phase[71], or DEAE-Sephadex columns[52]. Lafont and co-workers have improved their retention and resolution in RP-HPLC by buffering the aqueous part of the mobile phase to acid[44] or alkaline pH[51]. In an investigation by Isaac et al., they have been partially purified and separated by HPLC on a anion exchange column and RP column[92]. Another useful technique in handling these very polar compounds in RP-HPLC is to use an aqueous buffered medium containing a large counter-ion[172,173] (technique known as ion-pair chromatography, briefly IPC). Although all these techniques have been applied to the analysis of the conjugated ecdysteroids, there have been no reports on their use for direct separation, identification and quantification of conjugates present in insect material.

During this study a chromatographic method has been devised for the qualitative and quantitative analysis of conjugates in eggs of S. gregaria. The method is based on RP-IPC analysis using a C_{18} bonded phase column eluted with a buffered aqueous (pH 7) methanol solution containing a lipophilic counter ion[174]. Since the conjugates present in S. gregaria eggs have been shown to bear a

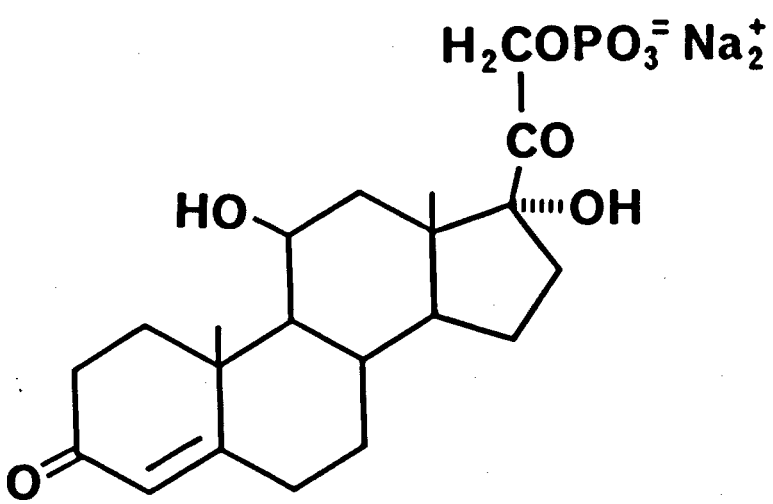
negative charge at neutral pH[53-55], a cation has to be chosen as counter ion. The retention mechanism of conjugates in RP-IPC can be described in the following way: the sample (E^- aq.) and counter ion (C^+ aq.) molecules soluble only in the aqueous mobile phase form an uncharged ion pair which then partitions into the lipophilic stationary phase:

$$E^- \text{ aq} + C^+ \text{ aq} \rightleftarrows [EC]\text{aq} \rightleftarrows [EC]\text{org.}$$

In this way the retention of the highly polar conjugates on a non-polar stationary phase can be increased.

Before using the RP-IPC technique for the analysis of biological extracts, we have applied it to the chromatography of a model compound in order to verify if ion pair formation was occurring. Hydrocortisone 21-phosphate (XXIII) (Fig. 14) was chosen.

Figure 14



Hydrocortisone 21-phosphate disodium salt (XXIII)

It was demonstrated that on an RP column, the compound was eluted with the solvent front when the mobile phase was 40% methanol in phosphate buffer (pH 7), whereas when tetrabutylammonium ion (TBA^+ 0.003M) was added to the mobile phase the retention volume was 26.6 ml. In order to optimise the RP-IPC method for the assay of conjugates from eggs, several buffers (pH 7, i.e. carbonate, citrate, phosphate and ammonium acetate) and counter ions (i.e. TBA^+, hexadecyltrimethylammonium ion) were examined. Phosphate and ammonium acetate buffers were found to give satisfactory results. However, the former was discarded since it interferes with the enzymic hydrolysis step necessary for the identification of the ion-paired conjugates. Furthermore, ammonium acetate evaporates and sublimates easily, so that salt contamination of the ion-pair material collected from the column is avoided. With regard to the counter ion, TBA^+ was chosen because it gave better peak shape than cetrimide*. Moreover, the ion-paired conjugates from S. gregaria eggs eluted too far apart under isocratic conditions, therefore a gradient elution system was adopted to avoid unnecessarily long separation times.

The sample preparation for the RP-IPC analysis of the hydrolysable conjugates involves the extraction of the eggs with methanol, followed by two solvent partitions (i.e. methanol-water-hexane and n-butanol-water). The aqueous layer from the latter partition system is suitable for RP-IPC without further purification. The conjugates present were base line separated (see Fig. 16, 17, pages 55, 56) and quantified by recording the UV absorbance of the effluent from the column. The scheme of the RP-IPC method is shown in Figure 15 where it is compared with the enzymic hydrolysis

* cetrimide = hexadecyltrimethylammonium bromide

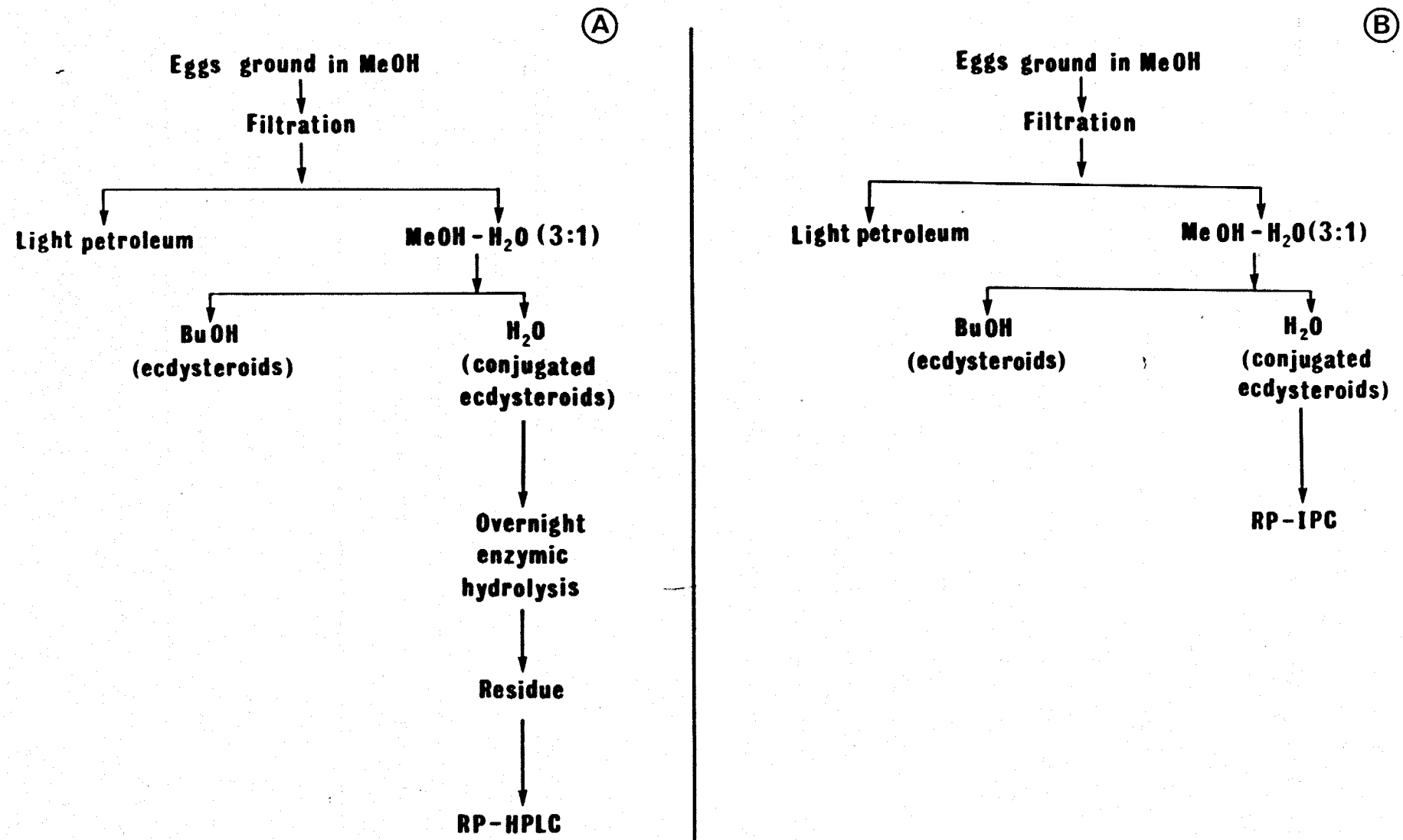


Figure 15. Scheme of the enzymic hydrolysis procedure (A) and RP-IPC method (B) for the analysis of the hydrolysable conjugates.

procedure. A typical RP-IPC trace of 1 day old egg extract is shown in Figure 16. Because UV absorbing impurities are usually present in the biological extract, UV detection is poorly specific. Nevertheless the method permits accurate and rapid quantification of the hormones, suitable for routine analysis. The levels of the conjugated ecdysteroids have been measured by a standard curve obtained with known amount of free ecdysteroids[174]. This indicates that the conjugating ionic group itself does not absorb in the ultraviolet region, nor does its attachment involve the unsaturated ketone chromophore.

To verify that this method for the rapid determination of the levels of conjugates in the eggs of S. gregaria gave results consistent with the previously used enzymic hydrolysis coupled with RP-HPLC analysis[55], the two methods were compared on the same sample of eggs. The values obtained are given below (Table 2).

Table 2

Comparison of results obtained by direct RP-IPC method and by chromatography after hydrolysis.

Compound	RP-IPC method (ng/egg)	After hydrolysis (ng/egg)
Ecdysone	382	384
2-Deoxyecdysone	230	218
20-Hydroxyecdysone	33	40

Values represent the quantities of conjugated ecdysteroids present in 1-day-old eggs of S. gregaria.

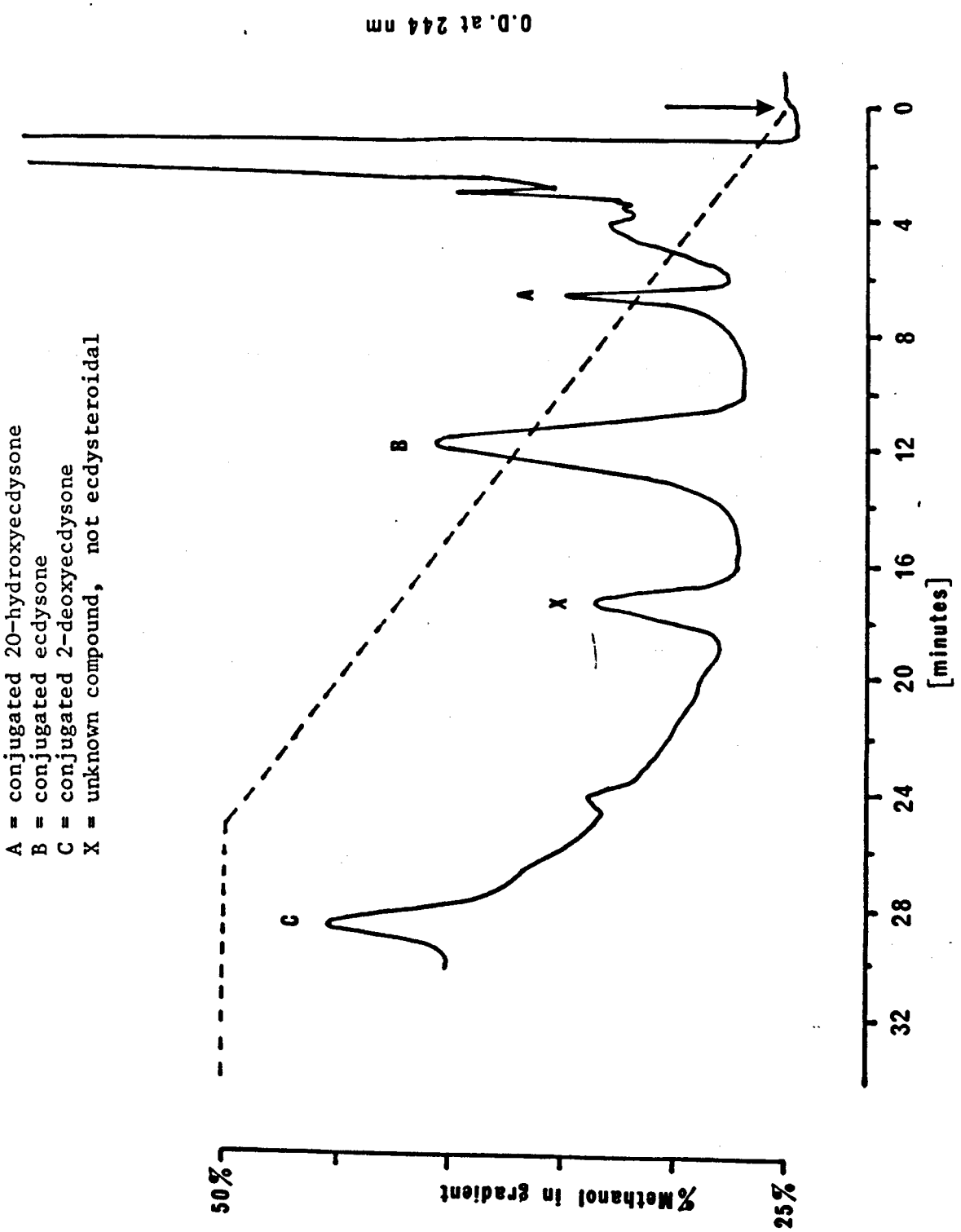


Figure 16. Typical RP-IPC separation of conjugated ecdysteroids from 1 day old eggs (2 g), using TBA^+ (0.003M).
Operating conditions: 10 cm x 5 mm i.d. column of Spherisorb-ODS.
Primary solvent: 25% methanol in 40 mM ammonium acetate buffer (pH7).
Secondary solvent: 50% methanol in 40 mM ammonium acetate buffer (pH7).
Linear gradient: 0 to 100% secondary solvent in 25 min.
Flow rate: 1 ml min^{-1}.

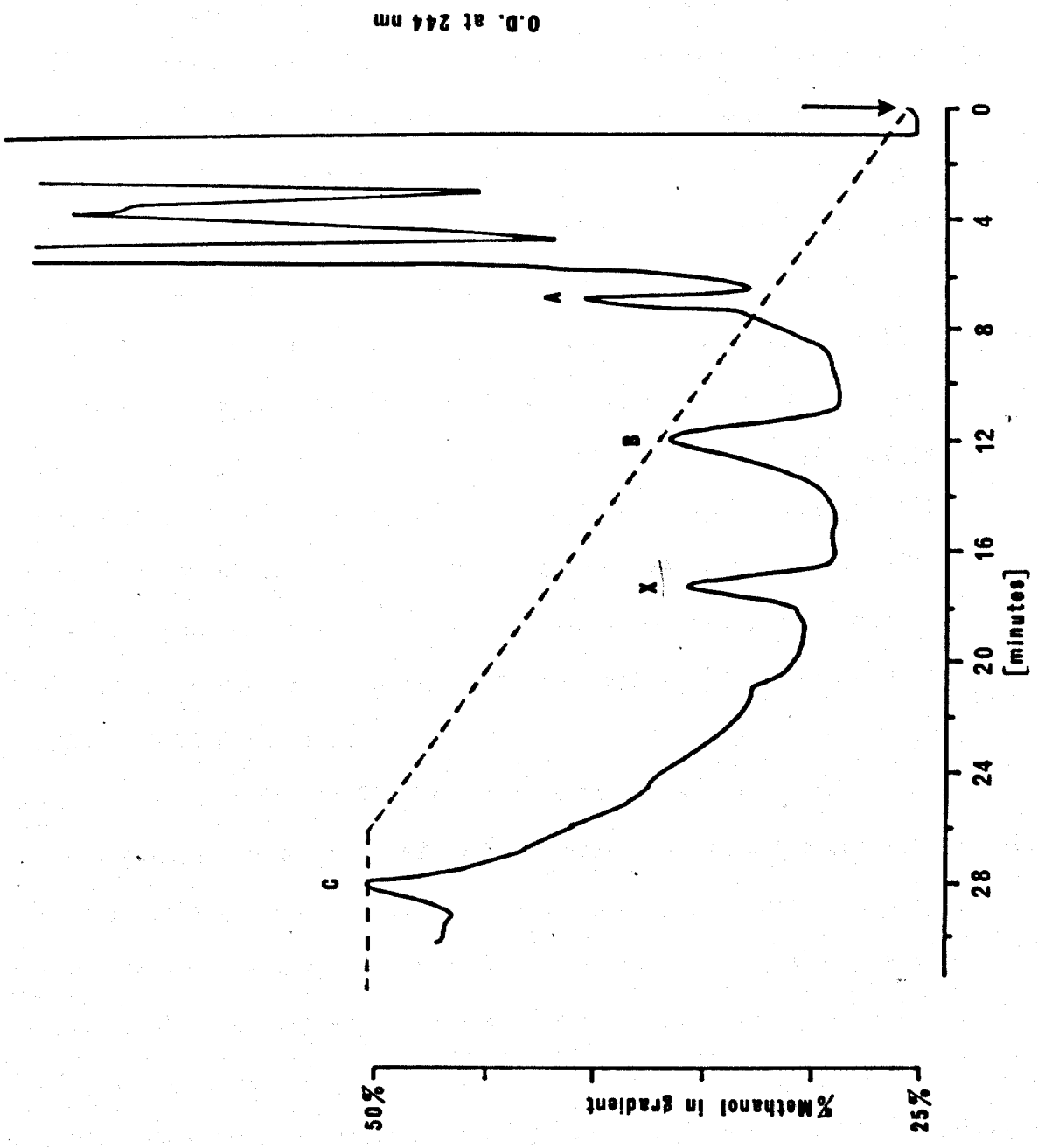


Figure 17. Typical RP-IPC separation of conjugated ecdysteroids from 11 day old eggs (2 g.).

