terminal acetylation (*7*). Although all are glycoproteins, their widely scattered putative carbohydrate attachment sites occur at nonequivalent locations. All four sequences were analyzed by computer programs that predict secondary structure (*10*). Within the limitations of this approach, the distribution of α-helical segments and β-sheet sections was found to be reasonably similar. Similarly, the hydropathy profiles (*11*) of all four sequences were found to be equally undistinguished and to be characteristic of soluble globular proteins.

The four sequences were optimally aligned by pairs, and the statistical significance of the alignment scores was determined (Table 1). Angiotensinogen most resembles α_1-antitrypsin and is least similar to antithrombin III. The similarities are less than were observed previously for the antitrypsin-ovalbumin-antithrombin triad, but they are still highly significant in two cases (Table 1). Comparisons of alignments among the four sequences (Fig. 1) reveal that "invariant" residues occur at 31 of the aligned positions in the 400-residue bloc that spans them. At 27 other positions, angiotensinogen is identical to two of the other three, and at an additional 63 places it matches one of the three. Thus, at more than a third of the positions angiotensinogen has a residue in common with at least one of the other three proteins. The case for common ancestry is, in my view, indisputable.

On the basis of distantly related proteins whose primary and tertiary structures are both known, it can be expected that the three-dimensional resemblance of angiotensinogen and the protease-inhibitor group will be strong. In the case of serine proteases, for example, comparisons of α-carbon backbone coordinates are about twice as effective as sequence matching in detecting homology (*12*).

The evolution of a biologically active peptide as a portion of a protein of large molecular weight is of considerable interest. Although not enough data are available to make a judgment about which of the four related proteins appeared first, a possible scenario could involve chance mutations in some protease inhibitor that rendered the protein susceptible to cleavage near its amino terminus. If the amino-terminal segment so removed was not essential for the inhibitor function, then the gradual generation of a biologically active constellation of amino acids, comprising the removable portion, does not seem unreasonable, as long as a suitable receptor for the peptide existed. Alternatively, the biologically active peptide could conceivably have evolved as the result of an internal duplication of a signal peptide. It is perhaps noteworthy that the angiotensinogen segment destined to be released as the biologically active angiotensins occurs at a point which is proximal to the initiation points of two of the other three proteins, but which overlaps the signal peptide of the third (Fig. 1). In any event, the question arises as to whether the present protein—before or after the release of angiotensin—is a protease inhibitor, and, if so, what proteases does it regulate?

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Male Esterase 6 Catalyzes the Synthesis of a Sex Pheromone in *Drosophila melanogaster* Females

Abstract. *Esterase 6, a component of the seminal fluid of* Drosophila melanogaster *males, hydrolyzes* cis*-vaccenyl acetate, a lipid made only by males, to* cis*-vaccenyl alcohol. This reaction occurs in the female reproductive tract and is virtually complete within 6 hours after copulation. Both the alcohol and the acetate decrease the number of matings among pairs of virgin flies in which the female is treated topically with these substances. Although females tested 10 minutes after copulation elicit less courtship than virgin females, females tested 6 hours after copulation stimulate even less courtship if they received active esterase 6 in the seminal fluid of their respective mates. Either the alcohol or a derivative appears to be an antiaphrodisiac that decreases courtship elicited by inseminated females and thus reduces the probability of further mating. Thus the activity of the pheromone depends on a final reaction which occurs in the female, using both substrate and enzyme provided by the male.*

Esterase 6 (EST 6) is a carboxylesterase (E.C. 3.1.1.1) of *Drosophila melanogaster*, and its locus (*Est-6*) is polymorphic for two or more alleles in most natural populations (*1*). The enzyme is concentrated in the anterior ejaculatory duct (*2*), a secretory and propulsive organ of the male reproductive system, and is transferred to females within 2 to 3 minutes of the initiation of copulation (*3*). The presence of EST 6 in the male ejaculate is associated with effects on sperm utilization, female fertility (progeny production at 18°C), and the timing of female remating (*4*).

An acetate ester, *cis*-vaccenyl acetate (cVA), is found in the male ejaculatory bulb of the mature adult and is transferred to females during mating (*5*). The presence of cVA in the female reproductive tract may inhibit or prevent further courtship of inseminated females by acting as an antiaphrodisiac pheromone (*6*). The close proximity of EST 6 and cVA in the male reproductive system, the transfer of the two compounds to the female during copulation, and the chemical structure of cVA suggested that it might be an in vivo substrate for EST 6 in the female reproductive tract.

