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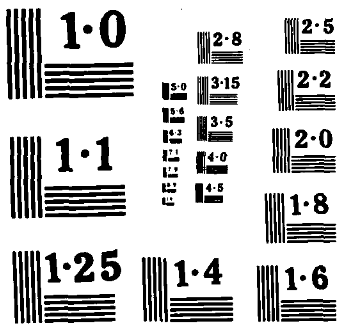
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ANNUAL TECHNICAL REPORT NUMBER 5

HORMONAL INTERFERENCE WITH PHEROMONE SYSTEMS IN

PARASITIC ACARINES, ESPECIALLY IXODID TICKS

By

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The biosynthesis and metabolism of ecdysone was investigated in the ticks Dermacentor variabilis and Hyalomma dromedarii. Following synthesis from cholesterol, ecdysone is metabolized to 20-hydroxyecdysone and, subsequently, a spectrum of predominantly apolar ecdysteroids. Despite the large variety of ecdysteroids present in these ticks, only one active hormone, 20-OH ecdysone, occurs. Most of the ecdysone and 20-OH ecdysone is conjugatqed with unknown compounds, resulting in much more apolar compounds. Saponification or

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered) treatment with esterases resulted in breakdown of the apolar compounds, releasing 20-OH ecdysone and other more polar unknown compounds. The The results implicate esterification of the tick ecdysteroids with fatty acids. However, no evidence of fatty acids was found in the hydrolysates following saponification or enzymatic hydrolysis.

The matabolism of exogenous juvenile hormone (JH) was also investigated by incubating authentic ^{3}H JH with tick hemolymph. Chromatography of the products revealed 2 labelled metabolites, characteristic of the acid and diol. These findings suggest the presence of JH specific enzymes as well as general esterases capable of hydrolyzing this hormone. The variety of tick hemolymph proteins, including esterases, is described.

The potential use of ecdysteroid analogues to disrupt development and sex pheromone activity was investigated. Previous tests with the analogue BSEA-28 indicated substantial reduction in the ecdysial period in H. dromedarii nymphs and some disruption of feeding, as expressed by reduced body weight. Treatment with the analogue BSEA-1 (22,25 dideoxyecdysone) led to increased nymphal mortality following feeding, but only slight changes in the ecdysial period. No evidence of a hormonal effect was observed.

New evidence of a JH-like gonadotropic hormone was found in mites and ticks. Studies done by Dr. Oliver and his colleagues demonstrated partial loss of reproductive capacity in Dermanyssus gallinae treated with Precocene-2, and partial restoration of reproductive capacity by application of JH-III to the treated mites. In Ornithodoros parkeri, inoculation of JH stimulated vitellogenesis, but not oviposition. Preliminary evidence also implicated catecholamines in the initiation of vitellogenesis. Hemocoelic injections of male reproductive tissues into fed virgin females stimulated egg maturation and oviposition. Significant but less pronounced stimulation was also induced by inoculation of male salivary gland homogenates, but not by synganglial homogenates. Vaginal insertion of these extracts also induced oviposition. Other studies concerned the weight relationships between nymphal stages and subsequent stages and the processes of permatogenesis and spermiogenesis. The number of chromosones, autosomes vs. sex chromosomes, and the genetics of parthogenesis is reviewed in several species.

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I. INTRODUCTION

This report reviews new progress in our studies on hormones in ticks and their role in regulating sex pheromone activity and other basic physiological and developmental phenemona in ticks. Our finding that ecdysteroids, particularly 20-hydroxyecdysone (20-OH ecdysone), excites 2,6-dichlorophenol secretion and increases biosynthesis is a major new discovery, the importance of which is buttressed by evidence of ecdysteroid regulation of sex pheromone biosynthesis in the housefly. In the latter case, as reported by Blomquist and his colleagues, 20-OH ecdysone alters the biosynthesis of cuticular hydrocarbons, leading to the production of Z-9 tricosene and subsidiary pheromone components. Thus, in 2 widely disparate arthropods, the hormone 20-OH ecdysone regulates the same fundamental event, sex pheromone production. Presumably, this process is widespread throughout tne phylum. Knowledge of the role of gonadotropic hormones, the regulation of 20-OH ecdysone production, and site(s) of hormone biosynthesis, take on increasing importance in ticks.

In view of the role of 20-OH ecdysone in regulating sex pheromone activity, it became increasingly important to determine the metabolism of this hormone and its parent, ecdysone. Of special interest was the question of the kinds of metabolites that are produced, whether the same metabolic pathways found in insects occur in ticks, and whether the active hormone is conjugated or esterified and retained for later use, inactivated by catabolic pathways and conjugated or esterified for elimination. We were also interested in the role of juvenile hormone (JH), and whether it persisted in the animal, or was also metabolized rapidly into inactive metabolites and eliminated.

Much of the work of the past year was dedicated to study of the metabolism of the hormones, 20-OH ecdysone and JH. Early in the course of these investigations, we discovered that ecdysone was converted into 20-OH ecdysone and several unknown ecdysteroids, the majority of which were much less polar than the parent molecule. Using radiotracer methods, we were able to demonstrate most of the tritium labelled molecule was converted into a group of at least 2 and possibly 3 apolar fractions of unknown identity, metabolites that were non-reactive in our RIA. Much smaller proportions were converted into polar metabolites, at least 2 of which were immunoreactive. These findings paralleled evidence of similar types of ecdysteroids in the soft tick, Ornithodoros moulata by Swiss workers. Recently, two important reports by workers in England and Switzerland have described these metabolites, particularly the apolar fractions, which represent a novel class of ecdysteroids in which the parent molecule is esterified with fatty acids. Thus, ticks appear to utilize a very different pathway for inactivation of ecdysone or 20-OH ecdysone than insects, which inactivate these hormones by hydroxylation, oxidation and epimerization and conjugation to form relatively polar metabolites. The details of our work on this important aspect of tick hormone biology and comparisons with the findings of other workers are described in one of the sections of this report.

The significance of these new discoveries on ecdysteroid metabolism for our understanding of physiological processes in ticks is enormous. Instead of being viewed merely as inactive metabolites destined for excretion, the fatty acyl esters of the hormones may accumulate in cells and tissues, dissolving readily in membranes, and release active hormone when cleaved by esterases in the cytosol. Moreover, this process probably occurs in the target tissues, and the finding of these metabolites in specific body organs

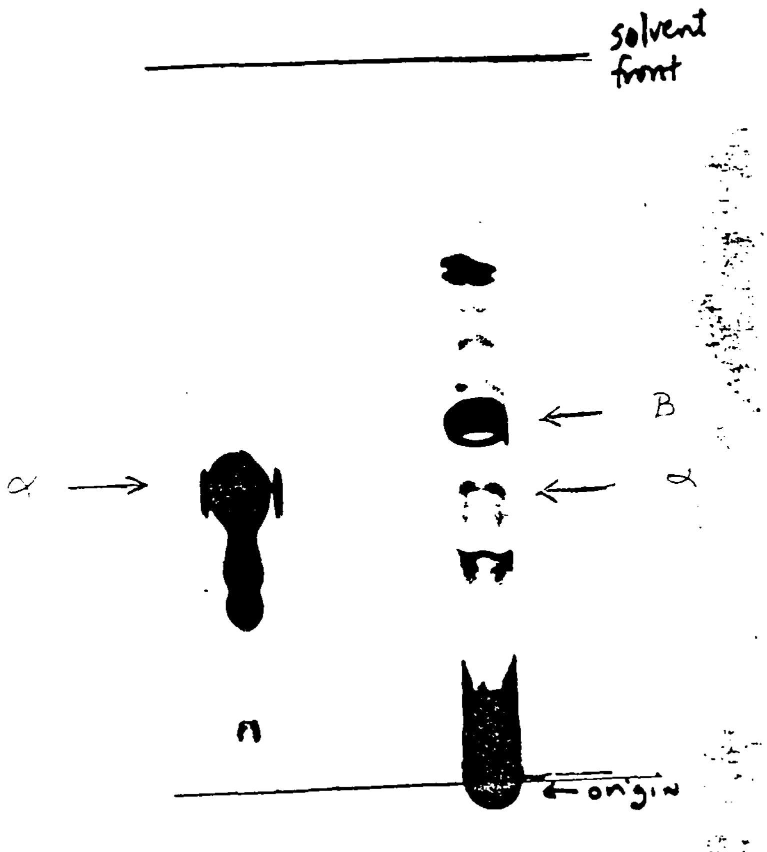

Figure 3. X-ray autoradiograph illustrating the distribution of ^3H radioactivity in a crude extract developed on a C-18 TLC plate. The hand lane shows the distribution of radioactivity in the standard, ecdysone, representing a sample containing 0.01 uCi. The dominant spot, representing ^3H ecdysone migrates together with the authentic standard. The right hand lane shows the distribution of radioactivity in a sample of 30 ul containing an estimated 0.02 uCi of ^3H radioactivity. The location where ecdysone (α) and 20-OH ecdysone (β) migrate are shown by the arrows.

peak of activity was found immediately following the retention time of ecdysone. No evidence of Makisterone, 22, 25-DDE, or Ponasterone was found in any of these extracts. The highest concentration of radioactivity was found in a group of apolar fractions eluting from approximately 32 to 40 minutes post-injection (Fig. 2). When the chromatographic separations were repeated using the tris buffer system in the mobile phase, these fractions were resolved into two dominant peaks of radioactivity (see below).

Metabolism of ^{3}H ecdysone. Fig. 3 is an X-ray autoradiograph illustrating the distribution of radioactive substances in an aliquot of the ^{3}H ecdysone extract vs. the ^{3}H ecdysone standard. The samples were chromatographed on a C-18 TLC plate. Some degradation has taken place in the standard, despite the use of re-purified (by TLC) material, especially the formation of 2 apolar substances. Nevertheless, many more radiolabelled compounds appear in the extract. The most intense radioactivity is at the origin, indicating one or more apolar substances, in the zone immediately above the origin, and in the spot that co-chromatographs with ecdysone (arrow). A weakly active spot co-chromatographs with 20-OH ecdysone and is probably this compound (symbol). Some activity also occurs near the solvent front (very polar); however, the radioactive substances less polar than ecdysone (symbols) coincide with the breakdown products seen in the ecdysone standard.

To determine the polarity characteristics and solubilities of the compounds in the ecdysteroid extracts, the TLC was repeated using a variety of different solvent systems. Table 2 summarizes the results of these assays. When the extract was developed on C-18 with methanol and water, 65:35, 47.9% of the radioactivity remained at the origin while the second highest amount,

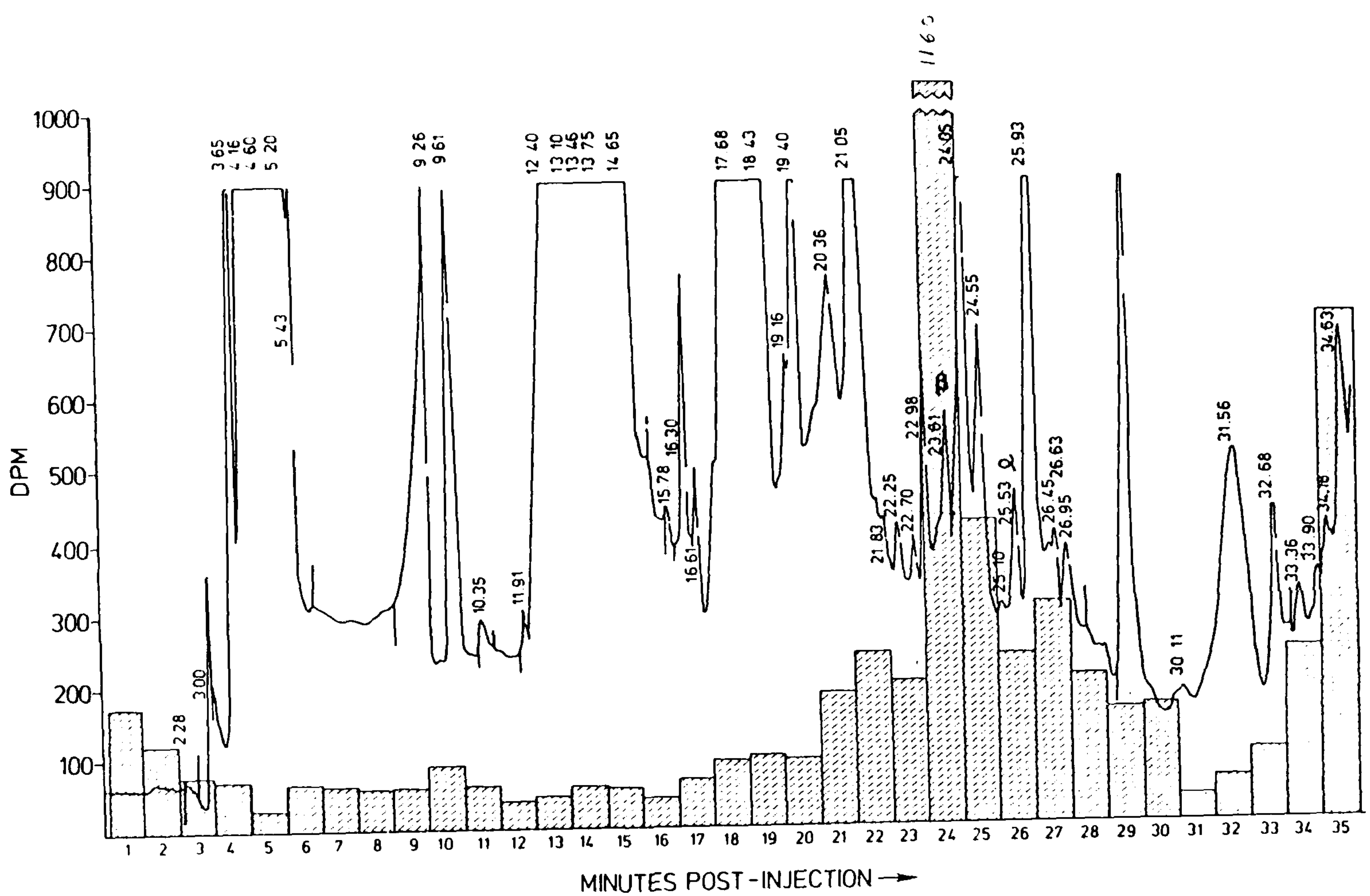

Figure 2. Distribution of ^{14}C radioactivity in 1 min collections superimposed on an HPLC chromatogram to illustrate the distribution of radioactivity in relation to the location of sample peaks. The sample was a crude extract made from adults inoculated as engorged nymphs (pooled samples). The retention times for "B" (20-OH ecdysone) and "a" (ecdysone) are shown on the chromatograms. HPLC conditions were: linear gradient from 100:0 water: methanol to 35:65 water:methanol over 20 minutes, constant conditions from 20 to 25 minutes, 0:100 water:methanol, 25 to 35 minutes, returning to 100:0 water:methanol from 35 to 50 minutes.

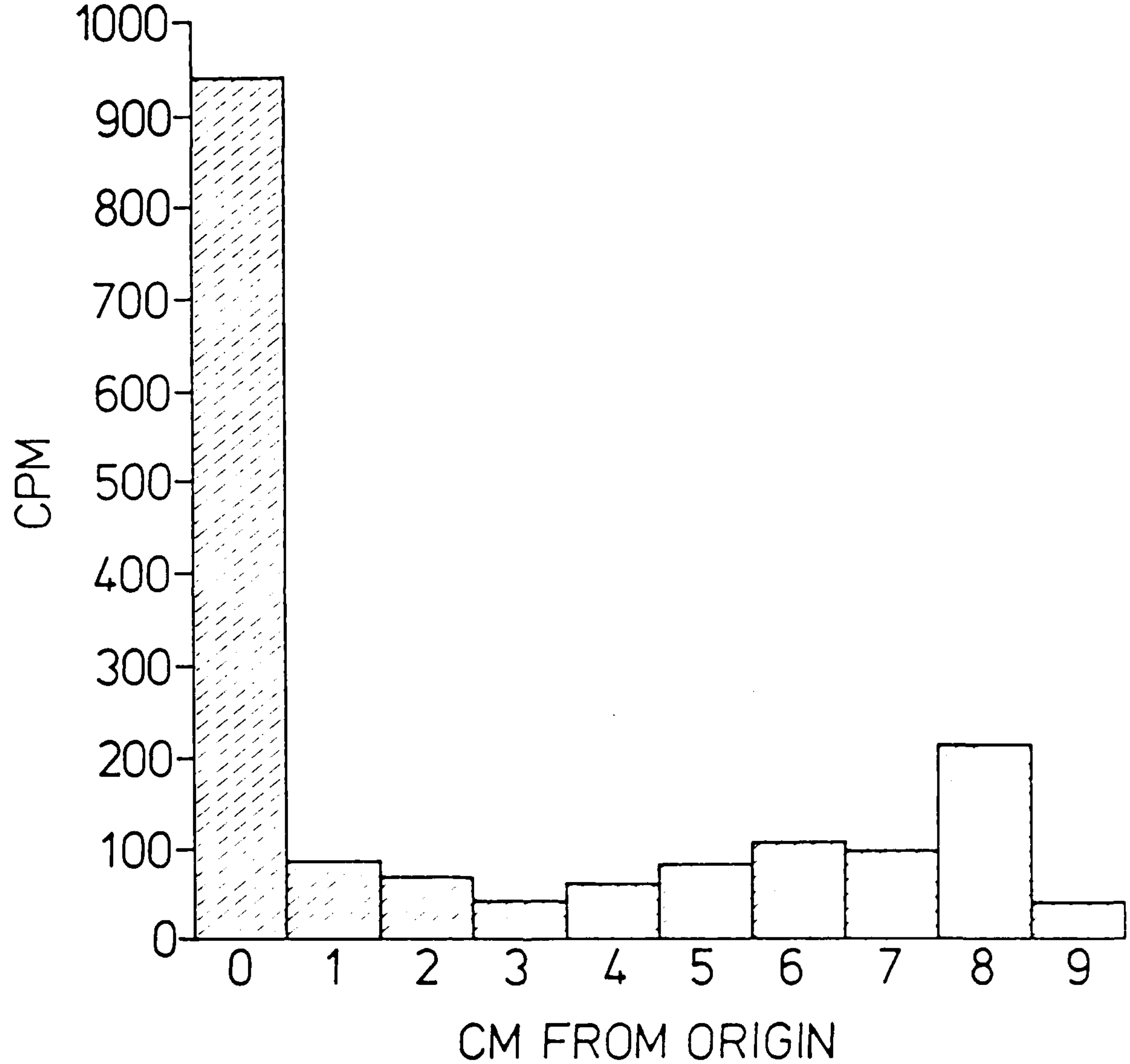

Figure 1. Histogram illustrating the distribution of ^{14}C radioactivity in H. dromedarii males inoculated with ^{14}C cholesterol as engorged nymphs. The crude extract was spotted onto a reversed phase (C-18) thin layer chromatography (TLC) plate and developed with 65% HPLC grade methanol:35% distilled water, v/v. Radioactivity was determined by scraping the silica-C-18 from 1 cm zones in each lane, depositing the material in scintilant solution and assaying for radioactivity with a Beckman LS-250 liquid scintillation spectrometer.

8, these 2 ecdysteroids represent 7.0% of the total radioactivity, in contrast to only 3.0% on day 1. Radioactive compounds more polar than 20-OH ecdysone also occurred, and increased in amount during the 8 day period; from 7% on day 1 to 15.6% on day 4 and 16.1% on day 8.

Fig. 1 illustrates the distribution of radiolabelled material when an aliquot of an extract of 10 H. dromedarii males was spotted onto a C_{18} TLC plate. These males were inoculated with ^{14}C cholesterol as engorged nymphs. Most of the radioactive material, 54.5%, remained at the origin, although this was much less than in the nymphs. Radioactive spots were found in zones 4 and 5, co-chromatographing with ecdysone and 20-OH ecdysone, respectively. Unidentified highly polar radioactive compounds, more polar than 20-OH ecdysone, were found; an unusually large concentration, 12.2% of all radioactive material, occurred at zone 8, near the solvent front. The radioactive compounds more polar than 20-OH ecdysone increased to 26.0% in the males. The test was repeated 2 X with similar results.

HPLC was used to separate and collect fractions from female and male H. dromedarii extracts for radioassay. To facilitate the detection of significant radioactivity, the extracts were pooled and collected as a single extract. Fig. 2 shows the radioactivity found in the different fractions superimposed on the HPLC chromatogram. The highest count was in the fraction coinciding with the elution of 20-OH ecdysone, 928 CPM, eluting at 23.61 min., representing approximately 11.5% of all of the radioactivity in the entire sample. Traces of radioactivity occurred in fractions eluting as early as 10 minutes, and another small surge of activity occurred at 17 and 18 minutes. A significant rise was noted with a peak at 21 minutes, followed by the very sharp rise coincident with 20-OH ecdysone. The small peak of 14-C activity at 21 minutes represents unknown polar compounds. A second

Results

^{14}C incorporation into ecdysteroids. Tests were done using extracts of engorged nymphal ticks killed 1, 4, or 8 days post-injection. Table 1 compares the distribution of radioactivity in the different locations on the

Table 1. Distribution of ^{14}C radioactivity in different 1 cm zones on reversed phase (C-18) TLC plates spotted with extracts of *H. dromedarii* nymphs killed 1, 4, and 8 days post-inoculation.