All conditions as in Figure 16.

The results shown give good agreement between the methods and prove the validity of the RP-IPC titre determination of the hydrolysable conjugates. The limit of detection of the RP-IPC method is the conjugate equivalent to approximately 10 ng of ecdysteroid.

In the absence of authentic reference conjugated ecdysteroids, the identity of the compounds separated by RP-IPC analysis was established by collecting the different UV absorbing peaks from the column and then hydrolysing and analysing them on RP-HPLC and NP-HPLC, as described in the experimental section.

After hydrolysis with Helix pomatia digestive juice, peak A (Fig. 16, page 55) gave 20-hydroxyecdysone, peak B ecdysone, and peak C 2-deoxyecdysone[174]. The amount of ecdysteroid obtained by collecting the effluent, corresponding to each peak in Figure 16, and then hydrolysing it, corresponded to an efficiency of 82-91% of the amount expected from the same sample of eggs, when the sample was directly hydrolysed and the ecdysteroids determined by HPLC with UV detection.

When a sample of 11 day old eggs (towards the end of embryogenesis) was subjected to the RP-IPC analysis, the chromatographic pattern obtained was similar to the 1 day old eggs, with peaks at the same retention volume (Fig. 17, page 56), showing that the same hydrolysable conjugates are present at the beginning and at the end of embryogenesis. Moreover, at this time too, good agreement was found between titre determination by the RP-IPC method and by enzymic hydrolysis coupled with RP-HPLC analysis.

The direct conjugates analysis was also carried out without added TBA^+, in which case the peaks are less well resolved (Fig. 18). However the latter result may have been affected by TBA^+ irreversibly adsorbed on the column after the IPC. Using RP-IPC for the analysis

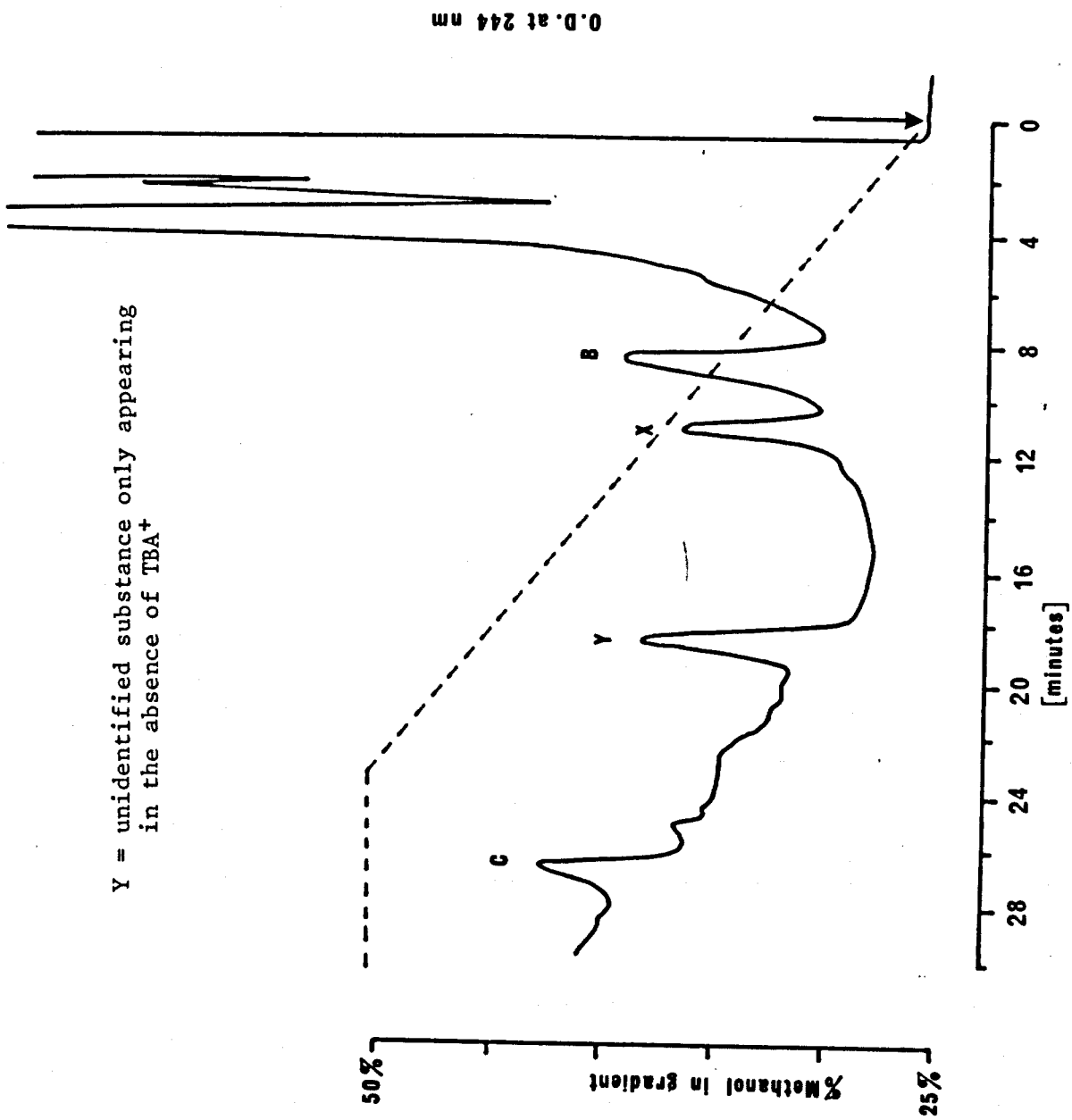


Figure 18. Separation of conjugated ecdysteroids from 11 day old eggs (2 g.) without TBA in the mobile phase.

All other conditions as in Figure 16.

Conjugated 20-hydroxyecdysone is not resolved with these conditions.

of polar metabolites of ecdysteroids from Pieris pupae, Lafont et al.[175] have found that TBA^+ (present in the mobile phase) was not removed from the column after several hours of elution with a strong solvent such as methanol.

No deterioration of the column due to TBA^+ was noticed over several weeks of continuous use at pH 7. At more basic pH other authors have found the column performance deteriorated[176,177].

The concentration of buffer and TBA^+ were not found to be critical. The appearance of the chromatogram was not altered if the buffer was changed from 0.4 M to 0.04 M and the TBA^+ from 0.003 M to 0.0015 M.

RP-Ion Suppression Analysis of Conjugates

The direct chromatography of conjugates was also attempted by RP-ion suppression HPLC, a procedure which consists of using an acidic (pH 2.6) mobile phase. Since the conjugates behave like weak acid[32], acid pH should suppress their ionization and consequently increase their retention. However when the method was applied to the analysis of the conjugated ecdysteroids, the resolution obtained was not satisfactory; therefore the method was abandoned.

Titre of the Free Ecdysteroids in the Whole Eggs

During the investigation, the levels of free ecdysone have been measured in each of the 14 days of the development of the eggs of S. gregaria (Table 3). Starting from day 3, the day and the corresponding stage of embryonic development are reported according to the scheme of Shulov and Pener[178].

Table 3

Mean levels of free ecdysone in the developing eggs of S. gregaria

DAY	Embryonic stage	Free ecdysone ng/egg	Free ecdysone µg/g
1	-	37.7	3.0
2	-	28.2	2.2
3	VI	32.5	2.6
4	XI	17.5	1.4
5	XIV-XV	15.3	1.2
6	XV	11.4	0.9
7	XVI	9.5	0.7
8	XVI-XVIII	11.1	0.4
9	XVIII	24.9	0.9
10	XIX	26.8	1.0
11	XX	44	1.7
12	XXI	55.1	2.2
13	XXII	10.2	0.4
14	XXIII	17.8	0.7

Each determination is the mean at 2 separate extractions.

Levels of free 20-hydroxyecdysone and 2-deoxyecdysone were below 5 ng/egg throughout.

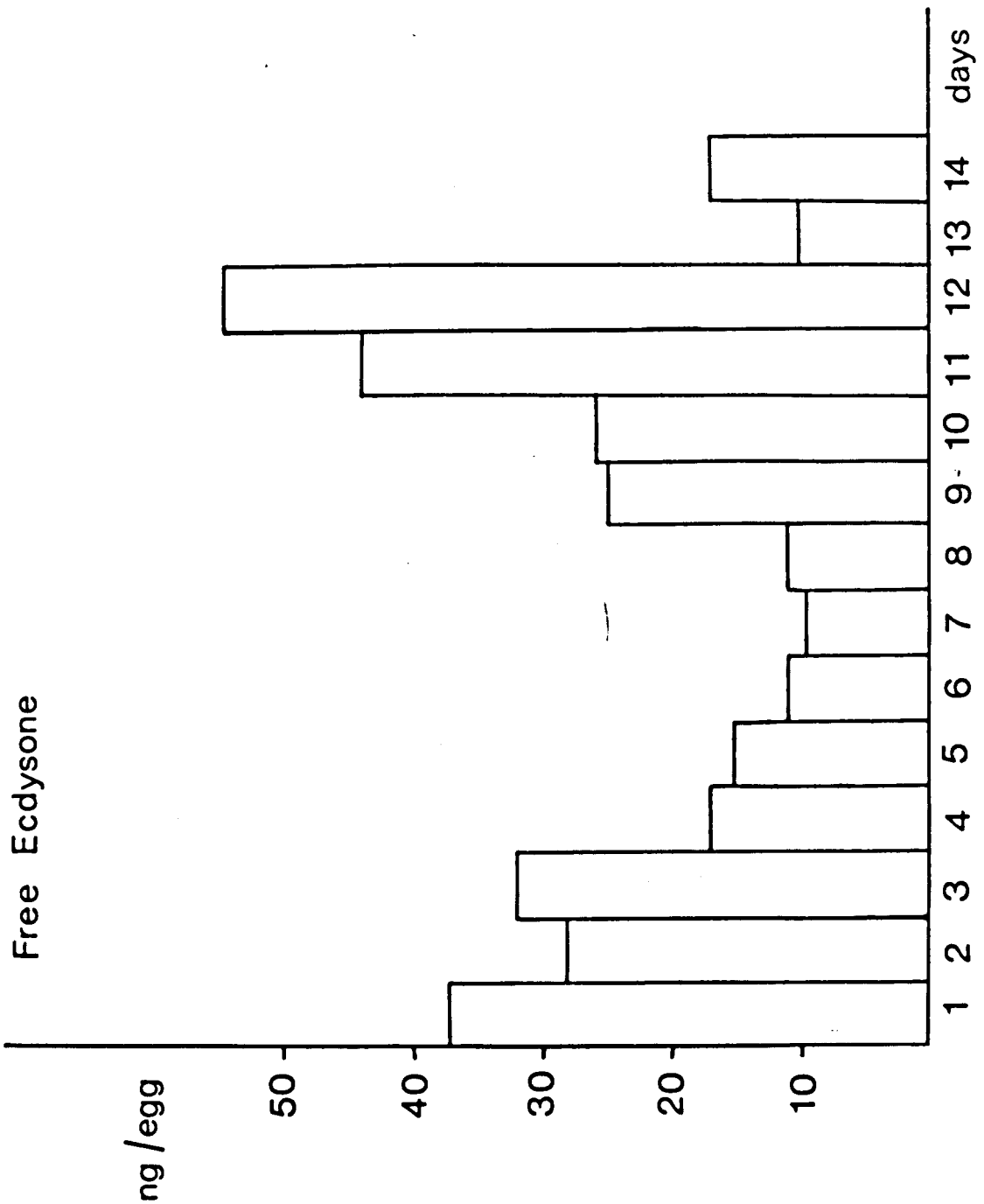


Figure 19. Levels of free ecdysone in the developing eggs of S. gregaria from oviposition to hatching.

A decrease in amount of hormone during the first 8 days is followed by a peak on days 11-12 (55 ng/egg). Thereafter the level falls to the minimal values reported (10 ng/egg) (Fig. 19). The total weight of each egg increases by 200% during the first 8 days due to the absorption of water, therefore the difference between the variation of the levels of the hormone expressed in ng/egg and µg/g (Table 3).

Free 20-hydroxyecdysone and free 2-deoxyecdysone were also detected. Because of the small amount (1-5 ng/egg) found, the quantification of these two ecdysteroids in the free state, during embryonic development, has not been undertaken.

Titre of Free Ecdysteroids in Embryo and Yolk

As ecdysteroids have been found in the eggs of S. gregaria[53,55] and there was no information from previous work to say whether the hormones are external or internal to the embryo, we began an investigation on the location of these substances within the eggs.

The free ecdysteroids have been assayed in the separated embryo and yolk of groups of eggs at the same stage of the embryonic development[179]. In this study the eggs have not been dated by the number of days elapsed from oviposition but by stages, assigned using the scheme of Sbrenna-Micciarelli[180]. The latter method provides a more precise way to define a particular point in the development process, since it has been found that as many as 5 different embryonic stages are present in 4 and 5-day-old eggs of S. gregaria[181]. Although the investigation is still in progress, the data already available are shown in Table 4 (page 63). Also in this case, only ecdysone was quantified, the amount of 20-hydroxyecdysone and

Table 4

Levels of free ecdysone in embryo and yolk of developing eggs of S. gregaria.

Embryonic stage	Free ecdysone (ng/egg) Embryo	Yolk
1	*	*
2-4	0	40
6-7	*	*
9-10	*	*
11	*	*
12	0	39.1
12-13	*	*
16	0	21.6
17-18	*	*
19-20	0	28.5
21-22	4.9	18.5
23	5.3	*
24	*	9.2
25	12.6	*
26	45	0
27	48.4	0
28	9.3	0
30	5.4	0

Results are single determinations. Zero value means an amount below the detection limit (0.6 ng/egg).

* Biological sample not available.

2-deoxyecdysone being below the limit of accurate detection on the sample size available (100 eggs; i.e. the limit of detection is 50 ng/g or 0.6 ng/egg). Furthermore the values obtained for ecdysone are consistent with the quantities determined in the previous investigation on the whole eggs.

The titre determinations show that in the preblastokinetic stages (from stage 1 to 19) ecdysone which is lacking in the embryo, is located in the yolk. After blastokinesis (i.e. after stage 19) the amount of ecdysone in the yolk progressively diminishes while in the embryo it increases and then returns to zero shortly before hatching. The results are expressed graphically in Figures 20 and 21.

The rapid increase of the titre of ecdysone in the embryo takes place at stage 27, close to the cuticulogenesis of the larval procuticle[100] (stage 28).

Blastokinesis indicates the movement, into the egg, of the developing embryo which undergoes a partial revolution with enclosure of the yolk.

The term cuticulogenesis has already been described in the introduction.

Possible Function of Free Ecdysone During Embryogenesis

The fluctuation of the titre of ecdysone during embryogenesis in S. gregaria is characterized by a decrease in the level of the hormone in the early stages of the embryonic development followed by an increase after blastokinesis when the prothoracic glands have differentiated. Blastokinesis occurs between stages XV and XVII, according to the scheme of Shulov and Pener[178] that corresponds to stages 18 and 20 in the scheme of Sbrenna-Micciarelli.