A mixture of the electrophoretically fast and slow allozymes of EST 6 purified from an Ore R strain of *Drosophila melanogaster* (*7*) was incubated with

^{14}C-labeled cVA (*8*); the hydrolysis of cVA is a function of both enzyme concentration and incubation time (Fig. 1, A and B). Similar experiments with purified slow or fast allozyme preparations (data not shown) reveal that the rates of hydrolysis by these variants do not differ. Gas-liquid chromatography was used to confirm that cVOH is indeed a product of the hydrolysis of cVA (*9*).

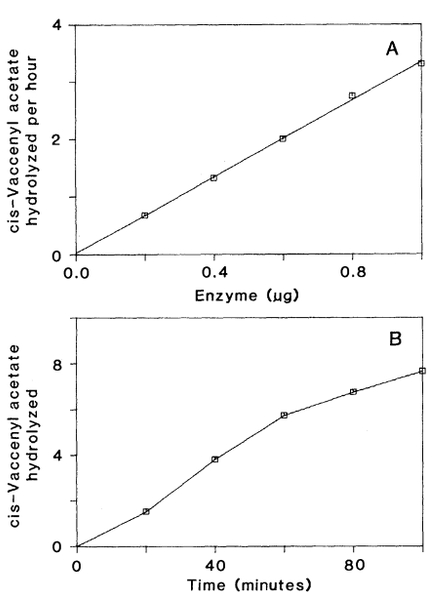
EST 6 activity increases dramatically in males between 1 and 3 days after eclosion from the pupal case (*2*). As expected, the activity of the cVA-hydrolyzing enzyme increases in parallel to that of EST 6 assayed by standard procedures (*2*). Homogenates of the reproductive tracts of males from an *Est-6*^{o} strain (6^{o}) do not hydrolyze either cVA or other artificial esterase substrates.

We used two strains of *D. melanogaster* which differ in EST 6 activity to determine whether homogenates of either inseminated or virgin-female reproductive systems can hydrolyze cVA. The females were obtained from the 6^{o} strain and the males were from the 6^{o} strain and an *Est-6*^{s} strain (6^{s}) that is homozygous for the slow electrophoretic mobility variant of EST 6 (*2*). EST 6 activity is readily detectable in homogenates of 6^{o} females mated to 6^{s} males (*3*). Females were mated either to 6^{o} or 6^{s} males, and 15 to 20 minutes after the initiation of mating their reproductive tracts were removed and homogenized. Hydrolysis of cVA was monitored by incubating homogenates with ^{14}C-labeled cVA. Virgin 6^{o} females and females mated to 6^{o} males show equivalent low levels of cVA hydrolysis. In contrast, homogenates of female reproductive tracts inseminated by 6^{s} males hydrolyze four times as much cVA (data not shown). Thus, enhancement of cVA hydrolysis by homogenates of inseminated-female reproductive tracts depends on the presence of active EST 6 in the seminal fluid of the mate of the female.

Male *D. melanogaster* transfer approximately 200 ng of cVA to females during copulation (*11*). The rate of disappearance of cVA from the female reproductive tract is rapid; approximately 70 percent of the lipid is lost within 6 hours of mating. Our data show that purified EST 6 can hydrolyze 55 pmole of cVA per minute per gram of protein. From the rates of cVA hydrolysis by homogenates of 6^{s} inseminated-female reproductive tracts, we estimate that males transfer about 40 ng of EST 6 to the female during copulation. This amount of enzyme is sufficient to account for the loss of cVA from inseminated-female reproductive tracts.

Drosophila courtship has been shown to be modulated by chemical cues (*12*). *cis*-Vaccenyl acetate has been shown to inhibit a spontaneous male courtship behavior when this substance is present in a test chamber with a single virgin male (*6*). We have tested the behavioral effects of both cVA and cVOH by topically applying these substances either singly or in combination to the abdomens of wild-type (Ore R) virgin females. Treated and control females were placed with virgin males of the same strain for 1 hour; females treated with either cVA, cVOH, or a combination of both substances mated less frequently than untreated or solvent-treated controls (Table 1). There is, however, no evidence of statistically significant differences among the three experimental groups. In this assay system, cVA is as potent an inhibitor of copulation as cVOH.