	Percent Radioactivity		
cm from origin	Day 1	Day 2	Day 3
0	85.7	70.0	75.4
1	3.0	4.9	6.1
2	1.7	2.9	3.3
3	1.4	4.1	3.3
4	1.6	3.0	4.2
5	1.4	2.6	2.8
6	1.6	4.8	3.3
7	1.4	3.4	4.7
8	4.0	3.9	4.5
9	1.7	3.5	3.6

TLC plates in the 3 different age groups. Most of the radioactivity remained at or near the origin, primarily due to ^{14}C cholesterol which does not move in the solvent system used. On day 1, 85.7% of the radioactivity remained at the origin, and only 1.6% and 1.4%, co-chromatographed with ecdysone or 20-OH ecdysone, respectively. On days 4 and 8, the amount of radiolabelled compounds remaining at the origin declined, whereas the amount that co-chromatographed with ecdysone and 20-OH ecdysone increased. Thus, by day reinjected to determine fraction purity, and purified as required. The process was continued until aliquots estimated to contain 50 ug of each fraction were collected, dried, and reconstituted in a mixture of deuterated methanol and deuterated acetone. Nuclear Magnetic Resonance (NMR) was done with a Bruker WH-400 Fourier Transform NMR (Magnetic Resonance Laboratory, University of South Carolina, Columbia, S.C.). Proton NMR spectra were obtained for 3 fractions more polar than 20-OH ecdysone as well as the authentic 20-OH ecdysone and ecdysone standards. Other aliquots were dried and ground in KBR for infrared studies. Infrared spectroscopy was done with a Nicolet Model 5000 (Minneapolis, MN) (Department of Chemical Sciences, Old Dominion University). The same fractions noted above, as well as 2 other fractions less polar than 20-OH ecdysone, was assayed. Finally, aliquots were injected onto a Whatman 5 um Partasil S-ODS-3 RAC semi-preparative column, 9.4 cm O.D. by 10 cm long (Whatman Chemical Separations, Inc., 9 Bridewell Place, Clifton, NJ), and collected as described above. These fractions were submitted for Mass Spectrometry using the Fast Atom Bombardment attachment at Squibb Pharmaceutical Laboratories, Princeton, NJ. Both FAB and negative ion spectra were collected.

To test for the presence of amine radicals, the extracts were chromatographed by TLC and the plates sprayed with ninhydrin. In addition, the fractions separated by TLC were saponified, the aqueous extracts derivatized (OPA) and the derivatized products analyzed by HPLC. Authentic standards were prepared by derivatizing amino acids and determining their specific retention times with the same HPLC parameters. Comparisons were made with unsaponified extracts of the same TLC fractions. Controls were done with solvents collected from TLC plates that were spotted and developed with the same solvent systems.

Gas chromatography. Aliquots of the saponified extracts were injected on a Varian series 1400 gas chromatograph with a flame ionization detector. The glass column, ___ M x -- mm., was packed with 3% SE-30 on Chromsorb 80/100 mesh. Column temperature, injector port temperature gas flow rates, etc., all to be spelled out. The detector signal was integrated by a Shimadzu CR3A chromatopac integrator.

Radioimmunoassay (RIA). This was done as described by Dees et al. (1984). The Horn I-2 ecdysone antiserum (from J. D. O'Connor, UCLA) was used in the assay.

Enzymatic hydrolysis. To determine whether the more polar compounds in the tick extracts that reacted in the radioimmunoassay were sulfate or glucuronate conjugates, 100 ul aliquots of the crude extracts were dried, reconstituted in 10% methanol and loaded onto preconditioned c-18 Sep Paks (Waters). The contents were eluted with 5 ml volumes of increasing proportions of HPLC grade methanol in water, namely, 10%, 30%, and 100%. The eluates were concentrated and aliquots assayed by HPLC; the remainder was dried, reconstituted in borate buffer, and assayed by RIA. The process was repeated but the reconstituted eluates were subjected to enzymatic hydrolysis with a mixture containing of beta-glucuronidase, types H-1 and L-II (with sulfatase activity), and beta-glucuronidase type-1 (no sulfatase activity) in a ratio of 7.4 mg, 7.4 mg, and 4.0 mg, respectively (Sigma). The 3 eluates were incubated with the enzyme mixture at pH 5 (0.2M acetate buffer) for 24 hrs. The reaction was stopped by the addition of methanol: butanol, 1:3, v/v, dried (N_2), reconstituted and assayed as described below.

Structural analysis. Aliquots of the non-radioactive extracts were pooled and injected into the HPLC. Fractions were collected at 1 min intervals with a Pharmacia Frac-100 collector. Tne fractions were concentrated, and the different solvents separated for assay.

Liquid scintillation spectrometry. This was done with a Beckman Model LS 250 spectrometer with automatic quench compensation. Quench corrections were applied where appropriate.

Autoradiography (AR). To detect the presence of labelled metabolites on TLC plates, the developed plates were sprayed with Enhance (NEN) and exposed to X-ray film (Kodak Exomat no screen AR-2, 20 x 25 cm) for 3 days at -80°C.

High Performance Liquid Chromatography (HPLC). This was done with a Waters System comprised of a pair of Model 510 pumps, Model 721 System Controller, Model 730 Data Module, Model U6K septum-less injector, a Z-module for column support and a Model 441 fixed wave length detector equipped with a 254 nm filter (Waters & Assoc., Milford, MA). For analytical work, the column was a 5-um Novapak C-18 cartridge, 8 mm. I.D. by 15 cm long. The solvents were HPLC grade methanol and water, without buffers. Although a variety of isocratic and gradient mixtures were used, optimal separation was achieved with the following gradient, at 1 ml/min: (1) 0% to 65% methanol, from 0- 20 min; (2) constant conditions from 20 - 25 min; (3) 65% to 100% methanol, from 25 to 35 min; (4) 100% to 0% methanol, from 35 to 50 min. Separations were improved by use of a 20 mM Tris/$HClO_4$ buffer as suggested by LaFont (pers. commun.). Samples were filtered (Acrodisc filters, 0.2 um, Gelman Sciences, Inc., Ann Arbor, MI) prior to injection. The column was flushed for 10 -20 min between injections. All assays of each sample were repeated at least 3 X. Ecdysone and 20-hydroxyecdysone were identified by comparison of their retention times with authentic standards by observing their co-elution when co-injected with the standards, and by RIA (see below).

surviving the inoculations were killed and extracted for ecdysteroids 1, 4 and 8 days after inoculation. Other treated nymphs were allowed to molt and the adults extracted separately by sex as described previously. In another experiment, ^{14}C cholesterol (0.42 uCi/ tick) was injected into 50 partially fed feeding females on day 3 of feeding, detached on day 7, and the 28 surviving individuals extracted for ecdysteroids. The ^{32}P solution, in Shen's saline, was also inoculated into ca 90 attached feeding ticks, ca. 10 uCi/tick, on day 3, detached on day 7, and the surviving 85 individuals extracted for ecdysteroids. Finally, the ^{14}C acetate in Shen's solution was inoculated into engorged nymphs on the day of drop off, ca. 1 uCi/tick, allowed to molt, and the unfed females extracted for ecdysteroids.

Thin layer chromatography (TLC). Aliquots of the tick extracts were applied to (1) silica gel plates, IB2, 20 x 20 cm (Arthur H. Thomas, Philadelphia, PA) and chromatographed with petroleum ether:diethyl ether:acetic acid, 80:20:1, v/v (Kates, 1972) (this same system was used to assay for free fatty acids), to (2) reversed phase C-18 plates (J. T. Baker Co., Phillipsburg, NJ) and chromatographed with methanol:water, 65:35, v/v, and (3) microcrystalline (cellulose) plates (Eastman Kodak Co., Rochester, NY) and developed with butanol:acetic acid:water, 80:20:20, v/v, for free amino acids Visualization was done long wave UV or 50% H_2SO_4 (spray), or, in the case of (3), with 0 04% Ninhydrin in acetone. UV spectrometry of samples eluted from the TLC plates was done with a Beckman Model DU scanning UV spectrometer.

Saponification. The material remaining at the origin following TLC on C-18 plates (with methanol:water) was eluted, dried (N_2) redissolved in a mixture of benzene and sodium methoxide, heated at 50°C, and extracted with diethyl ether:water, 1:1. The mixture was mixed (Vortex mixer), centrifuged specific radioactive compounds as described below.

Chemicals. All solvents, including those used for extraction (except ethanol), were HPLC grade (Burdick & Jackson, Muskeegan, MI). Authentic standards included ecdysone, 20-hydroxyecdysone (20-OH ecdysone), makisterone A (Sigma Chemical Co., St. Louis, MO) and inoksterone (from M. J. Thompson). ^{3}H ecdysone (80 mCi/mmol) (New England Nuclear, Boston, MA) (NEN) was dried under N_2, reconstituted in methanol, and assayed by thin layer chromatography (TLC) as described previously (Dees et al. 1984a); ^{14}C cholesterol (C-4 position, 50-60 mCi/mmol, NEN) was dried under N_2 and assayed by TLC on Bakerflex IB-2 silica gel plates, 250 um (benzene:ethyl acetate, 9:1, v/v). ^{32}P orthophosphoric acid (mCi/mmol, NEN) and ^{14}C acetic acid (mCi/mmol, NEN) were dried (separately) and reconstituted in Shen's saline (Oliver, 1974?). ^{3}H-ecdysanoic acid (eoic) and 20, 26-dihydroxyecdysone (20, 26E) were obtained as a gift from Dr. R. LaFont); 22, 25-dideoxyecdysone (22, 25 DDE) and Ponasterone were obtained as a gift from Dr. J. L. Connat.

Inoculations. Labelled ecdysone was purified by TLC, reconstituted in Shen's solution and inoculated (3 ul/tick) into attached feeding adult virgin females (while still attached) on day 3 of feeding. To facilitate the treatment, the rabbit host was tranquilized (Acepromazine, AVECO Co., Fort Dodge, IA) and the ticks inoculated using a 50 ul Hamilton syringe and 30 gauge needle. A total of 57 ticks were inoculated with 0.5 uCi/tick. The treated ticks were detached 4 days later and extracted in accordance with procedures described previously (Dees et al. 1984a). The ^{14}C cholesterol was dried and reconstituted in a mixture of ethyl oleate and olive oil, 1:1, v/v. Aliquots (3 ul) containing ca. 0.25 uCi were inoculated into 200 engorged nymphs on the day of drop off. Groups of 10 nymphs as conjugation with phospate (Isaac et al. 1984) and the formation of highly polar, non-hydrolyzable ecdysanoic acids (LaFont et al. 1983). In contrast to the sulfate or phosphate conjugates, which may provide a source of active hormone by enzymatic hydrolysis in a later life stage, conversion to the acid forms appears to be irreversible.

In ticks, a mixture of polar and apolar compounds have been reported which may be endproducts of ecdysteroid metabolism. In the soft tick, Ornithodoros moubata, Bouvier et al. 1982 described evidence implicating the presence of 20, 26-dihydroxyecdysone, as well as a mixture of metabolic endproducts much less polar than ecdysone, but no C-26 acids. In Boophilus microplus, the major metabolites were found to be a mixture of unknown fatty acyl esters of ecdysteroids (Wigglesworth et al. 1985).

This study was undertaken to determine the metabolic fate of ecdysteroid hormones in adults of the camel tick, Hyalomma dromedarii Koch.

Materials and Methods

Ticks. The camel tick, Hyalomma dromedarii, was colonized from a stock originally from the U.S. NAMRU-3 Medical Zoology Department, Cairo, Egypt (HH No. 59723, U.S. APHIS license No. 9433) and reared as described previously (Dees et al. 1984b). Ticks were held in an AMINCO AIRE Climate Lab environmental chamber at 27 ± 0.5°C and 90 ± 2% RH during their non-parasitic periods.

Tick extracts. These were prepared from 20 partially fed virgin females, 20 attached feeding males (fed 8 days), or 100 engorged nymphs (whole body extracts), as described previously (Dees et al. 1984a, b). All extracts were reconstituted in 100% ethanol and stored at -5°C until needed for assay. Extracts were also prepared from specimens inoculated with

II. METABOLISM OF ECDYSONE AND 20-HYDROXYECDYSONE IN THE CAMEL TICK, *HYALOMMA DROMEDARII* (ACARI:IXODIDAE)

Introduction

The occurrence of the steroid hormone ecdysone and 20-hydroxyecdysone, has been demonstrated in several species of ixodid and argasid ticks (Solomon et al. 1982; Delbecque et al. 1978; Dees et al. 1984). These hormones are probably the common molting hormones in ticks (Solomon et al. 1982), functioning in a manner similar to their role in insects and crustaceans. The associations of these hormones with the ecdysial process in ticks was established by correlating the changes in hormone titre with cuticle synthesis during the nymphal-adult molt (Germond et al. 1982). Ecdyteroids are also believed to regulate vitellogenesis during the gonotrophic cycle (Solomon et al. 1982) and sex pheromone synthesis/secretion in the female (Dees et al. 1984).

Much less is known about the metabolism of ecdysone in ticks. In insects, ecdysone is hydroxylated by c-20 hydroxylase to 20-hydroxyecdysone, mostly in peripheral tissues such as the fat body. Ecdysone and 20-hydroxyecdysone may be hydroxylated further to 20, 26-dihydroxyecdysone. Ecdysone and 20-hydrozyecdysone may be degraded to their 3-dehydro forms (Riddiford and Truman, 1978), as well as 3-epi and/or 26-hydroxy derivatives (Dinan et al. 1981) and/or 2-deoxyecdysone and 2-deoxy- 20-hydroxyecdysone (Isaac et al. 1983). In some insects, e.g., the desert locust, *Schistocerca gregaria*, inactivation is also accomplished by the formation of highly polar conjugate, primarily as sulfates of ecdysone, 20-hydroxyecdysone, or their metabolites (Koolman et al. 1973), or as phosphate esters (Isaac et al. 1982, 1983), or even as adenosene monophosphoric esters (Tsoupras et al. 1983). Other routes of inactivation involve acetylation at the 3-position as well and tissues may provide a powerful new tool for determining the tissues that are affected by ecdysteroids. New evidence, presented in this report, suggests that apolar metabolites are also the predominant inactivation products in *H. dromedarii* and *D. variabilis*.

Similar efforts were directed to analysis of the metabolites produced by tick hemolymph and tissues in order to determine evidence of JH specific enzymes in these parasites. Direct assays for confirmation of JH-III in these ticks were done in collaboration with Dr. Baehr, as reported previously, and new studies were undertaken using RIA and mass spectrometry to confirm the identity of the hormone. Indirect methods were used in our program at Old Dominion University (ODU) to determine whether the metabolites of JH could be demonstrated in these ticks, and whether known JH precursors were utilized to synthesize the hormone. The results of these studies are also described in this report.

This report also reviews new studies with ecdysteroid analogues, especially 22,25 dideoxyecdysone and their effects on sex pheromone activity and developmental processes in ticks. As our present studies have shown, merely administering exogenous hormones to ticks is ineffective in view of the enzyme systems that inactivate these molecules. Use of analogues that are not susceptible to enzymatic degradation may offer new insights into hormone regulation of sex pheromone activity, if these analogues are active.

Table 2. Distribution of ^3H radioactivity following TLC of the ^3H ecdysone inoculated adult H. dromedarii extract with different supports and solvent systems.

System Cm. from origin	System No. 1 CPM	%	System No. 2 CPM	%	System No. 3 CPM	%	System No. 4 CPM	%	System No. 5 CPM	%
0	21026	47.9	3962	33.3	226	63.8	51	0.8	63	1.1
1	3733	8.5	2002	16.9	21	5.9	45	0.7	27	0.5
2	2980	6.8	2426	20.4	11	3.1	50	0.8	31	0.5
3	2498	5.7	556	4.7	14	3.9	100	1.5	87	1.5
4	5460	12.5	2052	17.2	15	4.2	147	2.3	209	3.5
5	1247	2.8	723	6.1	19	5.4	1893	29.1	177	3.0
6	942	2.2	153	1.3	11	3.1	3983	61.2	2597	43.9
7	1769	4.0	21	0.2	9	2.5	239	3.7	2174	36.8
8	4069	9.3	0	0.0	15	4.2	0	0.0	529	9.0
9	130	0.3	0	0.0	13	3.7	0	0.0	16	0.3

1) System No. 1. C-18 plate; solvent system methanol:water, 65:35, v/v.

2) System No. 2. Silica gel (IB2) plate; solvent system chloroform:methanol, 4:1, v/v.

3) System No. 3. Silica gel (IB2) plate; solvent system hexane:ethyl acetate, 65:35, v/v.

4) System No. 4. Silica gel (IB2) plate; solvent system methanol, 100%.

5) System No. 5. C-18 plate; solvent system methanol, 100%.

12.5%, co-chromatographed with ecdysone. Only 2.8% co-chromatographed with 20-OH ecdysone. Approximately 18.6% of the radioactivity appeared in the zones above the Rf for ecdysone, suggesting polar compounds. Separating the extract on silica gel with apolar solvents was expected to reverse this distribution, since apolar compounds move toward the solvent fron. However, in the least polar system used, Petroleum ether:diethyl ether:acetic acid, 80:20:1, all of the radioactivity remained at the origin (not shown in the table). In system 2, moderately polar, most of the radioactive material remained at or near the origin except for a large fraction, 17.3%, which co-chromatographed with ecdysone (Rf 4.3). In systems 4 and 5, pure methanol, almost all of the radioactive compounds migrated extensively, leaving only trace amounts at or near the origin. Clearly, ^{3}H ecdysone and its metabolites comprise moderately polar compounds, with low solubility in the least polar solvents (e.g., hexane or diethyl ether). When the test using system No. 1 were repeated with the tick extract inoculated with ^{14}C acetate, 89.3% of the radioactivity remained at the origin, and 6.1% chromatographed in zones less polar than ecdysone; however, a significant rise in radioactivity occurred just below the solvent front and may represent a highly polar compound or compounds (Table 3).

Fig. 4 illustrates the ^{3}H radioactivity observed in a crude extract separated by HPLC and collected at 1 min intervals. A small peak of radioactivity occurred with the void volume and probably represents ^{3}H labelled water or low molecular weight ions resulting from the metabolism of the inoculated ecdysone. Maximum radioactivity was coincident with the elution of ecdysone (25.72 min), with a second peak of radioactivity coincident with 20-oH ecdysone (23.46 min). If all of the radioactivity in the fraction at 23 and 25 min is due to 20-OH ecdysone and ecdysone, respectively, the

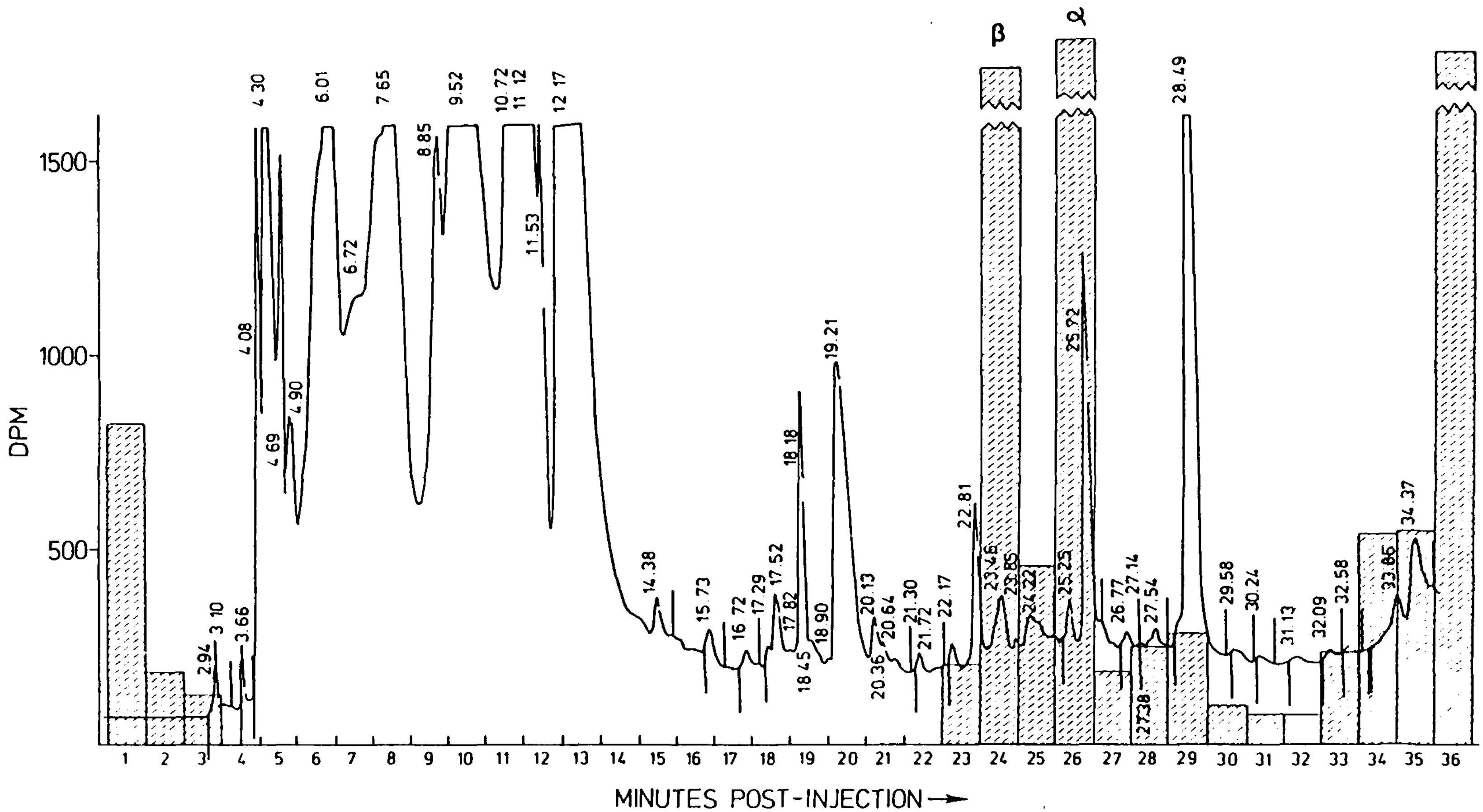

Figure 4. Distribution of ^3H radioactivity in 1 minute collections superimposed on an HPLC chromatogram to illustrate the distribution of ^3H labelled metabolites in relation to the sample peaks. Chromatographic conditions the same as in Figure 2. The retention times for 20-OH ecdysone (β) and ecdysone (α) are noted on the figure. Major peaks of radioactivity also occured in fractions collection after 36 min, but were deleted from the figure.

