

AN ABSTRACT OF THE THESIS OF

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Title: Carboxylesterases in the House Fly (*Musca domestica*, L.),
Flesh Fly (*Sarcophaga bullata*, Park.), and Black Blow Fly (*Phormia
regina*, Meig.)

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The carboxylesterases of three species of diptera, the flesh fly (*Sarcophaga bullata*, Park.), the black blow fly (*Phormia regina*, Meig.), and the house fly (*Musca domestica*, L.) were compared in relation to hydrolytic activity against three substrates, response to inhibition and induction, isozyme composition, and age-dependent variation in activity. Similar studies were also conducted with an insecticide-resistant house fly strain. Most of the experiments were with adult female flies but male flies as well as larvae and pupae were included in several experiments.

Naphthyl acetate was the substrate common to all experiments and the esterases hydrolyzing this compound were further characterized according to their inhibition by eserine, parahydroxymecuri-

benzoate (PHMB), and paraoxon. The hydrolytic activity observed in the presence of eserine and PHMB or blocked by paraoxon was considered to be due to carboxylesterases of the B-type. Two other esters, hydroprene, an insect growth regulator similar in structure to the natural juvenile hormone, and juvenile hormone-1, were also used as substrates.

Neither the hemolymph nor the cytosol esterases of the three species were susceptible to eserine inhibition, indicating that the samples contained minor quantities of the choline esterases. PHMB had little inhibitory effect on the flesh fly esterases of both hemolymph and soluble fraction. However, this inhibitor reduced the activity of the blow fly esterases by more than 50 percent, indicating a high content of aryl esterases in this species. The house fly esterases were also inhibited by PHMB but to a less extent than those of the blow fly. As expected, paraoxon was a strong inhibitor of the esterases in both hemolymph and soluble fraction of the three species.

The total enzyme content of the flies was estimated from the hemolymph volumes determined earlier and the activity per unit volume found in the age-dependency experiments. The daily average activity estimated in this way was highest in the flesh fly, about four times that of the blow fly, and lowest in the house fly, about one-seventh that of the flesh fly. The values were even lower in the case of the resistant house flies, about 1/40th those of the flesh fly.

When hydroprene was used as a substrate in the age-dependency experiments, the activity profiles were similar to those found with naphthyl acetate as substrate indicating that the same enzymes were involved in the hydrolysis of this JH analogue. Of the three species, the flesh fly esterases were most active against hydroprene and the blow fly and resistant house fly enzymes were the least active. The flesh fly hemolymph enzymes averaged, on a per-day basis, up to three times more hydroprene cleaving activity than those of the resistant house flies.

The electrophoresis experiments with hemolymph and cytosol revealed the presence of several carboxylesterase isozymes. As many as 14 different esterase-active bands were found in flesh fly hemolymph, 11 in blow fly hemolymph, and 12 in house fly hemolymph. Similar results were obtained with the cytosol. These bands were placed in five groups according to their mobilities in the polyacrylamide gels and it was evident that the isozymes migrating to the midpoint of the gels were responsible for most of the carboxylesterase activity. Some slow-moving isozymes were also thought to be responsible for this type of activity. Other evidence obtained during the electrophoresis experiments indicated that the isozymes hydrolyzing hydroprene were from the same groups as those cleaving naphthyl-acetate while those which attacked JH-1 had different electrophoretic characteristics.

Both hydroprene and naphthyl acetate esterases were induced by

topically applied hydroprene. The response was dose dependent in the range 1-10 ug for flesh flies and 1-5 ug for house flies and blow flies. However, the response was variable and appeared to depend on the age of the flies at the time of the treatment. Increases in esterase activity ranged from 1.5 to 4-fold with the flesh fly enzymes being the most responsive and those of the blow fly the least. The possibility of JH-1 esterase induction by hydroprene was not investigated.

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Flesh Fly (*Sarcophaga bullata*, Park.), and Black
Blow Fly (*Phormia regina*, Meig.)

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Carboxylesterases in the Housefly (*M. domestica*), Flesh
Fly (*S. bullata*), and Blowfly (*P. Regina*)

INTRODUCTION

Insect esterases are a poorly defined group of enzymes with both a protective and a physiological role. They catalyze the hydrolysis of various groups of insecticides, insect growth regulators, and the juvenile hormone (JH) and are thus of considerable importance in insecticide action as well as the growth and development of insects. The general term, esterase, can be assumed to include the enzymes which hydrolyze carboxyl esters. However, the term carboxylesterase (CE-ase) has been assigned to the latter sub-group. Typical substrates of these enzymes, in the fields of insect toxicology and physiology, are organophosphate esters containing carboxylester linkages, such as the insecticide, malathion, both synthetic and natural juvenile hormones (JHs), synthetic pyrethroid insecticides and various common carboxylic esters such as naphthyl acetate and acetyl choline.

These esterases, known as carboxylic ester hydrolases (E.C.3.1.1.1) by the International Union of Biochemists, cleave their substrates with the addition of water to yield alcohol and acid. They are widely distributed in insect tissue and hemolymph and occur in multiple forms in the species examined so far. Their substrate specificities are generally broad and overlapping (Myers, 1959).

Carboxylesterases are also known as aliesterases or B-esterases (report of the Commission on Enzymes of the International Union of Biochemistry, 1961). The B-esterases are further subdivided into

two main types, B' and B'', by means of their differential inhibition by eserine. B''-esterases are eserine sensitive and hydrolyze cholinesters (but also aromatic esters) and are designated as "cholinesterases". The B'-esterases usually hydrolyze aliphatic as well as aromatic esters, are relatively insensitive to eserine, and are usually named "aliesterases" although lipases and peptidases all perform ester hydrolysis (Cunningham, 1965). It is the author's desire to use the common name "carboxylesterase" to avoid the unnecessary compounded view of this esterase group.

The mechanism of the degradation of such esters as the insecticide malathion and the resistance to these compounds by various insect species has been intensively investigated in the last decade. It has been generally acknowledged that these enzymes are responsible for the selective toxicity of this group of compounds, favoring mammals over insects (Krueger and O'Brien, 1959) and resistant insects over susceptible ones (Matsumura and Brown, 1961; Matsumura and Hogendijk, 1964).

Carbamate insecticides are another group of compounds that can be degraded by carboxylesterases (CE-ases) of insects. However, it is considered that this group of insecticides are poor substrates for these enzymes (Matsumura, 1975). It has also been found that hydrolysis of synthetic pyrethroids is a major detoxication mechanism in Lepidopteran insect larvae (Bigley and Plapp, 1978) and in house flies (Miyamota and Suzuki, 1973).

Recent studies have concentrated on the regulation of juvenile

hormone-1 by both nonspecific and specific CE-ases in the hemolymph of the potato beetle and the tobacco hornworm (Kramer and de Kort, 1976; De Kort *et al.*, 1978; Sanburg *et al.*, 1975). There is evidence that JH control of insect metamorphosis is also regulated by these enzymes. Yu and Terriere (1975) studied the esterases of the housefly in relation to hydrolysis of juvenile hormone analogs (JHAs) and have suggested that these enzymes may be involved in the regulation of endogenous JH in this species. The diapause of adult and larva appears to be under the control of CE-ase isozymes in a scheduled physiological program during the life cycle of some insects (Kramer and De Kort, 1976; Vince and Gilbert, 1977).

There is evidence that cuticular wax synthesis and transport are related to the esterases (Locke, 1974). The esterases were also found to be responsible for the breakdown of some short-chain fatty acids in the insect gut. It is probable that in the gut these enzymes function in digestion (Ahmad, 1970a,b; Cook *et al.*, 1969).