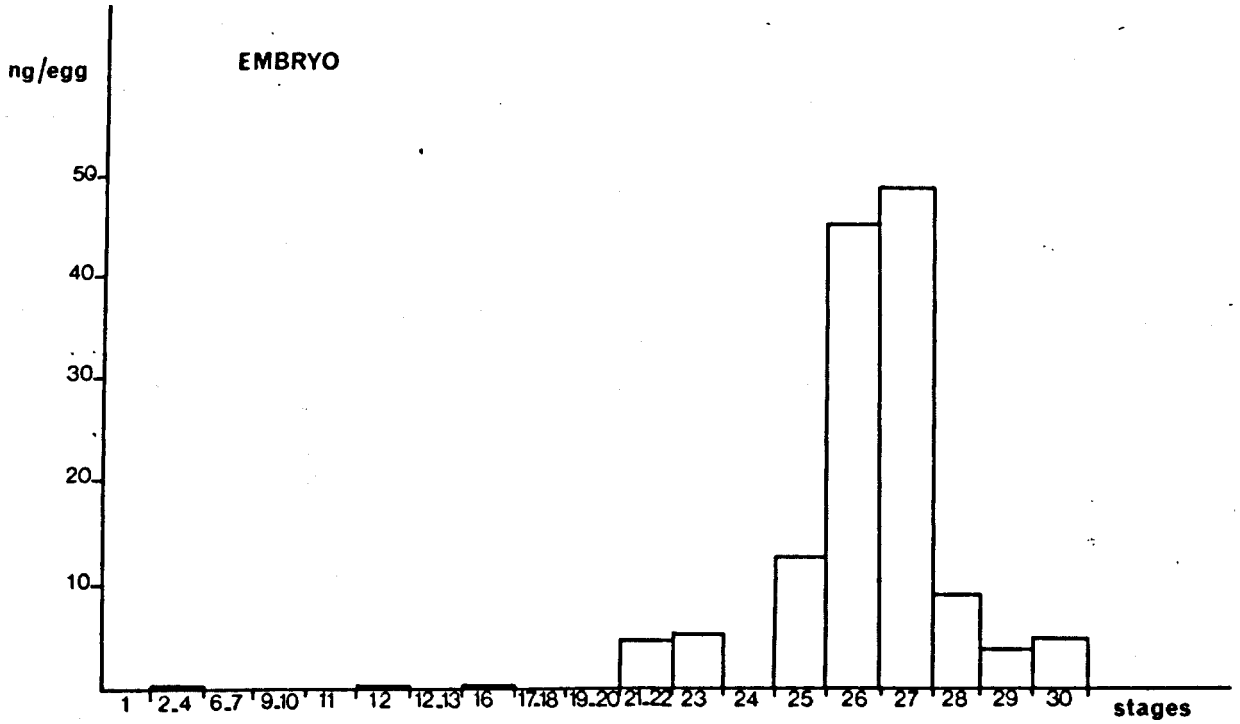


Figure 20. Levels of free ecdysone in the embryo of developing eggs of S. gregaria, at different developmental stages.

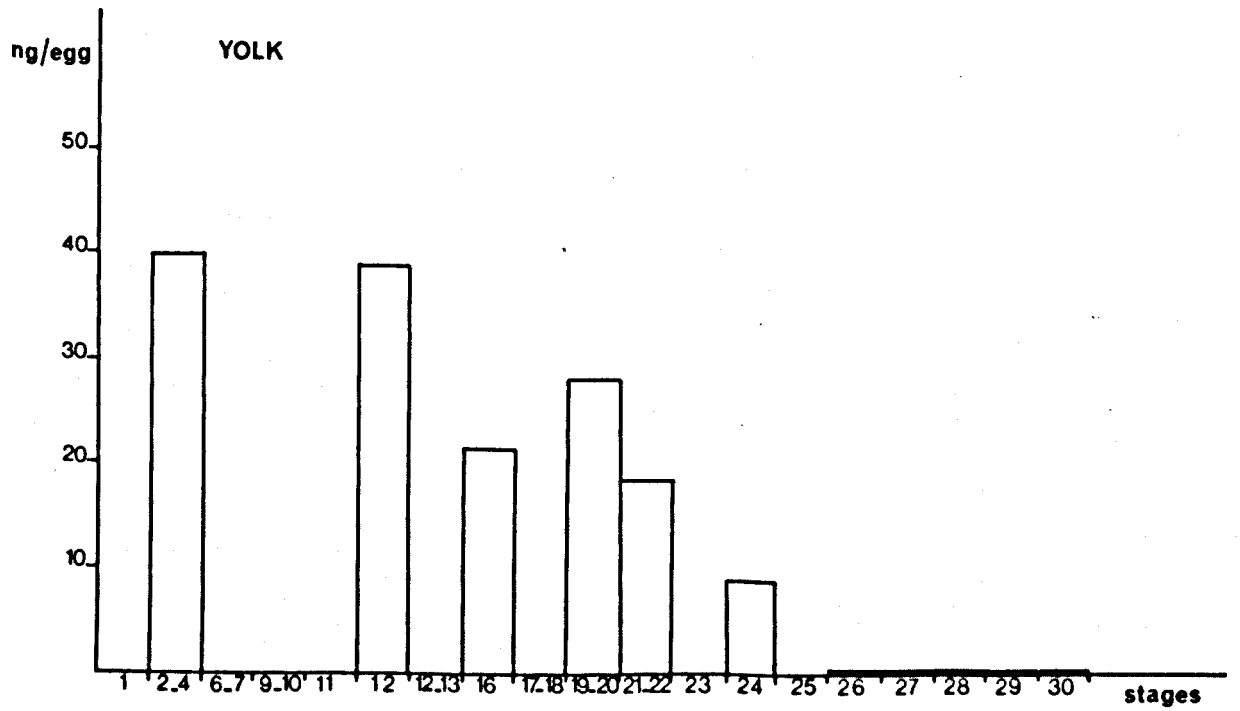


Figure 21. Levels of free ecdysone in the yolk of developing eggs of S. gregaria at different developmental stages.

The single ecdysone peak found in this study, compares favourably with previously published results[53,80,83,101], although there is other evidence of a more complicated pattern with several peaks[49].

It is not possible to formulate any conclusive explanations of the role of ecdysone in embryonic development from the data so far available. However the sharp hormone peak within the embryo suggests that the rising ecdysone titre in the later phase of embryogenesis is due either to de novo synthesis by the embryonic neuroendocrine glands or to an embryonic enzymic system responsible for the interconversion of hormone precursors or metabolites. Furthermore the correlation between the ecdysone peak and the cuticulogenesis of larval procuticle (stage 28 in the study of Sbrenna-Micciarelli and Sbrenna, which corresponds to stage XXI of Shulov and Pener) supports the hypothesis of embryonic moults being controlled by ecdysone in a manner similar to the control of the larval moults.

Titre of Hydrolysable Conjugated Ecdysteroids in the Whole Eggs

In the developing eggs of S. gregaria considerable amounts of conjugates were found. Conjugated ecdysone, 20-hydroxyecdysone and 2-deoxyecdysone were detected and the amount of each ecdysteroid at each of the 14 days of the embryonic development is shown in Table 5. Starting from day 3, the day and the corresponding stage of embryonic development are reported according to the scheme of Shulov and Pener[178]. Since the mass of the eggs increases during development due to absorption of water, results are expressed both in terms of ng per egg and µg per g. The total amount of conjugates

Table 5

Mean levels of conjugated ecdysteroids in the developing eggs of S. gregaria.

DAY	Embryonic stage	Conjugated ecdysone		Conjugated 20-hydroxyecdysone		Conjugated 2-deoxyecdysone	
		ng/egg	µg/g	ng/egg	µg/g	ng/egg	µg/g
1	-	465	37.2	48.4	3.8	242	19.3
2	-	391	31.2	44.6	3.5	222	17.7
3	VI	304	24.3	21.5	1.7	138	11.0
4	XI	218	17.4	27.0	2.1	127	10.2
5	XIV-XV	285	22.8	25.0	2.0	144	11.5
6	XV	187	15.0	27.3	2.1	100	8.0
7	XVI	236	9.4	32.3	1.25	162	6.5
8	XVI-XVIII	326	13.0	40.6	1.6	198	7.9
9	XVIII	338	13.5	51.7	2.0	243	9.7
10	XIX	372	14.8	88.3	3.5	342	13.7
11	XX	411	16.4	84.9	3.4	374	14.9
12	XXI	477	19.0	61.0	2.4	315	12.6
13	XXII	314	12.5	57.7	2.3	234	9.3
14	XXIII	282	11.2	54.2	2.1	298	11.9

Each determination is the mean of at least 3 different extractions.

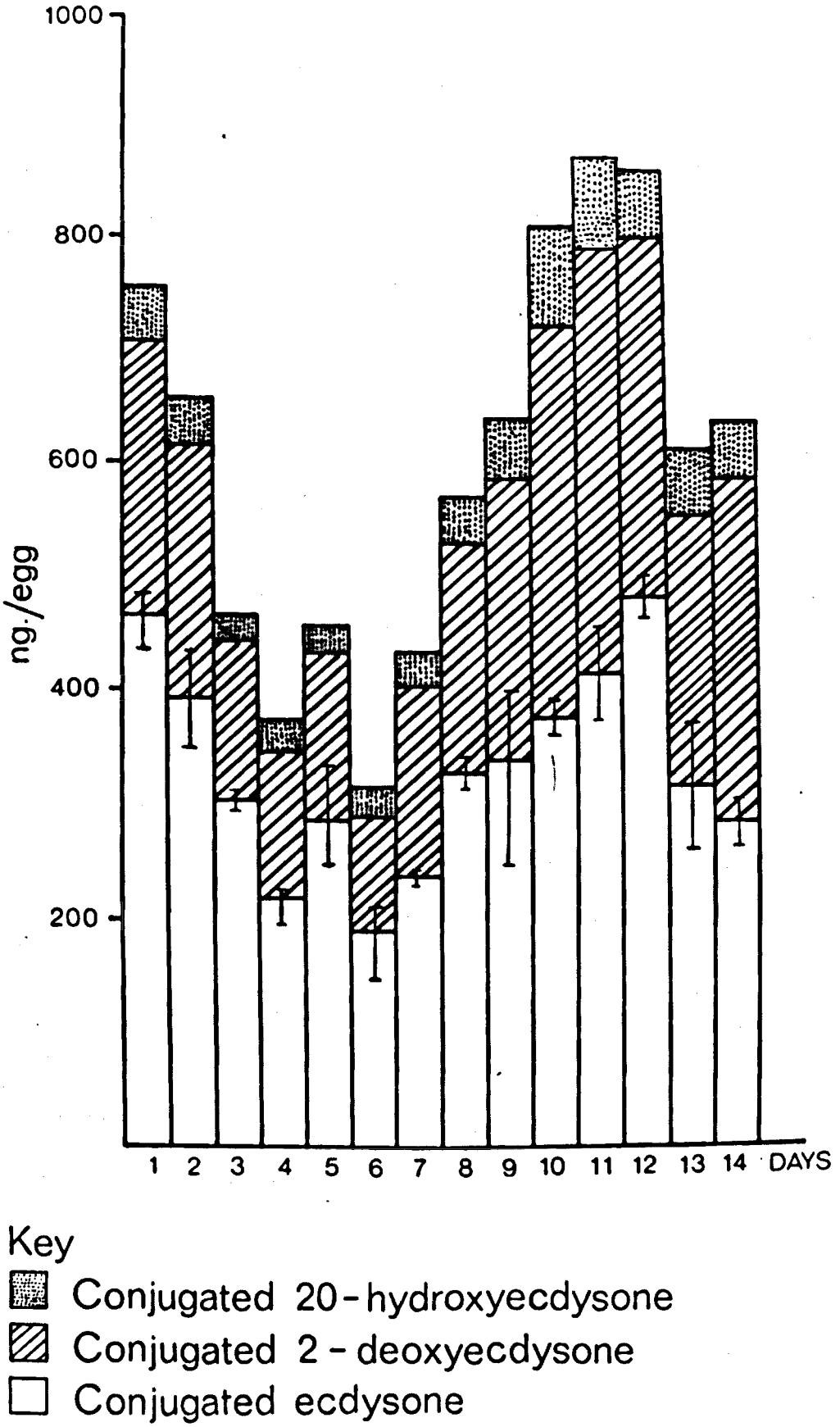


Figure 22. Levels of conjugated ecdysteroids in the developing eggs of S. gregaria from oviposition to hatching.
Vertical bars indicate the range of results for conjugated ecdysone.

in newly laid eggs is 755.7 ng/egg, it decreases to a value of 513.4 ng/egg at day 6 and then it rises again to a maximum on day 11-12 (870 ng/egg). Ecdysone is the main hormone present (60% of the total amount of conjugates detected) followed by 2-deoxyecdysone (30%) and 20-hydroxyecdysone (10%). The fluctuation of the level of the three conjugates is shown in Figure 22.

Titre of Hydrolysable Conjugates in the Embryo and Yolk

As the assay of conjugates in embryo and yolk is still in progress, only conjugated ecdysone has been determined and its quantification has not been completed yet. However, the values so far obtained are reported in Table 6 (page 70). As for the free form, conjugated ecdysone is present only in the yolk at the beginning of embryogenesis; thereafter the level of the hormone in the yolk decreases and eventually falls to zero at stage 26 (Fig. 23). A different pattern is found in the embryo where the conjugated ecdysone is absent from egg laying up to blastokinesis (stage 18-20). Only from stage 19-20, the hormone occurs in the embryo where its quantity increases and reaches a maximum at stage 26 (Fig. 24). Although the levels measured are very similar to those reported for the whole egg, this time the total amount of conjugated ecdysone does not rise during late embryogenesis (Table 6) as it was found in the previous investigation on the whole eggs (Fig. 22, page 68). There are two possible reasons for this discrepancy: the efficiency of the column deteriorated by the continuous use in the RP-IPC mode, and also the biological sample had been refrigerated for several months before being assayed. These analyses should be repeated with fresh samples using a new RP-column.

Table 6

Levels of conjugated ecdysone in embryo and yolk of developing eggs of S. gregaria.

Embryonic stage	Conjugated ecdysone (ng/egg) Embryo	Yolk
1	*	*
2-4	0	334.8
6-7	*	*
9-10	*	*
11	*	*
12	0	288
12-13	*	*
16	0	262.4
17-18	*	*
19-20	39.9	241.5
21-22	43.5	205.6
23	74	*
24	*	114.6
25	85	*
26	228	0
27	172	0
28	110.4	0
29	93.7	0
30	0	0

Results are single determinations. Zero value means an amount below the detection limit (0.6 ng/egg).

* Biological sample not available.

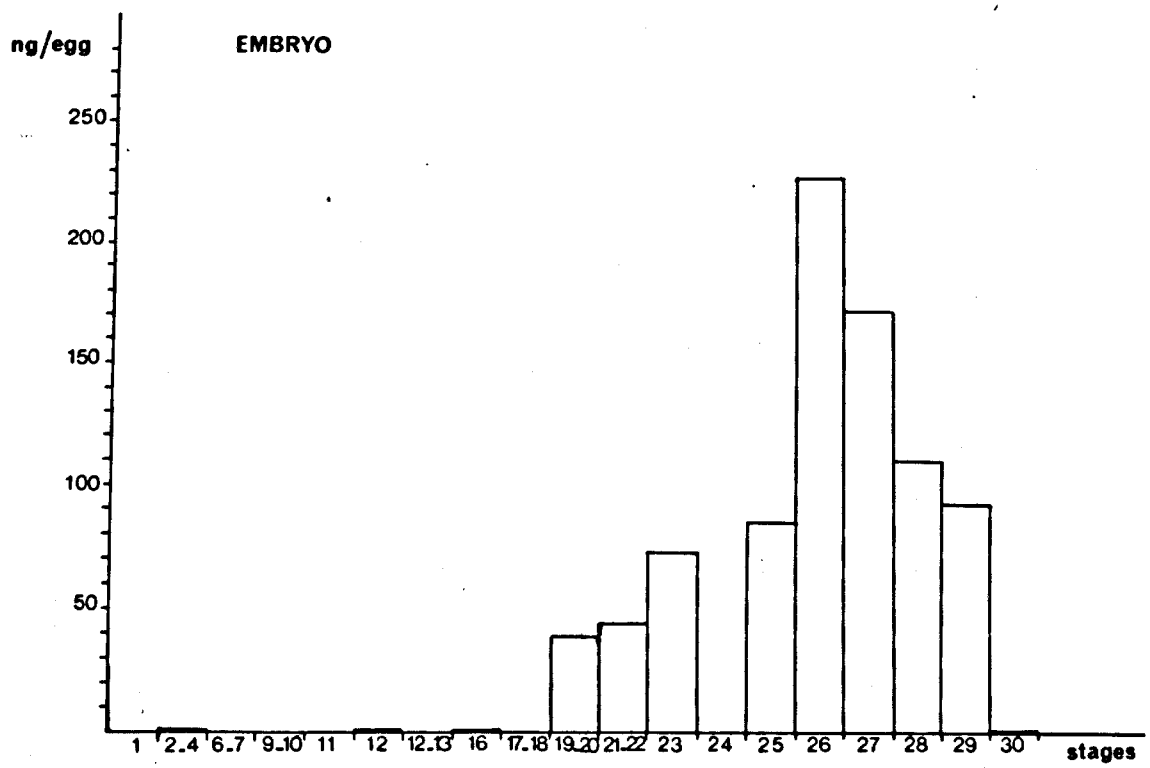


Figure 24. Levels of conjugated ecdysone in the embryo of developing eggs of S. gregaria at different developmental stages.

