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A REEVALUATION OF THE ROLE OF *CIS*-VACCENYL ACETATE, *CIS*-VACCENOL AND ESTERASE 6 IN THE REGULATION OF MATED FEMALE SEXUAL ATTRACTIVENESS IN *DROSOPHILA MELANOGASTER*

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Abstract—Gas–liquid chromatography was used to investigate the role of *cis*-vaccenyl acetate, *cis*-vaccenol and esterase 6 in the inhibition of male courtship in *D. melanogaster*. Results indicate that (1) *cis*-vaccenyl acetate is not converted to *cis*-vaccenol, (2) esterase 6 has no effect on the rate of *cis*-vaccenyl acetate loss from the reproductive tracts of mated females, (3) *in vivo* concentrations of *cis*-vaccenyl acetate transferred to females during copulation fall below effective courtship-inhibitory levels within 4 h, (4) *cis*-vaccenyl acetate is not translocated from the female reproductive tract to the abdominal cuticle, and that (5) mutant male flies that do not reduce the post-mating sexual attractiveness of females contain and transfer normal amounts of *cis*-vaccenyl acetate. Therefore, direct chemical analyses do not support the contention that esterase 6, *cis*-vaccenyl acetate and its hydrolysis product, *cis*-vaccenol, act in concert as an anti-aphrodisiac pheromone system.

Key Word Index: *Drosophila melanogaster*, *cis*-vaccenyl acetate, *cis*-vaccenol, esterase 6, pheromone, anti-aphrodisiac, courtship inhibition, don giovanni mutant

INTRODUCTION

The sexual attractiveness of *Drosophila melanogaster* females changes radically immediately following mating. Mature virgin females elicit a high level of courtship from mature males and generally mate rapidly. However, immediately after mating, female sexual attractiveness declines by 50% or more, falls further over the next 18 h, and then gradually returns to pre-mating levels over a period of 9 days (Bastock and Manning, 1955; Cook and Cook, 1975; Siegel and Hall, 1979; Tompkins and Hall, 1981a; Jallon *et al.*, 1981; Scott and Richmond, 1985). Even copulation interrupted after 3 min (normal copulation time is about 20 min) is sufficient to effect the decline in female attractiveness (Tompkins and Hall, 1981a).

Investigation of post-mating courtship inhibition in *D. melanogaster* has led to the hypothesis that a chemical mechanism(s) is involved. Particular interest has been focused on the role of *cis*-vaccenyl acetate (*cis*-11-octadecen-1-ol acetate), a lipid found specifically in male ejaculatory bulbs (Butterworth, 1969; Brieger and Butterworth, 1970; Jackson *et al.*, 1981). The observation that males transfer *cis*-vaccenyl acetate to females during copulation in conjunction with behavioural data led Jallon *et al.* (1981) to suggest that *cis*-vaccenyl acetate had an inhibitory (anti-aphrodisiac) effect on male courtship (measured as the proportion of an observation period during which a male courts a female). They also found that the quantity of *cis*-vaccenyl acetate in extracts of mated females falls rapidly within 6 h after mating. This implied that *cis*-vaccenyl acetate could not directly account for courtship inhibition observed over several days. However, the amounts of courtship-stimulating compounds produced by virgin females are significantly reduced by 3 days after mating (Tompkins and Hall, 1981a; Jallon *et al.*, 1982). Accordingly, Jallon (1984) proposed that male-derived *cis*-vaccenyl acetate in the female inhibits male courtship for a period of hours immediately after mating, and a concomitant decrease in the level of female courtship-stimulating compounds results in a continuing reduction in female sexual attractiveness. However, Tompkins and Hall (1981b) proposed that the courtship-inhibitory substance extractable from mated females was not the male-derived compound *cis*-vaccenyl acetate, but rather a substance(s) produced by the female.

A resolution of these conflicting hypotheses was suggested by the presence of a carboxyl esterase, esterase 6, in male seminal fluid (Sheehan *et al.*, 1979). Mane *et al.* (1983) demonstrated that *in vitro*, esterase 6 hydrolyzed *cis*-vaccenyl acetate to its alcohol, *cis*-vaccenol, and that *cis*-vaccenol inhibited copulation. Behavioural tests employing the olfactory-deficient mutant, *olfactory C*, (see also Tompkins and Hall, 1981b; Tompkins *et al.*, 1983) suggested that this reaction also occurred *in vivo*.