From this overview of the various functions of the CE-ases in insects, their importance is easily understood. Much additional information is needed to complete our understanding of their biochemistry and physiology. The questions I have attempted to answer in this study are: What categories of CE-ases are present in the house fly, blow fly and flesh fly: what is the age dependency of these esterases in the three species; i.e., how do they fluctuate during growth and development: are these esterases susceptible to induction

by exogenous chemicals? I was also interested in whether the enzymes which hydrolyze a JHA are also active against JH-1. Finally, I wished to compare the characteristics of these enzymes among three closely related species of Diptera and between an insecticide resistant and susceptible strain of house flies.

HISTORICAL REVIEW

Aldridge (1953) separated serum esterases of vertebrate species into two distinct types, A- and B-esterase. The A-type is not inhibited by 1×10^{-3} M paraoxan and cleaves p-nitrophenyl acetate at a higher rate than p-nitrophenyl butyrate. The B-type is inhibited by 1×10^{-7} to 10^{-8} M paraoxon and hydrolyzes p-nitrophenyl butyrate at the same or higher rate than p-nitrophenyl acetate. Mounter and Whittader (1953) using the same technique, found that there are three groups of esterases with overlapping substrate specificities. Similar overlap in substrate specificity was also found in many insect species (Metcalf and March, 1949, 1950; Casida, 1955). Phosphorous insecticides, thus, were being widely used as inhibitors to detect and characterize the biochemical properties of the esterases in insects (Lord and Potter, 1953).

Casida (1955) used the Warburg manometric technique to measure the esterase activity in twelve species of insects and one species of mite. Homogenate of insects and mite were incubated with choline chloride, tetraethyle pyrophosphate (TEPP) and eserine salicylate in the Warburg flask containing bicarbonate buffer. The results indicated that the enzyme consistently inhibited (67% - 85%) at the prostate stage was acetylcholinesterase. Therefore, Casida concluded that the primary cause of organophosphate (OP) insecticide poisoning in insects was blockage of the nerve acetylcholine-cholinesterase (AChE-ase) system. Casida's concept is currently accepted.

March *et al.* (1956) used malathion as inhibitor to study the

comparative enzymatic activity of esterases between the hen, white mouse, and insects. He found that the hen and the mouse have a fast rate of metabolism of malathion by the progressive hydrolysis of the ethyl ester moities to produce ionic and water soluble compounds. O'Brien (1957) followed March and found that the selective toxicity of malathion was due to the balance of activity (oxidation) and hydrolytic enzymes in the susceptible and nonsusceptible species which led to much greater levels of malaaxon in susceptible animals. Insect poisoning by this group of OP compounds is due to the oxidized derivatives which accumulated in insects. O'Brien, therefore, concluded that the most important factor in this observed result was the more extensive CE-ase activity in the mammals (nonsusceptible species). Later on, Krueger and O'Brien (1959) demonstrated that CE-ases were responsible for the selectivity of malathion, favoring mammals over insects.

The enzymes which hydrolyze the $-\text{COOC}_2\text{H}_5$ groups in malathion-resistant *Culex tarsalis* have been isolated and purified 100 times by Matsumura and Brown (1961, 1963). They found that the CE-ase from malathion susceptible and resistant strains of mosquito (*Culex tarsalis*) purified by DEAE column chromatography had the same molecular weight (16,000). Properties such as K_m , energy activation and temperature coefficient were also similar and n-propyl paraoxon and oxygen analogues of EPN inhibited the formation of malathion monoacid in both strains. It was concluded that, in this case, the interstrain

difference was of a quantitative nature.

On the other hand, a twenty-fold difference in K_m relative to malathion between partially purified CE-ase from resistant and susceptible strains of the two-spotted spider mite indicated a qualitative difference between the two enzymes (Matsumura and Voss, 1965).

Many other insects with esterase involvement in OP resistance also appear to have unusually high levels of CE-ase as measured by a variety of techniques including substrate hydrolysis *in vitro* with polyacrylamide gel electrophoresis, acetone extraction, column chromatography, and gel filtration. These insects with CE-ase activity measured are: *Musca domestica*, *Oocopeltus fasciatus*, *Tetranychus telarius* (Afshar-pour and O'Brien, 1963), *Dermestes maculatus* (Dyte *et al.*, 1966), *Tribolium castaneum* (Dyte and Rowlands, 1968), *Heliothis virescens* (Bull and Whitten, 1970, 1972), *Myzus persicae* (Needham and Sawicki, 1971; Sudderuddin, 1973), *Nephotettix cincticeps* (Ozaki and Kassai, 1970), and *Laiideophax straitellus* (Ozaki, 1969).

Townsend and Busvine (1969) using malathion-C¹⁴ and thin layer chromatography and gas liquid chromatography observed that the resistant strain of blow fly (*Chrysoma putoria*) had only 15 percent as much aliesterase as that in the susceptible strain. Oppenoorth (1959, 1972), Van Asperen (1958, 1962), Van Asperen and Oppenoorth (1959) have extensively studied the detoxication of parathion, diazinon, malathion, and acethion in susceptible and resistant house flies in relation to aliesterase enzymes. Based on the finding of low aliesterase levels which were correlated in several strains with

high OP resistance, they proposed the intriguing "mutant aliesterase" theory, in which the original gene on the fifth chromosome of susceptible flies controlling aliesterase production has mutated in resistant flies to form alleles which hydrolyze these phosphate insecticides. On the other hand, other strains of OP resistant flies which are very active in degrading methyl paraoxon, show no indication of lowered aliesterase activity. Also, the low turnover number of the "mutant aliesterases" in the Oppenoorth study indicates that other enzymes must carry out the major portion of OP metabolism in the flies.

The mechanism of resistance seems to be an increase in ability to degrade the AChE-ase-inhibiting oxygen analogs of phosphorothioate insecticide (Oppenoorth, 1965) or an increase in ability to degrade malathion via CE-ase hydrolysis.

Studies of esterases and their inhibition by different inhibitors have been reported numerous times. A few authors have compared esterase patterns after electrophoresis in insecticide-resistant and susceptible strains of various insects. Ogita and Kasai (1965) and Van Asperen and Oppenoorth (1959) could not detect any qualitative differences in *Musca domestica*. There seemed to be less activity of the B-esterase in resistant flies.

Menzel, *et al.* (1963) mentioned that in house flies the great variation of soluble esterases occurring during the development of a given strain is evidence for the unique character of the individual esterase bands. In both resistant and susceptible strains the esterases appeared to vary in a unique manner with the stage of

development of the insect.

Electrophoretic analyses of hemolymph and homogenates of house fly have demonstrated the existence of a number of CE-ases either in hemolymph or in tissues of different organs (Menzel, et al., 1963; Van Asperen, 1964; Cook and Forgash, 1965; Ahmad, 1970, 1974, 1976). In fact, inconsistent electrophoretic zymograms of esterases have been found by many researchers in different dipteran species like *Drosophila virilis* (Korochkin, 1975), *Culex pipiens pipiens* (Pasteur and Gilbert, 1975), and *Musca domestica* (Collins and Forgash, 1968; Narang, et al., 1976; Ahmad, 1976).

Ahmad (1974) characterized 10 esterase zones as aliesterases in electrophoresis study of house flies. Ahmad (1970, 1974) indicated that the level of CE-ase was negatively correlated with OP resistance in the house fly. He also indicated that two extra esterase bands were present in normal (susceptible) strains. Quantitative differences were apparent between the resistant strain and the normal strain. The absence of two bands (out of 10) and the reduced enzyme activity level of the other esterases (with the exception of one) is related to the "low esterase" phenomenon.

Based on the behavior of these electrophoretic esterase zones to various esters and inhibitors, one can conclude that the nonspecific CE-ase consist of a heterogeneous mixture of enzymatic proteins with a wide range of catalytic properties. It is therefore possible that one or more components of this soluble enzyme preparation are responsible for the hydrolysis of organophosphorus carboxylic esters

such as malathion. Clearly, this area requires additional study in support of this suggestion.