In the behavioral assay just described, cVA and cVOH presumably are perceived by the male as they evaporate from the female's abdomen. The kinetics of the release of these compounds from the reproductive tract of an inseminated female may be substantially different. Another limitation of this assay is that measurements of the number of matings occurring during a 1-hour period provide only indirect information about the effect of inhibitory pheromones on courtship. Accordingly, we took advantage of the fact that in females inseminated by 6^{o} males, cVA will not be converted to cVOH, while such a conversion does occur in 6^{s} inseminated females. The



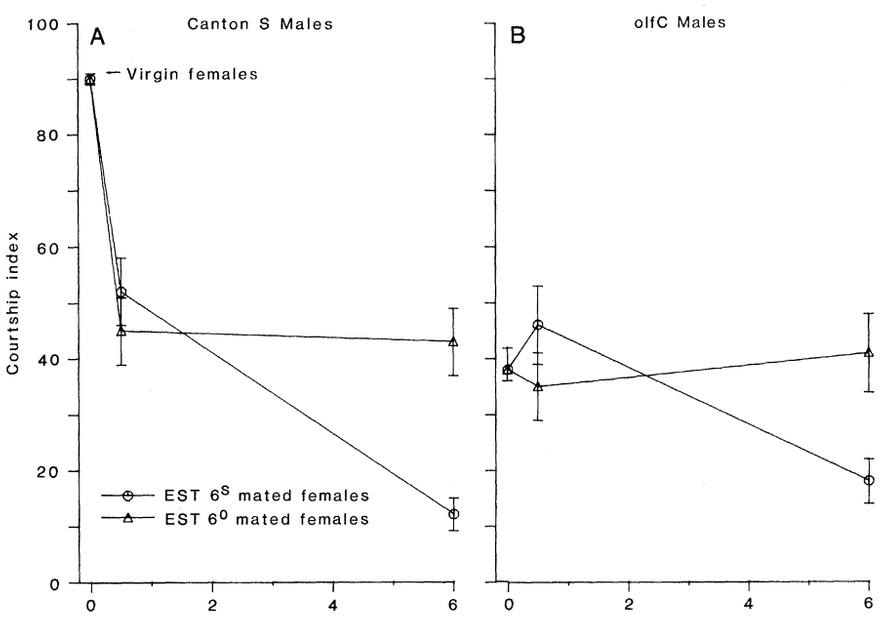


Fig. 1 (left). Hydrolysis of *cis*-vaccenyl acetate (cVA) by purified EST 6. For (A), 0.2 to 1 μg of purified EST 6 was incubated with $1.61 \times 10^{-4}M$ [^{14}C]cVA in a volume of 100 μl for 1 hour at 25°C (*10*). For (B), 6 μg of purified EST 6 was incubated with $8.05 \times 10^{-4}M$ [^{14}C]cVA and portions of the reaction mixture were removed at 20-minute intervals and assayed (*10*). Points shown are the averages of two to three determinations and have coefficients of variation ranging from 0 to 28.2 percent. Fig. 2 (right). Average proportion of 10-minute observation periods (courtship index) that mated females were courted by wild-type Canton S males (A) or olfactory-deficient males (B). Canton S females were either virgin (0 hours) or inseminated by esterase 6–deficient (EST 6^{o}) or active (EST 6^{s}) males at the beginning of the experiment. Courtship indices were determined at 10 minutes and 6 hours after females were inseminated. Error bars show standard errors of the means.

results of Jallon *et al.* (*6*) show that approximately 70 percent of the cVA present in a female 30 minutes after insemination is lost by 6 hours after insemination. Therefore, we compared the ability of females to elicit male courtship behavior either 10 minutes or 6 hours after they began to copulate with either 6^s or 6^o males. The inseminated females were tested with virgin males (*11*). The cumulative time that a male performs courtship in response to a female during a 10-minute period was recorded and a courtship index (CI), which is the percentage of the observation period that a male spent courting, was calculated (*11*). In addition to testing the response of wild-type Canton S (CS) males to inseminated females, we tested olfactory C (*olfC*) mutant males, which are unable to detect acetates but are capable of detecting alcohols (*12*). The results (Fig. 2) show that soon after mating, the CI of females by CS males is reduced. There is no statistically significant difference between females inseminated by the two EST 6 male types. However, by 6 hours after mating, there is a clear difference in the amount of courtship elicited by the two female types. Females receiving EST 6^s in the male ejaculate elicit less courtship than 6^o inseminated females. This effect is not always obtained when different strains and experimental conditions are used. The *olfC* males can detect the difference between 6^s and 6^o inseminated females 6 hours after mating but do not detect a difference between virgin and very recently inseminated females, as predicted from their inability to detect acetates. Tompkins and Hall (*11*) observed that extracts of volatile compounds from mated females inhibit courtship and predicted that cVA and EST 6 might be responsible for the effect of copulation on the sexual attractiveness of females. Moreover, courtship of *olfC* males is inhibited by extracts from females mated 24 hours earlier but not by extracts from mature males (*13*).