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Mane *et al.* (1983) concluded that esterase 6 converts *cis*-vaccenyl acetate to *cis*-vaccenol in the reproductive system of the female, and that *cis*-vaccenol is the female-produced anti-aphrodisiac discovered in the experiments of Tompkins and Hall (1981b).

We report a series of studies designed to determine whether or not *cis*-vaccenyl acetate is converted to *cis*-vaccenol in the reproductive tracts or on the cuticle of mated females and whether esterase 6 influences the amount of *cis*-vaccenyl acetate found in the female's reproductive tract.

MATERIALS AND METHODS

Drosophila *stocks*

Flies were maintained on a cornmeal–agar–molasses medium at 25°C on a 12-h light–dark cycle. The following stocks of *Drosophila melanogaster* were used for chemical and behavioural analyses of strain differences with respect to esterase 6, *cis*-vaccenyl acetate and *cis*-vaccenol: (A) Oregon-R (Ore-R), a wild-type strain derived from the P2 stock maintained at the Institute for Cancer Research in Philadelphia. (B) Dm 100, a wild-type strain homozygous for the *esterase* 6^0 (null) allele (see Sheehan *et al.*, 1979, for a complete description of the origin of this strain). Males do not produce esterase 6. (C) Dm 189, a wild-type strain homozygous for the *esterase* 6^s (slow) allele. This stock carries a genetic background essentially identical to that of Dm 100 as a result of 28 generations of backcrossing to the Dm 100 line. Males produce esterase 6. Stocks D to H were supplied by Dr D. G. Gailey, Brandeis University. Female *D. melanogaster* mated to don giovanni (*dg*) males (stocks E, F and G) retain full sexual attractiveness after mating (Tompkins *et al.*, 1983). (D) Wild-type, Canton-S line from which the *dg* mutant was isolated. (E) Wild-type stock carrying the original *dg* allele extracted by inbreeding and backcrossing from the Canton-S stock. (F) Strain marked with X chromosome mutants *crossveinless* (*cv*) and *forked*. Males express the *dg* phenotype. The *dg* mutant(s) maps to the X chromosome between yellow and *cv* (Tompkins *et al.*, 1983; Gailey *et al.*, 1984).

Preparation of tissues for analysis of cis-*vaccenyl acetate and* cis-*vaccenol*

Prior to dissection etherized flies were weighed on an analytical balance. Male ejaculatory bulbs and female reproductive tracts (ovaries not included) were dissected from adult flies (5 days old) in sterile *Drosophila* Ringers (Ephrussi and Beadle, 1936) using a dissecting microscope. Insect pins were used for a single dissection and discarded. Following dissection, tissue from individual flies was immediately transferred to ice-cooled glass ampules containing 200 μl of *n*-hexane (Sigma H-9379). An internal standard (11-eicosenyl acetate, Sigma E-5377) was added to the ampule (1 μg per male ejaculatory bulb and 0.25 μg per female reproductive tract), after which the ampule was sealed.

Gas chromatography (GC)

Sample ampules were broken and the solvent evaporated to 5–20 μl under a nitrogen stream. Approximately 1 μl of this solution was analyzed by gas chromatography, after which hexane (100–200 μl) was added to the ampule and the sample transferred to a vial for storage. Samples were analyzed on a Varian Gas Chromatograph Model 3700 fitted with a flame ionization detector. The following columns and stationary phases were used in the analysis:

(A) A 1.8 m × 2.0 mm i.d. glass column packed with 3% Silar 10C on 100/120 Gas-Chrom Q (Applied Science Laboratories, State College, Pa); Kovat Indices (150°C): *cis*-vaccenyl acetate = 2654, *cis*-vaccenol = 2764. (B) A 1.8 m × 2.0 mm i.d. glass column packed with 3% OV-17 on 120/140 Gas-Chrom Q (Applied Science Laboratories, State College, Pa); Kovat Indices (170°C): *cis*-vaccenyl acetate = 2300, *cis*-vaccenol = 2175. (C) A 15.0 m × 0.32 mm i.d. DB-1 fused silica capillary column (J. and W. Scientific, Inc., Rancho Cordova, Calif.); Kovat Indices (185°C): *cis*-vaccenyl acetate = 2177, *cis*-vaccenol = 2040. *Cis*-vaccenyl acetate and *cis*-vaccenol were identified using analytical standards (Sigma Chemical Company). The GC data were quantitatively analyzed with a Varian Vista 401 data processor (lower limit of detection for *cis*-vaccenyl acetate and *cis*-vaccenol = 0.5 ng).

Quantity of cis-*vaccenyl acetate and* cis-*vaccenol in virgins of both sexes and in mated females at various times after mating*

Virgin females from the Dm 100 (esterase 6^0) stock and virgin males from the Dm 100 and Dm 189 (esterase 6^s) stocks were individually aged for 5 days at 25°C. Virgin males of strains D–F were aged for 6 days. Ejaculatory bulbs from virgin males and reproductive tracts from virgin females were dissected, extracted in hexane as described, and analyzed for *cis*-vaccenyl acetate and *cis*-vaccenol. Matings between esterase 6^0 females and either esterase 6^0 or esterase 6^s males were accomplished by pairing flies in vials and observing copulation. At the completion of mating, the male was removed and the female retained for dissection and extraction of her reproductive tract. Individual female reproductive tracts were obtained at 0, 1, 2, 3, 4, 6, 12, 24 and 48 h after the completion of copulation and analyzed for the presence of *cis*-vaccenyl acetate and *cis*-vaccenol.

Cuticular washes of stock C (Dm 189) flies

Surface washes of virgins of both sexes, mated females and immature males were obtained by immersing an etherized fly in 200 μl of hexane in a glass ampule for 5, 10, 20, 30 or 60 s and then removing the fly, introducing the internal standard and sealing the ampule. All mature flies tested were 4 days old; immature males had eclosed less than 5 h previously. Mated females had copulated about 2 h before immersion. Two flies of each type were analyzed for each of 5 immersion times.

Quantity of cis-*vaccenyl acetate*, cis-*vaccenol and esterase 6 in don giovanni stocks*

Male ejaculatory bulbs from strains expressing the *dg* phenotype were removed, extracted and analyzed for *cis*-vaccenyl acetate and *cis*-vaccenol as described above. Esterase 6 expression in these strains was assayed by starch gel electrophoresis (Cochrane and

Richmond, 1979). Additionally, courtship index bioassays (Tompkins *et al.*, 1980) were used to verify the report by Tompkins *et al.* (1983) that 4-day virgin females from the Ore-R strain remained sexually attractive when mated to *dg* males.

RESULTS

Quantity of cis-*vaccenyl acetate and* cis-*vaccenol in virgins of both sexes and in mated females at various times after mating*

No *cis*-vaccenol was detectable in the reproductive tracts of virgins of either sex or in the reproductive tracts of mated females at any time after mating (Fig. 1). However *cis*-vaccenyl acetate was present in the reproductive tracts of virgin males and mated females. Females inseminated by esterase 6^s males initially received significantly more *cis*-vaccenyl acetate from their mates than females inseminated by esterase 6^0 males ($\bar{X} \pm SEM = 361 \pm 26$ vs 243 ± 22 ng, $n1 = n2 = 5$; $t = 3.42$, d.f. $= 8$, $P = 0.005$). The difference in the amount of *cis*-vaccenyl acetate present in the two mated female samples may reflect one or a combination of the following: (1) Esterase 6^s male ejaculatory bulbs contained significantly more *cis*-vaccenyl acetate than ejaculatory bulbs of esterase 6^0 males ($t = 4.66$, d.f. $= 8$, $P < 0.001$; Table 1), although males from each strain did not differ significantly in weight ($\bar{X} \pm SEM = 0.66 \pm 0.05$ vs 0.70 ± 0.08 mg, $n1 = n2 = 5$; $t = 0.61$, d.f. $= 8$, $P = 0.28$). (2) Females mated to esterase 6^s males were somewhat heavier ($\bar{X} \pm SEM = 1.20 \pm 0.09$ mg, $n = 5$) than females mated to esterase 6^0 males ($\bar{X} \pm SEM = 0.96 \pm 0.13$ mg, $n = 5$; $t = 1.46$, d.f. $= 8$, $P = 0.09$). (3) The amount of *cis*-vaccenyl acetate transferred to females at mating may be correlated with mating duration, a variable not measured. In these experiments esterase 6^0 males transferred about 55% of their *cis*-vaccenyl acetate, whereas esterase 6^s males transferred 33%. Significant strain differences in amounts of *cis*-vaccenyl acetate per male were also obtained in comparisons between Canton-S males and Ore-R males ($t = 7.28$, d.f. $= 6$, $P < 0.001$; Table 1).