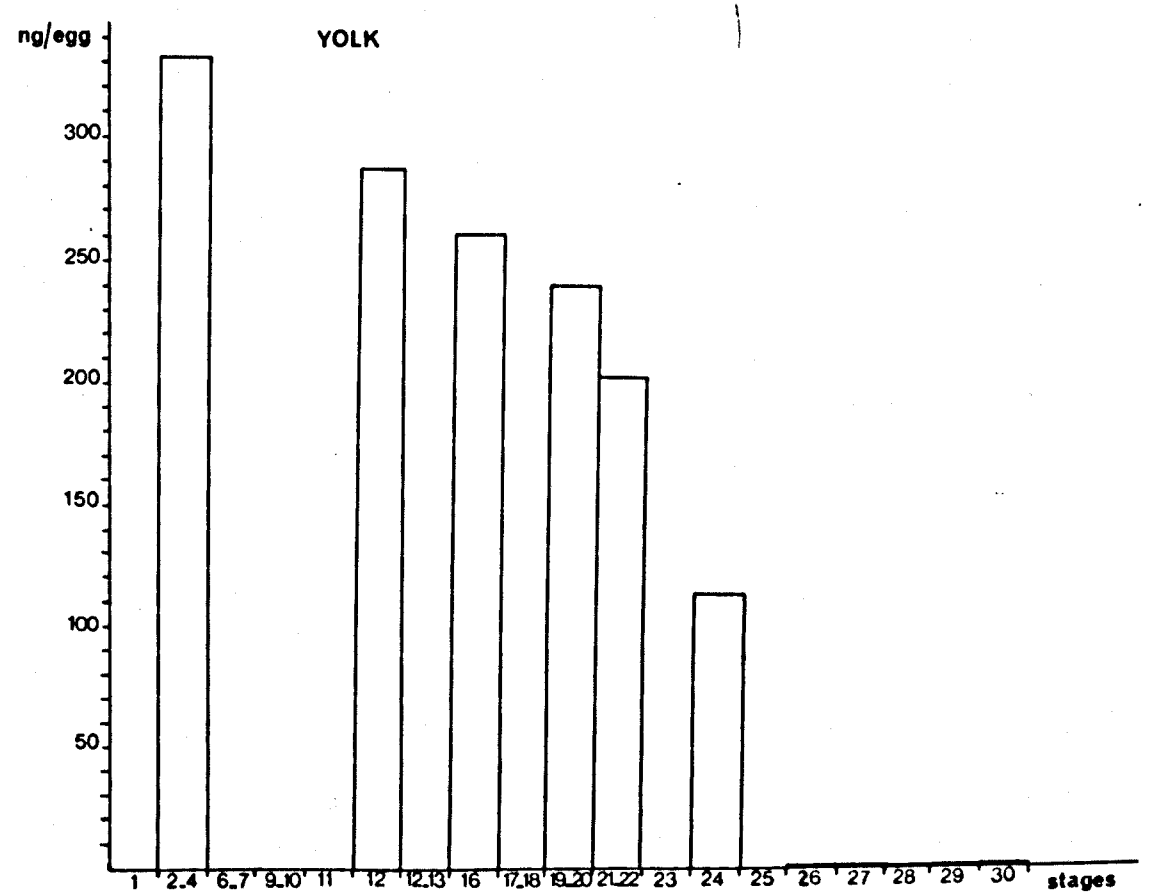


Figure 23. Levels of conjugated ecdysone in the yolk of developing eggs of S. gregaria at different developmental stages.

Physiological Significance of the Conjugated Ecdysteroids in the Embryo.

The results obtained in this investigation show that by far the greater part (90%) of ecdysteroids exists in the eggs of S. gregaria in the conjugated form. Moreover the nature of the ecdysteroids released from the conjugates by enzymic hydrolysis reflects the pattern of the free hormones. These data are in agreement with previous reports on the same species[53,54] and on other insects[49,71].

In spite of the relatively large amount of conjugates present in the eggs of S. gregaria, from this correlative study, it is not possible to formulate any hypothesis on their physiological relevance during embryogenesis.

Other authors[53,93,103] speculated that the metabolic interconversion of conjugates could be responsible for the production of the free hormone peaks in the developing eggs. However this role of conjugates as inactive 'storage' compounds of the moulting hormones is questionable in S. gregaria eggs since there is no correlation between free ecdysone peak (which occurs at day 12 of the developmental period, see Figure 19) and the falling level of the corresponding conjugate (which is observed on day 13 of development, see Figure 22).

Conjugation as main inactivation route of moulting hormones has been proposed in Locusta embryo by Lagueux et al.[49]. These authors suggested that the decrease in free hormone concentration was brought about by conjugation since an increase of the conjugate fraction was found after each peak of free ecdysone. However the values detected in our investigation on S. gregaria eggs show a

coincidence of the free and conjugated ecdysone peak, ruling out conjugation as a means of inactivation of the hormone.

The conjugated ecdysteroids present at the beginning of the embryo development of S. gregaria have been shown to be of maternal origin[72]. During embryogenesis their titre fluctuation is very similar to that of the free form of the hormone. A decrease in the level of conjugates in the early stages of embryonic development is followed by a rise after blastokinesis, when the prothoracic glands have differentiated. We speculate that the increase of the amount of hydrolysable conjugated ecdysteroids, occurring at day 12, originates from de novo synthesis by the embryonic endocrine glands which show a peak of activity at that stage of the developmental period[99].

Comparison of HPLC and GC Methods for the Assay of the Ecdysteroids in Eggs

A previous investigation has been carried out, in our group, on the variation in ecdysteroid levels in eggs of S. gregaria from the time of oviposition to hatching[53]. Determinations were by GC with ECD after silylation of the ecdysteroids. Ecdysone and 20-hydroxyecdysone, present both as free and, mainly, as polar conjugates were identified. However, in the course of comparison of GC and HPLC methods, large differences were found in the amount of conjugated ecdysteroids assayed in the eggs with the two different procedures[53,55]. As much as 477 ng/egg of conjugated ecdysone was found in this study by HPLC in contrast to 28.4 ng/egg, the maximal value measured for the same compound by the GC technique. In order to find the reason for this discrepancy we have checked GC determination

against HPLC on a single sample from the hydrolysed conjugates. It was easily demonstrated that GC and HPLC methods of detection gave consistent results[55].

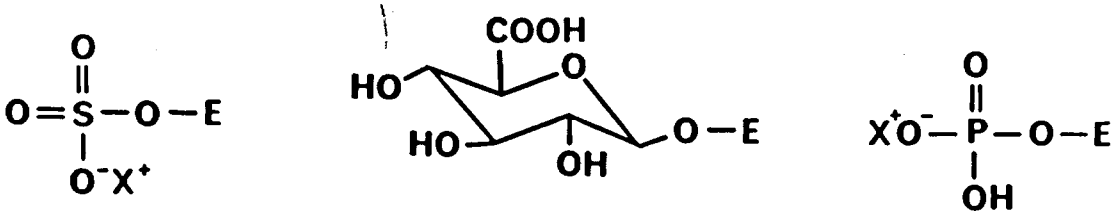
Also the enzymic hydrolysis step was re-examined to account for the reported difference. The acetate buffer, normally used for the hydrolysis of the conjugates was not suitable for GC-ECD analysis since it caused problems with the conversion of 20-hydroxyecdysone to its trimethylsilyl ether[168]. (The acetate has a catalytic effect and causes the formation of more than one derivative). Phosphate buffer was not found to interfere with the GC method, therefore it was used instead of the acetate[53]. Phosphate ions caused some inhibition of the H. pomatia enzymes[182], but satisfactory conditions were found using a five fold increase in the amount of enzyme. To determine whether phosphate buffer affected the rate of hydrolysis of conjugates, experiments were carried out comparing the yield of ecdysteroids released from the conjugates in the conditions adopted in the HPLC procedure (i.e. 0.1 M acetate buffer (pH 5.2), 5 µl/ml of enzyme) and in the GC procedure (i.e. 0.1 M phosphate buffer (pH 5.2), 2.5 µl/ml of enzyme) respectively. The same amount of substrate was found to give, with the former method, 436 ng/egg for ecdysone, 217 ng/egg for 2-deoxyecdysone, 44.6 ng/egg for 20-hydroxyecdysone, whereas with the second one the values were: 52.5 ng/egg for ecdysone, 23.8 ng/egg for 2-deoxyecdysone, 5 ng/egg for 20-hydroxyecdysone. Therefore the error in the GC investigation was caused by incomplete hydrolysis of the conjugates due to enzyme inhibition by phosphate buffer.

Another divergence evinced during the comparison of the two techniques (HPLC and GC) involved the qualitative analysis of the

conjugates. In the HPLC method the three major ecdysteroids found in the eggs of S. gregaria were ecdysone, 2-deoxyecdysone and 20-hydroxyecdysone. However only ecdysone and 20-hydroxyecdysone were identified by GC[53]. It was demonstrated that with the parameters adopted in the GC analysis, 2-deoxyecdysone elutes with the solvent front; this explains why it was not detected.

Inhibition of Enzymatic Hydrolysis of Conjugates

When the present work was undertaken, the exact nature of the conjugating group was not established. A phosphate, sulphate or glucuronide had been suggested (Fig. 25), but it was uncertain whether one or several such groups were present in the egg conjugates.



E = ecdysteroid moiety

Figure 25. Suggested structure of the conjugated ecdysteroids

In this study we have tried to shed some light on the chemical nature of the conjugated ecdysteroids present in the eggs of S. gregaria by an indirect approach; namely by studying the effect, on the rate of the hydrolysis, of a selective inhibition of one of the enzymes present in the H. pomatia juice: i.e. β-glucuronidase. Although the H. pomatia juice contains a large number of non-specific enzymes[165], only β-glucuronidase, arylsulphatase and acid phosphatase activities have been measured in this study.

We have examined several factors affecting the hydrolysis of the conjugates, including the nature, pH and ionic strength of the buffer, and a specific glucuronidase inhibitor.

Nature of the Buffer

In addition to phosphate, sulphite ions have been shown to inhibit the enzymic activities of the H. pomatia juice. However the inhibition of these ions was not selective, i.e. all the three activities measured were inhibited, therefore it was not suitable for the purpose of this investigation.

Influence of pH

Since enzymatic activity varies markedly with pH, the effect of the pH of the medium (acetate buffer) on the activity of the H. pomatia enzymes was studied in the range 3.1-5.6. At pH 3.1, both aryl sulphatase and β-glucuronidase were partially inhibited (30%), whereas the activity of the acid phosphatase was not altered. Therefore the pH did not provide a means of specifically inhibiting one of the three enzymes examined, so this line of investigation was not pursued.

Influence of the Ionic Strength

Buffered solutions of different ionic strength (i.e. acetate buffer 0.1, 1 and 1.8 M) were tested on the standard assay for the β-glucuronidase, aryl sulphatase and acid phosphatase activity of the digestive juice of Helix pomatia. Acetate buffer 1.8 M was found to inhibit completely the β-glucuronidase activity and to produce 89% inhibition of the aryl sulphatase activity, whereas

it did not affect the acid phosphatase activity. The effect of 1.8 M acetate buffer was then studied on the hydrolysis of the conjugates by comparing the amount of ecdysteroids released by the H. pomatia enzymes in presence of 1.8 M acetate buffer (pH 5.2) and 0.1 M acetate buffer (pH 5.2) which is normally used as incubation medium. The values obtained (see Table 7) show that the efficiency of the hydrolysis of the conjugates is little affected (10%) by the higher ionic strength of the 1.8 M acetate buffer[55].

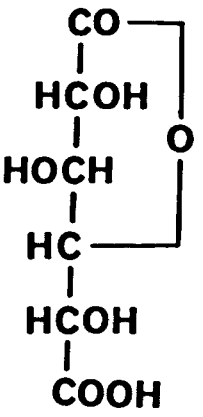
Table 7

Influence of ionic strength on the hydrolysis of conjugates.

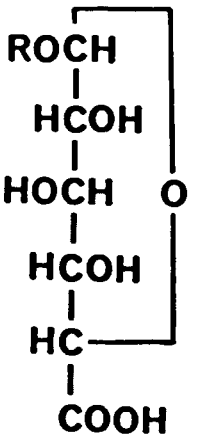
Hormone	Amount released (ng/egg) in 0.1 M acetate buffer	Amount released (ng/egg) in 1.8 M acetate buffer
Ecdysone	124	112
2-Deoxyecdysone	48.1	43.6
20-Hydroxyecdysone	13.7	12

Specific Glucuronidase Inhibitor

D-saccharic acid-1,4-lactone is known to be a competitive inhibitor of β-glucuronidase, acting as a substrate of the same configuration[183,184] (see below, Fig. 26).



D-saccharic acid 1-4 lactone



β-D-glucuronide

Figure 26

Its high specificity has been confirmed by showing that only the β-glucuronidase of the H. pomatia digestive juice is powerfully inactivated. It lost 95% of its activity, in the standard assay with 1 mM D-saccharic acid-1,4-lactone, whereas a concentration of 20 mM did not affect the aryl sulphatase and acid phosphatase activity present in the digestive juice.

Before investigating the influence of D-saccharic acid-1,4--lactone on the hydrolysis of the eggs conjugates, the effect of different concentrations of inhibitor has been measured in the standard assay for the β-glucuronidase, using as substrate phenolphthalein β-glucuronide, the substrate with the highest known affinity[185]. As shown in Figure 27, 95% of the enzymic activity is lost when the concentration of the inhibitor is 1 mM. This value was then chosen for the investigation on the hydrolysis of the conjugates.

Bearing in mind that the β-glucuronidase is not specific with respect to the aglycone[184], we have studied the effect of 1 mM D-saccharic acid-1,4-lactone on the hydrolysis of the ecdysteroid

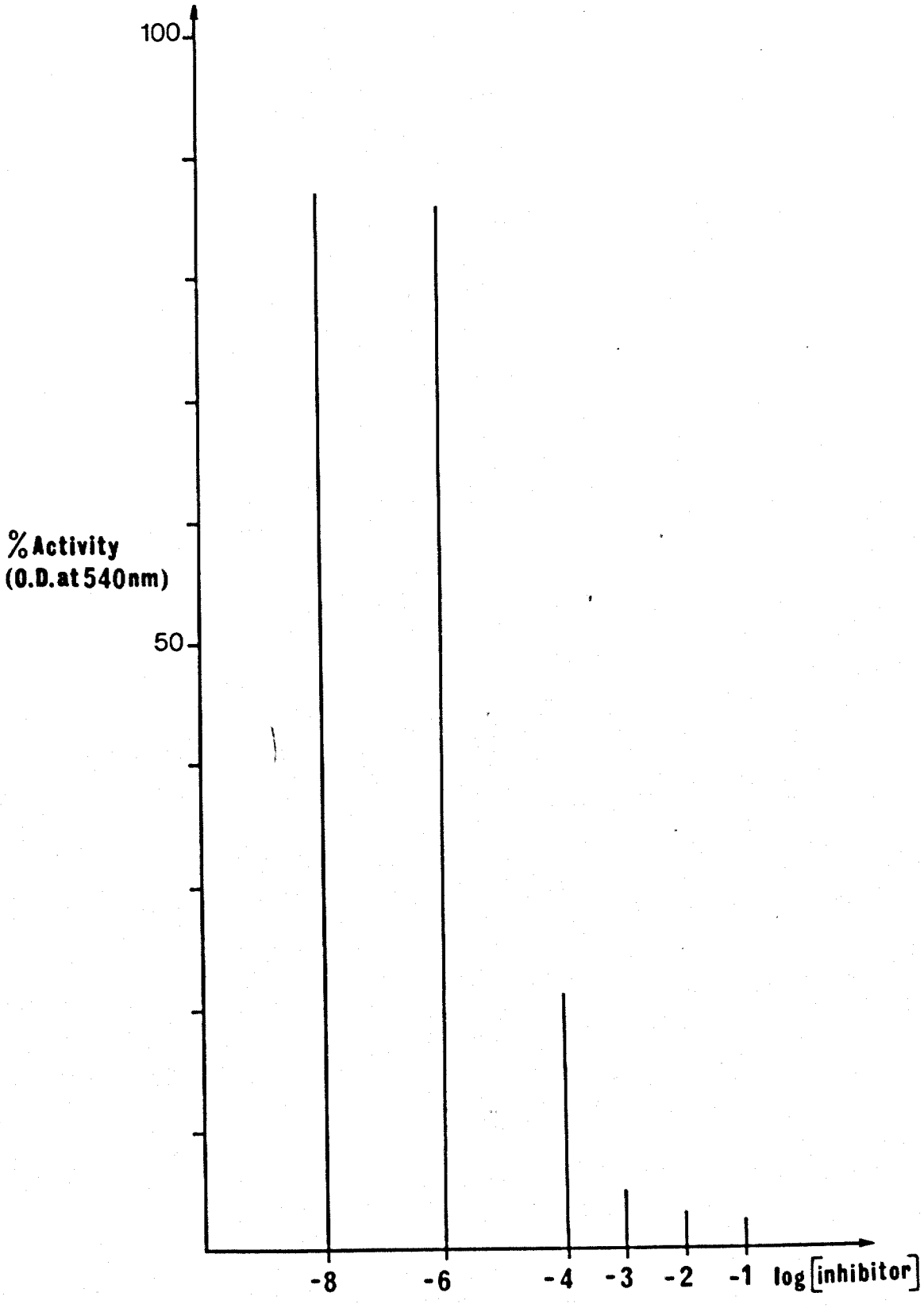


Figure 27. Inhibition of β-glucuronidase activity from H. pomatia juice by D-saccharic acid-1,4-lactone.