Fertilized females remain unreceptive to further copulation for several days (*14*), and their ability to elicit vigorous courtship increases gradually over a similar time period (*11*). In spite of the coincidence of these two effects of mating on female reproductive behavior, the presence of cVOH in the reproductive tracts of mated females is not the only factor involved in the control of receptivity. Manning (*14*) postulated that copulation per se and the presence of stored sperm affect the receptivity of inseminated females, and our previous analyses (*4*) of the remating kinetics and number of sperm stored by EST 6^o– and EST 6^s–mated females also support the hypothesis that sperm storage is involved in receptivity. We postulate that cVOH within the reproductive tract of the mated females reduces the amount of courtship that she stimulates, which in turn decreases the probability that she will mate again, but that other factors such as sperm storage cause the mated female to remain unreceptive to copulation regardless of her sexual attractiveness.

Table 1. The effect of the topical application of *cis*-vaccenyl acetate (cVA) and *cis*-vaccenyl alcohol (cVOH) on the proportion of *D. melanogaster* females mating within 1 hour. The test substances (200 ng of cVA or cVOH or 200 ng each of both compounds in 0.1 μl of acetone) were applied to the abdomens of anesthetized (ether) 4-day-old virgin Ore R females. After a recovery period of 30 minutes the females were placed individually in a glass mating chamber (0.5 ml in volume) with a virgin male for 1 hour. A χ^2 test of homogeneity rejects the null hypothesis ($\chi^2_4 = 75.2$, $P < 0.001$) that these data sets were drawn from the same distribution.

Treatment	Number of pairs			
	Mated		Number unmated	Total
	Number	Percent		
None	239	70	101	340
Acetone	304	72	119	423
cVA	130	49	134	264
cVOH	129	52	118	247
cVA + cVOH	107	47	121	228

Carboxylesterases are a heterogeneous group of enzymes and are widely distributed in both plants and animals. The physiological substrates for these enzymes are largely unknown with the exceptions of acetylcholine esterase (*15*) and the juvenile hormone specific esterases of insects (*16*). Our data identify a physiological substrate for EST 6 and suggest a metabolic role for this enzyme in the regulation of the sexual attractiveness of mated *Drosophila* females. The mechanism for male control of female reproductive behavior elucidated here has counterparts in at least two other insect systems (*17*) and may be of general significance.

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9. The products of a 3-hour incubation of purified EST 6 with cVA (*10*) were extracted three times with chloroform, and the reduced extract was subjected to gas-liquid chromotography (Varian aerograph 1400, flame ionization detector). The following conditions were used: column, 2 m by 2 mm, inner diameter; packed with Gas Chrom Q with 3 percent SE-30; temperature program, 8°C per minute, 140° to 300°C; helium carrier gas, 40 ml/min. Retention times for cVA and cVOH were 8.5 and 7 minutes, respectively.
10. Our assay for the EST 6–catalyzed hydrolysis of cVA utilized a 100 μl reaction volume containing 0.1*M* tris-HCl, *p*H 7.0, 0.01 percent Triton X-100, $10^{-3}M$ to $10^{-4}M$ ^{14}C-cVA and enzyme preparation. Homogenates of reproductive tracts were first incubated for 10 minutes at 25°C with $10^{-4}M$ *p*-chloromercuribenzoate and $10^{-5}M$ eserine sulfate; the labeled cVA was added, and the mixture was incubated at 25°C for 1 to 4 hours with agitation. Chloroform (200 μl) and 0.1*N* NaOH (50 μl) were added sequentially, and the mixture was shaken (Vortex) and centrifuged for 3 minutes (Eppendorf Microfuge). Portions (100 μl) of the upper layer (aqueous phase) were assayed for labeled acetate in 15 ml of Aquasol (New England Nuclear) in a Beckman LS 350 liquid scintillation counter with a 90 percent counting efficiency. Conditions for the assay of β-naphthyl acetate hydrolysis by EST 6 are described in (*2*).
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