Table 1. Amount of *cis*-vaccenyl acetate present in the ejaculatory bulb of virgin males of various *D. melanogaster* strains

Strain (n)	ng *cis*-vaccenyl acetate ($\bar{X} \pm SEM$)
Esterase 6^0 (4)	442 ± 109
Esterase 6^s (5)	1116 ± 96
Canton-S (3)	1595 ± 25
Ore-R (5)	520 ± 110
dg mutant E (3)	1458 ± 205
dg mutant F (4)	1284 ± 163

A comparison of the loss of *cis*-vaccenyl acetate from mated female reproductive tracts for esterase 6^0 and esterase 6^s-mated females is presented in Fig. 2. In order to correct for possible effects of female size (see above), amounts of *cis*-vaccenyl acetate are expressed as nanograms per milligram female. These results clearly demonstrate that the loss of *cis*-vaccenyl acetate from the reproductive tracts of females is independent of the esterase 6 type of their mate. There was no significant difference between the two treatments in the amount of *cis*-vaccenyl acetate detected at each time period (unpaired t-test, 1 tailed, $\alpha = 0.05$), except for the 6 h point (Fig. 2). A multiple regression analysis of the full data set for mated females using *cis*-vaccenyl acetate as the dependent variable and mate type, hours after mating, and

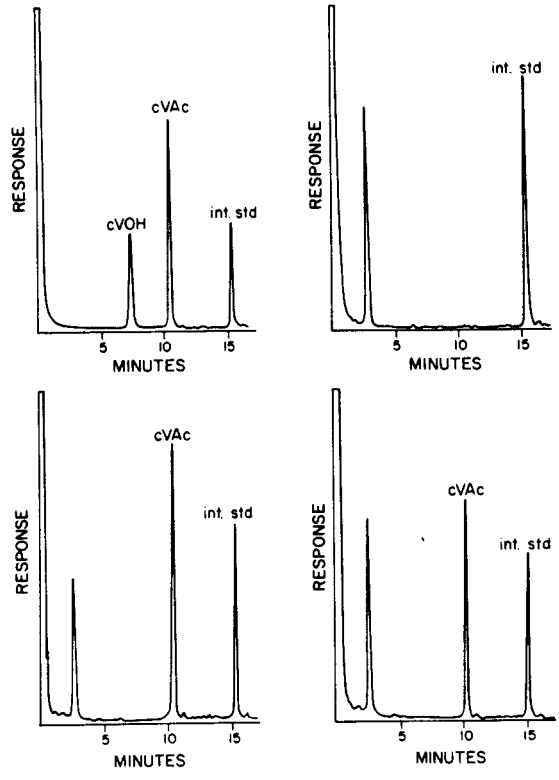

Fig. 1. Representative capillary column GC profiles of (A) standard compounds, (B) reproductive tract of virgin *D. melanogaster* female, (C) male ejaculatory bulb, and (D) reproductive tract of mated female (see Materials and Methods). Note the presence of *cis*-vaccenyl acetate in males and mated females, and the absence of *cis*-vaccenol in flies of both sexes.