The CE-ases are also involved in the detoxication of synthetic pyrethroid insecticides. House flies susceptible to pyrethroids were found to metabolize tetramethrin by hydrolytic cleavage (Miyamota and Suzuki, 1973). They claimed that the ester bond cleavage is one of the principal metabolic pathways of tetramethrin in house flies *in vivo*, whereas oxidative degradation may play a minor role. Jao and Casida (1974a) indicated that many of the newer pyrethroids, primary alcohol esters, are detoxified by esterases. It was found that acetone powder preparations of the house fly hydrolyzed the (+)-trans and (+) cis isomers of resmethrin and tetramethrin but not S-bioallethrin. Homogenate fractions were less suitable for assaying the insect esterases due to interfering reactions of inhibitors (Jao and Casida, 1974b).

Shono *et al.* (1979) found that microsomal esterases of house flies were more active against the trans isomer than the cis isomer of permethrin. They also found species differences in hydroxylating the 2', 4' or 6 position of the alcohol moiety.

In recent years, progress in understanding isozymes has afforded considerable insight into the genetic and structural interrelationships of multiple enzyme forms, the physiological significance of this multiplicity, and the control of enzyme synthesis during the ontogenesis of the insect. Ontogenetic changes of esterase activity

have been reported in many dipteran insects including *Anopheles albimanus* (Vedbrat and Whitt, 1975), *Drosophila* species (Korochkin and Matveeva, 1974; Pasteur and Kastritsis, 1971), and *Musca domestica* (Menzel, et al., 1963; Ahmad, 1974, 1976).

The appearance and the disappearance of an isozyme in the hemolymph or in the tissue fraction reflects a special physiological condition of the insect. For example, the larval isozyme E_x in the house fly, isozyme E_1 in various larval tissues and adult gut of house fly (Ahmad, 1976) and prepupal esterase in the *Drosophila virilis* (Rauschenbach, et al., 1977), add biological significance to these isozymes. The prepupal esterase and the isozyme E_1 were thought to be related to JH metabolism in these insects.

The insect JH has been described as a modulator determining the quality of the molt of the insect. This hormone is hydrolyzed by a specific group of CE-ases in the hemolymph of *Manduca sexta*, *Hyalophora cecropia*, *Schistocerca gregaria*, and *Sarcophaga bullata* (Slade and Zibitt, 1972). Hydrolysis of JH-1 by these CE-ases was further confirmed in *Schistocerca gregaria* and *Rhodnius prolixus* (White, 1972) *hydrophora glorer* pupa (Whitemore, et al., 1972) and some seventeen other species in eight orders of insects, including three dipterans, *M. domestica*, *D. melanogaster* and *C. americanus* (Ajama and Riddiford, 1973). Since then, more insect species either in larval, pupal, or adult stages have been examined, and it is now generally accepted that the major metabolic pathway of this hormone of insect in the hemolymph is by hydrolysis of the methyl ester by the CE-ases. This

enzyme is mostly found in the hemolymph of the last instar larval stage of Lepidoptera, Coleoptera, Hemiptera, Orthoptera, and Homoptera (Whitemore, *et al.*, 1972; Slade and Wilkinson, 1974; Sanburg, *et al.*, 1975; Pratt, 1975; Kramer and De Kort, 1976; Brown, *et al.*, 1977; Hammock, *et al.*, 1977; Edward and Rowland, 1978; Reddy, *et al.*, 1979).

On the other hand, it has been reported that the hemolymph of some Dipteran insects as well as the honey bee and the American cockroach contain little or no juvenile hormone esterase (JHE-ase) activity (Slade and Zibitt, 1972; Weirich and Wren, 1973; Weirich and Wren, 1976; Hooper, 1976; Wilson and Gilbert, 1978; De Kort, *et al.*, 1979; Mumby, *et al.*, 1979). However, Klages and Emmerich (1979) did find that the JHE-ase activity drastically increased in the prepupal hemolymph of *Drosophala hydei*.

Weirich, *et al.* (1973) found a major esterase peak on day three of the 5th instar larvae of *M. sexta* and that the hemolymph of 4th instar larvae and of pupae was inactive to JH. The changes were independent of fluctuations in general esterase activity. By using gel filtration to resolve *M. sexta* larval hemolymph, Sanburg (1975) found two types of esterases. The type I esterase which hydrolyzed free JH in hemolymph could not attack JH bound to carrier protein (s) that were released by the fat body when the larva were in the early stage. In the 5th instar larva, the type II esterases (s) were released into the hemolymph. These esterases could hydrolyze both the free and protein-bound JH into acid for normal metamorphosis.

That the two esterase activity peaks merged during the last instar and during the larval-pupal molt was confirmed by work on *Drosophila hydei*, *Leptinotarsa decemlineata*, *M. sexta*, and *Trichoplusia ni* (Klages and Emmerich, 1979; Sparks and Hammock, 1979; Vince and Gilbert, 1977; Weirich, et al., 1973; Kramer and De Kort, 1976).

Isoelectric focusing of the hemolymph esterase in *M. sexta* revealed that there are three forms of the type II esterases with equal rates of hydrolysis of the JH. As Sanburg, et al. (1975) indicated, type I esterase (molecular weight: 10^5) activity can be totally inhibited by 10^{-4} M diisopropylphospho-rofluoridate (DFP) whereas those in type II esterases (molecular weight: 6×10^4) were unaffected under the same condition. It was concluded that these esterases are JHE-ases. These esterases and the special binding protein have been found in all insect species examined (Kramer and De Kort, 1976; Kramer and Childs, 1977; Hammock, et al., 1977). The major specific JHE-ase has a sedimentation coefficient of 4.98 S and a diffusion coefficient of $6.4 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ (Agosin, 1978). These esterases were later to be inducible by JH injected into *Hyalophora gloveri* pupae (Whitemore, et al., 1972, 1974). Induction of these esterases was also found in other insect species.

Nowock and Gilbert (1976) demonstrated that cultured fat body, after treatment of JH, immediately released JHE-ases into the culture medium. Spark, et al. (1979) found that JHE-ases could be artificially induced to appear in the hemolymph of the larvae of *Trichoplusia ni* by topical treatment with JH-I, JH-II, or dihomobranched juvenoids.

ETB, at high dose, is also an inducer of JHE-ase.

Kramer (1977) found that enzyme activity to JH-III can be induced by topical treatment of three juvenoids: methoprene, hydroprene, and JH-II on diapausing Colorado potato beetles. Injection or topical application of the JH-I caused identical effect (Kramer and De Kort, 1976a). They also demonstrated that two JH analogues (JHA) (ZR 512 and ZR 515) were not degraded by JHE-ases. Therefore, it was assumed that this induction phenomenon is not specific for JH.

The profile study of esterase activity to naphthylacetate (NA) and to JH-I or JH-II showed that these two esterase systems were different in substrate specificity and in sensitivity to DFP inhibition. Reddy, et al. (1979) demonstrated that JH did not induce general esterases in the pupa of *Galleria mellonella*, and only JH specific esterases were induced by the agent. Other workers found that the specific hormone esterases could break down both the free and protein bound JH (Nowock and Gilbert, 1976; Spark and Hammock, 1979; De Kort, et al., 1979). The half-life of exogeneous JH *in vivo* was found to be in the range of 15 to 90 minutes in different insect species (Nowock, et al., 1975; Kramer, et al., 1976; Mitsui, et al., 1979), whereas it is 25 minutes in house fly (De Kort, et al., 1978).

Besides the hemolymph of various stages of insects, soluble fraction JHE-ases from tissues or organs have also been tested for hydrolytic activity. In general, fat body and body wall (including integument, wing discs, and epidermis) contained more JHE-ase activity

(Nowock, et al., 1975; Mitsui, et al., 1979; Klages and Emmerich, 1979). Midgut, malphigian tubes, and other organs also contained the enzyme but at a lower level (Slade, et al., 1976; Brown, et al., 1977; Slade and Wilkinson, 1974).