Table 3. Distribution of ^{14}C radioactivity following TLC of the ^{14}C acetate inoculated adult H. dromedarii extract.[1]

Cm. from origin	CPM	% activity
0 - 1	9896	89.3
1 - 2	446	4.0
2 - 3	229	2.1
3 - 4	0	0.0
4 - 5	0	0.0
5 - 6	0	0.0
6 - 7	77	0.7
7 - 8	146	1.3
8 - 9	298	2.7
9 - 10	69	0.3

[1]TLC done with same C-18 reversed phase plates described previously, developed in methanol:water, 65:35, v/v.

amounts observed represent 8.1% and 16.9% of the total radioactivity incorporated into these 2 compounds. Thus, only 16.9% of the ^{3}H ecdysone originally inoculated into the ticks has remained in that form; 8.1% has been converted to 20-OH ecdysone. In addition, considerable radioactivity was found in the fractions coincident with the less polar compounds eluting after ecdysone, including one at 27.38 min coincident with the authentic standard, Inokosterone, which is similar to 2-deoxyecdysone. This latter fraction contained 2.0% of the total radioactivity. In addition, a large peak of radioactivity, 66% of the total, was found coincident with the compounds eluting from 31 to 39 minutes. Overall, these collections accounted for virtually all of the total radioactivity in the sample. The remainder was eluted only after extensive flushing of the column with 100% methanol.

Fig. 5 shows the ^3H radioactivity observed in a second crude extract, also from ticks injected with ^3H ecdysone, but processed with Tris/HCLO4 buffer:methanol instead of water:methanol as the mobile phase. The most noteworthy differences are the presence of substantial ^3H radioactivity in polar fractions that elute ahead of 20-OH ecdysone, and the separation of the apolar ecdysteroids into at least 2 fractions. No significant radioactivity was found coincident with 20-OH ecdysanoic acid, which resolves sharply with this buffer system (arrow), and this metabolite is probably not present in these ticks. Activity was found where 20,26-diOH ecdysone elutes, and the point of peak radioactivity (258 CPM) was consistent with the retention time of this compound (arrow). Radioactivity at 18 and 19 minutes may represent other unknown polar ecdysteroids. A sharp peak of radioactivity was found consistent with 20-OH ecdysone and is clearly identified with this compound (which eluted earlier in the buffer system than when water was used in the mobile phase). Somewhat more ^3H ecdysone was converted to 20-OH ecdysone in these ticks than in the previous example (Fig. 4); 20-OH ecdysone represented 7.0% and ecdysone only 1.3% of the total radioactivity in the sample. Again, a small peak of radioactivity (173 CPM) occurred after ecdysone, and may represent a less polar ecdysteroid such as a deoxyecdysone. The great majority of the ^3H radioactivity was associated with two very large fractions, AP_1, containing 1 or more compounds with a major peak at 32.61 min, and AP_2 with a major peak at 37.3 min. AP_1, from 30-36 minutes, contained 60.5% of all of the radioactivity in the sample while AP_2 contained an additional 13.5% of the radioactivity. Thus, the great majority of the original ^3H ecdysone inoculated into the ticks, 75.6%, was converted to compounds less polar than ecdysone, especially 2 very apolar fractions. In addition, 7.0% was converted to 20-OH ecdysone, 8.5% was converted to compounds distinctly more polar than 20-OH ecdysone, and only 1.3% remained in the original form.

Figure 6 illustrates the ^{14}C radioactivity observed when an aliquot of a crude extract of H. dromedarii females inoculated with ^{14}C acetate was separated by HPLC, using the same gradient and Tris/HCLO4 buffer as in the previous example. Radioactivity was found coincident with the 2 apolar fractions described above but nowhere else. The ^{3}H and ^{14}C radioactive peaks coincide for AP_1 but not for AP-(2), where the ^{14}C peak appears to coincide with a peak at 34.9 min. Nevertheless, the profiles for both ^{3}H and ^{14}C radioactivity show good agreement suggesting ^{14}C labelling of the same or similar compounds.

Figure 7 and 8 illustrates the distribution of radioactivity, ^{3}H and ^{14}C in the origin fraction separated by TLC from the same extracts described above. AP_1 and AP_2 are more clearly delineated by the ^{3}H radioactivity profile than the ^{14}C profile. AP_1 contained 55.2% of the incorporated ^{3}H radioactivity, AP_2, 44.8%. Analysis of the ^{14}C profile shows 60.4% in AP_1, 39.6% in AP_2.

Effects of saponification. Following this reaction, radioactivity was found in both the aqueous and ether extracts. Table 4 summarizes the results of the saponification reaction and assay of the products. TLC was done with the same C-18 plates and development system (methanol:water) described previously. The results show that relatively polar radioactive products were liberated by the reaction. Most of the radioactive substances migrated 6 and 7 cm from the origin, indicating compounds more polar than 20-OH ecdysone. Small amounts also co-migrated with the authentic standards, as confirmed by visualization with H_2SO_4.

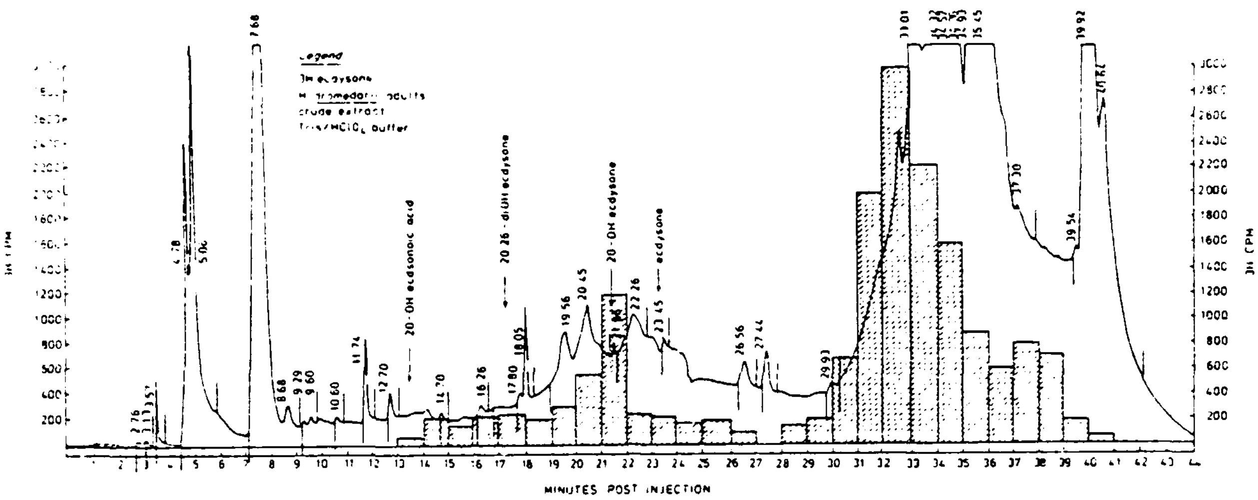

Figure 5. Distribution of ^3H radioactivity in 1 min collections superimposed on an HPLC chromatogram to illustrate the distribution of ^3H labelled metabolites when the crude extract was chromatographed using a 20 mM Tris/$HCLO_4$ buffer, pH 6.9. The retention times of ecdysone (23.45 min) and 20-OH ecdysone (21.66 min) as well as the more polar metabolites, 20,26-dioH ecdysone (17.46) and 20-OH ecdysanoic acid 913.36 min) are indicated by arrows. No peaks were observed that co-eluted precisely with the latter two compounds. Chromatographic conditions were the same as in Figure 2.

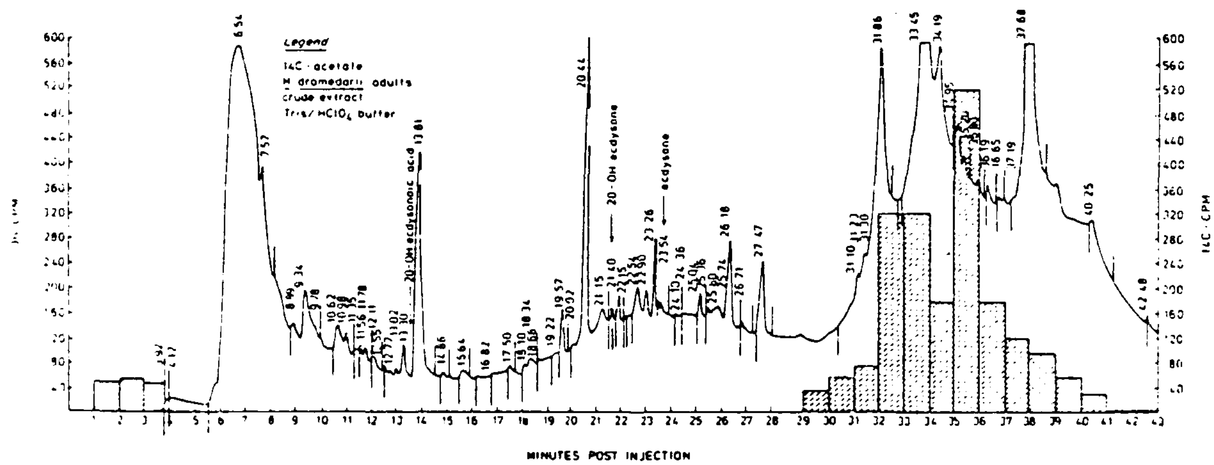

Figure 6. Distribution of ^{14}C radioactivity in 1 min collections superimposed on an HPLC chromatogram to illustrate the occurrence of ^{14}C compunds when the crude extract was chromatographed using the same buffer system as described in Figure 5.

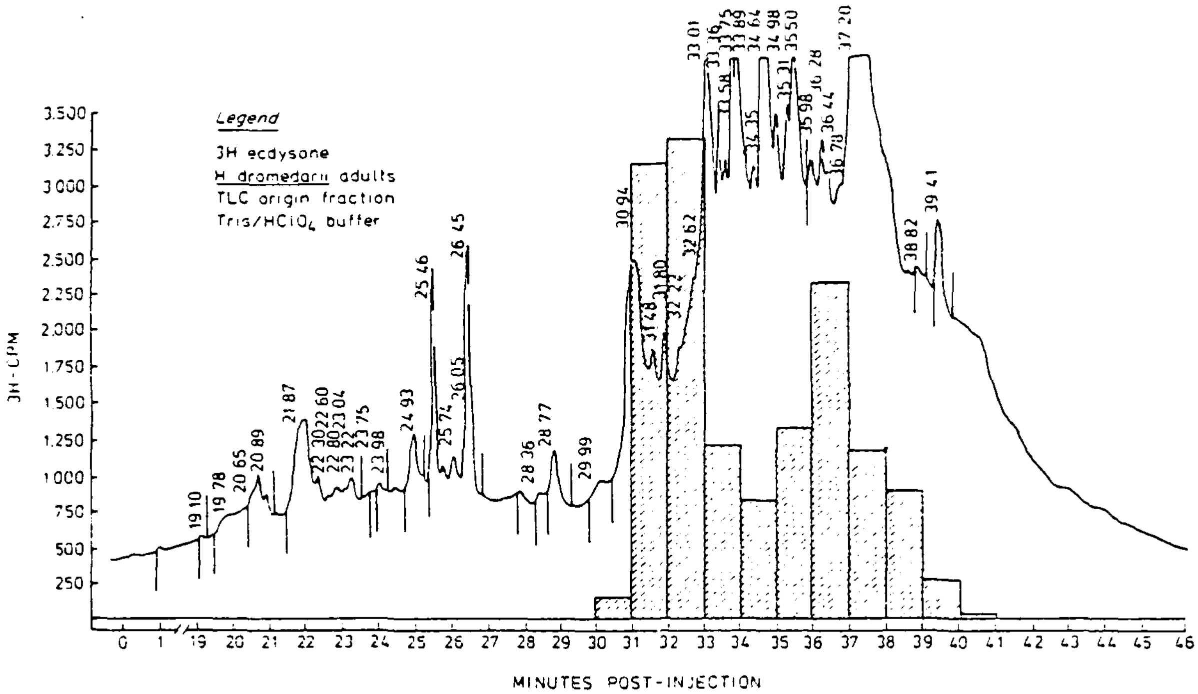

Figure 7. Distribution of ^{3}H radioactivity in 1 min collections superimposed on an HPLC chromatogram when crude origin origin extract was collected from a C-18 TLC plate after an aliquot of the tick extract (from ticks inoculated with ^{3}H ecdysone) was developed under the same conditions as described in Figure 1. HPLC conditions were the same same as described in Figure 5.

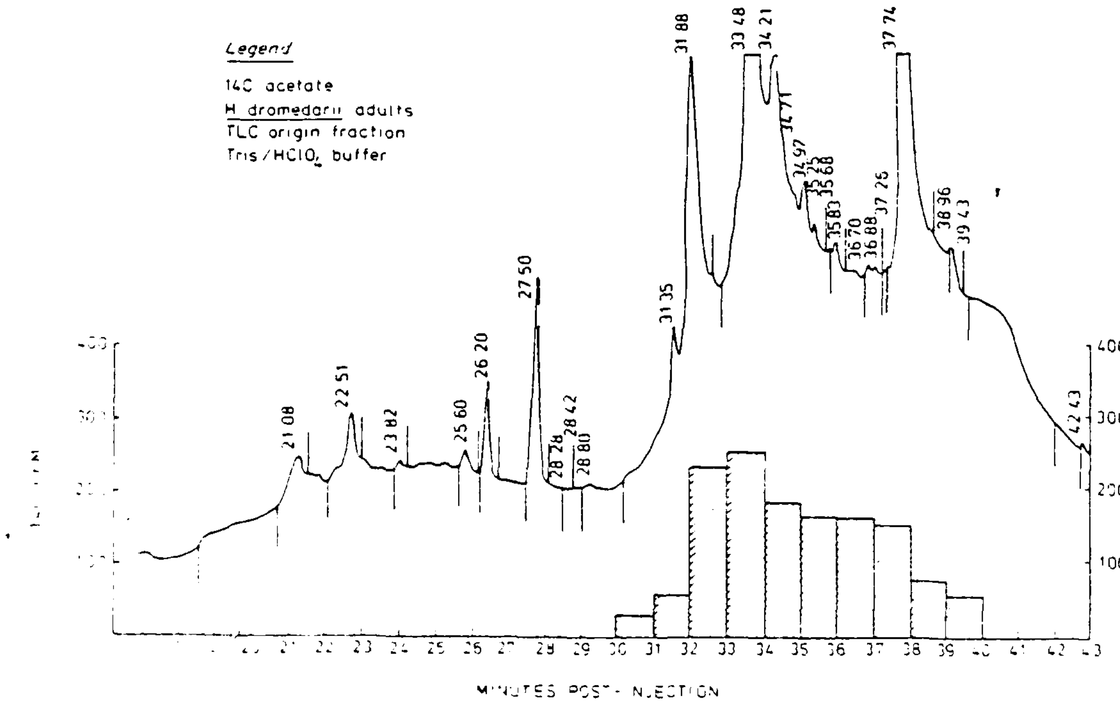

Figure 8. Distribution of ^{14}C radioactivity in 1 min collections superimposed on an HPLC chromatogram when the crude origin extract was collected from a C-18 TLC plate after an aliquot of the tick extract (from ticks inoculated with ^{14}C acetate) was developed in the same system as described in Figure 1. HPLC conditions were the same as described in Figure 5.

Table 4. Radioimmunoassay of eluates of crude extracts subjected to enzymatic hydrolysis and fractionated with a C-18 cartridge (Sep Pak)[1]

		Amount of ecdysteroid prior to hydrolysis ng ecdysteroid (% of total)		Amount of ecdysteroid after hydrolysis ng ecdysteroid (% of total)	
Fraction	methanol	Exp. No. 1	Exp. No. 2	Exp. No. 1	Exp. No. 2
1	10%	1.6 (1.3%)	2.3 (1.9%)	3.5 (2.8%)	0 (0.0%)
2	30%	2.8 (2.2%)	3.0 (2.5%)	6.1 (4.8%)	3.4 (3.9%)
3	100%	122.1 (96.5%)	114.5 (95.6%)	116.9 (92.4%)	83.5 (96.1%)

Analysis of the saponfied extract by HPLC revealed a very large amount of 20-OH ecdysone, ca. 500 ng, in the extract, or an estimated 15% of the total mass of the pre-saponified material. (Fig. 9). The identity of this compound was verified by repeating it with the addition of the standard and observing their co-elution.

Clearly, saponification released 20-OH ecdysone, indicating that this compound was present in a different chemical form prior to this treatment, probably esterified with a fatty acid. Further studies with saponification of these extracts are still in progress.

No evidence of free fatty acids was detected when the saponified extract was assayed by TLC or GC.

Effects of enzymatic hydrolysis. RIA of the original crude extract prior to enzymatic hydrolysis revealed an estimated 1.3 ng/tick. Followig enzymatic hydrolysis of the unfractionated crude extract, the RIA estimate was 2.0 ng/tick.

Table 5 summarizes the changes in the amounts of RIA positive ecdysteroids in the fractions obtained by sequential elution on C-18 Sep Paks.

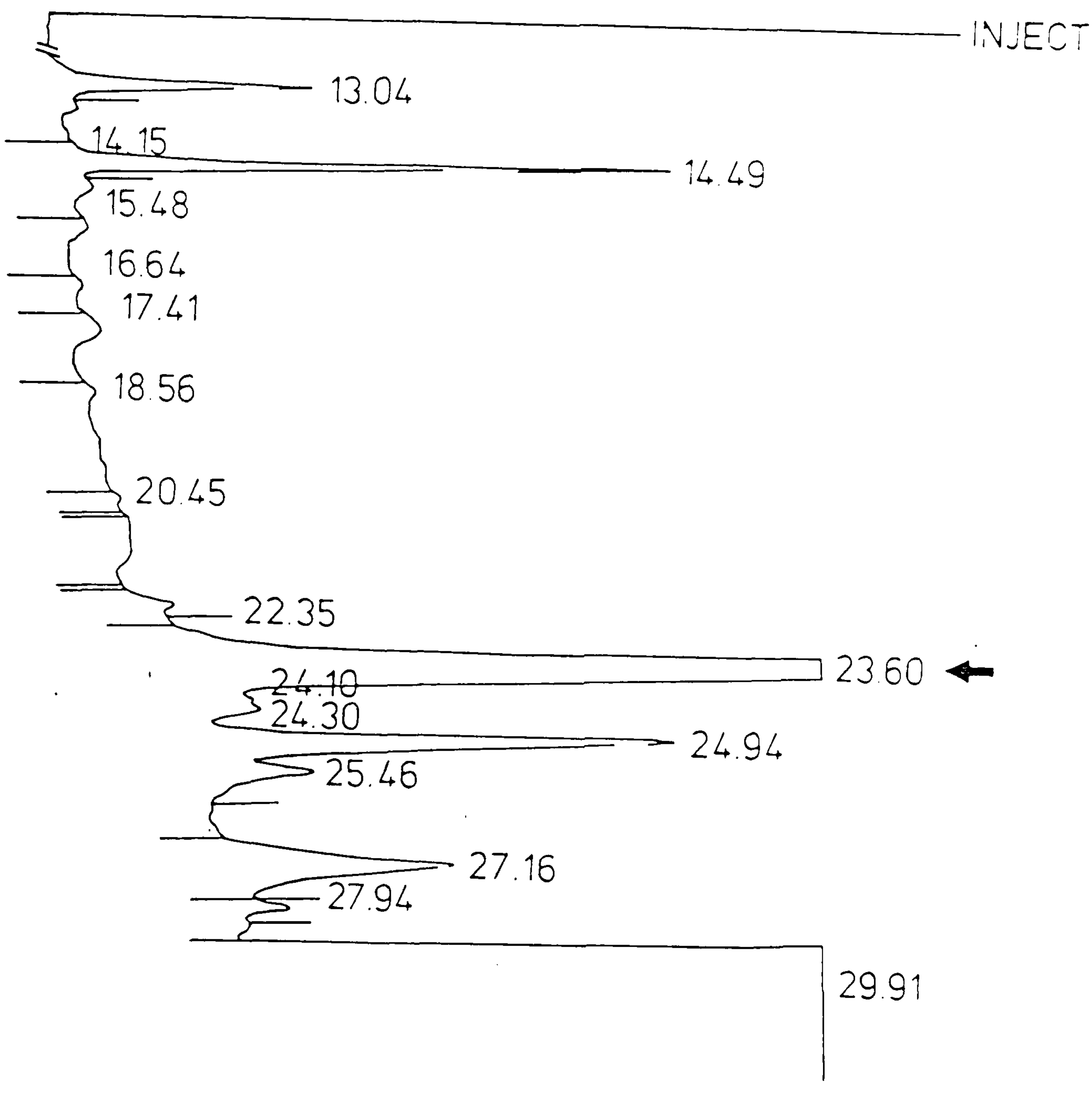

Figure 9. HLPC chromatogram illustrating the components found when the origin extract collected from a C-18 plate spotted with the tick extract (entire crude extract) was saponified. The arrow indicates the large peak coincident with the retention time for 20-OH ecdysone. HPLC conditions were the same as described in Figure 2.

Table 5. Distribution of ^{3}H radioactivity following saponification of the TLC origin fraction of the extract *H. dromedarii* adults inoculated with ^{3}H ecdysone.[1,2]

Cm. from origin	CPM	% of total
0	60	1.0
1	29	0.5
2	34	0.6
3	87	1.5
4	202	3.4
5	172	2.9
6	2617	44.0
7	2201	37.0
8	523	8.8
9	17	0.3
	5942	

[1]Material remaining at the origin following TLC of crude extract removed, saponified, and extracted with diethyl ether.