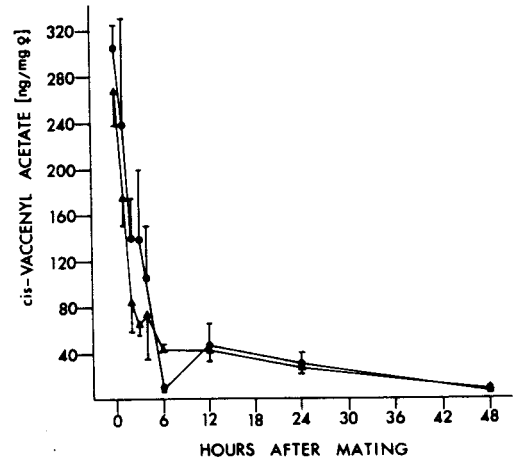

Fig. 2. Loss of *cis*-vaccenyl acetate in reproductive tracts of females mated to either esterase 6^s (●) or esterase 6^0 (▲) males. Quantities of *cis*-vaccenyl acetate ($\bar{X} \pm SEM$) are expressed as weight per mg of female.

female and male weights as independent variables revealed that only time after mating was significantly correlated with the amount of *cis*-vaccenyl acetate found in mated females.

Quantity of cis-*vaccenyl acetate and* cis-*vaccenol in cuticular washes*

The amount of *cis*-vaccenyl acetate present in cuticular washes of mature males increased from about 8 ng for a 5 s immersion to about 50 ng for a 60-s immersion. No *cis*-vaccenol was present in any of these washes. Washes of virgin female cuticles contained no *cis*-vaccenyl acetate or *cis*-vaccenol. Washes of mated female cuticles revealed a trace (< 10 ng) of *cis*-vaccenyl acetate at short immersion times (5 and 10 s) and between 20 and 22 ng of *cis*-vaccenyl acetate for 60-s immersions. These samples contained no *cis*-vaccenol. No *cis*-vaccenyl acetate or *cis*-vaccenol was detected in cuticular washes of immature males, a result confirming the finding of Jallon (1984). The increasing amounts of *cis*-vaccenyl acetate found in mature males with longer immersion times indicate that cuticular washes of more than a few seconds begin to extract material stored in the reproductive tracts of flies.

Analysis of dg *strains*

Analysis of the amounts of *cis*-vaccenyl acetate and *cis*-vaccenol in the *dg* strains unambiguously demonstrates that the quantity of *cis*-vaccenyl acetate in the ejaculatory bulbs of males from these stocks (Table 1) is comparable to (if no greater than) the levels found in the esterase 6^s stock (Table 1). No *cis*-vaccenol was detected in *dg* strain ejaculatory bulbs. Starch gel electrophoretic analysis of these stocks revealed that all were homozygous for the estease 6^s allele with approximately normal levels of expression. Females mated to *dg* males elicited about 35% more courtship 24 h after mating (i.e. male wing-vibration) than females mated to wild-type males ($F(1,75) = 5.04$, $P = 0.027$). These results confirmed the description of the *dg* phenotype by Tompkins *et al.* (1980).

DISCUSSION

The role of cis-*vaccenyl acetate*

This study confirms the finding of Butterworth (1969) and Jallon *et al.* (1981) that *cis*-vaccenyl acetate is localized in the male ejaculatory bulb (Table 1) and transferred to females at mating (Fig. 1). Based on cuticular rinses, Jackson *et al.* (1981) and Jallon *et al.* (1981) reported that mature adult males contain about 200 ng of *cis*-vaccenyl acetate. Ejaculatory bulb extracts indicated that (1) males contain significantly more *cis*-vaccenyl acetate than previously obtained using cuticular rinses and that (2) strain differences exist in the amount of *cis*-vaccenyl acetate present per male. Specifically, esterase 6^0 males possess less than half the amount of *cis*-vaccenyl acetate present in esterase 6^s males (Table 1). Similarly, males of the Ore-R strain contain about one-third the *cis*-vaccenyl acetate obtained from Canton-S males (Table 1). Our figure of 1595 ng/fly for 5-day old Canton-S males compares favorably with the 1400 ng/fly obtained from 8-h extractions by R. Bartelt, Montana State University (pers. commun.).