The mitochondria fraction (12,000 g pellet), the microsomal fraction (100,000 g pellet), and the soluble fraction (100,000 supernatant) of house fly, blow fly and flesh fly were active in hydrolyzing JH-I as well as certain alkyl 3,7,11-trimethyl-2,4-dodecadienoate type JHA (Yu and Terriere, 1975, 1977a, 1977b; Terriere and Yu, 1977, 1978). The hydrolytic activity of these fractions has also been found in other species such as *M. sexta*, *Diatrea grandiosella*, *Culex pipens*, *Drosophila hydei* (Hammock, et al., 1975; Nowock, et al., 1975; Hooper, 1976; Klages and Emmerich, 1979).

The highest level of the esterase which hydrolyzes the JHA may be present in the microsomal fraction or the soluble fraction, depending on the insect species. This fact indicates the presence of both membrane-bound and soluble esterases (Hammock and Quistad, 1975).

The level of JH hydrolytic activity in the hemolymph or in the microsomal fraction is totally age or stage-dependent.

Yu and Terriere (1975) reported that microsomal esterases of the house fly seem to fluctuate in activity during the last 15-20 hours of the larval period, dropping first to a low level then rising sharply just before pupation. In the pupal stage the enzyme activity remained low. The increased hydroperene-esterase activity in the pharate adults was assumed to be necessary for eliminating traces of

the endogenous JH in order to complete the pupal-adult transformation.

A similar fluctuation of JHA esterase in the microsomal fraction was also found in different stages of blow fly and flesh fly (Yu and Terriere, 1977a, 1977b; Terriere and Yu, 1977). Interesting was the higher esterase activity in the soluble fraction than the microsomal fraction in blow fly.

The microsomal hydroperene esterase activity of adult house flies was higher and more constant (Yu and Terriere, 1975, 1978). In *D. hydei* hemolymph this enzyme was found only in the prepupal stage (Klages and Emmerich, 1979). In lepidoptera insects the highest hemolymph JHE-ase activity was restricted to the last instar larvae (Slade and Wilkinson, 1974; Kramer, et al., 1974; Sanburg, et al., 1975). In Colorado beetle, JHE-ases occurred during all the stages tested but showed characteristic changes in activity during development (Kramer and De Kort, 1976).

Weirich and Wren (1973) first indicated that the JHE-ase in *M. sexta* hemolymph rapidly hydrolyzed JH-I. The ethyl ester of JH-I was hydrolyzed comparatively slow. In Colorado beetle, JHE-ases degraded both JH-I and its ethyl ester at the same rate (De Kort, et al., 1978, 1979). They also indicated that the natural 2 E isomer of JH-I was very rapidly hydrolyzed while the 2 Z isomer was not. Peter, et al. (1979) found that male *Locusta migratoria* hemolymph contain JHE-ases. Exposure to racemic mixtures of JH-I and JH-III resulted in preferential hydrolysis of the natural enantiomer of JH-I but the unnaturally enantiomer of JH-III. These findings

indicate a rather marked substrate specificity to the JHE-ases.

Metabolism of insect growth regulators' ester function by esterases in insect has been intensively studied in dipteran species, especially in *Musca domestica*, *Phormia regina*, and *Sarcophaga bullata* (Yu and Terriere, 1973, 1975, 1977a, 1977b; Terriere and Yu, 1977; Quistad, et al., 1975; Weirich and Wren, 1973; Georghiou, et al., 1978; Mumby, et al., 1979). *Culex pipien pipiens*, *Aedes aegypti*, *Munduca sexta*, *Hyalophora cerpia*, *P. brunnea*, *Schistocerca vaga*, *Samia cynthis*, *Tenebrio moltor*, *Blaberus giganteus*, and *Solenopsis invicta* were also tested for their esterase activity against the juvenoids (Weirich and Wren, 1973; Weirich and Wren, 1973; Hooper, 1976; Kramer and De Kort, 1976; Hammock, et al., 1977a, 1977b; Brown, et al., 1978; Mumby, et al., 1979; and Bigley and Vinson, 1979).

Weirich and Wren (1973) reported on juveniod metabolism by hemolymph esterases of *M. sexta* indicating that the general trend in esterase hydrolysis rate was often methyl > ethyl > propyl > isopropyl although hydrolysis of the isopropyl ester, methoprene, could not be compared directly with the methyl and ethyl esters of JH. Yu and Terriere (1975, 1977a, 1977b) and Terriere and Yu (1977) have indicated the importance of esterases in the metabolism of the ester moiety, ethyl, propynyl, and thioesters, and pointed to the resistance to degradation of all branched juveniods tested in the three dipteran species. The microsomal esterases could hydrolyze the straight chain analogs tested.

Methoprene, the isopropyl analog, was not only resistant to hydrolysis by *M. sexta* hemolymph esterases but also resistant to the esterases from other *M. sexta* tissues (Nowock, et al., 1975). Similar results were found by Yu and Terriere (1975, 1977a, 1977b) in three Dipteran species. Hydroprene, an ethyl ester, was metabolized by both soluble and microsomal esterases of these dipeteran species in the order of house fly > flesh fly > blow fly, with the susceptible house fly being most active (Yu and Terriere, 1977a).

Other species can hydrolyze methoprene, however, because Solomon and Metcalf (1974) found that a compound co-chromatographing with the methoxy acid of methoprene was the major *in vivo* metabolite of methoprene in *Oncopeltus fasciatus* and a minor metabolite in *Tenebrio molitor*. Quistae, et al. (1975) found that in *Aedes aegypti* and *Culex pipiens quinque-fasiatus* larvae the methoprene ester is cleaved to give hydroxy acid. Hooper (1976) found very low esterase activity to JHA(s) in *Culex pipiens* larvae. The estimated esterase activity to NA was high in the mitochondrion fraction, but was low in the soluble and microsomal fraction. Bigley and Vinson (1979) also found larvae and pharate pupae of *Solenopsis invicta* metabolized methoprene principally by esterase cleavage of the isopropyl ester. During the pupal molt and during the pharate pupal transformation, metabolism of methoprene occurred in the cuticle, resulting in the accumulation of metabolites within and on the cuticle.

Quistad, et al.(1975) also indicated that house fly and mosquito larvae were able to isomerize the 2 E form of methoprene to the 2 Z

form. Yu and Terriere (1978) were unable to detect the isomerization of the 2 E methoprene *in vitro*.

As to the reaction of these enzymes with inhibitors, Quistad, *et al.* (1975) also showed that Triorthocresyl Phosphate (TOCP) decreased the proportion of methoprene esterases present in the larvae. Yu and Terriere (1977a) found that DFP inhibited microsomal esterase activity in flesh fly and blow fly, but not in the house fly, and that paraoxon 10^{-5} M had low inhibitory effect on house fly microsomal esterases, but inhibited these esterases in blow fly and flesh fly. They indicated that the house fly microsomal esterases should be classified as B-type while those of flesh fly and blow fly should be classified as C-type. It is assumed that the enzyme system metabolizing the JHA(s) composed of several esterases, each with a somewhat different substrate preference. In fact, multiple forms of esterases have been detected in many other insects including cockroach, tobacco hornworm, and *Prosophila melagartra*.

METHODS AND MATERIALS

Insects

Three Dipteran species were used in this study: Flesh fly (*Sarcophaga bullata* Parker), black blow fly (*Phormia regina* Meigen), and two strains of house fly (*Musca domestica* L.). The house fly strains were the CSMA, insecticide susceptible, and Rutgers, insecticide resistant. The latter is resistant to diazinon, an organophosphate compound and propoxur a carbamate compound. This resistance is due to a considerable extent to the increased metabolism of these insecticides by the microsomal oxidase system (Terriere and Yu, 1979).