[2]Ether extract of saponified material chromatographed on C-18 TLC plate and developed with methanol:water, 65:35, v/v.

Prior to enzymatic hydrolysis, only 1.6 and 2.7 ng was found in the 10% and 30% methanol elutes. These fractions contain material more polar than 20-OH ecdysone. Almost all of the ecdysteroid activity, 96.5%, remained on the Sep Pak and was not eluted until the cartridge was flushed with 100% methanol. Following enzymatic hydrolysis, slight increases in the amount of RIA positive material were observed with the 10% and 30% methanol elutions; however, 92.4% of the activity was still found in the 100% eluate. When the experiment was repeated, the amounts of RIA positive material detected after hydrolysis were very similar; 96.1% of the activity remained in the final eluate.

HPLC analysis of the C-18 Sep Pak elutions confirmed the results of the assays obtained by RIA. HPLC demonstrated that none of the 20-OH ecdysone and ecdysone was found in the 10% or 30% elutions, not even in trace amounts. In the 100% eluate, 10.87 ng of 20-OH ecdysone/tick was found prior to enzymatic hydrolysis; following hydrolysis, the amount found was 4.21 ng/tick.

These findings do not support the hypothesis of a sulfate or glucuronate conjugate serving as a product of ecdysone metabolism.

Tests of the extract fraction remaining at the origin after TLC (C-18 plates) with ninhydrin indicated a weak positive reaction. A single amine containing fraction was identified when the origin extract was saponified, derivatized and analyzed by HPLC. This fraction did not co-chromatograph with any of the known amino acids. No amine containing compounds were found in unsaponified fractions or in the controls.

Studies to determine the susceptibility of the ecdysteroid fractions to hydrolysis by esterases (e.g., porcine esterase) are in preparation.

Structural analysis. The similarity of certain of the polar compounds eluting near 20-OH ecdysone was confirmed by proton NMR (Fig. 10). The NMR spectra for the fractions eluting at 17.8, 21.7 and 23.5 min were compared with those of authentic ecdysone and 20-OH ecdysone. Comparisons are made in accordance with polarity, fraction 1 being the most polar. Excluding the signals for water or solvent, ecdysone had major peaks at +0.85, +0.90, +1.33, and a minor signal at +3.5; 20-OH ecdysone had strong signals at +0.90, +1.33, +3.55, and 4.05 ppm. In contrast, fraction No. 1 had the peaks at 1.33 and 3.55, as well as others at +0.96, +0.98, +1.00, +1.05, and +1.42. Fraction 2 had the peaks at +0.85, +1.33, and +3.50, as well as

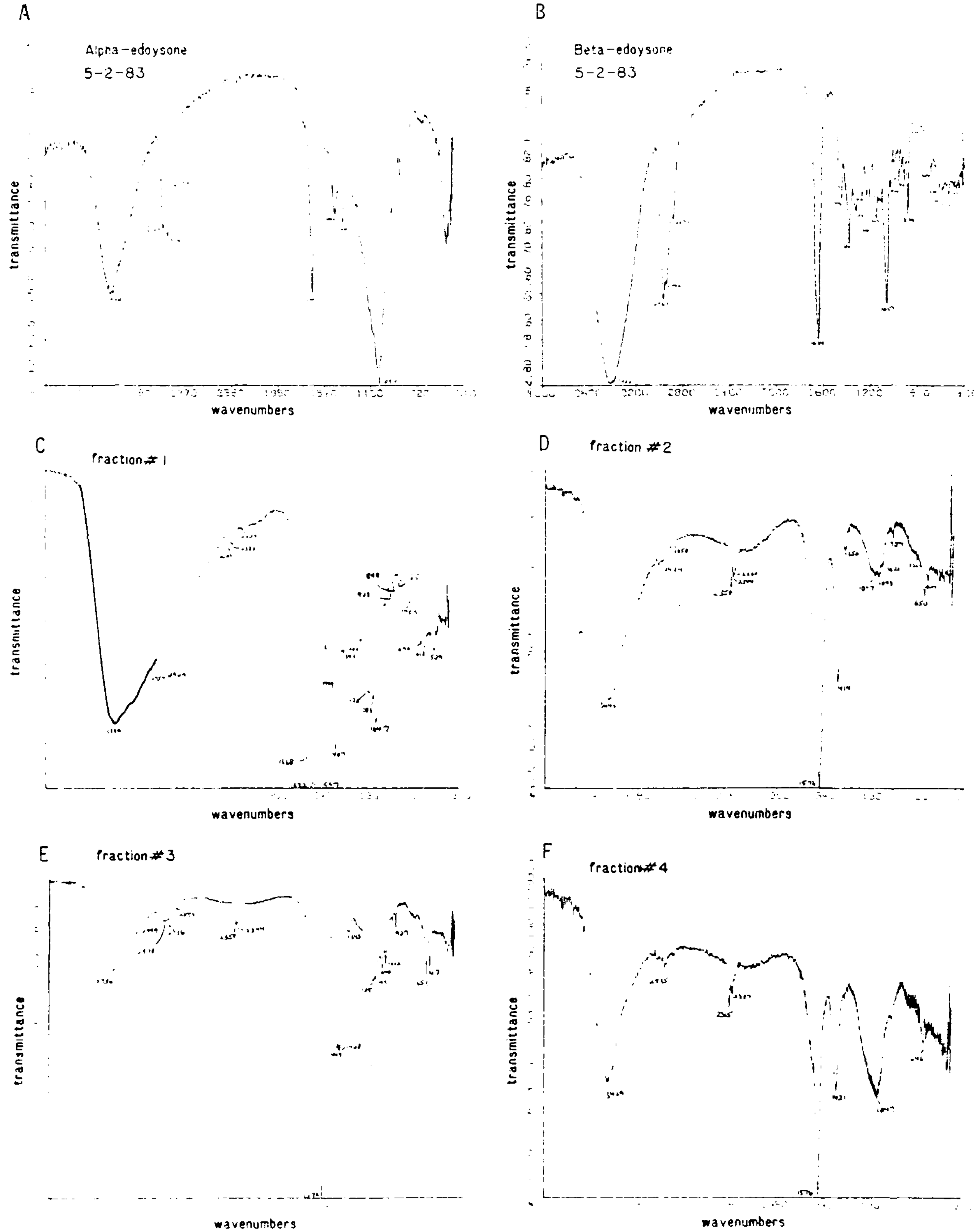

Figure 10. Infra Red scans of the authentic ecdysteroids, ecdysone (alpha-ecdysone) and 20-oh ecdysone (beta-ecdysone) and 4 unknown fractions eluted from the tick extracts by HPLC.

other peaks at +1.22, +1.60 and +4.25. Fraction 3 exhibited the least similarity, with peaks at +1.00, +1.32, +1.53 (a major peak), a doublet at +1.58, and others at +1.65, +2.13, +2.37, +2.50, +2.65, +3.65, and +4.15. The signals at (1) +0.90 to +1.00, (2) +1.30 to +1.35, and (3) +3.48 to +3.65 are present in all of the compounds and probably represent the same functional groups. In this respect, all of the compounds resemble one another and the authentic ecdysteroids. The 3 unknown compounds have additional signal peaks at (1) +1.53 - +1.65 and (2) +4.15 - +4.25. The latter is also shared with 20-OH ecdysone (but not with ecdysone) and may represent the C-20 moiety.

Infrared analysis revealed spectra with absorbance bands resembling those characteristic of steroids. Fraction 1 exhibited intense absorption at 1047, 1407, 1597-1632, 2929-2957, and 3380 mu, which resembles the spectra for ecdysone and 20-OH ecdysone, as well as the published spectrum for ecdysone (Hoppe et al. 1965). Weak absorbance bands which occur at 2330 - 2365 mu were absent in the authentic standards. Fraction 2 had weak absorbance bands at 1048 and 2929 mu, and a strong absorbance band at 1576 rather than 1639. Fraction 3 and a fourth fraction, not analyzed by NMR, gave infrared spectra similar to that of fraction 2, with very weak absorbance bands at or about 2942 to 2964.

The results of these analyses suggest that the compounds in these fractions are steroids. Fraction 1 exhibited the greatest similarity and is most probably an ecdysteroid.

Discussion

In those insects that have been examined, ecdysone and 20-OH ecdysone are metabolized and conjugated to form relatively polar compounds. In the

Table 6. Metabolism of juvenile hormone by hemolymph from part fed *Dermacentor variabilis* females (expressed as percent of amount administered found in aqueous or organic phase)

	ACTIVE HEMOLYMPH		INACTIVATED HEMOLYMPH		NO HEMOLYMPH	
Trial No.	Aqueous	Organic	Aqueous	Organic	Aqueous	Organic
1	67.0	33.0	6.4	93.6	8.7	91.2
2	82.5	17.5	17.5	82.5	1.6	98.4
3	58.6	41.4	4.4	95.6	3.2	96.8
Avg.	69.4	30.6	9.4	90.6	4.5	95.5

Table 7. Metabolism of juvenile hormone by hemolymph from Tobacco hornworm *Manduca sexta* larval hemolymph (expressed as percent of amount administered found in aqueous or organic phase)

PHASE	ACTIVE	INACTIVE	NO HEMOLYMPH
Aqueous	77.5	10.7	1.8
Organic	22.5	89.3	98.2

Results

Incubating radiolabelled juvenile hormone with tick hemolymph resulted in hydrolysis of this compound, with most of the polar end products appearing in the aqueous phase. Table 6 summarizes the results of a series of experiments. At the completion of the incubation, an average of 69.4% was found in the aqueous phase, vs an average of only 30.6% remaining in the organic phase. In contrast, almost all of the JH-3 remained in the organic phase following incubation with inactivated tick hemolymph (average of 90.6%) or Shen's saline without hemolymph (average of 95.5%). Similar results were obtained when juvenile hormone was incubated with THW larval hemolymph (Table 7). When an aliquot of the aqueous phases from the active hemolymph incubation was chromatographed on a reversed phase (C-18) plate, 19.9 and 39.9% of the radioactivity was found at 6 and 7 cm from the origin, respectively, indicating that most of the radioactivity was in the form of compounds more polar than JH-3, presumably JH acid, diol, and/or acid-diol. Chromatographing an aliquot of the organic phase from this same incubation revealed only 16.9% of the radioactivity co-eluting with JH-3, i.e., the radiolabelled JH-3 remaining from the original treatment. Very different results were obtained when the aqueous and organic phases from the controls (inactivated hemolymph or Shen's saline) were chromatographed on C-18 plates; almost all of the radioactivity, 86.3% and 94.1%, co-eluted with JH-3. The same general trends were obtained when D. variabilis PFV female hemolymph was used (Table 9). However, considerably greater amounts of undigested JH-3 remained than was found with the THW larval hemolymph, with a major peak, representing 56.1% of the sample radioactivity, co-chromatographing with JH-3. Only one major polar peak of radioactivity, represent-

For isoelectric focusing, slab gels with BioLyte 3-10 were prepared in accordance with the manufacturer's instructions. Samples were applied on 3 X 4 mm oval pads near the cathode and electrophoresis was continued until the voltage became constant. Staining and destaining was done as described above.

Effects of authentic JH on tick development. Following trials with a variety of solvents and techniques, three methods of administration were used, (1) inoculation, (2) topical application, and (3) by contact, in Petri dishes (Bowers et al. 1976; Leahy and Booth, 1980). All ticks were treated on the day of drop off. Inoculations were done with 2 ul of ethyl oleate containing different concentrations of JH compounds. The concentrations used were 0.1, 0.5, 1.0, 2.0, and 10 ug/tick. In addition, others were inoculated with 0.5 female equivalents (FE) of PFV female hemolymph, reconstituted in ethyl oleate. Controls were done with solvents only. Topical applications were done with different concentrations of JH-3 dissolved in DMSO:acetone, 1:4, v/v, and administered in 1 ul aliquots onto the venter of 50 engorged nymphs immobilized with tape. The concentrations used were 1, 5, 10, 20, 50 and 100 ug/nymph. Controls included nymphs treated with (1) solvents only, or (2) were untreated. To treat by the contact method, solutions of JH-3 dissolved in DMSO:acetone, 1:9, were applied to filter paper circles placed in glass Petri dishes, 9-cm diam, top and bottom. The total treated area was 127 cm^2. Nymphal ticks, 50/treatment, were released to each treated dish and allowed to contact the treated surfaces for 3 hr. The concentrations used were 0.1, 0.5, 1, 2, 5 and 10 ng/cm^2. Controls included (1) nymphs exposed to solvents only and (2) untreated nymphs. The treated ticks in all 3 treatment types were monitored daily for mortality and time of molting.

was recovered and dialized (VWR Scientific Co., Phillipsburg, PA, size 8) to remove salts and small peptides. Controls were done using (1) TBH larvae, and (2) porcine liver extract, Type I. Protein determinations were done using the BioRad protein assay (BioRad, Richmond, CA). The total protein concentration of the tick hemolymph was 115 ug/ul; of the TBHL hemolymph, 56 ug/ul; of the porcine liver extract, 1 ug/ul. Molecular weight markers, high and low (BioRad) were used to determine the approximate size of the hemolymph proteins.

Polyacrylamide (PAGE)-SDS gels were prepared as column gels or slab gels. SDS-PAGE column gels were prepared at a concentration of 5% with 0.5 M tris-HCL, Temed, and ammonium pursulfate (pH 8.9). Following loading of the samples (200 to 400 ug) or the standards (10 to 20 ug) onto the gels, electrophoresis was done in a Buchler Model 1004 electrophoresis cell (Buchler Instrument Co., Fort Lee, NJ) with tris-glycine buffer (0.04 M, pH 8.3) at 3 mA/tube for 3 hrs or until the tracking dye (BB) reached the bottom of the gel. Staining for proteins was done with CBB, 0.25% in 50% methanol, followed by destraining overnight in methanol acetic acid (30%:7%). To analyze for esterases, the gels were prepared in 0.03 M borate buffer, pH 8.0. Following loading of the samples or standards, electrophoresis was done as described above, but a 0.3 M borate buffer was used and staining was done with Fast Blue (FB) esterase stain with 0.1% alpha-naphthol acetate in acetone as the substrate. Slab gels were prepared by applying 10% separating gel solutions to 100 X 125 mm glass plates. Following polymerization (1 hr), a 4.75% stacking gel was used to cover the first layer. Samples or standards were loaded in wells cut at the top of each gel. Electrophoresis was done overnight in a BioRad Model 220 slab gel electrophoresis cell; the well buffer was 0.04 M tris-glycine (pH 8.3). Staining and destaining was done as described above.

elutions were also used. Under these conditions, JH1, JH2, and JH3 eluted at 12.42, 6.75 and 8.96 min, respectively. The acid and diol of JH3 eluted at 4.08 and 7.69 min, respectively. The sensitivity of the system, at 0.005 AUFS, was ca 2 ng.

JH esterase activity. Hemolymph was collected from PFV females as described above and held at ca 0°C. A sample of 150 ul of fresh hemolymph was added to 6 ml of 0.1M phosphate buffer and inoculated with a dilute ethanolic solution containing 1 uCi of ^{3}H JH-3 and 50 ug of non-labelled authentic JH-3. The mixture was incubated, with gentle shaking, for 60 min at 30°C. The reaction was stopped by addition of 250 ul of methanol:ammonia:water, 10:9:1, with vigorous shaking. Iso-octane, 15 ml, was added with vigorous shaking to extract the remaining JH and centrifuged 2 X (16,000 rpm, 2 min) to partition into organic and aqueous phases. The aqueous phase was separated, re-extracted with iso-octane, and the fractions combined. Emulsions at the interface of the 2 layers prevented complete separation. Controls consisted of (1) tick hemolymph + JH-3 and solvents, as described above, but inactivated by heating in a water bath at 80°C for 60 min, and (2) as described above, but without tick hemolymph. In addition, the same study was also done with hemolymph collected from tobacco hornworm larvae (TBH), with the same controls. Analysis of the extracts was done by radioassay with a Beckman model LS 250 liquid scintillation counter (estimated 37% efficiency for tritium). Aliquots of the aqueous and organic phases were also separated further by TLC and HPLC and eluates collected by these methods were assayed for radioactivity as described above.

Electrophoresis of tick esterases. A hemolymph sample containing 500 ul was collected from PFV females as described above and centrifuged for 10 min at 16,000 RPM to remove hemocytes and cellular debris. The supernatant ance with techniques described previously (Sonenshine et al. 1985).

Assays for JH by High Performance Liquid Chromatography (HPLC). Three types of tick materials were used for extracts, namely (1) hemolymph; (2) synganglia, all from part fed virgin females, fed 7 days (PFV females) or mated replete females (MR females); (3) whole body (WB) extracts. The hemolymph collections comprised 920 ul from PFV females and 750 ul from MR females; 150 synganglia were from PFV females. Extractions were made according to the method of Connat (1982), with modifications. Hemolymph was extracted with iso-octane. The synganglion or WB extracts were homogenized in cold acetonitrile (ACN) with the aid of celite, filtered (fritted glass funnel), and the filtrate re-extracted 3 X with hexane:water with 1% NACL. The organic and aqueous phases were separated, concentrated (N_2), and separated further on either (1) Bakerflex IB2 silica gel TLC plates (Arthur H. Thomas, Inc., Philadelphia, PA) or reversed phase C-18 plates (J. T. Baker Co., Phillipsburg, NJ). Typical solvent systems were benzene:ethyl acetate, 96:4, v/v, for the silica gel plates and methanol:water, 80:20, v/v for the C-18 plates. The zone expected to contain the JH was eluted (nexane:ethyl acetate, 90:10), dried, and reconstituted in ACN. JH acids and diols were prepared by incubation of the authentic standards plus ^{3}H labelled JH with THW hemolymph or acidification with HCL. The highly concentrated samples were assayed by HPLC, using a Waters Assoc. system (Milford, MA) consisting of a Model 441 UV fixed wavelength detector (214 nm filter), 2 Model 510 pumps, a U6K septumless injector, a Z-module to contain the column, a Model 721 Systems Controller, and a Model 730 Data-Module. The column was a Nov-Pak 5-um C-18 column, 8 um I.D. X 10 cm long (Waters). The solvents were ACN and water. Typical solvent ratios and pumping parameters were 75:25 and 1 ml/min, respectively for isocratic separations; a variety of gradient traction (except ethanol), were HPLC grade (Burdick & Jackson, Muskeegan, MI). Hexane was double distilled (Omni-Solv, Krackler Chemical Co., Albany, NY). Dimethyl sulfoxide (DMSO) (spectranalyzed) and iso-octane were obtained from Fisher Scientific Products, Inc., Fairlawn, NJ. Ethyl oleate and the authentic standards JH1, JH2, and JH3 were from Sigma Chemical Co., St. Louis, MO, while radiolabelled ^{3}H JH was from New England Nuclear, Boston, MA (NEN). To purify the radiolabelled JH, it was dried (N_2), reconstituted in 100% ethanol, and chromatographed by TLC as described above. Tobacco hornworm (THW) larvae Manduca sexta were from Carolina Biological Supply Co., Burlington, NC. The esterase standard was porcine liver extract, type I (Sigma). Acrylamide, tris, temed, Bromphenol Blue tracking dye (BB) and Commassie Brilliant Blue (CBB) for straining of proteins and BioLyte for isoelectric focusing were all from BioRad (Richmond, CA). Fast Blue was obtained from Sigma.

Inoculation of ^{3}H JH-3 and ^{14} mevalonic acid. ^{3}H JH-3 was reconstituted in a mixture of ethyl oleate:olive oil, 1:1, v/v, and inoculated into PFV females while attached to their rabbit host. The rabbit was anesthetized (name of anesthetic) and 3 ul was inoculated into each tick with a 50 ul Hamilton syringe and 30 gauge needle. The treated females were allowed to continue feeding, detached 4 days later, and extracted as described below. ^{14}C mevalonic acid was separated from the lactone in accordance with the manufacturer's instructions, dissolved in Shen's saline, and inoculated into H. dromedarii engorged nymphs. An estimated 0.25 uCi was inoculated into each tick. Following molting, the surviving adults were separated by sex, pooled and extracted as described above. Samples of the extract were assayed by TLC and HPLC as described above. In addition, tissues from 3 males and 3 females were sectioned and autoradiographs prepared in accordmay be expected to occur in these small arthropods makes this an unusually difficult goal to achieve. However, important information may be obtained by study of JH enzymes and metabolites. The latter may be expected to occur in considerably greater quantities than the parent compound. In insects, the various JH types are degraded rapidly into the corresponding acids and diols by non-specific hemolymph esterases and JH specific esterases and epoxidases, and the products can accumulate in considerable quantity. Thus, measurement of the degradation of the authentic hormone and/or study of the result acid and diol byproducts can provide useful information as well as compelling evidence of natural JH.

This study was done to determine (1) whether enzymes were present in tick hemolymph capable of metabolizing authentic JH and, (2) whether the end products of this metabolic degradation were similar or identical to the known acid and diol metabolites that result from degradation of the corresponding hormone in insects.

Materials and Methods

Ticks. The American dog tick, *D. variabilis* was colonized and reared as described previously (Sonenshine et al. 1977). Immature ticks were allowed to feed on albino rats (*Rattus norvegicus*), adults on rabbits (*Oryctolagus cuniculus*). The camel tick, *H. dromedarii* was colonized from a shock originally from the U.S. NAMRU-3 Medical Zoology Department, Cairo, Egypt (HH No. 59723, U.S. APHIS license No. 9433) and reared as described previously (Dees et al., 1984b). Ticks were held in an AMINCO AIRE Climate Lab environmental chamber at 27± 0.5°C and 90± 2% RH during their non-parasitic periods.