Phenolphthalein glucuronide was used as substrate.

conjugates present in the eggs of S. gregaria. Experiments were made on newly laid eggs and nine-day-old eggs (i.e. after blastokinesis) to compare the hydrolysis of the same amount of conjugates in the absence and presence of inhibitor. Under the same experimental conditions used for the standard assay, we observed no diminution of the amount of ecdysteroids released from the conjugated form by H. pomatia digestive juice when the incubation medium contains 1 mM D- saccharic acid-1,4-lactone[55].

Interpretation of the Results Obtained by Enzymic Hydrolysis Inhibition

No conclusive proof can be formulated from the study of the influence of high ionic strength of the buffer and β-glucuronidase competitive inhibitor, on the efficiency of the enzymatic hydrolysis of conjugates, because the H. pomatia juice contains such a large number of non-specific enzymes[165]. However the results obtained indicate the absence from the eggs of S. gregaria of ecdysteroids conjugated with glucuronic acid and suggests that the aryl sulphatase enzyme (measured in the assay) is not involved in the hydrolysis, but a sulphate or phosphate conjugate is possible.

After the present work was completed, in a preliminary communication, Isaac et al.[92], have identified the conjugates of ecdysone and 2-deoxyecdysone, in S. gregaria eggs, as simple 22-phosphates, providing confirmation of the correctness of our tentative conclusion.

However, the structure of conjugated 20-hydroxyecdysone has not been elucidated yet, probably because it is present in the eggs of S. gregaria in much lower amount than ecdysone (by a factor of

10) and 2-deoxyecdysone (by a factor of 5) (see Table 5, page 67). The results obtained in this study suggest that also 20-hydroxy-ecdysone is conjugated with phosphate.

Attempted Identification and Titre Determination of JH in Eggs of S. gregaria

Although JH1 and JH3 have been identified in S. gregaria larvae[143], there are no reports up to now on the presence of JHs (JH1, JH2, JH3) in the eggs of S. gregaria. However, because of the mutual interaction between moulting hormones and JHs during post-embryonic moults, and since ecdysteroids are present in S. gregaria eggs, it is plausible that JHs are involved with the ecdysteroids also in the control of the embryonic moults.

The first part of this study has concerned the identification and titre determination of moulting hormones in the developing embryos of S. gregaria. In the same line, it appears important to isolate and quantify JHs from the same biological material. Simultaneous titre determinations of ecdysteroids and JHs in the developing embryos will indicate if the correlation between JH and ecdysteroids activity peaks, found in the larva stage[149], occurs also in the eggs.

The Assay of JH in S. Gregaria Eggs

The assay of JHs in insect eggs is more difficult than for ecdysteroids, since JHs are present in much lower concentration[152,153]. This emphasized the necessity for a sensitive method of detection of JHs which would enable us to analyse small biological samples. A large size of the egg extracts would involve lengthy and tedious purification procedures which are not suitable for

routine determinations.

The method that was under investigation for the assay of JHs in S. gregaria eggs involved a solvent partition followed by a more rigorous purification step including Sep-Pak C_{18} and MPLC on a silicic acid column. The final detection was carried out by HPLC-UV and GC-FID. The scheme of the procedure is shown in Figure 28.

Solvent Partition

The biological extract, obtained by grinding the eggs in a glass mortar, was partitioned between aqueous methanol and hexane. The aqueous methanol-hexane partition coefficient of JH is 4:96 as found with standard samples; therefore two successive partitions allowed recovery of more than 99% of the JHs in the hexane phase.

Sep-Pak C_{18}

The residue from the hexane phase was redissolved in methanol and injected through a Sep-Pak C_{18} cartridge. This purification step corresponds to the removal of apolar substances, which are retained in the cartridge, whereas the JHs are quantitatively eluted.

Further Chromatographic Purification

The effluent from the Sep-Pak C_{18} was subjected to a further purification step which involved one of the following methods: TLC using 6% ethyl acetate in benzene as mobile phase, Sep-Pak silica eluted with 5 ml of 50% diethyl ether in hexane and MPLC on a silicic acid column eluted with 20% diethyl ether in hexane. Identical samples were purified by the three different methods.

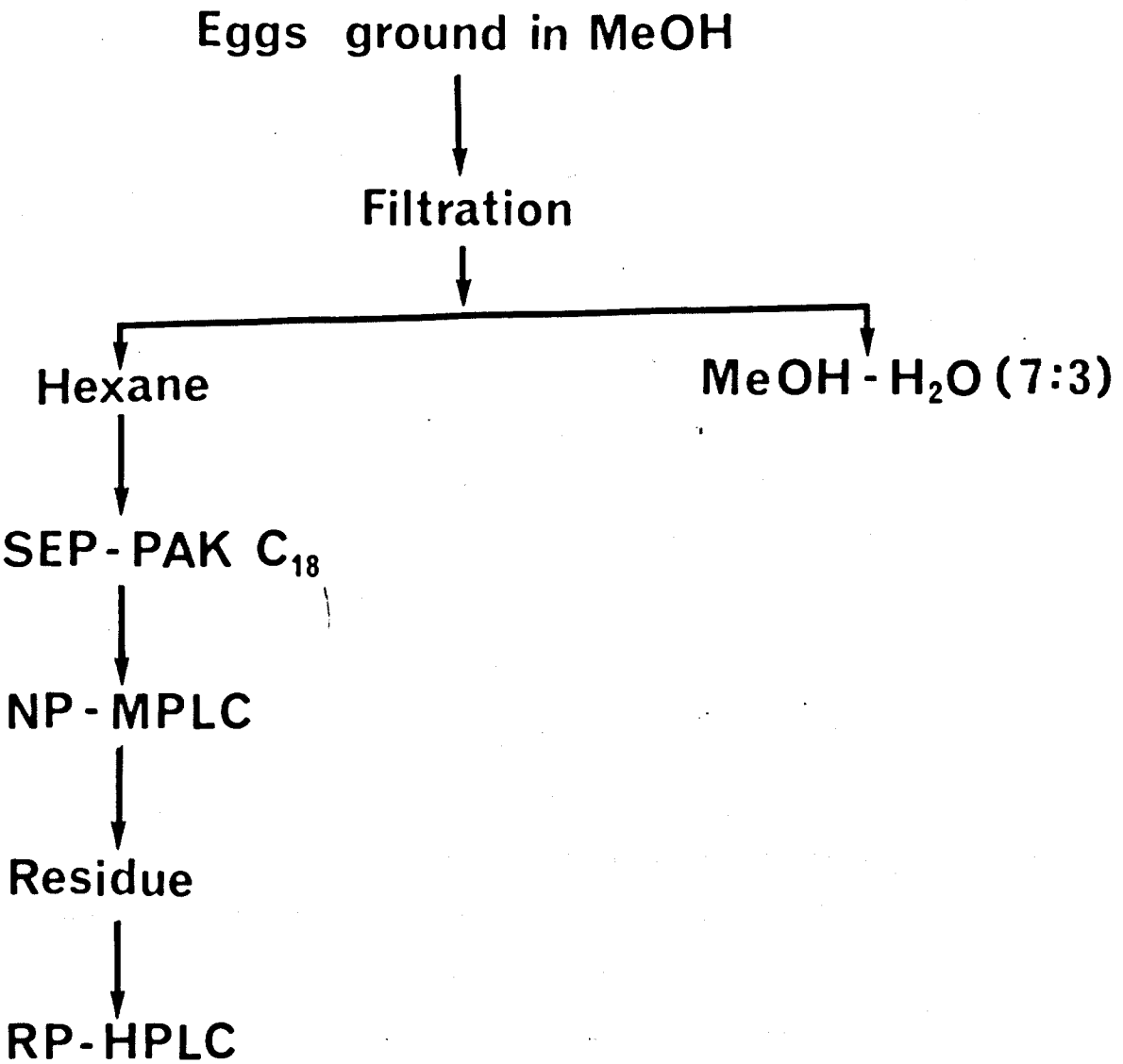


Figure 28. Extraction and purification procedure for HPLC analysis of JHs from S. gregaria eggs.

Only the latter one (i.e. MPLC on a silicic acid column) proved to be suitable for direct analysis by RP-HPLC with UV detection; TLC and Sep-Pak silica purification procedures being not able to remove the UV absorbing impurities coeluting with JHs in RP-HPLC.

Detection of JHs

Due to the presence of an unsaturated ester function, JHs show a strong absorption at 225 nm ($\varepsilon_{225\ nm} \simeq 13{,}800$) which is useful for detecting the hormones by UV monitoring the effluent from the RP-HPLC column[186]. The minimum amount of authentic JHs detectable by HPLC-UV was 10 ng per injection.

However when the fraction containing JHs from the MPLC was analysed by RP-HPLC, we did not find any UV absorbing peaks with the retention time of standard JHs (Fig. 29). The latter result indicated that the concentration of JHs (if present at all) in the eggs of S. gregaria was too low to permit direct HPLC analysis by UV monitoring at 225 nm on samples of 5 g or less.

GC-FID analysis provided lower sensitivity (30 ng of standard JHs) than HPLC-UV. Consequently it also was not suitable for the assay of the hormones in the egg sample taken for our study.

GC-MS analysis in the single ion monitoring mode (SIM) has also been examined. Figure 30 shows the electron impact mass spectrum of authentic JH3. The fragment ion at mass 81 was selected for the GC-SIM analysis since it carries the highest percentage of the total ion current. The selected ion current profile of JH3 is shown in Figure 31. Using standard solutions of JH3, the limit of detection of GC-SIM analysis was found to be 5 ng, which improved the sensitivity of the HPLC-UV method only by a factor of two.

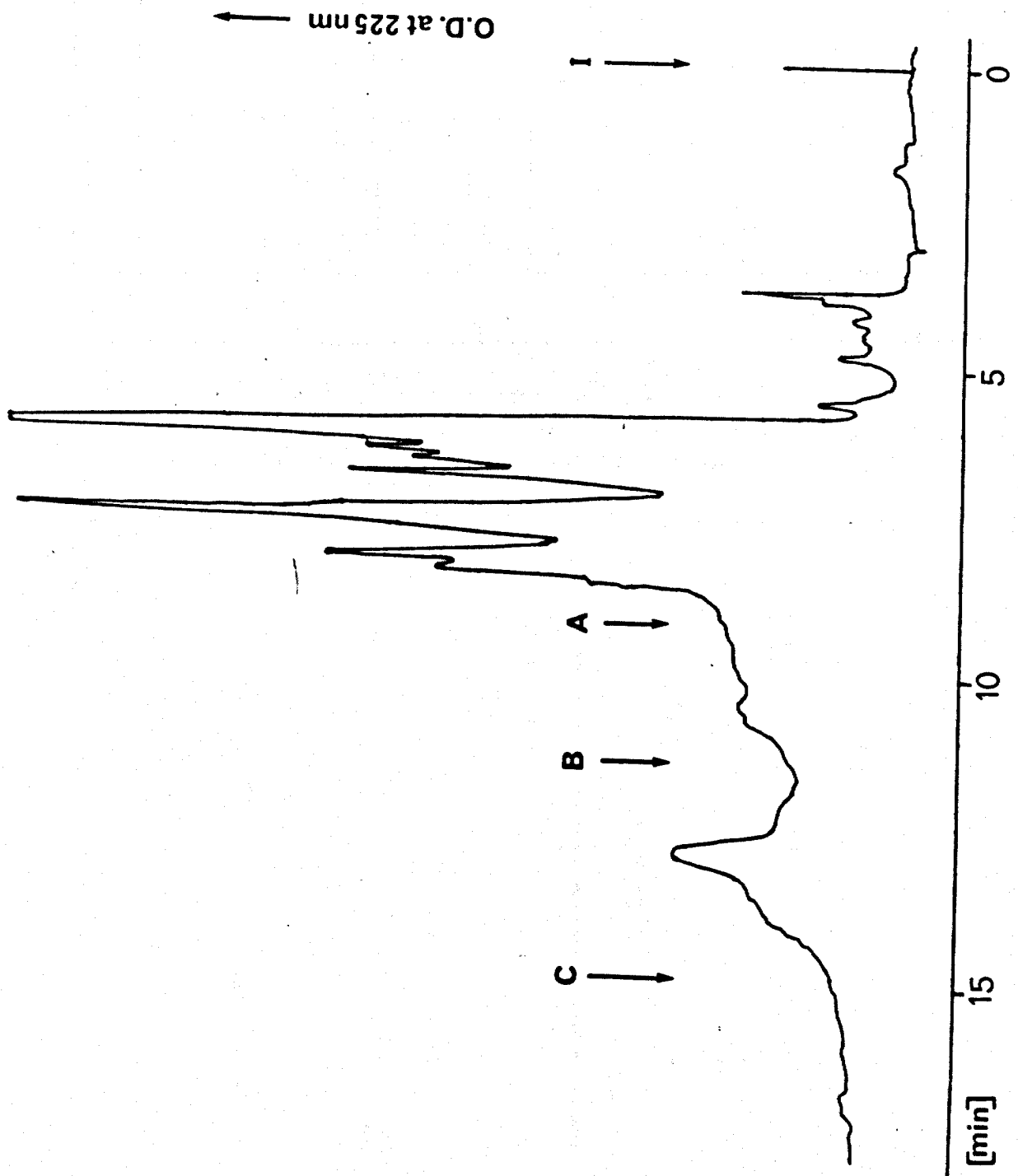


Figure 29. RP-HPLC trace of an extract from S. gregaria eggs, purified as in Figure 28.

Operating conditions: 25 cm x 4.6 mm I.D. column of Spherisorb 5ODS eluted with methanol-water (80:20, v/v; 0.8 ml min^{-1}).
Retention times of authentic JHs are indicated by (A) JH3; (B) JH2; (C) JH1.