The flies were reared under constant temperature $24 \pm 1^{\circ}\text{C}$ and light:dark conditions (16:8). House fly larvae were reared on an alfalfa meal-wheat bran mixture (1:3) and adults were fed a mixture of powdered milk, sugar, and powdered egg yolk (12:12:1). Blow fly and flesh fly larvae were reared on pork liver and adults were reared on the powdered milk:sugar:egg yolk mixture as above. All species reproduced normally under these rearing conditions. Stocks of newly emerged adults of each fly species were synchronized within six hours for the flesh fly and blow fly, and within twelve hours for the house fly. All of the experiments with larvae were with the third instar and those with pupae were at five days for house fly and blow fly and nine days for flesh fly. Adults were used at various ages depending

Chemicals

All chemicals and reagents were of analytical grade or the best grade available. Inulin-carboxyl-(carboxyl- ^{14}C), specific activity 1.91 mCi/gm., 98 percent pure as indicated by thin layer chromatography (TLC), power-like crystal, purchased from New England Nuclear Corporation, Boston, Massachusetts.

Juvenile hormone I [(cecropia- C_{16}) 10-14 1-JH-I (methyl-2E, 6E, 10Z); 10, 11-epoxy-7-ethyl-3, 11-dimethyl-2, 6-tridecadienoate], specific activity 13.5 Ci/mM, 98 percent purity from New England Nuclear Corporation, Boston, Massachusetts. Cold cecropia juvenile hormone was purchased from Eco Chemical Intermediates, Division of Eco-Control, Inc., Cambridge, Massachusetts. Hydroprene (ethyl (2E, 4E)-3,7,11-trimethyl-2, 4-dodecadienoate) 5-(^{14}C), specific activity 5-8 mCi/mM, containing 98.4 percent trans, trans and essentially no cis trans form, from Zoecon Corporation, Palo Alto, California. Cold hydroprene was also from Zoecon. Bray's counting solution for radioactive isotope labeled materials: naphthalene, PPO (2,5-diphenyl oxazole), POPOP (p-bis-(2-(5-phenyloxazolyl)) benzene), P-dioxane, scintillation grade, and methanol, ethylene glycol (1,2-ethanediol) were purchased from New England Nuclear Corporation. Silic gel G-1 was purchased from Sigma Chemical Company, St. Louis, Missouri. 2-naphthol, 2-naohthalene acetate, laryl sulfate (sodium laryl sulfate, dodecyl sodium sulfate, approximately 95 percent), diazoblue (O-dianisidine, tetrazotized ZnCl_2 complex, practical grade 30 percent

pure), phenylthiourea, eserine (Physostigmine), para-benzyate mercuribenzenate (para-hydroxy mercuribenzoate, crystalline sodium salt), Actinomycin D, and purchased from Sigma Chemical Company.

Acrylamide, bis acrylamide, N,N,N',N'-tetramethylenediamine, riboflavin, ammonium persulfate, bromophenol blue, all electrophoresis purity grade were purchased from Bio-Rad Laboratories, Richmond, California. Coomassie brilliant blue G-250, ethanol and phosphoric acid were purchased from Sigma Chemical Company.

Withdrawal and storage of hemolymph samples

Hemolymph samples were withdrawn from third instar larvae, pupae, and female adult flies of various ages. For the age-dependent (profile) study of CE-ases, the hemolymph was collected every 24 hours after emergence for six to eight days for CSMA and Rutgers house flies and for 13 days for blow flies and flesh flies. The flies were lightly anesthetized with carbon dioxide and the hemolymph collected under a binocular microscope using a 10x magnification. The right hind leg of each fly was cut off near its coxal base and the fly squeezed gently with a blunt forceps until a clear globule of hemolymph was forced out. The tip of a 10.0 ul capillary micropipette was applied to the globule which rose quickly up the bore. Little difficulty was encountered in collecting 10.0 ul from the larvae, and adults of female blow flies and flesh fly. It was more difficult to collect 10.0 ul hemolymph from female house flies since the hemolymph volume

of house flies is small, especially when vitellogenesis has just been completed. It was also difficult to withdraw enough hemolymph sample from male flies of all three species. At least 0.3 ul to 0.5 ul of hemolymph still can be collected per female house fly. More than 1.0 ul of hemolymph can be collected from male flesh fly or blow fly; and 1.0 to 6.0 ul from female blow fly; and 2.0 to even 10.0 ul from female flesh fly depending on the age. During the collection the micropipette was always chilled in an ice cup to prevent coagulation of the hemolymph and to prevent melanization.

The hemolymph was then blown into a centrifuge tube containing 0.9 ml of 0.1 M phosphate buffer, pH 7.5 and 10^{-4} M phenylthiourea. Phenylthiourea is a tyrosinase inhibitor which prevents blackening of the hemolymph with melanin which is formed as a result of tyrosinase activity.

At least 100 ul of hemolymph was pooled for each sample. The sample was then centrifuged in 1000 g for 15 minutes in an ultracentrifuge (Beckman, Spinco, Model L.). The supernatant was withdrawn and the upper lipid and bottom precipitate was discarded. The sample was then sealed in a parafin-covered tube and stored at -16°C . In this study the hemolymph sample was used immediately as soon as enough samples were collected.

Measurement of hemolymph volume of female adult flies

A biological dilution method derived from Tobe and Davey (1972)

was used for this study. In this method the ^{14}C -carboxyinulin is adequately dispersed in the hemolymph after one hour of incubation, and excretion or breakdown of the inulin does not occur within five hours after injection. In measuring the hemolymph volume of individual flies, one μl of labeled carboxy-inulin- ^{14}C (15,000 cpm) was injected into the fly through the scutellar suture. After 45 minutes the hemolymph was withdrawn from the coxal base of the fly and the volume was recorded. The hemolymph sample was blown into a vial containing 10.0 ml counting solution and was counted in an Isocap 300 6868 liquid scintillation counter (Searle Analytic, Inc.). Samples were counted two minutes twice. An internal standard was used for determination of the counting efficiency according to the manufacturer's manual. The counting efficiency for all samples was greater than 90 percent. The volume of hemolymph from ten flies was calculated and plotted against the age of the fly.

Tissue fractions

The following fractions were examined for enzyme activity in this study: Hemolymph—insect blood, collected as described above. Cytosol—the intracellular fluid, as free as possible of hemolymph. This fraction was obtained by dissecting 125 house flies, 50 blow flies, or 25 flesh flies under binocular microscope. Dissection was performed in ice cold 0.04 M phosphate buffer, pH 7.5. Damage to the organs of insects was at minimum level. Legs, wings, and heads were

cut off, and the headless flies were washed four times with 0.04 M buffer before homogenation. Cytosol fractions were collected after a sequence of centrifugations. The supernatant above the 188,000 x g pellet, containing possibly no hemolymph, was used for the location of intracellular CE-ases by electrophoresis.

Soluble fractions, headless flies

The supernatant above the 100,000 x g pellet, containing an unknown quantity of hemolymph. Obtained from homogenates of insects from which a hemolymph sample had been taken. There was no attempt to remove remaining hemolymph prior to the homogenization of the insect. Otherwise, this fraction is collected in the same way as the cytosol fraction.

Protein determination

Soluble fraction or hemolymph samples were diluted with phosphate buffer, 20:1 to 50:1, depending on the age of the flies. Three ml of protein reagent (Bradford, 1976) was mixed with 0.1 ml of diluted hemolymph or soluble solution. The mixture was held for 15 minutes at room temperature and the color was read at OD₅₉₅. The protein content was determined by plotting against a standard curve based on BSA (Yu and Terriere, 1975).