Chemicals and standards. All solvents, including those used for ex-

III. FATE OF JUVENILE HORMONES IN THE TICKS HYALOMMA DROMEDARII AND DERMACENTOR VARIABILIS (ACARI:IXODIDAE, WITH NOTES ON THE POSSIBLE OCCURRENCE OF JH IN THESE TICKS)

Introduction

Although the occurrence of juvenile hormones/gonadotropic hormones is generally assumed to occur in virtually all arthropods, no direct evidence of their presence in ticks has been obtained to date. Ticks are undoubtedly an ancient group of arachnids (Hoogstrall, pers. commun.) and their regulatory systems may not merely mimic those of the better known insects. Gonadotropic activity in ticks is well known (Solomon et al. 1982) and the gonadotropins may be similar or identical to the molecules that regulate development of the immatures, i.e., juvenile hormones (JH), as is the case in insects (references). Synganglia from fed-mated <u>Ornithodoros</u> <u>moubata</u> were found to induce oviposition in 41% of fed virgin females of this species (Aeschlimann, 1968). Other evidence of synganglion involvement in regulating oogenesis, all in argasid ticks, is summarized in recent reviews (Solomon et al. 1982, Diehl et al. 1982). Evidence implicating JH as the gonadotropin in ticks was described by Pound and Oliver (1979). These workers used authentic JH to restimulate oogenesis in soft ticks treated with the anti-allatotropin Precocene II. They hypothesized that this blocking agent removed the essential hormonal stimulus, whereupon the intervention of the exogenous JH "rescued" the ovary, restimulating the process. These and other findings reviewed elsewhere argue persuasively for but do not prove the existence of JH in ticks. Further progress is largely dependent upon identification of the specific hormone.

Although unequivocal identification of the hormone is the ideal solution to the problem of JH in ticks, the incredibly minute quantities that were found to contain ecdysteroid moeities when examined by proton NMR and infrared spectroscopy. Comparison of their retention times with known standards suggests that at least one of them may be 20,26-OH ecdysone. The identity of the apolar compounds is unknown, although the presence of 14-C labelling in the same fractions as those showing 3-H labelling certainly implicates fatty acids.

The physiological effects of the various ecdysteroids in ticks is unknown. It is not known whether these are merely inactivation products, storage products retained in an inactive form for later use, or some combination of the two. Moreover, it is not known to what extent these varied ecdysteroids may be sequestered preferentially in selected tissues or organs, e.g., the vitellogenic ovary, midgut, etc., where they may continue to exert an effect.

Acknowledgements

We are most grateful to Dr. R. LaFont, Ecole Normale Superieure, Laboratoire de Zoologie, Paris, France and Dr. J. L. Connat, Institut de Zoologie, Universite de Neuchatel, Neuchatel, Switzerland, for their generous gifts of authentic ecdysteroids and their most helpful advice.

entially into the vitellogenic ovary, passed to the embryos, and linked to the vitellogenic process in some unknown way. Clearly, these compounds may serve in some capacity other than merely as inactivation products. Diehl et al. (1985) postulated that this mechanism may occur in the midgut as a means of inactivating exogenous ecdysteroids present in the blood of herbivorous hosts, i.e., for coping with phytoecdysteroids.

In summary, both families of ticks possess enymatic pathways for inactivating ecdysteroids by esterifying them at the C-22 position, most probably with fatty acids. The ecdysteroid moiety undergoes little change, although the existence of other metabolites is not precluded. In this respect, ticks appear to differ greatly from most insects which degrade the parent molecule into a spectrum of acidic, dehydro and deoxy forms and further inactivate these metabolites as well as the parent molecules by conjugation. However, this mechanism is not unique to ticks, as similar apolar compounds have been found in _Drosophila_ (reported by Diehl et al. 1985).

Our studies with _H. dromedarii_ suggest a metabolic fate for ecdysteroids similar to that described in _B. microplus_ and _O. moubata_. In _H. dromedarii_, as in the other ticks, the two largest ecdysteroid fractions were apolar, representing almost 75% of the entire extract, and saponification led to the release of 20-OH ecdysone. However, we have been unable to detect evidence of high molecular weight fatty acids following alkaline hydrolysis, nor have we detected any indication of C-2 or C-3 acetate ecdysteroids. Hydrolysis of the tick extract with _Helix_ glucuronidase/sulfatase enzymic preparation had no apparent effect on the amounts of immunoreactive ecdysteroids detected, a finding which leads us to exclude ecdysteroid conjugation with polar moieties such as phosphate, sulfate or glucuronates. Significant amounts (up to 12%) of compounds more polar than 20-OH ecdysone from that observed with insects. At present, only 4 studies on this subject in ticks have been reported, one by Wigglesworth et al. (1985) on the cattle tick, Boophilus microplus and others by Bouvier et al. (1984), Connat et al. (1984) and Diehl et al. (1985) on the soft tick O. moubata. In the case of B. microplus, very little activity (approximately 6%) corresponding to polar conjugates or acidic forms of ecdysteroids were found. Rather, most of the ^{3}H radioactivity observed following injection of ^{3}H ecdysone was correlated with ecdysone, ecdysone 3-acetate, and 3 major peaks of relatively apolar material which were not immunoreactive. These occurred in both the parent females and the newly laid eggs (probably formed maternally). Presumably, the apolar fractions contain metabolites of the parent ecdysteroids. The susceptibility of the apolar compounds to hydrolysis by esterases and by alkaline saponification followed by the release of free ecdysteroids suggests that they are acyl fatty esters with ecdysteroid moieties. Moreover, the unhydrolyzed apolar compounds can be transformed into acetonide derivatives, which suggests that the C-2 and C-3 positions are not substituted. Consequently, the authors concluded that the apolar compounds are C-22 fatty acyl esters of ecdysteroids. Similar findings were made in O. moubata by Bouvier et al. (1982), Connat et al. (1984), and Diehl et al. (1985) who demonstrated that most (70-75%) of the ^{3}H ecdysone injected into the ticks was converted into 2 relatively apolar fractions, AP-1 and AP-2. Enzymatic hydrolysis (with esterase) liberated free ^{3}H ecdysone and ^{3}H 20-OH ecdysone. In subsequent studies, Diehl et al. (1985) and his colleagues reported evidence of 4 major conjugates consisting of 20-OH ecdysone esterified at C-22 with palmitic, stearic, oleic, and linoleic acids. Although it is not clear which of these fatty acids are present in which proportions in the 2 different classes, AP-2, the least polar of the 4 classes, is incorporated preferthese metabolites as well as the parent molecules form relatively polar conjugates with glucuronic acid, glucosides, sulfates and phosphates. In addition, LaFont & Koolman (1983) noted that conjugation with amino acids and even with glutathione also occurs in some insects, a reaction which transforms the ecdysteroids into very polar conjugates. This finding is noteworthy in view of our finding of an unknown amine containing product following saponification of the ecdysteroid extracts.

In locusts, C-22 phosphate conjugates of 2-deoxyecdysone, 2-deoxy-20-OH ecdysone, and 20-OH-ecdysone represent the predominant form of these ecdysteroids, although phosphate esters at C-2 and C-3 are also known. Moreover, ester formation with adenosinie monophosphate at the C-22 position and acetate at the C-3 position are also common. Rarely, side chain cleavage also occurs (forming poststerone). Finally, fatty acyl esters formed with various ecdysones at the C-22 position have been described in ticks (see below), leading to the formation of highly apolar ecdysteroids.

LaFont et al. also describe the major enzyme systems responsible for these pathways. Ecdysone-20-hydroxylase, found in the fat body, midgut, and many other tissues, hydroxylates ecdysone, forming 20-OH ecdysone. Ecdysone oxidase leads to the various deoxyecdysone species, while ecdysone epimerase generates the 3-epi-ecdysteroids and 3-dehydroecdysone reductases results in the various 3-dehydroecdysteroids. Conjugates are formed by the corresponding transferase enzymes, i.e., glucosyl, sulfo, phosphotransferases, and so on. The enzymes responsible for catabolism to the various ecdysanoic acids or esterification with fatty acids are unknown. All of these enzyme systems are cystolic enzymes, so that the sites of ecdysone activity and breakdown are intracellular.

The metabolism of ecdysteroids in ticks appears to be very different locust, Schistocerca gregaria, the major inactivation products are formed by esterification with inorganic phosphate at the C-22 position, and passed to the eggs; C-22 phosphate esters of ecdysone, 20-OH ecdysone, 2-deoxyecdysone and 2-deoxy-20-OH ecdysone have been reported (Isaac et al. 1982, 1983). The high reactivity of the C-22 position appears to account for the particular form of these conjugates. These conjugates are readily hydrolyzed by esterases, liberating the original ecdysteroid moieties. Thus, they can provide a convenient storage form for the active hormones for later use (e.g., in the late stage embryo) as well as a means of enhancing synthesis by removal of free steroid from the active synthetic tissues (Issac et al. 1983). Further studies with related orthopterans revealed other phosphate conjugates, especially 22-adenosine monophosphate-2-deoxyecdysone, 3-acetyl-ecdysone-2-phosphate, 3-acetyl-20-OH ecdysone-phospate, 3 and 2-acetyl ecdysone, and 3-epi-2-deoxyecdysone-2-phosphate (Isaac et al. 1983, 1984; Isaac & Rees, 1984). These latter compounds were considered as irreversible inactivation products of the parent molecules. Clearly, the C-2, C-3, and C-22 positions appear to be the major sites where conjugates may form. Further degradation yields ecdysanoic acid and 20-OH ecdysanoic acid (Isaac & Rees, 1984), described more fully below.

LaFont and his colleagues have shown that one of the principal routes of inactivation of ecdysteroids is oxidation to the corresponding acids. Following synthesis of ecdysone and its hydroxylation to 20-OH ecdysone by ecdysone 20-monooxygenase, these compounds are oxidized further to 26-OH ecdysanoic acid and 20,26-di-OH ecdysone. In those insects in which metabolites lacking a 25 hydroxyl group occur, e.g., Ponasterone A, the metabolite is converted to inokosterone by hydroxylation at the C-26 position and then to the corresponding acid, 25-deoxy-20-OH ecdysanoic acid. Frequently,

Table 8. Metabolism of ^{3}JH by Tobacco hornworm (Manduca sexta) hemolymph[1]; percentage of radioactivity eluted from each zone separated by TLC

Cm from origin	ACTIVE HEMOLYMPH aqueous	ACTIVE HEMOLYMPH organic	INACTIVE HEMOLYMPH aqueous	INACTIVE HEMOLYMPH organic	NO HEMOLYMPH aqueous	NO HEMOLYMPH organic
0-1	0.0	0.7	0.0	0.1	0.0	0.2
1-2	0.0	2.1	0.6	0.5	0.0	0.5
2-3[2]	2.1	25.0	5.8	86.3	1.7	94.1
3-4	1.3	0.5	4.1	0.0	0.3	
4-5	0.0	0.5	0.4	0.5	0.3	1.5
5-6	1.5	0.5	0.3	0.3	0.2	0.3
6-7	19.9	0.0	0.3	0.1	0.2	0.4
7-8	39.9	0.0	0.2	0.1	0.2	0.2
8-9	2.6	2.0	0.0	0.0	0.0	0.0
9-10	1.3	0.0	0.0	0.0	0.0	0.0

[1] Done on C-18 plate and developed with methanol:water, 80:20 (v/v).

[2] JH-3 migrate to this zone.

Table 9. Metabolism of ^{3}H JH-3 by part-fed virgin female *Dermacentor variabilis* hemolymph[2]; percent of radioactivity eluted from each zone separated by TLC

Cm from origin	ACTIVE HEMOLYMPH		INACTIVE HEMOLYMPH		NO HEMOLYMPH	
	aqueous	organic	aqueous	organic	aqueous	organic
0-1[1]	1.2	0.5	TO BE DONE		0.1	0.7
1-2	1.1	0.5			0.1	0.7
2-3[2]	6.6	56.1			1.4	86.3
3-4	3.5	4.3			0.2	4.3
4-5	3.3	1.1			0.4	1.6
5-6	1.4	0.8			0.2	1.6
6-7	14.5	0.4			0.2	0.6
7-8	2.0	0.5			0.2	0.5
8-9	0.8	0.4			0.1	0.4
9-10	0.9	0.4			0.1	0.4

[1] Done on C-18 plate and developed with methanol:water, 80:20 (v/v).

[2] JH-3 migrate to this zone.

Table 10. Distribution of ^{14}C labelled products in part fed female *H. dromedarii* extracts following inoculation of ^{14}C mevalonic acid into engorged nymphs.[1]

Cm from origin	Percent Radioactivity
0-1	31.1
1-2	1.1
2-3	1.2*
3-4	1.2
4-5	1.7
5-6	46.3
6-7	8.9
7-8	4.5
8-9	0.9
9-10	0.9

[1] Done on a C-18 reversed TLC plate and developed with methanol:water, 65:35, v/v.

*JH-III migrates to this location.

Table 11. Effects of treatment of H. dromedarii nymphs with JH-III in DMSO:acetone (1:4) on survival and ecdysis. (Topical Treatment)

Days Post-treatment	1 ng	5 ng	10 ng	20 ng	50 ng	100 ng
17	0	0	0	0	0	0
18	8	14	9	12	7	8
19	14	15	9	16	8	12
20	13	7	6	10	0	0
21	1	0	0	2	0	0
22	0	2	1	4	0	0
23	1	0	2	5	1	2
24	0	0	0	0	0	0
25	0	0	0	0	0	0
26	0	4	0	0	1	0
27	0	0	0	0	0	0
28	0	0	0	1	0	0
29	0	0	2	0	1	1
30	0	0	0	0	0	0
Total surviving	37	42	29	50	18	23
% mortality	74.0	84.0	58.0	100.0	36.0	46.0
Mean days to ecdysis	19.3	19.6	20.0	19.9	19.8	19.4

Table 12. Effects of treatment of H. dromedarii nymphs with JH-III in DMSO:Acetone (1:9) administered by the contract method on survival and the duration of ecdysis.

Days post-treatment	Concentration of JH-III in ng/cm^2					
	0.1 ng	0.5 ng	1.0 ng	2.0 ng	5.0 ng	10.0 ng
9	0	0	1	0	0	0
10	0	0	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	0	0	2	0	0	0
14	0	0	0	0	0	1
15	0	8	1	1	0	0
16	0	7	2	5	6	0
17	1	2	0	1	4	0
18	0	12	0	15	10	2
19	0	0	1	0	0	1
20	1	0	13	8	0	5
21	0	0	0	10	10	11
22	0	5	3	0	10	4
23	1	0	5	0	1	4
24	0	0	5	0	0	2
25	0	0	0	1	0	5
26	1	2	0	0	0	4
Total surviving	4	36	33	41	41	39
% mortality	92.0	18.0	34.0	18.0	18.0	22.0
Mean days ecdysis	21.5 ≠3.9	17.1 ±4.3	20.1 ±3.6	18.9 ±1.8	19.2 ±2.9	21.5 ±4.4

ing 14.5% of the sample radioactivity, was found in the aqueous fraction. This is in contrast to the THW larval hemolymph, where most of the radioactivity was found in the form of highly polar compounds. The total radioactivity recovered from the digests and from the TLC plates was only 46.5% of the total introduced at the start of the experiment, largely due to emulsions that formed at the interface between the 2 phases. Also of interest in the case of the tick hemolymph digests was the presence of weakly active spots at 3 and 4 cm, representing compounds more polar than JH, but not as polar as the much more prominent fraction at 7-8 cm.

HPLC of the aqueous phase eluted from the TLC plate (6-7 cm zone) revealed 2 peaks with retention times of 4.08 and 7.69 min, that were radioactive (Fig. 11). Together, these two fractions accounted for 71.7% and 20.8% of the total radioactivity of the sample. Both peaks co-eluted with similar fractions obtained by digestion of radioactive JH-3 with THW larval hemolymph. Fraction 1 is almost certainly JH-3 acid, based on the major metabolite obtained by acid hydrolysis of authentic JH-3; fraction 2 is JH-3 diol. HPLC of a sample of (500 ul) of *D. variabilis* PFV female hemolymph also revealed the same peaks (Fig. 2). Using the absorbance of JH-3 at 214 nm as the basis for comparison, the estimated amount of JH acid and JH diol in the hemolymph was 0.5 and 0.9 ng/tick, respectively.

Evidence of hemolymph esterases in feeding females is shown in Figures 12-16. Figure 12 is a composite of photographs of column gels; Part A reflects the distribution observed when the gels were stained with the nonspecific protein stain Comassie Brilliant Blue (CBB); Part B, when the gels were stained with the esterase specific stain Fast Blue B (FB). In Part A, gel no. 1 shows the occurrence and distribution of low molecular weight markers; gel no. 2 shows the high molecular weight marker proteins; gel No.

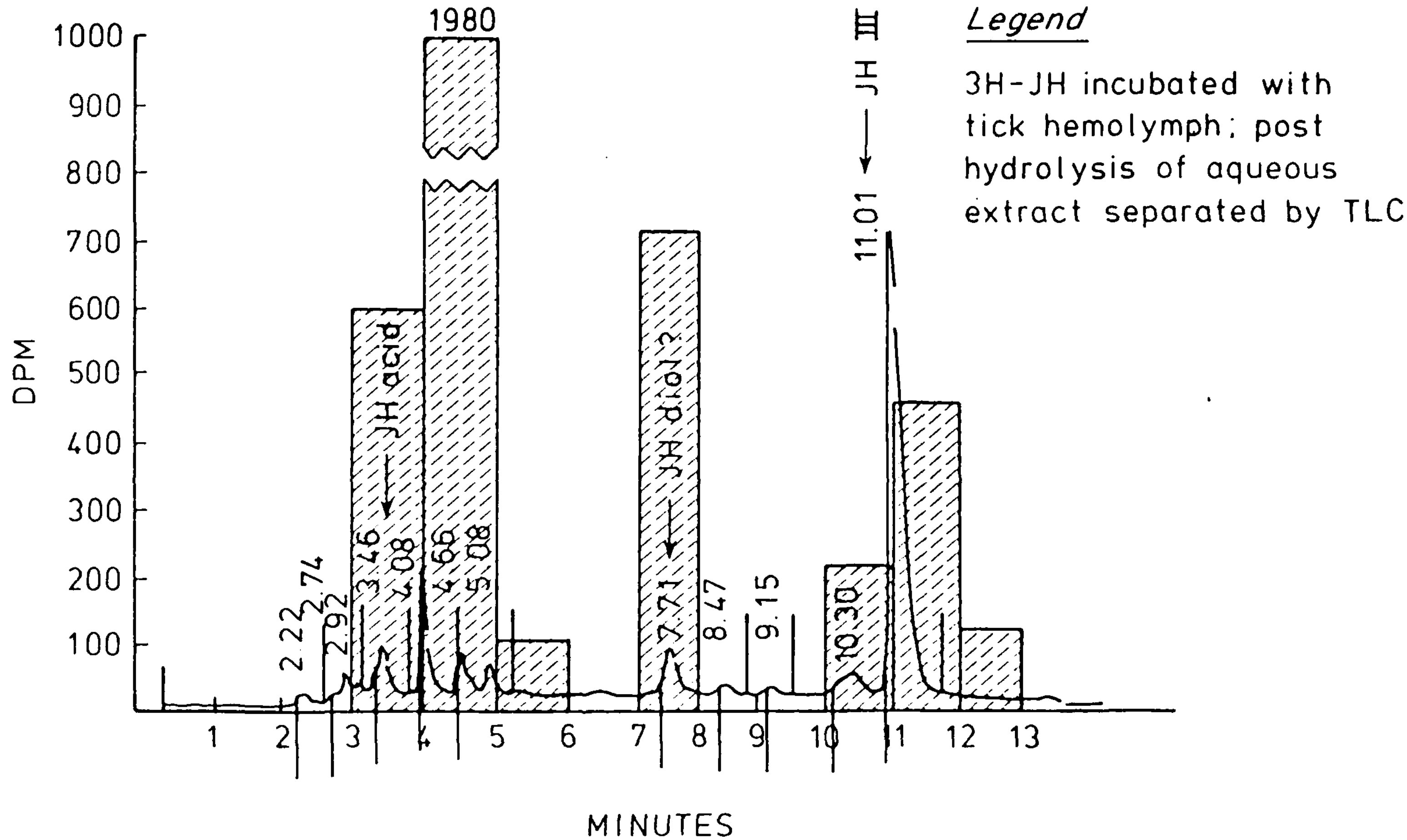

Figure 11. HPLC chromatogram illustrating the occurrence of 2 radioactive peaks more polar than ^{3}H juvenile hormone. Radioactivity is illustrated by the histogram superimposed on the chromatogram. The peak at 4.08 min co-elutes with the peak prepared by acid hydrolysis and is probably the acid. The peak at 7.71 min co-elutes with that obtained by incubation with THW hemolymph, and probably the diol.