Previous research conducted in several laboratories has led to the assertion that *cis*-vaccenyl acetate reduces the sexual attractiveness of mated *D. melanogaster* females. Siegel and Hall (1979) initially demonstrated that mated females immobilized by ether or heat (temperature-sensitive paralytic mutants) were courted significantly less than immobilized virgins. This suggested that a chemical mechanism regulated female attractiveness. It was subsequently demonstrated that male wing movement (Jallon *et al.*, 1981), a specific behavioral component of courtship, as well as total courtship (Mane *et al.*, 1983) were reduced when *cis*-vaccenyl acetate was present in a test chamber or placed on virgin females. More recently our laboratory (Zawistowski and Richmond, in press) has determined that courtship and mating are reduced most in the laboratory at *cis*-vaccenyl acetate levels (100–200 ng) corresponding to the amounts transferred to females by males in the experiments. Expanding the work of Siegel and Hall (1979), we have also demonstrated that males, conditioned by virgin females annointed with 200 ng of *cis*-vaccenyl acetate, court virgin females at the same, reduced level as males conditioned by mated females (cf. Tompkins *et al.*, 1983). *In toto* these data support the contention that *cis*-vaccenyl acetate functions as a female anti-aphrodisiac pheromone in this *Drosophila* system.

However, in light of quantitative chemical analyses presented in this paper, we feel that such a conclusion is premature. We have clearly demonstrated (Fig. 2) that the amount of *cis*-vaccenyl acetate in female reproductive tracts falls below optimal courtship reduction levels within 4 h after mating. One hypothesis to account for courtship reduction beyond 4 h after mating is that *cis*-vaccenyl acetate may be translocated from the reproductive system of the female to her abdominal cuticle. Here the *cis*-vaccenyl acetate would be available for a longer period of time and the increased surface area would presumably enhance the volatile signal detected by males. This hypothesis would in addition account for the rapid loss of *cis*-vaccenyl acetate from the female reproductive tract. However, only trace amounts of *cis*-vaccenyl acetate were present in the cuticular washes of mated females, and we believe that even these small amounts were leached from the mated females reproductive tract.

The *dg* mutant strains provided another approach to determining the role of *cis*-vaccenyl acetate in courtship reduction. Tompkins *et al.* (1983, p. 568) stated that "...females fertilized by *dg* males stimulate as much courtship as do virgins." This description led to the hypothesis that *dg* males would contain either significantly less *cis*-vaccenyl acetate than wild-type males or no *cis*-vaccenyl acetate at all. Our data clearly show that *dg* males produce normal levels of *cis*-vaccenyl acetate (Table 1), and Scott and Richmond (unpublished data) have demonstrated that *dg* males transfer equivalent amounts of *cis*-vaccenyl acetate to females as do normal males. It can therefore be concluded that *cis*-vaccenyl acetate is not responsible for the inhibition of courtship observed in females mated to normal males.

Additional evidence further complicates our efforts to determine whether *cis*-vaccenyl acetate mediates courtship in nature. Bartelt *et al.* (in press) have demonstrated that *cis*-vaccenyl acetate is a synergist of food odours in attracting both sexes of *D. melanogaster*. It is not inconceivable that reductions in courtship and mating observed in the laboratory and attributed to anti-aphrodisiac activity of *cis*-vaccenyl acetate are in actuality behavioural artifacts of presenting flies with a stimulus in an inappropriate context. When presented to flies in a small test arena, *cis*-vaccenyl acetate may stimulate non-courtship behaviours typically associated with the formation and/or response to aggregations. However, *cis*-vaccenyl acetate could, as suggested by Bartelt *et al.* (in press), be an example of pheromonal parsimony, mediating both aggregation and courtship inhibition. If so, we are faced with an intriguing problem. Specifically, how can we account for the evolution of a signal that attracts males to mating sites and subsequently inhibits their courtship activity? Studies addressing the behavioural ecology of *D. melanogaster* aggregation and courtship are needed.