Carboxylesterase assay

1-naphthyl acetate (NA) was used as the substrate for detecting

and measuring activity attributed to the CE-ases. All reagents used for the assay were made up fresh ten minutes before use to avoid photochemical formation of naphthol during the process. The assays were conducted in the dark to prevent a similar chemical reaction. The method was from Van Asperen (1962) with a modification of the stock solution of 1-naphthyl acetate. Instead of using 5.0 ml of 3×10^{-4} M substrate solution, 1.0 ml of 5×10^{-4} M was used. For the profile study of CE-ase activity in adult flies the hemolymph sample was pooled as described above. It was diluted to a specific concentration of 100:1 to 200:1 for flesh flies; 100:1 to 300:1 for the two strains of house flies; and 100:1 to 100:3 for blow flies, depending on the enzyme activity of the individual sex, species, age, and stage of the flies.

The diluted hemolymph sample which contained 0.5 to 1.5 μ l of hemolymph was first mixed with eserine and para-hydroxy-mercuribenzoate (PHMB) solutions and made up to final volume of 1.0 ml with 0.1 M phosphate buffer, pH 7.0. The contents were mixed by gently stirring for a couple of seconds and preincubated for 30 minutes at room temperature. Then, one ml of NA 5×10^{-4} M solution in phosphate buffer was added and stirred and gently shaken at room temperature in the dark to prevent naphthol formation photochemically (Van Asperen, 1962). After another 30 minutes one ml of DBLS solution (Diazoblue and laryl sulfate mixture) was added to the tube. After 15 minutes for color development the optical density of final mixture of the solution of

the solution of three ml was measured in a DB spectrophotometer (Beckman) at 600 nm. The results were compared with the standard curve to estimate the amount of 1-naphthol produced during the incubation.

Use of Inhibitors

Inhibitors were used throughout most of the study to block the action of non-specific esterases such as choline esterase and aryl esterases or to measure these enzymes in the presence of the CE-ases. The inhibitors were: eserine, to inhibit the choline esterases; PHMB (parahydroxymercuribenzoate), to inhibit the aryl esterases; and para-oxon, to inhibit the CE-ase. No inhibitors were used when it was desired to measure the general esterases, as in Part 1 of "Results".

The proper concentration of inhibitors for maximum inhibition of enzyme activity was determined to be 10^{-4} M (see Part 3, Results). When the inhibitors were used they were incubated with the enzyme sample (hemolymph, soluble fraction, or electrophoresis gels) for 30 minutes prior to the addition of NA.

Hydroprene and juvenile hormone-1 esterase assays

Several preliminary experiments were carried out to establish the conditions for these assays.

Hemolymph samples were first tested for their highest enzyme activity during the period of the life span investigated. This age of the fly was then used as a standard sample source of the rest of the

experiments. It was found that in Rutgers house flies CE-ases had the highest activity around day 3 and day 8; CSMA, day 4 and day 8; blow flies, day 6 and day 10; flesh flies, day 9 and day 15. The time for maximum enzyme activity also depended on how the flies used for the experiment were synchronized. Since male flies had limited volume of hemolymph, the experimental enzyme had to be obtained from the female flies.

Hemolymph samples were diluted with 0.1M phosphate buffer, pH 7.5, to concentrations of 3:1, 5:1, 8:1, and 10:1. The diluted samples were then incubated with hydroprene-¹⁴C or JH-1-³H for esterase assay. [The concentration of the two labeled compounds was 0.11 n mol (about 14,000 cpm) per ml for hydroprene and 3.18 p mol (about 95,500 cpm) per ml for JH-1.] The unlabeled chemicals were used for the determination of proper concentrations of the CE-ases. Three concentrations of hot hydroprene were tested, 0.11 n mol, and 0.44 n mol per test tube. Bovine serum albumen (BSA) was also added to the incubation tube for enzyme activity enhancement Yu and Terriere (1975). BSA was tested at 0.5 mg., 1.0 mg., and 2.0 mg., per test tube. A special sequence of adding the incubating materials was necessary for an accurate assay. One ul hot hydroprene was applied to the 10.0 ml incubating tube, and dissolved with 50.0 ul of hexane for dispersion. The solvent was evaporated under a nitrogen jet at room temperature and then cold hydroprene was added and dried. Then glass beads, BSA and hemolymph sample solution was added and incubated in a shaking

water bath on $34 \pm 1^\circ\text{C}$. Incubation times tested were 30 minutes, one, two, and three hours for hydroprene and 10, 20, 30, 45, and 60 minutes for JH-1. At the completion of the incubation 2.0 ml of distilled water and one and one-half ml of a 2:1 mixture of ethyl ether-ethanol were added to the mixture. After shaking and centrifuging, the solvent phase was removed. The extraction was repeated twice with ether-ethanol mixture. This procedure recovered all of the added radioactivity.

The extracts were combined and dried over sodium sulfate. Aliquots were then evaporated to approximately 0.25 ml and applied on silica gel G thin layer chromatoplates (0.25 mm thick 5 cm x cm). The chromatoplates were developed unidimensionally with hexane-ethyl acetate (100:15), then with benzene-ethyl acetate-acetic acid (100:30:3) (Quistad, *et al.*, 1975). The radioactive metabolites and unreacted substrates were detected by passing the plates under a Banguard automatic chromatogram scanner, Model 885. The zones of radioactivity were scraped from the plates, placed in scintillation vials with 10.0 ml of scintillation mixture and counted. Recovery of added radioactivity carried through the complete procedure was 75 percent.

The preliminary study of the assay revealed that a concentration of hemolymph of 20 μl for flesh fly and 20 to 25 μl for blow fly and house fly in a 10:1 dilution was suitable for JH-1 and hydroprene esterase assays. This volume of hemolymph represented approximately one volume of hemolymph of flesh fly, or total hemolymph volume of two

blow flies, or five house flies. The concentration of the substrate and the radioactive to non-radioactive ratio was 3.18 p mol (94,500 dpm) over 16.980 n mol for JH-1 and 110 p mol (14,000 dpm) over 10.07 m mol for hydroptene. The addition of 2.0 mg of BSA was effective in enhancing the enzyme activity if the incubation volume was around 0.2 to 0.5 ml. All incubations were carried out at $34 \pm 1^\circ\text{C}$. (Yu and Terriere, 1973, 1974).

For the assay of the soluble fraction, a concentration of 0.25 of a flesh fly, 0.50 of a blow fly, and 1.0 of a house fly equivalent was preferable. The other incubation conditions for assay were as above.

Enzyme induction

Groups of 25 flesh flies, 50 blow flies or house flies were anesthetized with CO_2 and topically treated with hydroptene in 1.0 ul acetone. The micro drops were applied to the tip of the abdomen. Controls were treated with 1.0 ul acetone. The flies were then released into the cages with food and water provided. Doses of hydroptene were 10.0, 5.0, 1.0, and 0.1 ug per fly. The age of the flies were day 1 through day 6. A group of 50 flies was used for hemolymph samples and for soluble fractions every twelve hours. Soluble fractions of headless flies were obtained according to Yu and Terriere (1971). Protein content of both fractions was determined.

When actinomycin D was used to characterize the induction, the flies were anesthetized with CO_2 and injected into the thorax through

the scutellar suture with 1 ul of an aqueous solution containing 0.1 ug of the compound. Controls were injected with 1 ul water. Neither treatment caused mortality in flies.

Electrophoretic procedures

The disc electrophoretic procedure of Davis (1964) was used. The electrophoresis was carried on for four hours at 4°C. All the procedures were at low temperature except the staining procedure. This was modified from Ahmad (1974). Inhibitors were also used before the staining procedure, using the same concentrations as in the CE-ase studies. The volume of hemolymph of these flies that applied to each electrophoresis tube is 5 ul of flesh fly, 10 to 15 ul of blow fly and house fly. In case of soluble fraction, flesh fly, one-fourth to half fly equivalent; blow fly, half to one fly equivalent; house fly, one to one and one-half fly equivalent were applied to each tube on electrophoresis. Esterase bands were visualized by treating for 30 minutes with 0.1 percent (w/v) solution of NA in 10 percent aqueous acetone and staining with 0.1 percent (w/v) diazo-dye, fast blue RR in 0.1 M phosphate buffer, pH 7. Upon development of the brown colored esterase bands (30 minutes) the gel was washed in three changes of distilled water and stored in seven percent acetic acid. The zymogram of the CE-ases was examined two days and two weeks after the two changes of acetic aqueous solution. When necessary of quantitation, the gel was scanned in a photovolt Model densitometer. The

isozyme bands in the gel were visible to the eye.