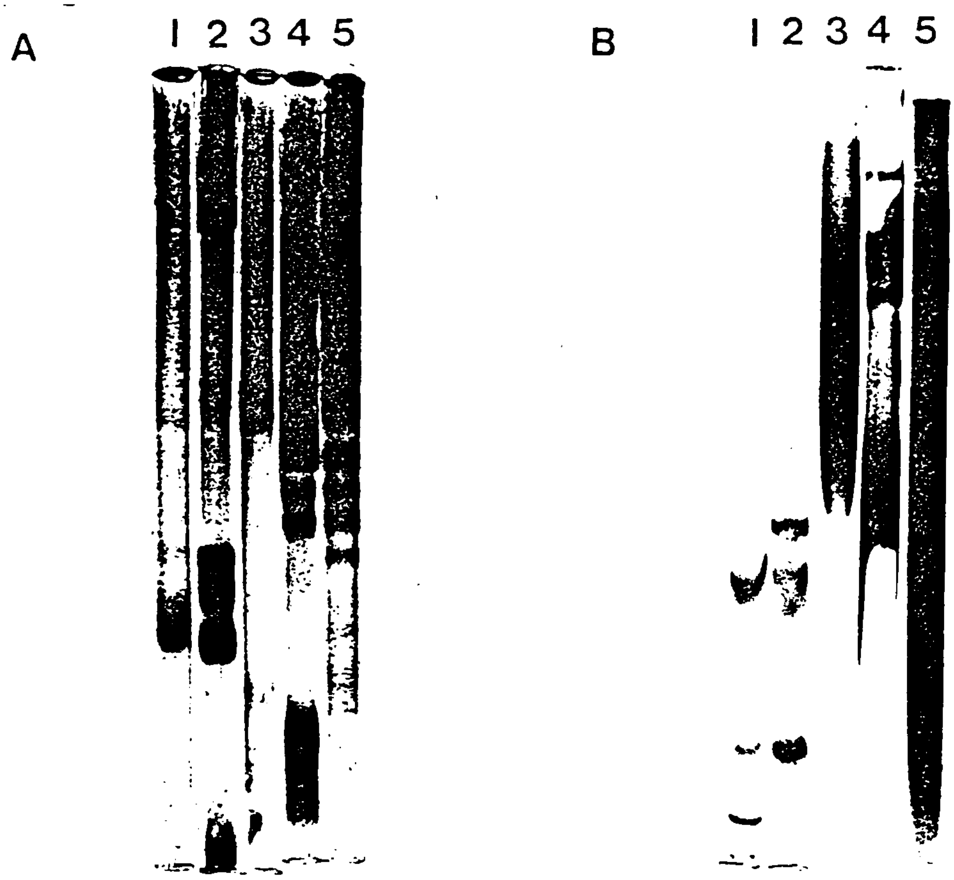

Figure 12. Distribution of tick hemolymph proteins detected by column gel electrophoresis and stained by different methods. Gels spotted with standards or samples of hemolymph extract. Part A, gels stained with Commassie Brilliant Blue (CBB); Part B, same gels, but gels 3, 4 and 5 stained with Fast Blue B (FB). Gel 1 = how molecular weight markers, 15 ul sample; gel 2 = high molecular weight markers, 10 ul sample; gel 3 = porcine esterase standard, 15 ul sample; gel 4 = D. variabilis hemolymph, 15 ul sample; gel 5 - tobacco hornworm larval hemolymph, 15 ul sample. Considerable overlap occurs between the low molecular and high molecular weight markers. Gels in part exposed to 48 mAMP, 170 volts for 2 hrs at ±2°C; gels in part B exposed to 54 mAMP, 130 volts for 2 hrs at ±2°C.

3 shows the location of the porcine esterase standard; gels 4 and 5 show the occurrence of tick and THW hemolymph proteins remaining after centrifugation and electrophoresis, respectively. The gels are shown side by side to facilitate comparison of the locations of the protein bands in different specimens. To facilitate interpretation of these electrophoretic separations, the same gels were photographed separated and the photographs reassembled with arrows indicating the location of specific protein bands. Figure 13 is a composite of the same gels, stained with CBB, but separated to allow labelling. Gels 1 and 2 show the locations of the molecular weight markers with known weights from 31,000 to 200,000 daltons; gel 3 shows the relative position of the porcine esterase proteins. In the adjacent tick hemolymph sample, at least 11 bands of varying intensity are evident, in contrast to 8 in the THW sample. The pattern of protein distribution shows considerable differences in the two different species. The tick hemolymph sample shows a group of very small proteins, less than 31,000 near the solvent front, as well as a broad band of intensely staining proteins in the range of 92,000 to 116,000. In contrast, the THW larval hemolymph samples lack the very small proteins and has intensely staining bands from 59,000 to 211,000 Daltons. Figure 14 shows the same gels as in Figure 13, but the gels separated to allow labelling. Gels 1 and 2, the molecular weight markers, were stained with CBB, since these proteins do not react with FB. This figure shows the occurrence of esterase enzymes, stained with Fast Blue, in tick and THW hemolymph. Clearly, esterases occur in the hemolymph of both species. In the case of the tick hemolymph, 4 esterase bands are evident, ca 218,000, 205,000, 191,000 and 118,000 daltons. In the THW hemolymph, 3 esterase bands were found, namely, a very large band of ca 190,000 daltons, a small band at 118,000 daltons, and a very large band of ca 92,000 daltons.

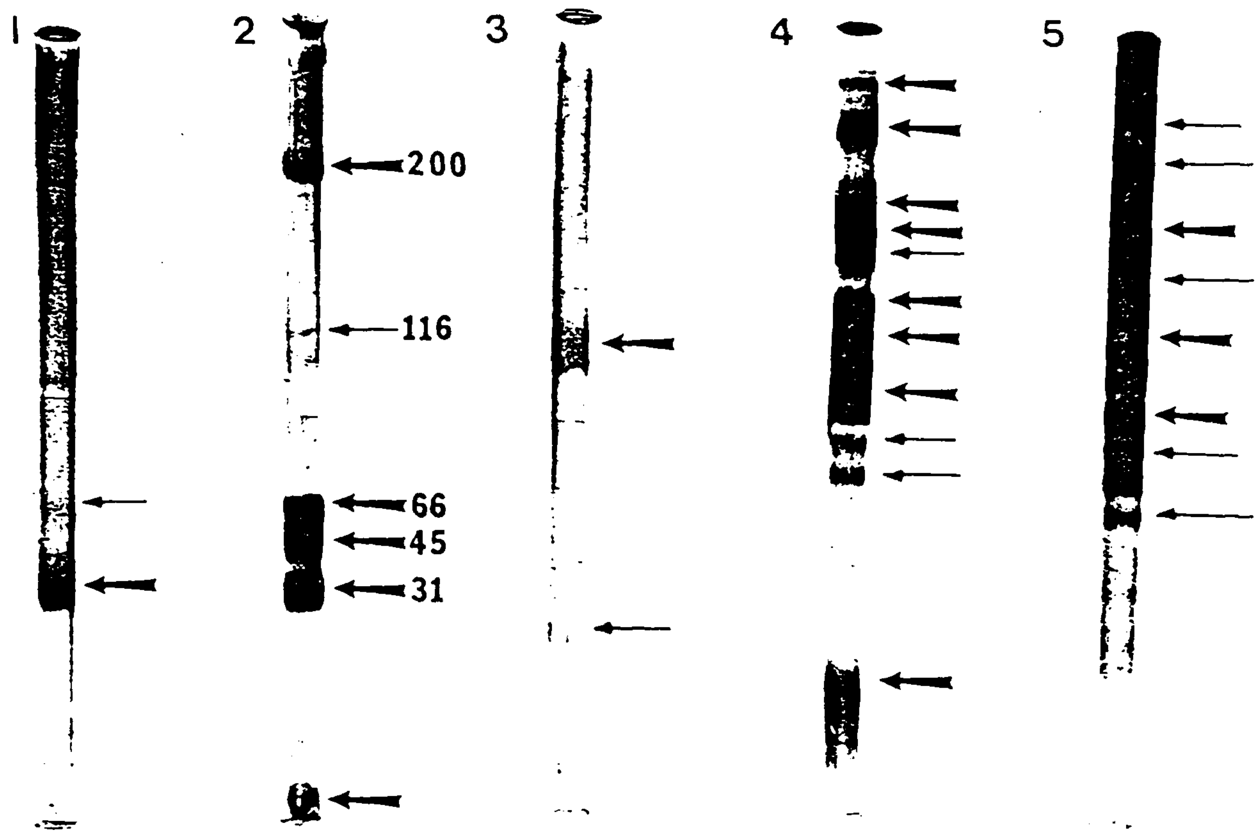

Figure 13. Distribution of tick hemolymph proteins and standards as shown in Fig. 2a, but gels separated to facilitate labelling. Gel 1 = low molecular weight markers; gel 2 = high molecular weight markers; gel 3 = porcine esterase standard; gel 4 = D. variabilis hemolymph, 15 ul sample; gel 5 = tobacco hornworm larval hemolymph, 15 ul sample. All gels stained with Commassie Brillian Blue. Arrows indicate location of the detectable proteins; thick arrows, most prominent proteins, thin arrows, faint protein bands.

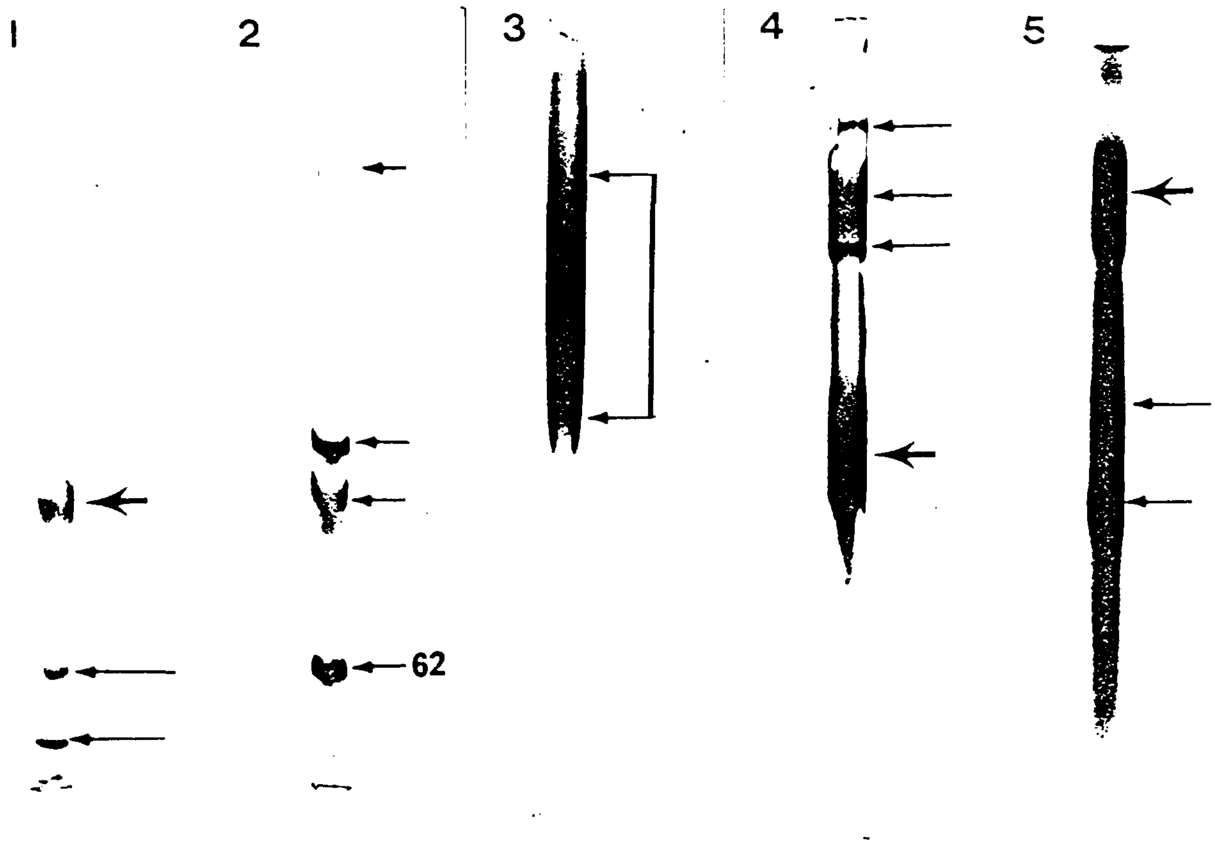

Figure 14. Distribution of tick hemolymph proteins and standards as shown in Fig. 2b, but gels separated to faciliate labelling. Gel 1 = low molecular weight markers, 15 ul; gel 2 = high molecular weight markers, 15 ul sample; gel 3 = porcine esterase standard, 20 ul sample; gel 4 = D. variabilis hemolymph, 20 ul sample; gel 5 = tobacco hornworm larval hemolymph, 30 ul sample. Gels 1 and 2 stained with Commassie Brilliant Blue, gels 3, 4 and 5 stained with Fast Blue B.

The tick and the THW esterases appear to be quite different. In the tick, the most intensely reacting band has a molecular weight of 118,000 daltons; in the THW, the most intensely reactive band has a molecular weight of 190,000 daltons.

Isoelectric focusing revealed many more proteins than were detected by column gel electrophoresis. Figure 15 is a composite photo illustrating esterases in D. variabilis and THW hemolymph as determined by isoelectric focusing on a slab gel; at least 8 bands, including 1 very dense band near the negative pole, are apparent. In the tick also, many more esterases are detected by this technique than by other methods. At least 10 are evident, including 3 that are intensely reactive. The concentration of esterase proteins appeared to be much greater in the tick hemolymph than in the THW hemolymph. Figure 16 is a composite photo illustrating the distribution of all hemolymph proteins in these 2 species as determined by isoelectric focusing on slab gels. At least 16 hemolymph proteins were resolved in the D. variabilis hemolymph samples, including one or more intensely reactive bands at near neutral pH; at least 14 protein bands were resolved in the THW hemolymph samples.

Treatment of H. dromedarii nymphs by the topical method had no apparent affect on the duration of the ecdysial period; the means for the different treatment groups were remarkably uniform, from 19.3 to 20.0 days. Survival was unaffected by the lower dose treatments. However, substantially reduced survival occurred with the 50 and 100 ng treatments. No higher doses were administered, and it is not known whether this represents a dose response curve or not.

Treatment of H. dromedarii nymphs by the contact method did not appear to have any effects on the ecdysial period or survival of the treated ticks.

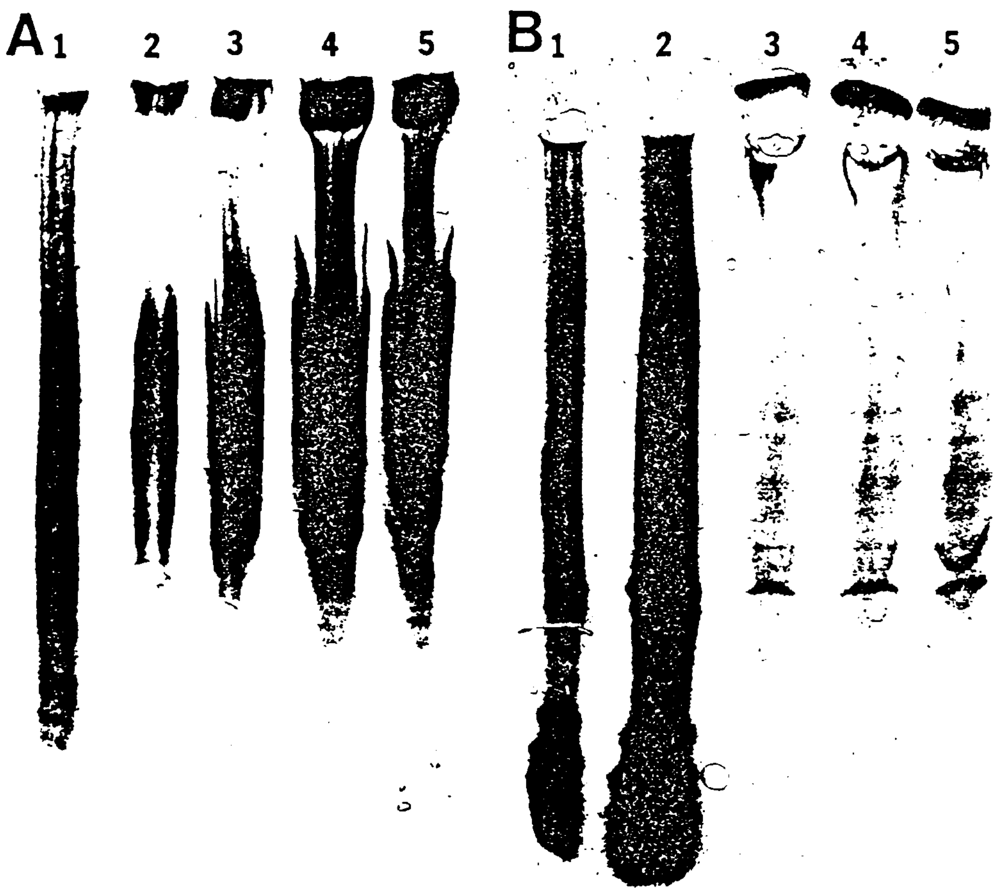

Figure 15. Distribution of tick and tobacco hornworm larval hemolymph esterases detected by isoelectric focusing on slab gels and stained with Fast Blue B. Part A, tick hemolymph: gel 1 = porcine esterase, 0.5 ul, gels 2-5 = D. variabilis hemolymph, 0.5, 1, 2, and 3 ul samples, respectively. Part B, tobacco hornworm larval hemolymph: gels 1 and 2 = porcine esterase standard, 1 and 5 ug, respectively; gel 3, 4 and 5 = tobacco hornworm larval hemolymph, 5, 10 and 15 ul samples, respectively. Gels run at 2 watts, constant power, for 1 1/2 hrs.

6

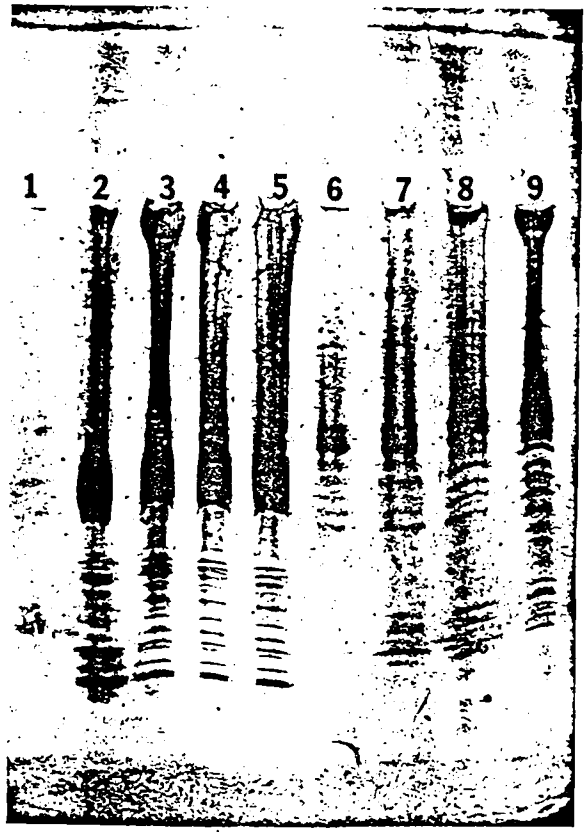

Figure 16. Distribution of tick and tobacco hornworm proteins detected by isoelectric focusing on slab gels and stained with Commassie Brilliant Blue. Gel 1 = porcine esterase standard, 5 ug; gels 2-5 = D. variabilis hemolymph, 0.5, 1, 3, and 5 ul samples; gels 6-9 = tobacco hornworm larval hemolymph, 0.5, 1, 3 and 5 ul samples. Gels run at 4 watts, constant power, for 1 1/2 hrs.

: 14. Effects of inoculated ecdysteroid hormone analogues on survival and molting in engorged _H. dromedarii_ nymphs.

of :ment	Amount Inoculated	No. nymphs died	No. nymphs molted	Mean ecdysial period (days)	t-test
!5 DDE	1 ug/tick	18	32	20.5 ±0.70	0.6, p 0.5 n.s.
:eroid	5 ug/tick	21	29	16.75 ±0.41	10.32, p 0.01 114 d.f.
·ol ·eated)	——	0	66	20.34 ±1.68	——
·ol ı's ine)	IN PROGRESS				

al Progress Report), the effects of BSEA-28 were restricted to acceler- molting and elevated sex pheromone activity. Mortality, at least of engorged nymphs, was not affected, with one exception. Greatest sur- l was found with nymphs fed on animals with the largest amount of im- ted analogue material, thereby dismissing the possibility of any toxic :ts on the feeding ticks. In the present case, DDE treatments resulted ignificant mortality both in engorged nymphs fed on treated rabbits and administered by direct inoculation. Also of interest are the effects ·ved with azasteroid, which were similar to those observed with 22, 25 except that they were induced by a 5 fold higher concentration than observed with 22, 25 DDE. The azasteroid, which closely resembles the ·al hormone except for the substitution of a nitrogen atom among the carbons, may be less susceptible to metabolism than the authentic steroids.