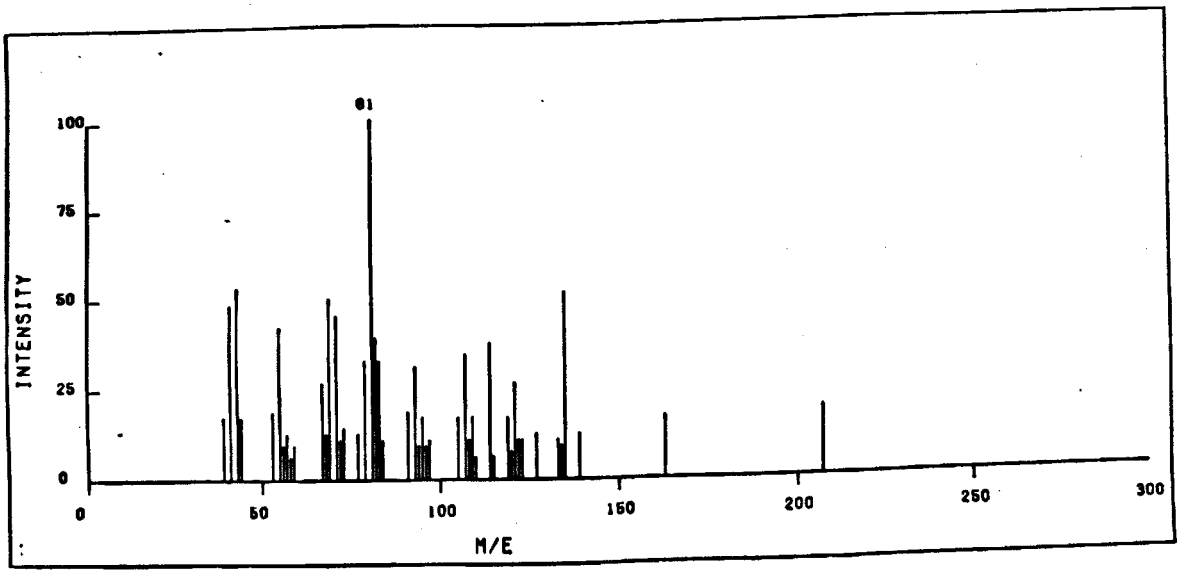


Figure 30. E1 mass spectrum of JH3 ($C_{16}H_{26}O_3$, M^+266).
Operating conditions: ionizing energy, 70 eV; accelerating voltage, 7.4 Kv; trap current, 60 μA.

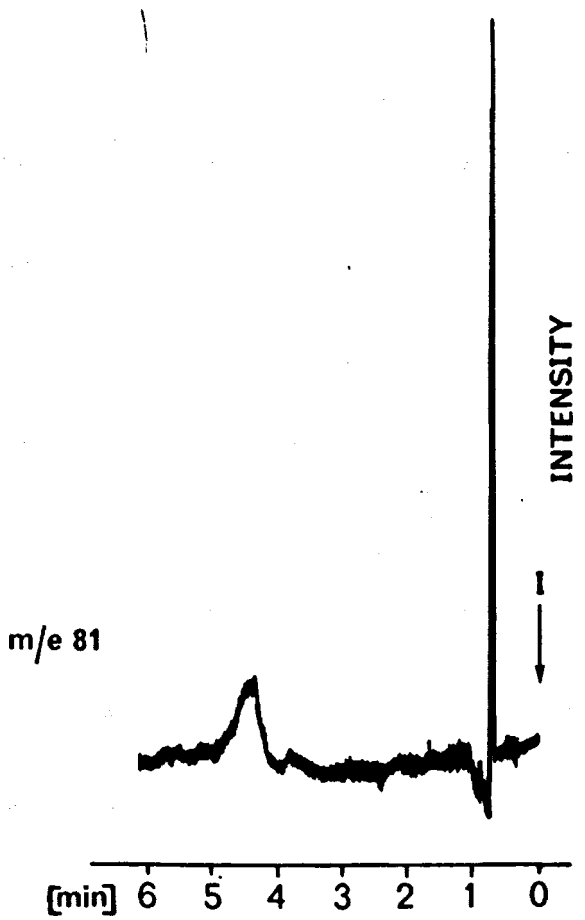


Figure 31. Selected ion chromatogram of JH3 from GC-MS analysis.
Operating conditions: 1.5 m x 4 mm column of 3% OV101 on Chromosorb W 100-120 mesh, oven temperature 200°C, helium carrier at 20 ml min^{-1}.

Since a higher sensitivity was necessary for the assay of JHs with the size of biological sample available, also the GC-SIM procedure was unsatisfactory for the present investigation.

Most of the recent work on the determination of unchanged JH by SIM has been carried out using CI[140,144,146] instead of EI[147]. Mauchamp *et al.* have found that CI affords higher sensitivity than EI, because the fragment ions produced are less numerous and therefore of high intensity[144]. Time has not allowed us to examine the CI procedure for the detection of JH by SIM.

Recovery

The recovery of JHs, after the purification procedure, was determined by adding a known amount of internal standard to the sample, before the extraction.

The ethyl ester of JH3 was chosen as internal standard. It was synthesized from JH3 using a mild transesterification method[187] (see below)

$$\text{JH3} + \text{EtOH} \underset{\rightleftharpoons}{\overset{\text{KCN}}{}} \text{JH3-ethyl ester} + \text{MeOH}$$

The recovery of JH3 ethyl ester added at 50 ng per gram was 74%.

The sensitivity of the HPLC-UV method with biological material was 35 ng/g of JH3 ethyl ester. Taking into account the latter value and the recovery obtained, the failure to detect JHs by HPLC-UV means that the level of the hormones in the eggs of *S. gregaria* is below 47 ng/g.

EXPERIMENTAL METHODS

Insect Rearing

Schistocerca gregaria Forskål were reared at Keele in crowded conditions in a 12:12 hours light/dark photoperiod day. The ambient temperature during the light phase was 32°C falling to 28°C in the dark phase. Illumination was provided by a 40 watt bulb in the cages. The locusts were fed on fresh grass and dry bran. Under these conditions mature female adults S. gregaria laid their eggs within 3-4 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 in aluminium tubes (1½" in diameter, 5" in length) containing moist sand. The sand was sterilised by heating at 100°C. The cages in which the insects were kept were of wood or metal construction and were cleaned regularly with o-chlorophenol to prevent the spread of disease.

Collection of Egg Samples

The eggs are laid in a group known as an egg-pod containing an average of 80 eggs and weighing about 1 g. The egg-pods were removed from the sand and allowed to develop for different lengths of time at a constant temperature of 30°C in moist cotton-wool. The eggs were dated by the number of days elapsed from oviposition to the extraction and also by the scheme of Shulov and Pener.[178] The latter required microscopic examination of the embryo and its classification according to external features. Under the conditions chosen for the incubation of the eggs, it required 14 days from oviposition to the hatching of the first instar Schistocerca larvae.

Each batch of egg-pods was dated and analysed (or stored at -20°C until analysed).

Another group of samples of S. gregaria eggs were obtained from Sbrenna and Micciarelli (Zoology Department, Ferrara University, Italy). The eggs were staged by the scheme of Sbrenna and Micciarelli[180], and separated into embryo and yolk. The biological samples were then freeze-dried and sent to Keele.

Purification of Solvents

"ECD grade" toluene for gas chromatography was purified by shaking it twice with small portions of concentrated sulphuric acid, then washed with distilled water and 5% aqueous sodium hydrogen carbonate to remove all traces of acid. The toluene was dried over anhydrous magnesium sulphate, distilled from phosphorus pentoxide and stored over 4 A molecular sieves.

Pyridine for silylation reactions was distilled from calcium hydride and stored over 4 A molecular sieves.

HPLC grade methanol was prepared by fractional distillation through a "knit mesh" stainless steel column.

HPLC grade hexane was obtained by shaking "hexane fraction" repeatedly with concentrated sulphuric acid and then with 0.1 N solution of potassium permanganate in 10% sulphuric acid. The hexane was washed to neutrality with water, dried over sodium sulphate and distilled under nitrogen from sodium. The fraction boiling between 65°-70°C was collected and stored over 4 A molecular sieves.

HPLC grade dichloromethane was prepared by washing the solvent with concentrated sulphuric acid, then with sodium carbonate and finally with water. The washed material was dried over calcium chloride, distilled from calcium hydride and stored over 4 A molecular sieves.

All other solvents were of analytical grade; where dry solvents were used (e.g. ether, chloroform and acetone) drying was effected using sodium wire or molecular sieves as appropriate. Water used for partitions, buffers and HPLC analysis was glass distilled.

Plates for Thin Layer Chromatography

Silica (normal-phase) TLC plates were prepared by coating 5 x 20 cm glass plates with a 0.3 mm layer of silica gel G (E. Merck, Darmstadt) which had been washed in excess methanol to remove UV absorbing impurities. After the silica had been slurried onto the plates, they were allowed to dry in air and stored over calcium chloride.

High performance silica TLC plates (silica gel 60 F254) were obtained from Merck.

Reversed-phase TLC plates were obtained from three commercial sources and were also made in the laboratory. Precoated plates (10 x 10 cm) containing short, medium and long carbon chains bonded to the silica surface (RP-2, RP-8 and RP-18 respectively) were obtained from Merck. One type of plate (20 x 5 cm) KC_{18}, precoated with C_{18} carbon chains was obtained from Whatman (Springfield Mill). Three types of Macherey, Nagel & Co. (Duren) plates were used which have different degrees of silanization with octadecylsilane. These plates (10 x 10 cm) are 50, 75 or 100% silanized. RP-TLC plates were prepared in the laboratory as follows. Precoated silica TLC plates (10 x 10 cm, Whatman high-performance silica, Type HP-K) were immersed in a solution of 2% trichlorooctadecylsilane in dry toluene. The plates were

sonicated three times for 30-sec. intervals in the first 15 min. of immersion and left in the bath overnight, then washed for 10 min. in each of toluene, acetone and methanol. Plates were dried at 110°C for 1 hour before use. Capping was carried out in the same way using trimethylchlorosilane.

All plates had an acid-stable fluorescent indicator incorporated, absorbing light at 254 nm.

TLC of Ecdysteroids

Different amounts of pure ecdysteroids (from 10^{-6} to 10^{-7} g) were spotted on the plates using a 10 µl SGE syringe. Ascending development was carried out in a Shandon Southern rectangular tank, saturation of the chromatography chamber being achieved by lining it with filter paper impregnated with the mobile phase.

The silica plates were developed in chloroform-methanol (8:2) or dichloromethane-methanol-hexane (80:20:5). The reversed-phase plates were conditioned for 30 min. in the atmosphere of the tank before use. They were then developed in 95% ethanol-water (1:1).

Recovery of Ecdysteroids from TLC and RPTLC Plates

After development, the plates were dried with the aid of a hair dryer. The ecdysteroids were visualised under UV fluorescence as dark spots against the fluorescent background and the appropriate R_f bands scraped off. The silica was transferred to a test-tube by means of weighing paper and extracted with methanol (1 ml). The silica gel was separated by centrifugation and the clear supernatant liquid decanted into a Reacti-vial, (Pierce & Warriner, Chester) where it was evaporated to dryness with a stream of warm nitrogen. The

residue was redissolved in a known volume of methanol (200 µl) and 5 µl of this solution were injected into a RP column and quantified by HPLC with UV absorbance monitoring of the effluent, as described below.

Biological Sample Processing

Extraction of Ecdysteroids and Ecdysteroid Conjugates from the Eggs

The eggs (from 1 to 5 g) were ground in methanol in a glazed or glass mortar and the resulting slurry filtered through a sintered glass funnel (porosity 3). The filtrate was evaporated down and partitioned between light petroleum (b.p. 40-60, 120 ml) and methanol/water (3:1 V/V, 120 ml) to remove apolar impurities. The aqueous methanol phase was then reduced to dryness in a rotary evaporator at 40°C. The residue from this was partitioned between countersaturated n-butanol (50 ml) and water (50 ml). Each fraction was backwashed with a small volume of the appropriate counter phase which was added to the main fraction. During this partition the free ecdysteroids were found in the butanol phase, whereas the polar conjugates were in the water phase.

Hydrolysis of Conjugated Ecdysteroids

The aqueous phase from the butanol-water partition was reduced to dryness by rotary evaporation as an azeotropic mixture with butanol. The residue obtained was dissolved in 100 mM acetate buffer (5 ml, pH 5.2) and subjected to overnight enzymatic hydrolysis at 37°C in the presence of sufficient amount of the digestive juice of the snail Helix pomatia (5 µl/ml) (Koch-Light, Colnbrook) to give 4,000 Roy units/ml of aryl sulphatase (measured by a modification of the

method of Roy[188] as described later), 500 Fishman units/ml of β-glucuronidase (measured by a modification of the method of Levvy[183] as described later) and 100 Sigma units/ml of acid phosphatase (measured by a modification of the method of Sommer[189], as described later).

Analysis of Free Ecdysteroids from Conjugates

In order to purify the ecdysteroids, freed by the enzymatic hydrolysis, from the impurities present in the incubation medium, the latter was injected into a pre-washed (5 ml of MeOH and then 5 ml of H_2O) Sep-Pak C_{18} cartridge (Waters Associates, Milford) and eluted successively with 5 ml of 20% (v/v) methanol in water and 7 ml of 80% (v/v) methanol in water. The latter fraction, containing all the different ecdysteroids present in the egg extract, was reduced to a small volume and directly assayed by RP-HPLC with UV detection of the eluate, as described later.

The enzymic hydrolysis of the conjugates was carried out also in the presence of different enzyme preparations; namely β-glucuronidase (5 µl/ml) (Koch Light, Colnbrook), aryl sulphatase (1 mg/ml) (Koch Light, Colnbrook), β-D glucosidase (1 mg/ml) (Sigma, London). In each case the experimental conditions were those reported for Helix pomatia juice hydrolysis.

Examination of Helix Pomatia Juice for Ecdysteroids

Helix pomatia juice (briefly, Helicase) (25 µl) was dissolved in 5 ml of acetate buffer (0.1 M, pH 5.2) and the resulting solution injected into a pre-washed (5 ml of MeOH and then 5 ml of H_2O) Sep-Pak C_{18} cartridge and eluted successively with 5 ml of

20% methanol in water and 7 ml of 80% methanol in water. The latter fraction was reduced in volume and analysed for ecdysteroids by RP-HPLC.

Purification of the Free Ecdysteroids

The butanol phase, from the butanol-water partition, was evaporated *in vacuo* on a rotary evaporator at 40°C. Evaporation was assisted by distilling an azeotropic mixture of butanol and water. The butanol residue was purified by reverse-phase medium pressure liquid chromatography (RP-MPLC) which was performed with a MPL series II Micropump (Metering Pumps Ltd., London), a Tefzel slider valve (Magnus Scientific Instrumentation Ltd., Sandbach) injection system and a IEC column (Whatman Ltd., Maidstone) 10 mm i.d. x 45 cm dry packed with 30/40 μm particles of C_{18} Magnusil H (Magnus Scientific Instrumentation Ltd., Sandbach). The sample, dissolved in 50% (v/v) methanol-water (4 ml) was injected into the column by means of a gas-tight syringe (Hamilton Company, Reno) and eluted successively with 40% (v/v) methanol-water (80 ml) and 80% (v/v) methanol-water (70 ml) collecting in 10 ml fractions. The ecdysteroids were eluted in fractions 11 to 15, which were then reduced in volume and assayed by NP-HPLC with ultraviolet absorption detection, as described later.

Examination of the Butanol Phase for Conjugates

The butanol phase, from the butanol-water partition, was checked for the presence of conjugated ecdysteroids as follows. The butanol residue was dissolved in 10 ml of acetate buffer (0.1 M, pH 5.2) and divided in two equal portions (2 x 5 ml). One of them

was subjected to overnight enzymic hydrolysis (see above), whereas the other incubated without enzyme under the same conditions, as a control. After the overnight incubation, both fractions were separately purified by passage through a Sep-Pak C_{18} cartridge, as previously described, and assayed for ecdysteroids by RP-HPLC.

HPLC Analysis of Ecdysteroids

The HPLC analysis was performed with a LC3XP liquid chromatograph pump (Pye Unicam Ltd., Cambridge) equipped with a Pye Unicam gradient elution system. The samples (from 2 to 10 µl) were introduced onto the column via a Model 7125 syringe loading microsample injector valve (Rheodyne Inc., Cotati). Certain analyses were performed by direct injection of the sample into the pressurized column through a self-sealing septum injector (Shandon Southern, Runcorn). Injection was with a microsyringe designed to withstand high pressure (SGE Ltd., London). The eluent from the column was monitored either at 254 nm with a Model 220 fixed-wavelength absorbance detector (Chromatronix Inc., Berkeley) or at 244 nm using a LC-UV variable-wavelength absorbance detector (Pye Unicam, Cambridge). The mobile phase was filtered through a porous stainless steel filter (porosity, 2 µm) and degassed by sparging it with a constant stream of helium. The chromatography was performed at ambient temperature ($21^{o} \pm 1^{o}C$).

Five different types of column were used.

(a) A reverse-phase column 10 cm x 5 mm I.D., packed with 5 µm particles of Hypersil ODS (Shandon Southern) was eluted under isocratic conditions (methanol-water, 45:55, v/v) at a flow rate of 1.0 ml min^{-1} (operating pressure 40 bar). For the ion-suppression analysis the

mobile phase was 20% methanol in glycine buffer (0.1 M, pH 2.6) and the flow rate 1 ml min^{-1}.