In the combination studies of cytosol and hemolymph, 5.0 ul of hemolymph and one fly equivalent of cytosol solution were mixed and applied to the electrophoresis tubes. Results from this combination were compared with those from cytosol and hemolymph alone. The fly at the age with the highest enzyme activity was basically used for the gel-extraction recovery experiment.

Recovery of Carboxylesterase from Acrylamide Gel

Recovery of the enzyme from the gels was also done in the cold. The procedure was modified from Whitmore (1972). The gel was stained for five to ten minutes to locate the bands. The appropriate gel section was removed, chopped, and homogenized at 0°C. with 2.0 ml of distilled water. After 40 minutes for protein diffusion, the homogenate was centrifuged at 1000 gx for 15 minutes and the supernatant was used for protein and enzymatic assay. In most cases, four major sections of the gel were taken for CE-ase characterization. The soluble fraction as well as the hemolymph (one fly equivalent for house flies and blow flies, and half equivalent for the flesh flies) were used for each electrophoretic gel.

Basis of comparisons

Enzyme activity of the hemolymph was based on two parameters, volume and protein content. When it was desired to estimate total

activity on a per fly basis, this was done by multiplying total hemolymph volume, as determined by the isotope dilutions method, by the activity per ul of hemolymph. Cytosol esterase level was based only on cytosol protein content. As seen in Table 7, these protein values were rather constant.

RESULTS

Part 1. Age Dependent Variation in EsterasesHemolymph volume

In preliminary experiments with the flies, it was observed that the volume of hemolymph which could be obtained for enzyme assays varied with the age of the insects. Since it was planned to base the assays on both blood volume and protein content, it was necessary to know the total volume of hemolymph per insect and how this varied with age.

Figure 1 shows that, in male flies, the hemolymph volume had a tendency to fluctuate irregularly. The differences among these three flies may be due to the age, life span, or feeding behavior. The volumes measured here were obtained by a mechanical method (prior to the use of the isotope dilution method).

All measurements of the blood volume of female insects was by the isotope dilution method using inulin-C¹⁴ (see Methods). The fluctuation of hemolymph volume of female flesh flies during the first two pregnancy cycles is shown in Figure 2a. Each point on the graph is the mean of 10 single insect blood volume measurements. The bar represents standard deviation. Also shown are the dates of egg (or larval) deposition (circle). The volume was high the first day after emergence and dropped sharply at day 3. It increased gradually from the beginning of day 6 and reached its maximum at day 9. Measurements of

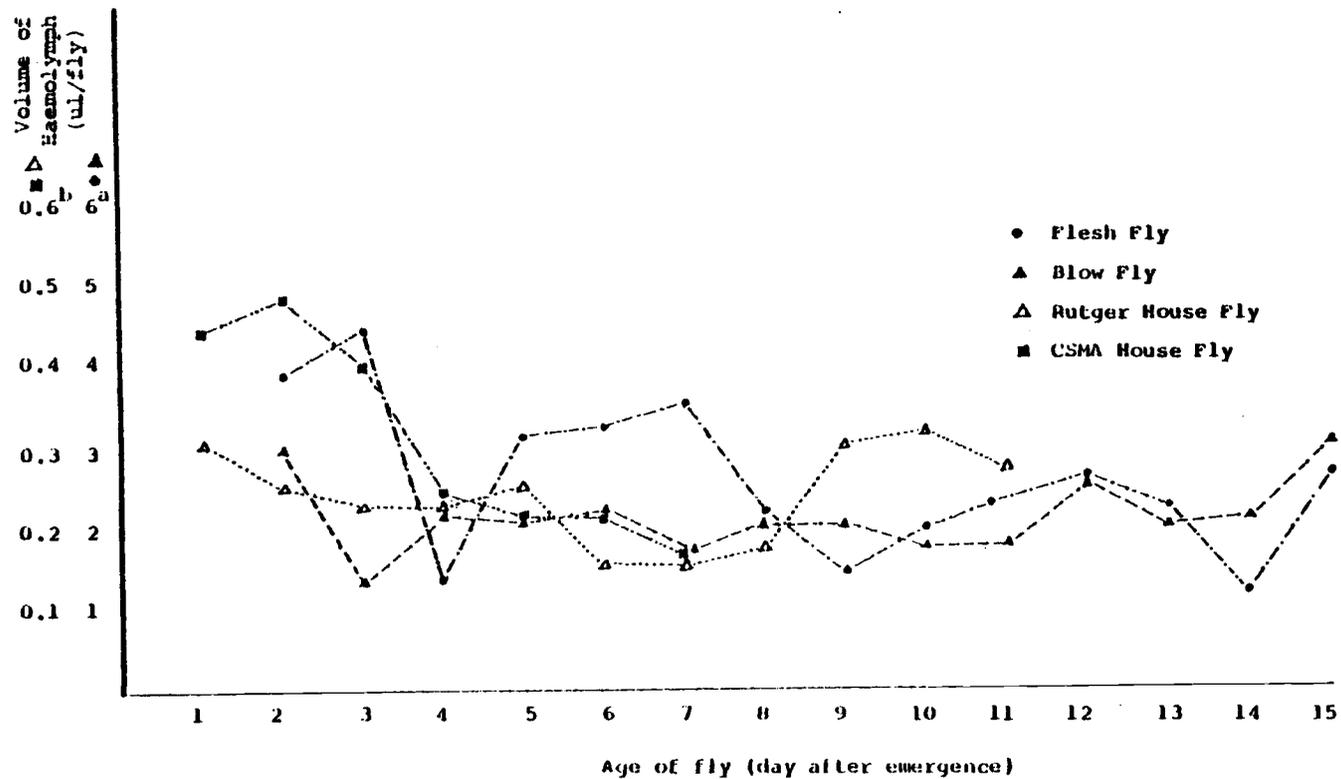


Figure 1
 Fluctuation of Haemolymph Volume of Male Flies Estimated Based on the
 Mechanical Method