Connat et al. (1983) reported that 22,25 DDE was highly effective when

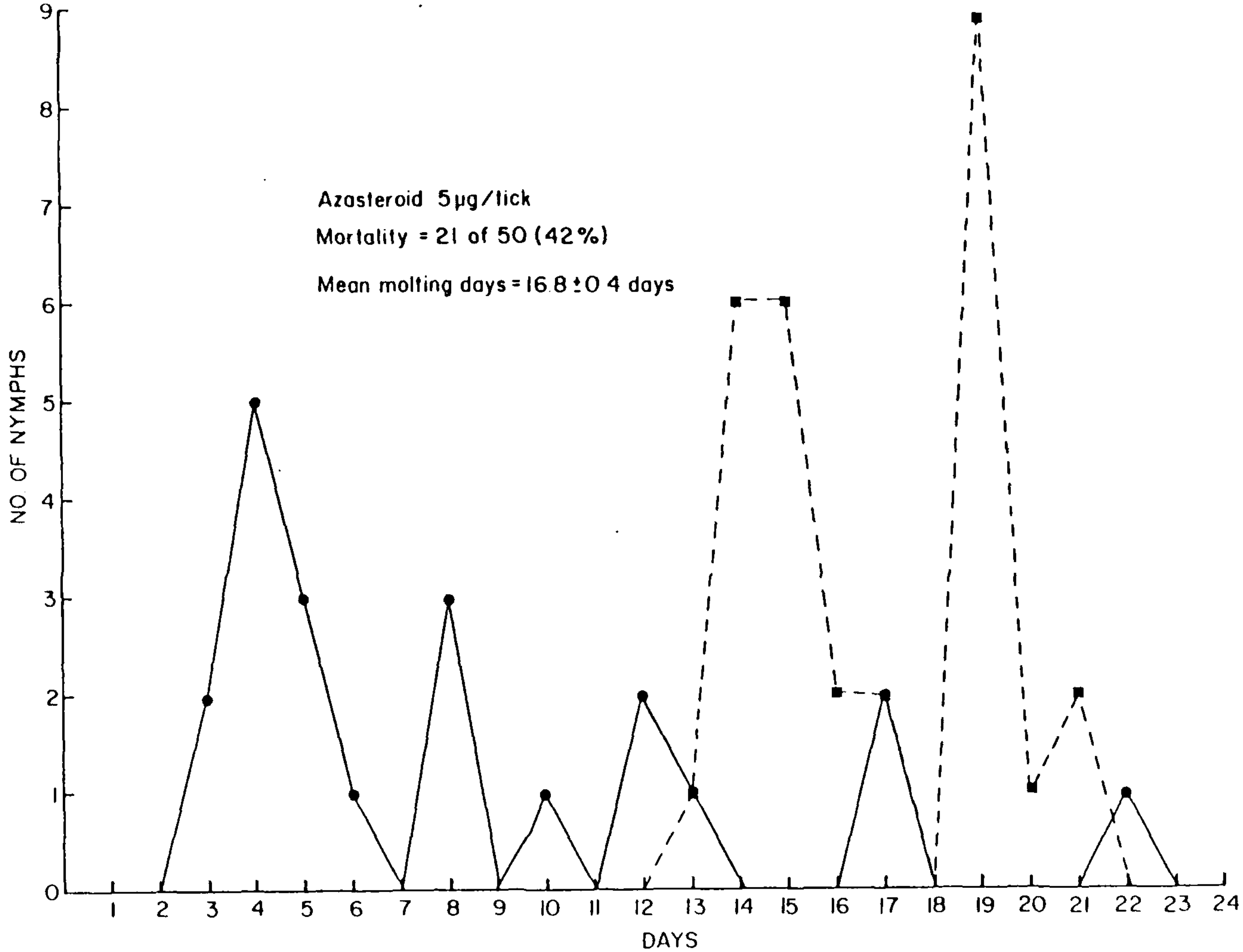

Figure 20. Effects of direct inoculations of azasteroid on survival and ecdysis of engorged nymphal H. dromedarii. Solid line = number dead on the days indicated; dotted line = number molting on the days indicated.

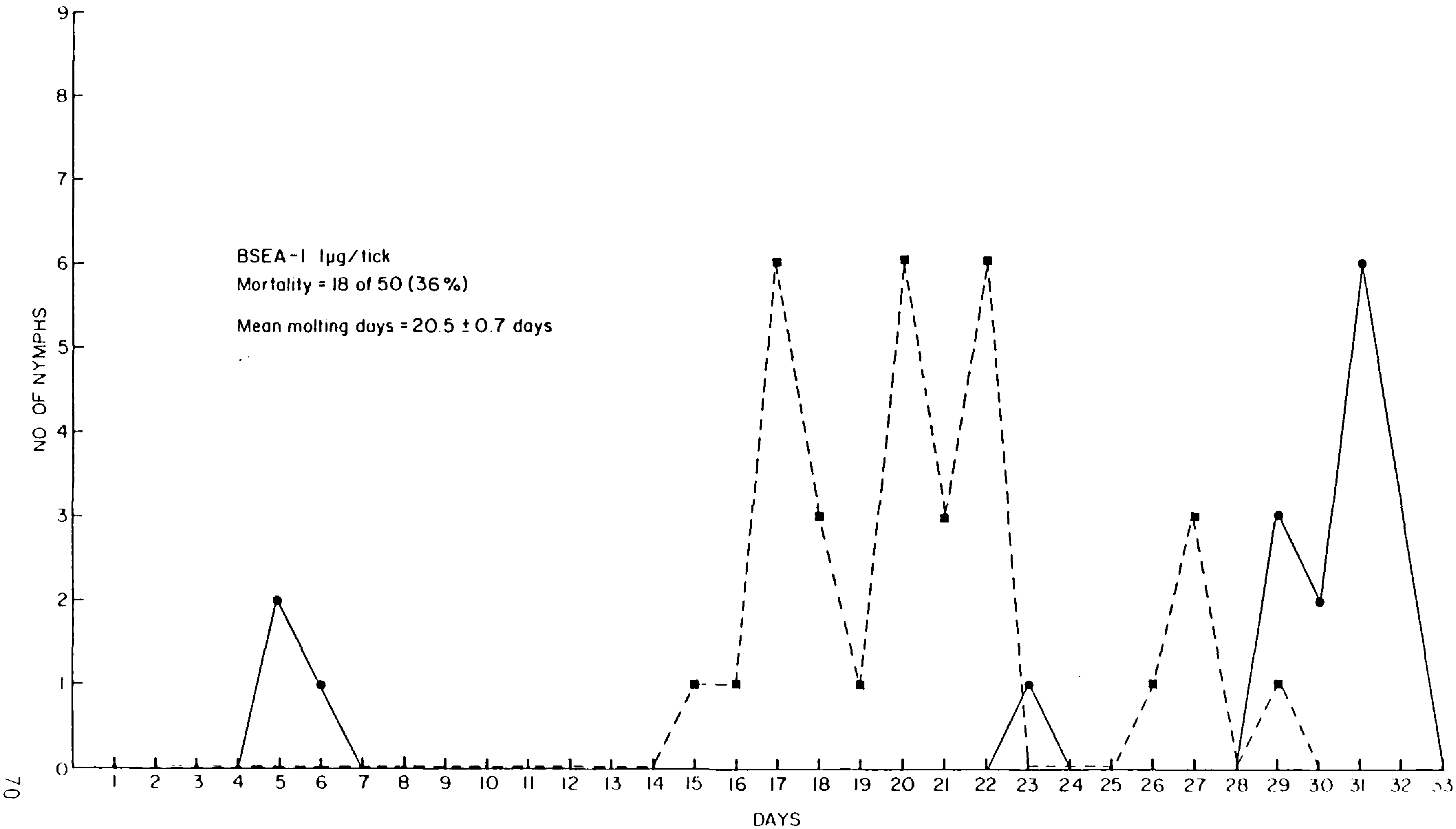

Figure 19. Effects of direct inoculation of BSEA-1 on survival and ecdysis of engorged nymphal H. dromedarii. Solid line = number dead on the days indicated; dotted line = number molting on the days indicated.

mean time for molting was reduced slightly, but significantly when compared with the controls ($p<0.01$ in both populations). Especially noteworthy is the high mortality observed with these ticks. Most of the mortality occurred early in the post-engorgement period. In the population from rabbit number 1, with 6 implants, 144 nymphs, or 27.4% of the 526 nymphs recovered from that animal died within the first 5 days post-engorgement; in the population from rabbit number 2, with 5 implants, 120 nymphs, or 27.0% of the 445 nymphs recovered died within the first 5 days.

Assays to determine whether BSEA-1 can be detected in host blood are in progress.

This study is being conducted with the same animals after a lapse of approximately 75 days to determine if the effects of the hormone analogue continue to be expressed.

The results of tests to determine the effects of ecdysteroid analogues on ticks by direct inoculation are summarized in Table 14 and Figures 19 and 20. Treatment of H. dromedarii nymphs with BSEA-1 at 1 ug/tick produced results similar to that observed with the implants described above, i.e., substantial mortality, but little effect on the ecdysial period; in this case, the ecdysial period was not reduced. Treatment with azasteroid at 5 ug/tick also induced considerable mortality, and there was a signficiant drop in the duration of the ecdysial period ($T=10.32$, $p<0.01$, 114 d.f.).

Similar tests with hormone analogues inoculated in D. variabilis nymphs are still in progress.

Discussion

These results contrast with previously reported studies for BSEA-28. In the case of BSEA-28, as described in our previous progress report (4th

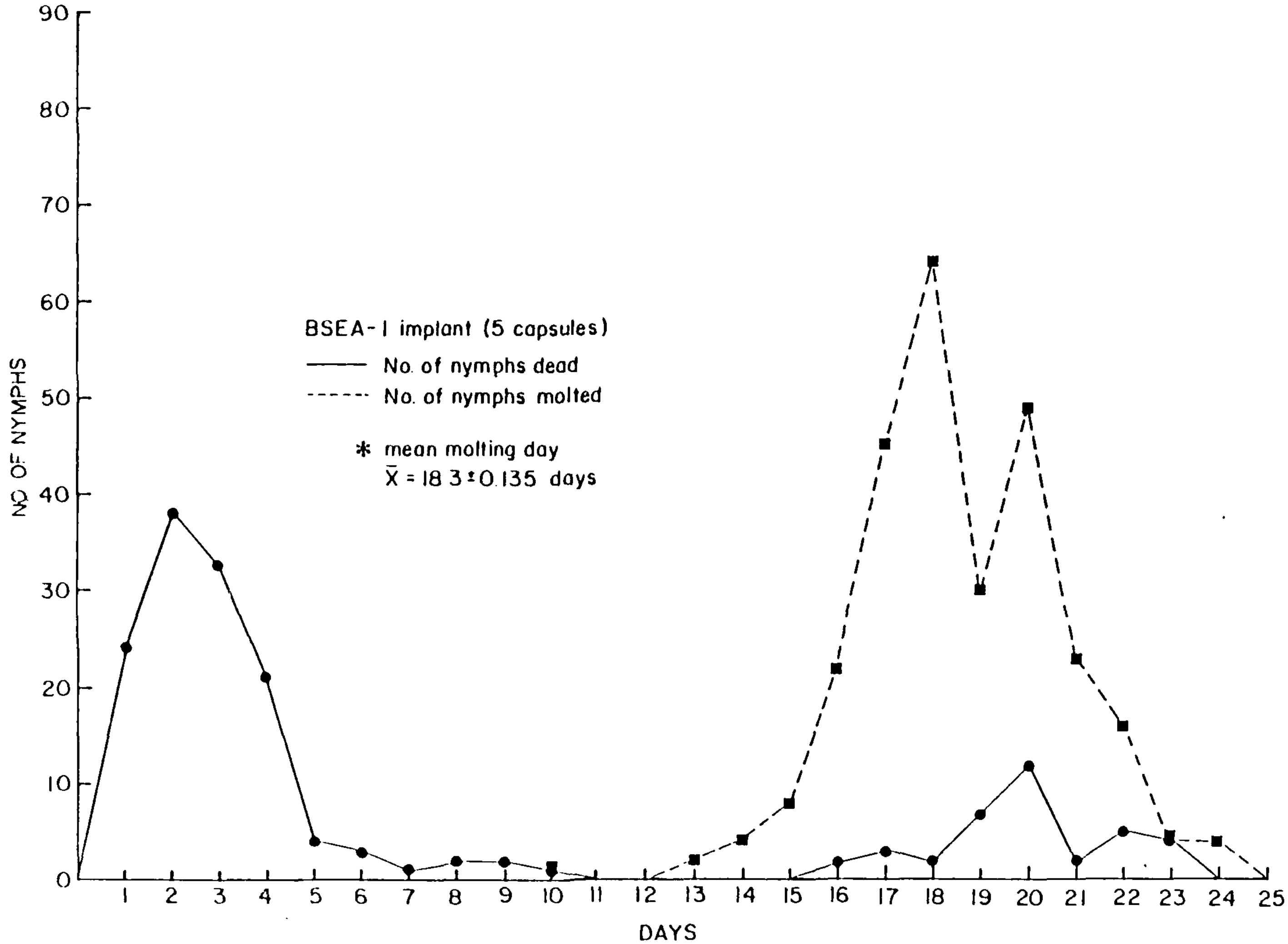

Figure 18. Mortality and ecdysis of survivors in H. dromedarii nymphs that engorged on a rabbit with BSEA-1 implants (5 capsules) containing 227 mg of active ingredient.

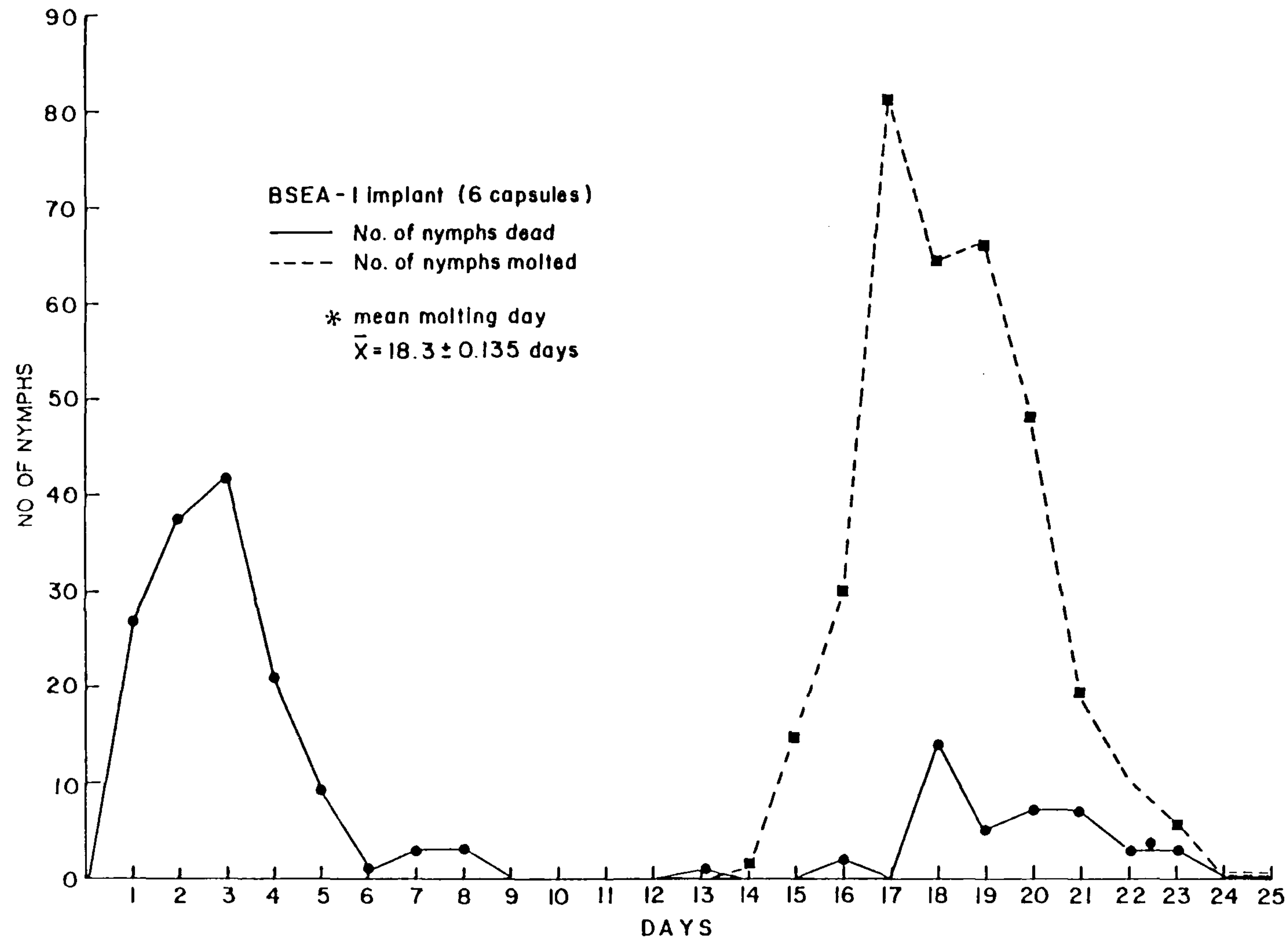

Figure 17. Mortality and ecdysis of survivors in H. dromedarii that engorged on a rabbit with BSEA-1 implants (6 capsules), containing 272 mg of active ingredient.

columns used in previous studies.

Results

Table 13 summarizes our results to date concerning the effects of the BSEA-1 treatment on ticks feeding on rabbits with implants containing this substance. A total of 971 engorged nymphs were collected from the 2 animals

Table 13. Effects of presence of the hormone analogue, 22, 25 dideoxy-ecdysone in rabbits serving as hosts to H. dromedarii immatures; effects on survival and duration of the ecdysial period.

Animal No.	Sex	Weight (kg)	Amount of active ingredient	Mean weight/ eng. nymph	No. nymphs dead	No. nymphs molting	Mean ecdysial period (days)	t-test
1	fem.	2.16	272 mg	20.4 mg ±1.5	186	340	18.30 ±0.14	13.44 P 0.01 970 d.f.
2	male	2.4	227 mg	21.6 mg ±2.9	173	272	18.60 ±0.24	9.67 P 0.01 902 d.f.
3	male	2.7	——	18.3 mg ±5.4	2	632	19.84 ±2.11	—— ——

(estimated 24.3% yield), 526 from one animal, 445 from the other. The mean weights for the 2 tick populations, 20.4 and 21.6 mg, respectively, were not significantly different from the controls described previously (4th Annual Progress Report, 20.53±3.94 mg/tick ($t=0.27$, $p>0.5$, n.s., and $t=0.1$, P 0.8, n.s.). However, survival of the nymphs from the treated rabbits was substantially reduced. Overall, 37.0% of the ticks died without molting (Table 13, Fig. 17, 18). The length of the ecdysial period for the nymphs from the 2 rabbits is summarized in Figures 17 and 18. The pattern of molting times reflects a more or less unimodal curve with molting from 13 to 24 days. The dromedarii larvae. Recovery of engorged nymphs, molting, and mortality were monitored as described in previous reports. The entire experiment was repeated with the same animals following an interval of 72 days, to assess the effects of continued long term delivery of these products. Controls were done with ticks feeding on a naive rabbit without implants or other study materials.

In addition to studies with BSEA-1 delivered via host blood, this compound was also inoculated directly into engorged nymphal ticks on the day of drop off. Both *H. dromedarii* and *D. variabilis* nymphs were used for these studies. Treatments were done with 1 ug and 5 ug/tick using both BSEA-1 and azasteroid.

Blood samples were collected from the rabbits with the BSEA-1 implants to be used for determination of detectable BSEA-1 in the circulating blood of these animals. Samples were collected at weekly intervals from the animals, extracted in accordance with the methods described by Dees et al. (1984), and held for analysis by RIA and HPLC.

To determine whether fecundity of fertility of the ticks exposed to the BSEA-28 treatments was affected, adults from the treated populations were selected at random and allowed to feed and mate on a normal host rabbit. Following mating and repletion, 5 replete females from each of the 2 populations were selected for these determinations. Total engorged weight, and egg mass weight are being measured in each case; hatching success will also be estimated. These studies are in progress.

Studies of the effects of BSEA-1 on sex pheromone activity in the emerged adults are also being continued, using the same procedures described by Dees et al. (1984) and in previous progress reports. The only change is the substitution of a capillary column (SE-54), instead of the packed blood. Consequently, we were interested in evaluating the effects of this analogue on _H. dromedarii_ and _D. variabilis_, which became possible when we succeeded in obtaining this compound as a gift from Dr. H. Jaffe.

This brief report describes preliminary results of studies commenced recently (1985) with the ecdysteroid analogue, BSEA-1 and a closely related analogue, azasteroid (in which a nitrogen atom replaces one of the ring carbons), to assess its effects when applied in different amounts, and its persistence in the host. Experiments were initiated using the same slow release delivery system described in previous reports. In addition, treatments were administered by direct inoculation to evaluate the effects of precisely known doses on tick survival and the ecdysial period.

Materials and Methods

BSEA-1 (22,25 dideoxyecdysone, or DDE) was synthesized by published methods (Thompson, et al. 1971), and was donated as a gift from Dr. M. Thompson (via Dr. Jaffe, USDA, BARC, Beltsville, MD). The material was suspended in ethyl oleate (25%, w/w of active ingredient, AI) and deposited in tubes of the permeable plastic, polycaprolactone. The filled tubes were cut into 4-cm lengths and sealed with silastic plugs. The resultant 4-cm tubes each contained an estimated 45.3 mg AI.

Implantation of the devices containing the ecdysteroid analogue was done by suturing them into the shoulder and back areas of normal, adult rabbits, _Oryctalagus cuniculus_, in accordance with methods described previously (Jaffe et al. 1984); 6 were placed in a female rabbit, providing 272 mg of active ingredient (ca 108.8 mg/kg body weight), and 5 were placed in a male rabbit, providing 227 mg of active ingredient (ca 75.6 mg/kg body weight). One week after treatment, each animal was infested with 2000 _H._

IV. CONTINUING STUDIES ON THE EFFECTS OF ECDYSTEROID ANALOGUES ON DEVELOPMENT AND SEX PHEROMONE ACTIVITY IN *HYALOMMA DROMEDARII* AND *DERMACENTOR VARIABILIS*

Introduction

Previous progress reports have reviewed studies in which an ecdysteroid analogues, BSEA-28 (Thompson et al. 1971) were delivered to immature *H. dromedarii* feeding on rabbits via slow release devices implanted into these hosts. These controlled release devices offer the opportunity to maintain uniform blood levels of the active ingredient over long periods, thereby providing continued efficacy for weeks or even months following their initial introduction. Using (BSEA-28 (beta, 5-beta-14-alpha-trihydroxy-5-beta-cholest-7-en-6-one), an analogue resembling the natural hormone, 20-hydroxyecdysone, we observed highly significant reductions in the time required for ecdysis, reduced body weight, and excitation of sex pheromone activity. These results were summarized in a recent paper by Jaffe et al. (in press).