(b) A reverse-phase column 25 cm x 5 mm I.D. packed with 5 µm particles of Hypersil ODS (Shandon Southern, Runcorn) was eluted under isocratic conditions (methanol-water, 60:40, v/v) at a flow rate of 0.8 ml min^{-1} (operating pressure 85 bar).

(c) A 10 cm x 5 mm I.D. column (Shandon Southern) was slurry packed in methanol with 5 µm particles of ODS Spherisorb (Phase Separations, Clwyd) using a Magnus P6000 slurry-packing unit. Elution was carried out with a linear methanol gradient in 40 mM ammonium acetate buffer (pH 7) containing 3 mM TBA^{+}. The flow rate was 1 ml min^{-1} (operating pressure = 40 bar).

(d) A normal-phase column 25 cm x 5 mm I.D. packed with 5 µm particles of Hypersil (Shandon Southern) was run under isocratic conditions at a flow rate of 1.5 ml min^{-1} (operating pressure = 35 bar). Two different solvent systems were used: methylene chloride/isopropanol/water (125:30:2) and methylene chloride/methanol (95:5).

(e) A 10 cm x 5 mm I.D. column (Shandon Southern) was slurry packed in 15% methanol in methyl iodide with 5 µm particles of Hypersil (Shandon Southern) using a slurry packing tube (Shandon Southern) connected via an off-on valve to a model PSP-1 gas pressurised solvent pump (Chromatronic Inc., Berkeley). The mobile phase consisted of methylene chloride-methanol (93:7) and was pumped at a flow rate of 1 ml min^{-1} (operating pressure = 10 bar). Because of its poor efficiency, the column was not used for the biological samples analysis.

Quantitative Analysis

The quantity of hormone present in each unknown sample was calculated by comparing its peak area with a calibration graph which was constructed by plotting peak area against injected quantity in ng of pure ecdysone (Simes, Milan), 20-hydroxyecdysone (Simes) and 2-deoxyecdysone (D.H.S. Horn, Melbourne). A typical standard curve for ecdysone, 20-hydroxyecdysone and 2-deoxyecdysone is presented in Figure 32. For accuracy, comparisons were made between standard and unknown at similar retention volumes, since sensitivity decreases with increasing retention. The least detectable amount was 10 ng.

The concentration of the standard solutions, used for the calibration plot, was measured for each hormone by determination of the absorption of the solution at 244 nm in methanol using a SP8-100 ultraviolet spectrophotometer (Pye Unicam). The resultant optical density was divided by the appropriate extinction coefficient to determine the molarity.

Preparation of Trimethylsilyl Ethers of Ecdysteroids

Samples of authentic ecdysteroids and of the purified hormones, from the egg extract, were evaporated to dryness in Reacti-vials (1 cm^3) (Pierce and Warriner, Chester) and dried under vacuum over boiling acetone for 1 hour. Purified pyridine (65 µl) and trimethylsilylimidazole (TMSI, 35 µl) were added to the vials which were then sealed with screw caps and heated at 100°C for 1 hour. 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 more vigorous conditions were

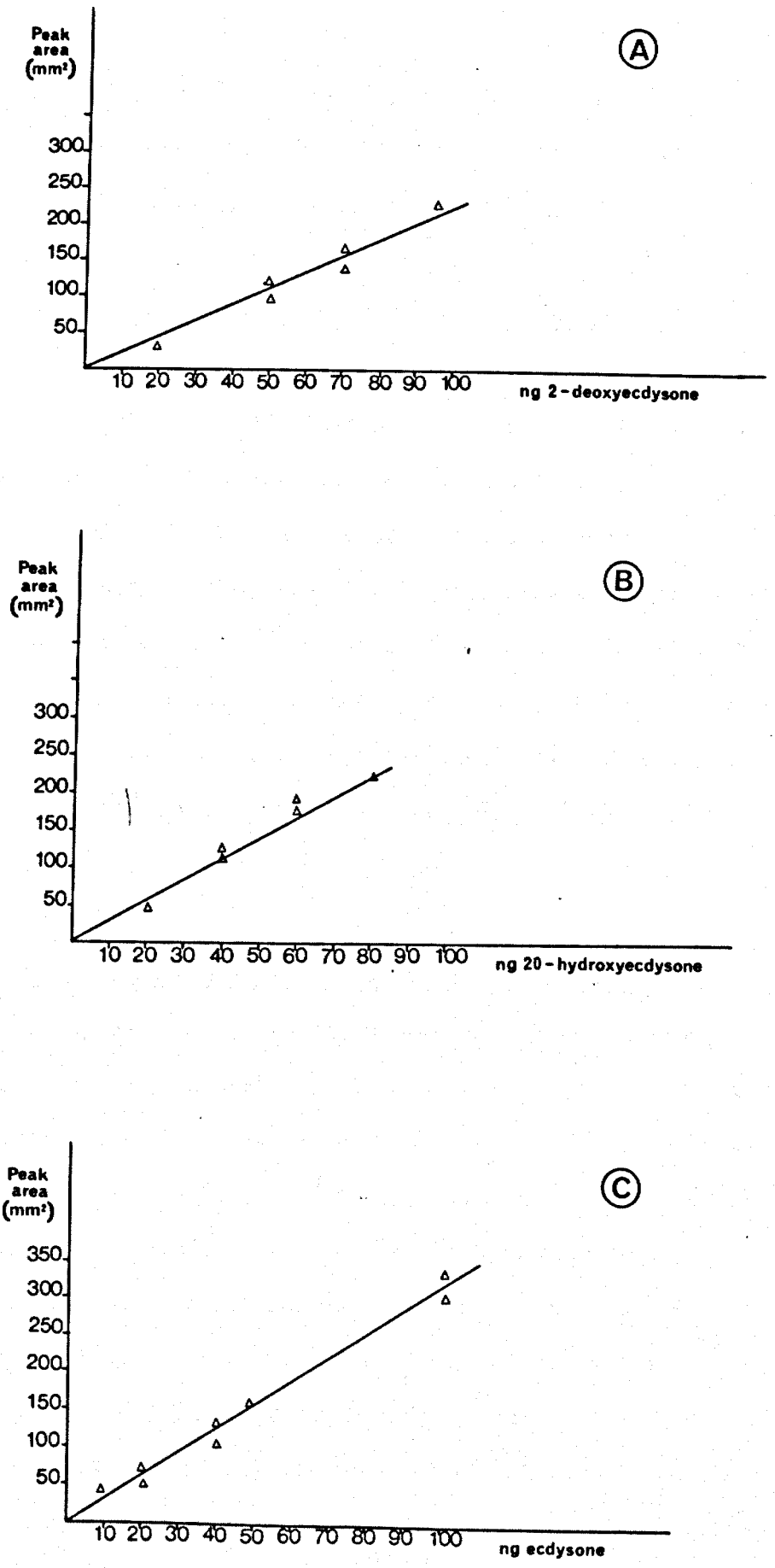


Figure 32. Calibration curves for 2-deoxyecdysone (A), 20-hydroxyecdysone (B) and ecdysone (C) determined by HPLC-UV.

required, such as heating at 140°C for 24 hours. The TMS ethers produced in this way were then either diluted with ECD grade toluene for GC analysis, or subjected to TLC.

Thin Layer Chromatography of the TMS Ethers of Ecdysteroids

The products of the silylation reaction, described above, were diluted with hexane (50 µl) and, after mixing, 10 µl of water was added in order to form a biphasic solution. The upper phase, containing the derivatized products, was removed and spotted on a silica TLC plate (5 x 20 cm) using a capillary tube. Solvent was evaporated from the silica with a hair dryer between applications. The plates were then developed in toluene-ethyl acetate (7:3). After development, the plates were dried with the aid of a hair dryer. Ecdysteroid derivatives were visualised under UV fluorescence.

Gas Chromatographic Analysis of Silylated Ecdysteroids

Gas chromatography was performed on a Pye Series 104 gas chromatograph equipped with a ^{63}Ni electron-capture detector and a Pye GCV variable-pulse amplifier. Conditions for analysis were: detector oven temperature, 300°C; injector temperature, 300°C; oven temperature, 280°C; nitrogen carrier gas flow rate, 60 ml min^{-1}. When the carrier gas was switched off, a purge of 15 ml min^{-1} of nitrogen was maintained through the detector. The column used was a 1.5 m by 4 mm silanized coiled glass column packed with 1.5% w/w OV-101 silicone phase on Chromosorb Q 100-120 mesh. Samples were injected directly "on column" with a microlitre syringe fitted with an 11 cm needle.

Mass Spectrometric Analysis

Mass spectrometry was carried out on a AEI MS12 single-focusing mass spectrometer in the solid sample inlet mode. The operating conditions were: ionizing energy, 70 eV; trap current, 60 μA; accelerating voltage, 7.4 Kv; electron multiplier, 3 Kv; solid inlet temperature, 240°C. Spectra were recorded in the 0-1,000 range with a resolution of 2 a.m.u. at 1,000 a.m.u. Authentic ecdysteroids and the purified hormones, from the biological sample, were dissolved in acetone. An aliquot of this solution was evaporated into a quartz probe and then directly introduced into the ionization chamber. When mass spectra of the TMS derivatives of ecdysteroids were taken, the silylation mixture was diluted with hexane (50 μl) and, after mixing, 10 μl of water were added. The upper phase was evaporated down, dissolved in acetone and introduced in the mass spectrometer as described above.

Comparison of HPLC and GC Methods for the Analysis of the Conjugates

After the butanol-water partition the aqueous layer, containing the conjugated ecdysteroids from insect eggs, was divided in two equal parts, which were then evaporated to dryness. One of them was redissolved in 0.1 M acetate buffer (5 ml, pH 5.2) to which 5 μl/ml of enzyme was added, the other one was redissolved in 0.1 M phosphate buffer (5 ml, pH 5.2) to which 25 μl/ml of enzyme was added. After the overnight incubation at 37°C, the acetate buffered fraction was analysed by HPLC as previously described, whereas the phosphate buffered fraction by GC as reported in Gande and Morgan.[53]

Inhibition of Enzymic Hydrolysis of Conjugates by Phosphate Ions

The aqueous phase, from the butanol-water partition, containing the polar ecdysteroid conjugates, was divided into two equal parts. The two portions were evaporated to dryness and redissolved, respectively, in 0.1 M acetate buffer (5 ml, pH 5.2) and 0.1 M phosphate buffer (5 ml, pH. 5.2). Both fractions were incubated overnight in the presence of Helicase (5 µl ml^{-1}) after which they were separately purified by Sep-Pak C_{18} cartridge and assayed for ecdysteroids by HPLC, as previously described.

Ion-Paired Reverse-Phase High Pressure Liquid Chromatographic Analysis of the Conjugates

The aqueous layer, from the butanol-water partition, containing polar ecdysteroid conjugates from eggs was reduced to dryness in vacuo at 40°C. The residue obtained was redissolved in a known volume of water (500 µl) and a portion of this solution (5 µl) was injected into a ODS Spherisorb HPLC column, eluting with a linear gradient (4% per minute) from 25% to 50% methanol in 0.04 M ammonium acetate buffer (pH 7). An amount of tetrabutylammonium hydroxide (TBA^+OH^-, 0.8 mg/ml) was added to the buffer solution to obtain a concentration of 0.003 M of counter ion in the mobile phase. The effluent from the column was monitored by UV detection at 244 nm.

Quantification of the conjugated ecdysteroids was obtained by reference to the calibration graph constructed for the free hormones.

Identification of the Ecdysteroids Present in the Conjugates

The column effluent corresponding to each of the conjugate peaks, in the previous section, was collected separately under

isocratic conditions. The solvent was removed by freeze-drying and the residue redissolved in 100 mM acetate buffer (5 ml, pH 5.2), hydrolysed with Helicase, purified by Sep-Pak C_{18} cartridge and assayed for ecdysteroids by HPLC. The identification of the individual hormones, obtained in this way, was confirmed by co-chromatography with the authentic compound on two different chromatographic systems, i.e. RP-HPLC and NP-HPLC.

Comparison of the RP-IPC and Enzymic Hydrolysis Methods for Conjugates Analysis

After the butanol-water partition, from extracts of eggs, the aqueous phase, containing conjugated ecdysteroids, was divided into two equal portions, which were evaporated to dryness. One of them was redissolved in water and analysed for conjugates by RP-IPC; the other portion was taken up in 100 mM acetate buffer (pH 5.2, 5 ml), subjected to overnight enzymatic hydrolysis, purified by Sep-Pak C_{18} cartridge and assayed for the amount of each of the hormones present by RP-HPLC with UV detection, as previously described.

RP-HPLC Analysis of Conjugates by Ion-Suppression

A portion of the aqueous phase, from the butanol-water partition, which contains the ionic ecdysteroid conjugates, was evaporated to dryness. The residue obtained was redissolved in a known volume of water (500 µl) and 5 µl of this solution were injected into a Hypersil ODS column and eluted isocratically with 20% (V/V) methanol in glycine buffer (0.1 M, pH 2.6). The eluate was monitored by UV detection at 254 nm.

The two major UV absorbing compounds were separately collected from the column, the solvent removed by evaporation and the residue obtained redissolved in 100 mM acetate buffer (5 ml, pH 5.2). The pH of the solution was then adjusted to 5.2 with aqueous NaOH and the Helix juice (25 µl ml^{-1}) added. After the overnight hydrolysis the incubation medium was purified by passage through a Sep-Pak C_{18} cartridge and assayed for ecdysteroids by HPLC, as in the previous section.

Procedure for the Analysis of Free and Conjugated Ecdysteroids in the Separated Embryos and Yolks

Groups of 100 eggs at the same stage of the embryonic development (according to the scheme of Sbrenna-Micciarelli[180]) were separated into embryo and yolk and, from these, the free and conjugated ecdysteroids were extracted and purified by a modification of the method previously reported for the analysis of the whole eggs. Briefly, the biological sample, to which 2-deoxy-20-hydroxyecdysone (500 ng) was added as internal standard, was ground in methanol with a tissue grinder and the resulting slurry filtered through a sintered glass funnel (porosity 3). The filtrate was evaporated down and partitioned between countersaturated n-butanol (30 ml) and water (30 ml). Each fraction was backwashed with a small volume of the appropriate counter phase which was added to the main fraction. In this partition, as before, the free ecdysteroids were found in the butanol phase, whereas the polar conjugates were in the water phase.

The latter was reduced to dryness in vacuo at 40°C. The residue obtained was redissolved in a known volume of water (500 µl)

and analysed for conjugates by RP-IPC, as previously described.

The butanol phase, containing the free ecdysteroids, was evaporated in vacuo on a rotary evaporator at 40°C. The residue obtained was redissolved in water (5 ml) and injected into a pre-washed (5 ml of MeOH and then 5 ml of H_2O) Sep-Pak C_{18} cartridge and eluted successively with 5 ml of 20% (v/y) methanol in water and 7 ml of 80% (y/y) methanol in water. The latter fraction, containing ecdysteroids, was then reduced in volume and analysed by NP-HPLC with UV absorption detection, as described earlier.

Determination of Enzymic Activities Contained in the Helix Pomatia Digestive Juice

Aryl sulphatase assay. The activity of aryl sulphatase was measured by a modification of the procedure of Roy[188]. Briefly, to acetate buffer (0.8 ml, 0.1 M, pH 5.2) and p-nitrocatechol sulphate solution (0.1 ml, 10 mg ml^{-1}, in acetate buffer) a dilute sample of enzyme (0.1 ml, in acetate buffer) was added. After incubation for 5 minutes at 37°C, the reaction was stopped and the colour developed by the addition of dilute sodium hydroxide (2ml, 0.1 M). The optical density was read in a UV-VIS spectrofotometer (Pye Unicam) at 520 nm against a suitable blank. The quantity of p-nitracotechol freed was estimated by reference to a calibration graph prepared using different concentrations of nitrocatechol (Fig. 33).

One Roy unit of arylsulphatase activity is defined as the amount of enzyme causing hydrolysis of 1 µmole of p-nitrocatechol sulphate per hour at 37°C.