Transformation of cis-*vaccenyl acetate to* cis-*vaccenol and the role of esterase 6*

It had previously been suggested (Mane *et al.*, 1983) that *cis*-vaccenyl acetate was hydrolyzed by esterase 6 to *cis*-vaccenol in the reproductive tract of the female, and that *cis*-vaccenol was primarily responsible for the reduction in female sexual attractiveness during the first few hours after mating. Data of Zawistowski and Richmond (in press) indicate that (1) 50 ng of *cis*-vaccenol causes a significant reduction in male courtship, and (2) that small quantities (3 ng) of *cis*-vaccenol additively enhance the effect of *cis*-vaccenyl acetate. We quantified the amounts of these substances in the reproductive tracts of females mated to males with active esterase 6 (esterase 6^s) as well as to males lacking active enzyme (esterase 6^0). If *cis*-vaccenyl acetate were indeed a substrate for esterase 6 *in vivo*, one would predict that at any time after mating, reproductive tracts of females mated to esterase 6^s males would contain proportionally less *cis*-vaccenyl acetate than reproductive tracts of females mated to esterase 6^0 males, and that *cis*-vaccenol would be detected in females receiving esterase. 6. Our results (Fig. 2) indicate that esterase 6 is not responsible for the depletion of *cis*-vaccenyl acetate in the reproductive tracts of mated females. Moreover, no *cis*-vaccenol (>0.5 ng) was detected in any of our analyses. Bartelt *et al.* (in press) have recently reported that 70% of the *cis*-vaccenyl acetate transferred to females during copulation is emitted into the rearing vial within 6 h after mating. These results argue strongly against the model of *in vivo* pheromone synthesis proposed by Mane *et al.* (1983).

There are at least two explanations for the failure of our experiments to detect *cis*-vaccenol in mated females. The first is that the hydrolysis of *cis*-vaccenyl acetate by esterase 6 does not occur *in vivo*. The reported *in vitro* hydrolysis may simply reflect the non-specificity of the enzyme (Danford and Beardmore, 1979). The second explanation is that the *cis*-vaccenyl acetate to *cis*-vaccenol conversion occurs elsewhere in the female, undetectable in our analyses of reproductive tracts. With this latter possibility in mind, we determined whether *cis*-vaccenyl acetate was transferred to the cuticle of the female, where the conversion to *cis*-vaccenol might be manifest. However, we failed to detect any *cis*-vaccenol in cuticular washes of female flies. The possibility exists that esterases known to be associated with insect olfactory sensilla (Ferkovich *et al.*, 1973a,b) could convert *cis*-vaccenyl acetate to *cis*-vaccenol in the microcosm of the male antenna. Until this possibility is investigated, we cannot conclude that *cis*-vaccenol does not mediate courtship inhibition.

Although the esterase 6 type of an inseminating male has been demonstrated to affect the probability of female remating (Gilbert *et al.*, 1981; Scott, unpublished), female fecundity (Gilbert and Richmond, 1982) and the rate of stored-sperm utilisation (Gilbert *et al.*, 1981), the function of esterase 6 (if any) in the control of mated female sexual attractiveness has not been conclusively demonstrated. Since there are many other components of the male ejaculate and the female haemolymph that are potential substrates for esterase 6, it is plausible that the effects of esterase 6 on female reproductive physiology and behaviour are mediated through substances other than *cis*-vaccenyl acetate and *cis*-vaccenol.

In summary, there is ample evidence that males synthesize *cis*-vaccenyl acetate and transfer it to females during copulation. Under laboratory conditions *cis*-vaccenyl acetate and *cis*-vaccenol do inhibit components of male courtship. However, chemical analyses have demonstrated that (1) *in vivo* concentrations of *cis*-vaccenyl acetate transferred to females during copulation fall below effective inhibitory levels within 4 h, (2) *cis*-vaccenyl acetate is not translocated from the female reproductive tract to the abdominal cuticle, (3) mutant male flies that do not reduce the post-mating sexual attractiveness of females contain and transfer normal amounts of *cis*-vaccenyl acetate, (4) esterase 6 has no effect on the rate of *cis*-vaccenyl acetate loss from the reproductive tracts of mated females, (5) no *cis*-vaccenol is detectable in the reproductive tracts or on the cuticle of mated *D. melanogaster* females. Therefore, direct chemical analyses do not support the contention that esterase 6, *cis*-vaccenyl acetate and its hydrolysis product, *cis*-vaccenol, act in concert as an anti-aphrodisiac pheromone system. The mechanism(s) underlying inhibition of courtship directed toward mated females remains to be elucidated.

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