Solomon et al. (1982), in their review of the potential for hormonal control of tick infestations on domestic animals, concluded that there was little reason for optimism regarding the use of ecdysteroids. This perspective has now changed as a result of the work done in Switzerland by Connat et al. (1983) with the ecdysteroid, 22,25 didexoyecdysone (DDE, or BSEA-1). These studies, done with the argasid tick, *Ornithodoros moubata*, suggested much greater activity for the ecdysteroid analogue, 22,25 dideoxyecdysone (DDE), also known as BSEA-1, than we observed with BSEA-28. These workers observed accelerated molting in *Ornithodoros moubata* with as little as 35 ng/ml of blood containing DDE, and substantial mortality in this species when the concentration of DDE was increased to 500 ng/ml of non-specific enzymes. Further study will have to be directed to the enzymatic activity of selected tick tissues, particularly the fat body, epidermis, the gonads, pheromone glands and other sites where specific high affinity binding sites (receptors) may be expected. In addition, autoradiographic methods might be useful in determining evidence of such high affinity binding sites, providing necessary clues to sites of JH activity and possible modes of action.

Exogenous JH may induce a variety of physiological effects in ticks. Pound and Oliver (1979) observed restoration of ovarian activity by JH administered after treatment of female Ornithodoros parkeri with the anti-allatotropin Precocene-II. More recently, Connat et al. demonstrated excitation of vitellogenesis in fed virgin O. moubata which normally do not oviposit unless mated. The most convincing evidence of JH as a gonadotropic/vitellogenic hormone has been obtained with argasid ticks (although Bassal and Roshdy, 1974, were unable to induce such action by JH in Argas arboreus). The role of JH in ixodid ticks is much more difficult to discern. Ioffe and Uspenskiy (1979) observed that Altozar, a JH analogue, delayed molting in non-diapausing Ixodes ricinus, but accelerated molting in diapausing nymphs, i.e., disrupted diapause. In their review of the literature on this subject, Solomon et al. (1982) noted that virtually all studies with exogenously administered JH in ixodid ticks reported no discernible juvenilizing effects. The sterilizing effects obtained with massive doses of Precocene-II in Rhipicephaus sanguineus (Leahy & Booth, 1980) may represent toxic rather than true hormonal effects. Moreover, true extension of the ecdysial period was not observed by these authors, who noted that treated ticks failed to molt well beyond the expected ecdysial period, but did not differentiate between delayed mortality and delayed ecdysis. Presumably, JH exists in ixodid ticks, affecting developmental processes as well as regulating spermatogenesis, vitellogenesis and oviposition in the adults. However, proof of the existence of this elusive hormone and its actions remains to be discovered.

At the present stage of our study, we have only been able to characterize the rapid degradation of JH by tick hemolymph. However, the formation of at least 2 metabolites implies the existence of JH specific as well as expect only one peak of radioactivity when JH-III is incubated and the products separated. This was the case when the reaction was performed with tobacco hornworm larval hemolymph but not when it was done with tick hemolymph. In the later case, TLC revealed two radioactive localities, a well defined spot at 6-7 cm, and a broader zone at 3-5 cm, both more polar than JH. When the aqueous extract of the reaction was extracted and separated further by HPLC, 2 radioactive fractions were detected, both more polar than JH-III. If JH metabolism was due solely to non-specific carboxyesterases, only one peak should have been found. These fractions may represent JH acid, JH diol, or JH acid/diol. In any case, the occurrence of 2 metabolites suggests the presence of more specific enzymes capable of cleaving the epoxide, i.e., an epoxidase, enzymes usually found only in cells and tissues. In view of the fact that ticks have a diffuse fat body, and hemocytes of various types were contained in the hemolymph samples, this is not unexpected. If confirmed, the presence of epoxidases would imply the presence of natural JH hormones.

In insects, JH inactivation is believed to begin in the hemolymph, where the free hormone is attacked by non-specific carboxyesterases and degraded to the corresponding acid. However, much JH is protected by JH specific binding proteins, and survives intact unless JH specific esterases capable of reacting with the bound hormone also occur (Riddiford & Truman, 1978). Further breakdown to the diol and acid/diol forms is accomplished by membrane bound enzymes, especially by epoxide hydratase. In insects, this enzyme is found primarily in the fat body, in the epidermis and, to a lesser extent, in other tissues. Protein bound JH is readily susceptible to attack by these intracellular enzymes. The various JH metabolites that result no longer bind to the carrier proteins and are excreted intact or as sulfate conjugates (Riddiford & Truman, 1978).

The ecdysial periods ranged from 17.1 days at 0.5 ng/cm^2 to 21.5 days at 10 ng/cm^2 treatments. However, the duration of the ecdysial period was just as long in the case of the nymphs exposed to 0.1 ng/cm^2, the lowest dose used, as at 10 ng/cm^2, the highest doses, and there was no evidence of a dose response curve. Survival remained high in most treated groups except those exposed to 0.1 ng/cm^2, the lowest dose used.

Tests with radioactive JH-III demonstrated that 22.7% of the compound administered topically in DMSO;acetone enters the body of the treated ticks.

Discussion

The results demonstrate that tick hemolymph has an impressive ability to enzymatically degrade exogenous JH. Non-specific carboxy-esterases are probably responsible for much of this metabolism of JH, resulting in acids of the corresponding JH molecules. Tick hemolymph is very similar to tobacco hornworm larval hemolymph with regard to this capability. Tick hemolymph contains numerous proteins with esterase activity, which are especially evident when they are separated by iso-electric focusing; at least 10 esterase bands are evident by this technique, including 3 with especially intense activity.

The presence of numerous esterases in tick hemolymph is not unexpected. This finding leads one to expect cleavage of the methyl ester of JH, including any JH administered exogenously, resulting in JH acid. This same activity can also be obtained with non-arthropod sources of esterase activity, e.g., porcine esterase preparations, indicating that free JH is readily susceptible to catabolic attack by any general esterase. Since this is the only cleavage product resulting from the action of such enzymes, one would administered by inoculation in inducing supermolting in the soft tick, Ornithodoros moubata; teratologic effects, reduced egg production, and inability to properly wax the eggs were also observed. Treatment of these ticks with the natural hormones induced supermolting only at very high doses, about 500 times that observed with 22,25 DDE. Topical treatment of the ticks with methanolic solutions of 22,25 DDE or other ecdysteroids had no effect. These workers concluded that in O. moubata, the hormone analogue remained in the tick's midgut because of the unusual internal anatomv of this species. O. moubata has a blocked midgut (no connection with the hindgut) and the ingested material would remain, diffusing slowly into the hemolymph.

Experience with argasid ticks (Ahmed & Bassal, 1982; Mango, 1979; Kitaoka, 1972) suggests that these ticks are relatively sensitive to exogenous ecdysteroids; some are extremely responsive to such treatments. Our experience with administration of exogenous ecdysteroids in ixodid ticks, at least in H. dromedarii and D. variabilis has been the reverse. Except for the effects on sex pheromone activity and several instances of accelerated ecdysis, the ticks are relatively insensitive to the natural hormone in low concentrations, e.g., 0.1 to 1.0 ug concentrations slightly above natural physiological levels. Consequently, the fact that any effects were found at all in H. dromedarii feeding on animals containing 22,25 DDE in their blood is noteworthy. Connat et al. found little or no effects when O. moubata were fed on blood containing ecdysone, 20-OH ecdysone, ponasterone, or makisterone A in amounts as high as 40 ug/ml of blood, doses many hundreds of times higher than those that were effective when O. moubata were fed on blood containing 22,25 DDE.

Although these studies are incomplete, the preliminary evidence suggests that _H. dromedarii_ and _D. variabilis_ may be sensitive to 22,25 DDE. However, toxicity rather than accelerated molting appears to be the predominant response.

V. HORMONAL REGULATION OF REPRODUCTION IN TICKS AND PARASITIC MITES

Introduction

Annual progress report on work completed at Georgia Southern College on ONR Project "Hormonal-Pheromonal Interrelationships in Ticks and Parasitic Mites," (Contract N00014-80-C-0546). James H. Oliver, Jr., Co-Principal Investigator (subcontractor).

Oocyte Maturation and Oviposition

Little is known about the hormonal control of oocyte maturation and oviposition in ticks. The lack of taomosis (organization of body into separate regions such as head, thorax, abdomen) and the extreme degree of fusion of nerve ganglia, etc. make selective ablations of tissues, ligations, etc. very difficult. The nature of the circulating hormones, site of vitellogenin synthesis, and the extract process of ovarian development are poorly understood.

In insects, juvenile hormone (JH) is known to function gonadotrophically in adults and is produced by the corpora allata (CA). Several reports suggest a role for JH-like compounds in the reproduction of ticks similar to that reported in insects. Two of these reports provide particularly strong evidence that a JH-like substance is necessary for egg development and oviposition in ticks (Pound and Oliver 1979; Connat et al. 1983).

1. An attempt to determine if JH plays a similar role in parasitic mites involved the use of the anti-JH compound precocene 2 (P2) applied to the chicken mite, Dermanyssus gallinae. Experiments were designed to determine if P2 would affect egg production, and if so, whether exogenous JH application would restore egg production. If application of JH to the mites restored egg production, this would be strong evidence for the natural presence and necessity of a JH-like compound in female mites and would lend credence to the hypothesis that JH (or closely related compound) functions

in a gonadotrophic manner in acarines similarly as in insects.

Two mg of P2 were exposed to fed female Dermanyssus gallinae for various lengths of time (24-96 h) and at several concentrations (0.25-4.0 mg). The P2 caused a reduction of progeny produced by the treated females. Reproductive capacity was significantly restored in P2 treated females by application of the insect juvenile hormone III (JH III), but was not restored to the full capacity of untreated control females. Although P2 reduces progeny production among treated females, and JH III partially restores it, the P2 doses tested were not strong enough to completely stop progeny production. Moreover, the doses of P2 used were not strong enough to permanently damage the tissue producing the putative JH-like gonodotrophic hormone as shown by the natural reproductive recovery of P2 treated females that were allowed additional feedings and recovery times. Results of this study suggest that a JH-like compound probably functions in egg development in mites.

2. Other experiments concerning the putative role of JH in egg maturation and oviposition involved the tick Ornithodoros parkeri. The purpose of these experiments was to determine the role of JH on egg maturation in fed-virgin females. Females of this species require a blood meal and mating for vitellogenesis to occur. Attempts were made to produce vitellogenesis in fed unmated female O. parkeri by topical applications and injections of JH and farnesol. Scoring of fed virgin female reproductive systems and fed mated female controls was done subsequent to treatment. Topical applications and injections of JH initiated vitellogenesis, but did not induce oviposition. Injection of JH stimulated vitellogenesis more than did topical application regardless of whether JHI, JHII or JHIII was used. The fact that JH stimulates vitellogenesis, but that oviposition did not occur, allows several different interpretations. Perhaps JH is necessary early in

the cycle of egg maturation, and some other compound(s) is necessary for later development and/or oviposition. Alternatively, perhaps the timing and concentrations of JH were not physiologically attuned to this species. Clearly, JH deteriorates rapidly and perhaps a pulse-type of JH treatment would more nearly simulate the normal condition. Preliminary experiments involving several catecholamines indicate that they may also play a role in stimulating vitellogenesis. More will be reported on these experiments in the next progress report.

3. Another series of experiments concerning stimulation of egg maturation and oviposition in O. parkeri involved hemocoelic injection and vaginal insertion of selected male reproductive and non-reproductive tissue homogenates into fed virgins. These treatments stimulated ovum maturation and/or oviposition to varying degrees. Mean times for oviposition and mean numbers of eggs laid per ovipositing female receiving hemocoelic injections of male reproductive tissue homogenates did not differ significantly from fed-mated controls. In addition, hemocoelic injection of male salivary glandular homogenate induced oviposition, yet synganglial homogenate did not. Although vaginal insertion induced both ovum maturation and oviposition, the effect was not as pronounced as when similar doses were administered by hemocoelic injection. These results indicate that a complex interrelated series of precopulatory and copulatory stimuli are necessary for oviposition to occur in fed O. parkeri.

Relationships Between Weights of Nymphal Stages and Subsequent Production of Nymphs, Adults and Sex Ratios of O. parkeri

From a practical standpoint, with laboratory colonies it is often necessary to be able to predict how many adult ticks of a specific sex will be available for experimental use. Moreover, certain experimental designs require treatment of ticks in the immature stages and scoring in subsequent nymphal or adult stages. In order to provide the kind of information needed the following experiments were conducted.

Unfed third stage nymphal (UN3) Ornithodoros parkeri were arranged into 7 weight classes, fed and reared to adults. Fed N3s produced 22.0% males, 3.8% females, and 57.2% fourth stage nymphs (N4s) (17.0% died or were lost). The resulting N4s were fed and subsequently produced 14.7% males, 56.4% females, and 9.5% fifth stage nymphs (N5s) (19.3% died or were lost). The N5s were fed and yielded 5.0% males, and 70.0% females (25.0% died or were lost). After rearing all UN3s to the adult stage the final sex ratio was 0.77:1 (43.5% males, 56.5% females). Unfed third stage nymphs weighing less than 2.6 mg produced males and N4s, but not females after feeding. A few females were produced from N3s heavier than 2.6 mg. N4s resulting from all N3 weight classes developed into males, females and N5s, and those originating from N3s in the lowest weight class (0.1-1.0 mg) produced more males than females. N4s originating from all other N3 weight classes produced more females.

In a second experiment, only females ecdysed from fed N4s weighing 25 mg and over, and those weighing 5-9 mg produced only males and N5s. Fifth stage nymphs in both experiments produced females almost exclusively (34 females and 1 male).

Spermatogenesis and Spermiogenesis

The processes and developmental timing of spermatogenesis and spermiogenesis in ticks and parasitic mites continues to be investigated as well as the stimuli initiating these events. Karyotypes, sex chromosome systems and sex ratios all relate to background information needed when considering genetic linkage groups and various parameters of reproduction. During the past 12 months we were able to make significant contributions to the data base on tick cytogenetics and developmental timing of sperm production. Four species of the medically important *Amblyomma* were studied. *Amblyomma inornatum* attached to the host for 1 to 3 days contain enlarging primary spermatocytes, but no meiotic divisions are evident.

Prophase I (especially diplonema and diakinesis), metaphase I, anaphase I and metaphase II are first seen on day 4 of attachment. These and subsequent meiotic stages as well as rounded spermatids are present on days 5 and 6 of feeding. Each subsequent day more cells develop into spermatids and fewer cells are seen in the meiotic divisional stages.

Cells at diakinesis and metaphase I reveal 10 autosomal bivalents plus one sex univalent. Reductional division occurs at anaphase I with the sex univalent going undivided to one pole along with 10 autosomal dyads, and 10 autosomal dyads without a sex chromosome go to the other pole. Anaphase II is equational. These 2 meiotic divisions result in the subsequent formation of 4 spermatids (2 with 10 + X and 2 with 10 chromosomes) from each primary spermatocyte.

Eleven pairs of chromosomes (bivalents) including 1 long pair are present in prophase I of oocytes. Later meiotic divisional stages of oogenesis are difficult to observe. Mitotic cells reveal 22 and 21 chromosomes from females and males, respectively. These include 20 autosomes (16 moderately long, 4 slightly shorter) in both sexes plus 2 long sex chromosomes (XX) in females and 1 long sex chromosome in males (X). Thus the diploid chromosome number of *A. inornatum* is 22 for females and 21 for males, and the sex determining mechanism is XX: XO.

The reptile feeding species *A. dissimile* (larvae feed on mammals also) has chromosal configurations indicating terminal centromeres on all chromosomes. Males have 20 autosomes plus 1 sex chromosome and females have 20 autosomes plus 2 sex chromosomes. Spermatogenesis is slightly delayed in this reptile feeding tick when compared to other Amblyomma that feed on mammals. A. dissimile normally reproduces bisexually, although there is a tendency for parthenogenesis in some individual females. The genetics of parthenogenesis is not understood in most cases. Another interesting aspect of the feeding and reproductive biology of this species is the habit of adults attaching to hosts in clusters. Although not tested, it seems likely that the clusters might be the result of an aggregation pheromone produced by the ticks.

Males of a new species of Amblyomma tick from the Galapagos Islands possess a diploid chromosome number of 21. Meiotic divisions reveal 10 autosomal bivalents and 1 long sex uivalent. Another Galapagos tick, Amblyomma darwini has a chromosome number of 20 consisting of 9 autosomal bivalents and 1 sex bivalent. The X chromosome is the longest of the complement and the length of the Y chromosome is uncertain. Anaphase I is reductional and anaphase II equational for both species.

Feeding as an adult is necessary prior to meiotic divisions and spermiogenesis in metastriata ticks. After feeding the subsequent factor(s) triggering the initiation of spermatogenesis is not known but our working hypothesis supported by preliminary data is that an ecdysteroid plays an early role in this process. We are currently analyzing several samples from ticks that fed for varying lengths of time in an effort to score changes in ecdysteroid titer.

VI. SUMMARY

These studies report new findings that contribute to our understanding of hormone-pheromone interactions in ticks and parasitic mites. Special effort was directed during the past year to an understanding of the metabolism of ecdysone and 20-OH ecdysone. We were especially interested in determining the variety of ecdysteroids formed, and the possibility that other metabolites of ecdysone (besides 20-OH ecdysone) may exhibit activity. At present, it is clear that the great majority of the parent molecule is converted to highly apolar moieties of unknown identity. Our evidence to date is consistent with the findings of other workers implicating conjugation with fatty acids.

We examined these apolar ecdysteroids in _H. dromedarii_ and _D. variabilis_ for evidence of similar conjugation, but without success. Preliminary evidence implicates a ^{14}C labelled compound in the formation of these conjugates, but its identity is unknown. Small quantities of polar ecdysteroids were also found. The sites of enzymatic degradation, whether in hemolymph or target tissues remains to be determined.

Other studies were directed to an analysis of the fate of exogenous juvenile hormone, in order to determine the probability of survival of this molecule in the environment of the living tick tissues and hemolymph. Clearly, JH is degraded rapidly by general esterases that appear abundantly in tick hemolymph. In addition, evidence of specific esterase activity is suggested by the formation of multiple peaks, consistent with both acid and diol formation. Non-specific esterases attack the carboxy methyl bond only, leading to the acid. Treatment of tick infested hosts with ecdysteroid

analogues administered by slow release, especially 22,25 dideoxyecdysone, led to high mortality among the engorged H. dromedarii nymphs, but no evidence of accelerated molting was found.

VII. FUTURE PLANS

The project will continue current studies on the identity, metabolism and role of selected hormones affecting mating and reproduction in ticks. Study of the metabolism of ecdysteroids has led to evidence of a family of apolar compounds which represent the majority of ecdysteroid metabolites. Studies in progress to identify these apolar metabolites will be continued. More important, studies will be done to determine where these changes take place in the tick body, i.e. whether in the midgut, hemolymph, or selected target organs. Special attention will be directed to the epidermis, pheromone glands and pre-vitellogenic ovary, i.e. the tissues and timing concerned with sex pheromone activity. Another important study will be done to determine the role of these other ecdysteroid metabolites (e.g., 20,26-diOH ecdysone, 22,25 dideoxyecdysone), as well as the active hormone, 20-OH ecdysone on sex pheromone activity. To study these effects, tick organ cultures will be prepared, where both hormones and other excitants can be added, together with ^{36}Cl as (Na ^{36}Cl) to monitor biosynthesis of the pheromone. We have been successful in maintaining tick organs and tissues alive for up to 7 days in culture, with explants of selected tissues with culture dishes as well as other indicators of viability. During this period, we have observed release (and/or synthesis) of ecdysteroids into the culture medium in cultures of tick midgut, synganglion and ovary. In addition to these treatments, these hormonal metabolites will be administered in vivo into the hemocoel, bypassing the midgut, and their effects on sex pheromone synthesis and secretion, if any, will be observed. Similarly, dopamine and dopamine plus ecdysteroids can be administered to determine whether excitation can be achieved.

Study of the metabolism and synthesis of juvenoid/gonadotropins will be ›ntinued and extended. We are especially interested in the effects of ıese molecules in the adult female, i.e., the stage when sex pheromone :tivity is evident. Studies to determine whether JH-III is synthesized in cks from known precursors will be done. Tests with ^{14}C Mevalonic acid :re unsuccessful, however other metabolites, such as methionine, may be ›re appropriate and can be considered. Assays will be done to determine ıether the acid, diol or acid/diol metabolites are formed. Using the cul- ıres described above, tests will also be done to determine whether direct .imulation of pheromone synthesis can be accomplished by JH.

Studies at Georgia Southern College on site(s) of ecdysteroid synthesis ıd storage will be continued. Similar studies on JH-like compounds, acting ; regulators of development processes and reproduction, will also be con- ınued.

IX. PUBLICATIONS AND MANUSCRIPTS

The following is a listing of all of the papers, published in press, or nanuscripts, produced by project personnel. New listings, produced since last progress report, are cited first, and identified with an asterisk the left of the citation. A total of thirty-six (36) papers have been duced with support from this contract as of this date. In addition, re have been numerous oral presentations at regional, national and interional scientific meetings.

New papers. (Prepared since May, 1984).

Sonenshine, D.E., M. Beveridge, L. Boland, and F. Scully. Metabolism of ecdysone and 20-hydroxyecdysone in the camel tick, Hyalomma dromedarii and Dermacentor variabilis (Acari:Ixodidae). Completed manuscript.

Sonenshine, D.E., M. Beveridge, and L. Boland. Fate of juvenile hormones in the ticks Hyalomma dromedarii and Dermacentor variabilis (Acari:Ixodidae), with notes on the possible occurrence of JH in these ticks. Manuscript in advanced stage of preparation.

Sonenshine, D.E. 1985. Tick pheromones: an overview. Proc. XVII Internatl. Congr. Entomol. Ellis Horwood, Ltd., Chichester, England. In press.

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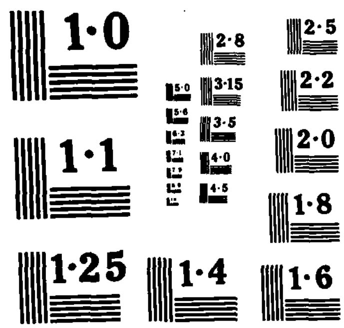

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