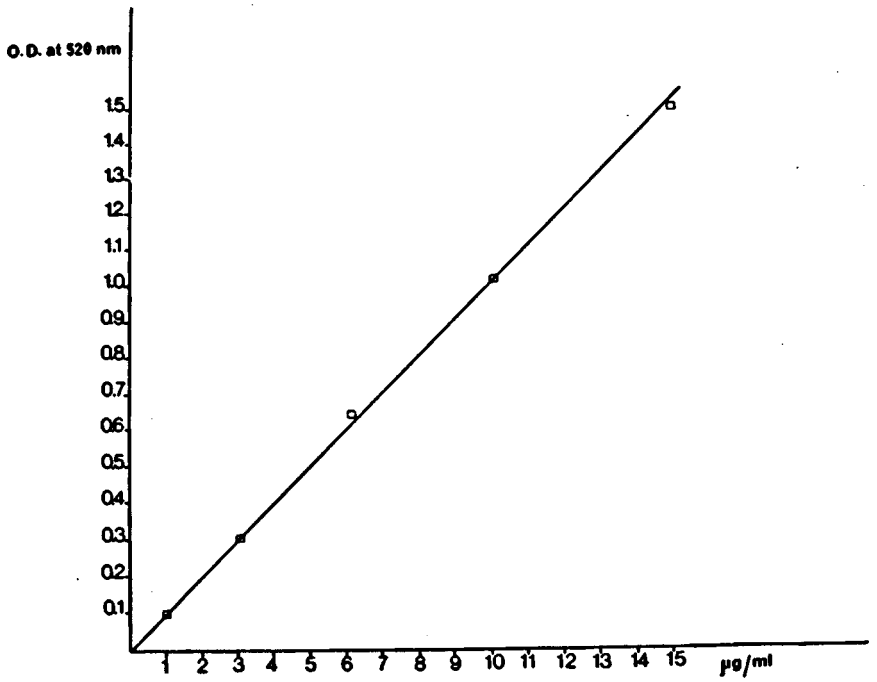


Figure 33. Optical density of p-nitrocatechol solutions

β-glucuronidase assay. The activity of the β-glucuronidase was determined by a technique developed from that of Levvy[183]. Phenolphthalein β-glucuronide (0.7 ml, 10^{-3}M in acetate buffer) was incubated with a dilute sample of enzyme (0.1 ml, in acetate buffer) for 30 minutes at 37°C in acetate buffer (0.7 ml, 0.1 M, pH 5). The reaction was stopped by the addition of glycine buffer (5 ml, 0.1 M, pH 10.4). The quantity of phenolphthalein liberated was determined by measuring the optical density of the resulting solution at 540 nm against a blank, and comparing the value obtained with a calibration graph prepared using known amounts of phenolphthalein (Fig. 34).

One Fishman unit of β-glucuronidase activity is defined as the amount of enzyme hydrolysing 1.0 µg of phenolphthalein β-glucuronide per hour at 37°C.

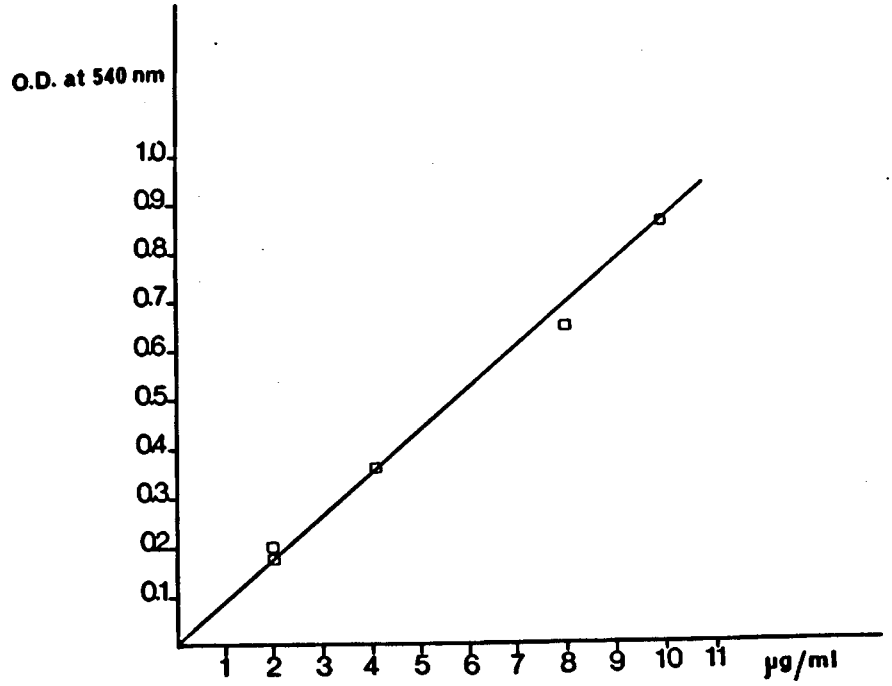


Figure 34. Optical density of phenolphthalein solutions.

Acid phosphatase assay. The activity of the acid phosphatase was determined by a modification of the method of Sommer[189], using a solution of p-nitrophenyl phosphate (0.5 ml, 4 mg ml^{-1} in buffer) in acetate buffer (0.5 ml, 0.1M, pH 5) to which a dilute sample of enzyme (0.1 ml in buffer) was added. After 30 minutes incubation at 37°C, NaOH (5 ml, 0.1M) was added to stop the reaction and the readings taken at 420 nm against a blank. The quantity of p-nitrophenol freed was estimated by reference to a calibration graph prepared with known concentrations of p-nitrophenol (Figure 35).

One unit of acid phosphatase activity is defined as that amount of enzyme which liberates 1 µmole of p-nitrophenol per hour, under the stated conditions.

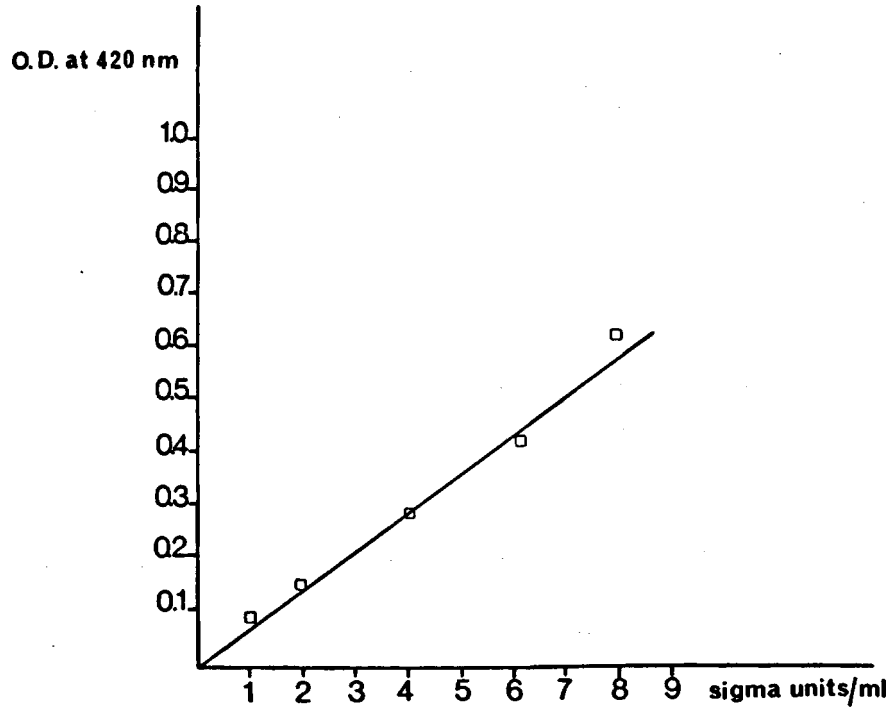


Figure 35. Optical density of p-nitrophenol solutions.

Measurement of Inhibition of β-Glucuronidase Activity by D-Saccharic Acid-1,4-Lactone

To 0.6 ml of 0.1M acetate buffer (pH 5.2) and 0.7 ml of phenolphthalein β-glucuronide solution (2.10^{-3} M in acetate buffer), 0.1 ml of buffer or D-saccharic acid-1,4-lactone (Sigma, London) solutions (in buffer) were added. A diluted sample of Helicase enzyme (0.1 ml, in buffer) was then added to the previously mixed substrate and inhibitor and the solution obtained placed in a shaking bath at 37°C. After 1 hour, 0.1M glycine buffer was added (5 ml, pH 10.4) and the liberated phenolphthalein determined as previously reported. Before being added to the incubation mixture, inhibitor solutions were adjusted to pH 5.2 with NaOH and suitably diluted. The final concentration of D-saccharic acid-1,4-lactone

ranged from 10^{-8} to 10^{-2} M. The activity was expressed relative to control determinations with no added inhibitor, this activity being taken as 100%.

Effect of D-Saccharic Acid-1,4-Lactone on the Rate of Hydrolysis of Conjugates

After the butanol-water partition, the aqueous layer, containing conjugated ecdysteroids, (2.8 μmoles) was evaporated to dryness, redissolved in 0.1 M acetate buffer (10 ml, pH. 5.2) and divided into two equal parts; to one of them digestive juice of Helix pomatia was added (5 μl ml^{-1}, to give 4,000 Roy units ml^{-1} of arylsulphatase, 500 Fishman units ml^{-1} of β-glucuronidase and 100 Sigma units ml^{-1} of acid phosphatase), to the other one D-saccharic acid-1,4-lactone was added (10^{-3} M after dilution in buffer). The pH of the buffered inhibitor solution was checked (glass electrode) and adjusted to pH 5.2, after which the enzyme (5 μl ml^{-1}) was added as in the other sample. Both samples were incubated for 1 hour at 37°C and then, separately, purified by Sep-Pak C_{18} and analysed by RP-HPLC, as already described.

Determination of Aryl Sulphatase, β-Glucuronidase and Acid Phosphatase Activities from Helicase at Higher Ionic Strength

The enzyme assays were carried out in 1 M acetate buffer (5 ml, pH 5.2) and 1.8 M acetate buffer (5 ml, pH 5.2) respectively, with an incubation time of 1 hour. All the other experimental conditions were kept as in the standard assays.

The activity of each enzyme was expressed relative to the control determinations containing 0.1 M acetate buffer (activity 100%).

Hydrolysis of Conjugates at Higher Ionic Strength

The aqueous phase, from the butanol-water partition, was divided into two equal portions which were evaporated to dryness and redissolved, one in 0.1 M acetate buffer (5 ml, pH 5.2) and the other in 1.8 M acetate buffer (5 ml, pH 5.2). Both samples were incubated for 1 hour in the presence of the same amount of enzyme and analysed for ecdysteroids by RP-HPLC.

Measurement of the Inhibition Effect of Sulphite Ions

To the incubation mixtures used for the test procedures of the enzyme activities a solution of Na_2SO_3 in acetate buffer (0.6 ml) was added to give a final concentration of sulphite ions of 2.10^{-1} M. Before being added to the hydrolysis medium, the inhibitor solutions were adjusted to pH 5.2 with acetic acid. The assays were carried out for each enzyme as reported above, and the results read from the appropriate calibration curves.

The activity was expressed as percentage of the values of the control determinations containing no inhibitor.

Measurement of the Inhibition Power of Phosphate Ions

The assay, for each of the three enzymes studied, was carried out in phosphate buffer (0.1 M, pH 5.2); all the other experimental conditions being as in the standard test procedures.

The activity was expressed relative to the control determinations containing acetate buffer (0.1 M, pH 5.2), activity 100%.

Measurement of Enzyme Activity at Different pH

The varying pH values were obtained with acetic acid - sodium acetate buffers (0.1 M).

For each of the three enzymic activities studied, the same amount of substrate and enzyme, used in the standard assays, was incubated for 1 hour at 37°C in the appropriate acetate buffer. The pH values ranged from 3.1 to 5.6. The control determinations were at pH 5.2.

Isolation of Juvenile Hormones from the Eggs of Schistocerca Gregaria

One day old eggs of the desert locust (5.7 g) were placed in a glass mortar containing methanol (45 ml), then the ethyl ester of JH-3 (50 ng per g of eggs) was added as internal standard and the biological sample homogenized using a glass pestle. The homogenate was filtered through a sintered glass funnel (porosity 3) and the cake returned to the mortar for a second homogenization. To the combined methanol filtrates (90 ml) water was added (30 ml) and the resulting mixture (3:1, methanol-water) was extracted twice with hexane (2 x 120 ml). The residue obtained by solvent removal, under reduced pressure, of the pooled hexane phases was redissolved in methanol (5 ml) and this methanol solution filtered through a pre-washed (10 ml of MeOH) C_{18} Sep-Pak cartridge. The eluent was concentrated to dryness, then dissolved in hexane (2 ml) and injected by means of a 5 ml gas-tight syringe (Hamilton Company, Reno) into a 10 mm i.d. x 45 cm IEC column (Whatman Ltd., Maidstone) dry packed with 40/63 µm particles of silica (Kieselgel 60, E. Merck, Darmstadt) and eluted with 80% hexane in diethyl ether. The solvent

was pumped through, using a MPL series II Micro-pump (Metering Pump Ltd., London) at a flow rate of 1.8 ml min^{-1} (operating pressure = 6 bar). The sample was introduced into the column via a Tefzel slider valve (Magnus Scientific Instrumentation Ltd., Sandbach). That fraction of the eluent which should contain JHs (as determined with authentic JHs) was collected, reduced to a small volume and analysed by HPLC, GC and GC-MS in the selected ion monitoring mode.

HPLC Analysis of JHs

The HPLC analysis was performed with a LC3XP liquid chromatograph pump (Pye Unicam Ltd) equipped with a Pye Unicam gradient elution system. The samples (from 2 to 10 µl) were introduced into the column via a Model 7125 syringe loading injector valve (Rheodyne Inc.). The column eluate was monitored at 225 nm with a LC-UV variable-wavelength absorbance detector (Pye Unicam). The mobile phase was filtered through porous stainless steel filters (porosity 2 µm) and degassed by a constant stream of helium. The chromatography was performed at ambient temperature (21 ± 1°C).

Two types of column were used:

(a) A normal-phase column 5 mm i.d. x 25 cm, packed with 5 µm particles of Hypersil (Shandon Southern) was eluted, under isocratic conditions with 7.5% dry diethyl ether in hexane at a flow rate of 1.2 ml min^{-1} (operating pressure = 21 bar).

(b) A reversed-phase column 25 cm x 4.6 mm i.d. packed with 5 µm particles of Spherisorb 5ODS (Severn Analytical, Gloucester) was eluted under isocratic conditions (80% methanol in water) at a flow rate of 0.8 ml min^{-1} (operating pressure = 80 bar).

The sensitivity of the HPLC with UV detector analysis was 10 ng of standard JHs.

Gas Chromatographic Analysis of JHs

Analyses by gas chromatography were performed on a Pye 104 chromatograph with flame ionization detector, using 1.5 m by 4 mm silanized coiled glass columns packed with either 3% W/W SE30 on Chromosorb W 100-120 mesh or 3% QF1 on CQ 100-120 mesh. Conditions for analysis were: detector oven temperature, 250°C, injector temperature, 230°C; column temperature, 200°C; nitrogen carrier gas flow rate, 60 ml min^{-1}. Samples were injected directly "on column" with a microlitre syringe fitted with a seven cm needle.

The least detectable amount was 20 ng of standard JHs.

Gas Chromatography with Selected Ion Monitoring Mass Spectrometry

Analysis by gas chromatography with selected ion monitoring mass spectrometry was performed with a Pye 104 gas chromatograph linked through a glass jet separator to a modernized AEI MS12 mass spectrometer.

For GC separations a 1.5 m by 4 mm packed column of 3% OV 101 on Chromosorb W 100-120 mesh was used. Other conditions were: injector temperature, 230°C; oven temperature, 200°C; helium carrier gas flow rate, 20 ml min^{-1}.

For mass spectrometry the GC-MS interface was kept at 250°C, and the ion source temperature at 280°C. Other conditions were: ionizing energy, 70 eV; trap current, 60 μA; accelerating voltage, 7.4 Kv; electron multiplier, 1 8 Kv. The ion at 81 mass units was selected by variation of the magnet current.

Samples were introduced into the mass spectrometer via the GC inlet, using a microlitre syringe fitted with a 7 cm needle.

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 was 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^{o} at 12 mm Hg. Average yield, 85%.

Synthesis of the Ethyl Ester of JH-3

JH-3 was transesterified according to Mori et al.[187] Briefly, authentic JH-3 (1 mg) was placed in a 0.3 cm Reacti-vial (Pierce & Warriner) containing 95% ethanol (200 µl) and potassium cyanide (0.1 mg). The vial was then sealed with a screw cap and heated at 70^{o}C in an oven for 24 hours.

The ethyl ester of JH-3 was purified by RP-HPLC as following. Several 10 µl portions of the reaction mixture were injected into a Spherisorb 5 ODS column eluted with 80% methanol in water at 0.8 ml min^{-1}. The eluent corresponding to the JH-3 ethyl ester peak was collected from the column, evaporated to dryness, under reduced pressure, and redissolved in hexane for storage. Yield obtained, 60%.

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