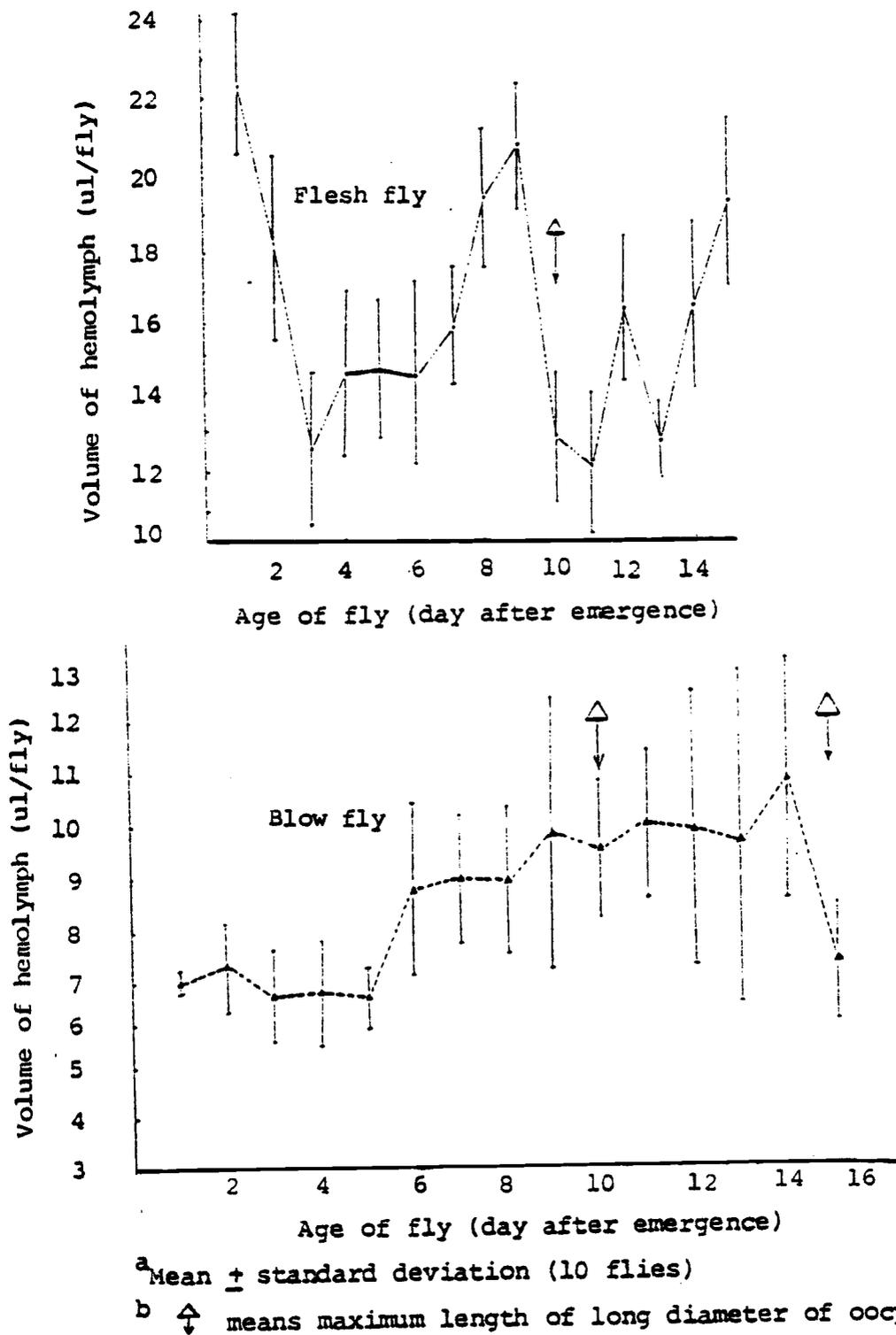


Figure 2a

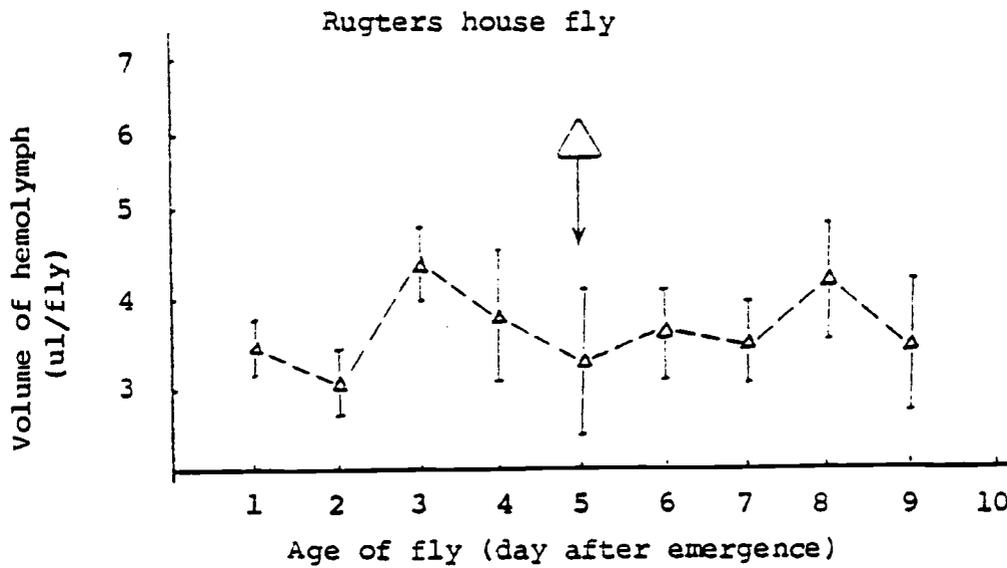
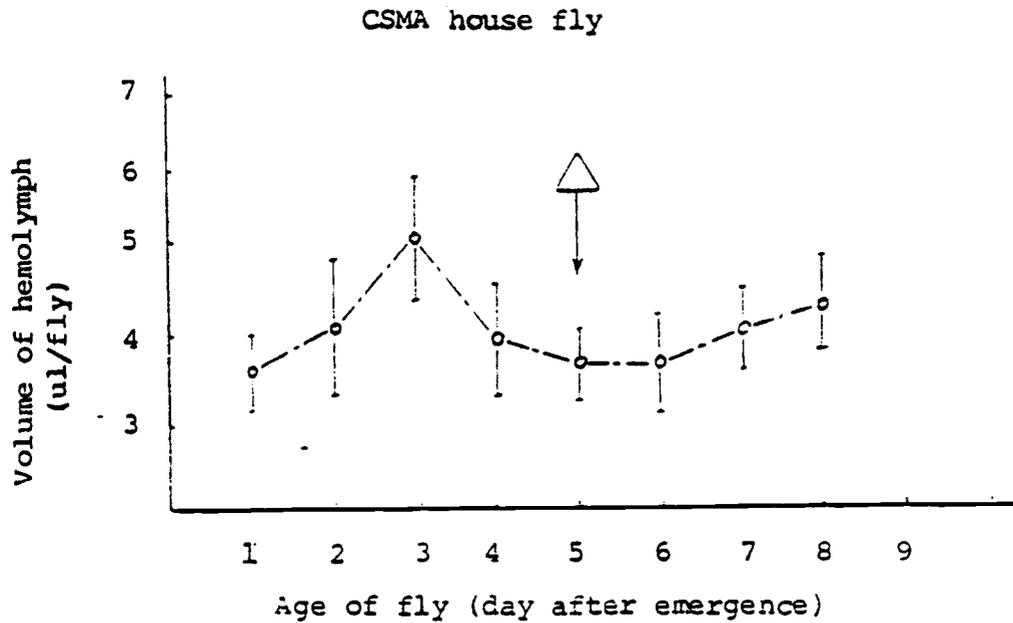
Fluctuation of hemolymph volume of female flesh fly and blow fly estimated based on the isotop dilution method a,b.

oocyte long diameters during this period showed that maximum length occurred at day 10, indicating that vitellogenesis was complete (triangle). The blood volume dropped at this point. The first batch of larvae was deposited by the female flies around day 12. At this time the hemolymph volume was at a low level and increased afterward. The second peak of volume was reached around day 15 and dropped again afterwards. Larger quantities of hemolymph can be obtained from flies at day 9. The hemolymph volume remained at a level no less than 12 ul.

In the female blow fly, the hemolymph volume increased gradually (Figure 2a). There was a plateau from day 9 to day 13 and a peak at day 14. The longest diameter of the oocyte occurred around day 9 to day 11, and day 14 and day 15. The maximum hemolymph volume was collected at day 9 and day 15. The volume was low around days 4 to 6.

In the female house fly, the hemolymph volume peaked at day 3 or 4 in each synchronized colony. However, the effectiveness of synchronization seemed to decrease as time elapsed. Figure 2b shows that the volume reached a plateau before the second peak appeared. This phenomenon was observed in both strains of house fly.

The cyclic fluctuation of the hemolymph volume in the three species indicated that to deal with the activity of CE-ases in hemolymph, volume of the hemolymph as well as the protein content should be considered especially in the case of flesh flies.



a Mean \pm standard deviation (10 flies)

b \uparrow means maximum length of long diameter of oocytes

Figure 2b

Fluctuation of hemolymph volume of female house flies estimated based on the isotop dilution method^{a, b}.

General Esterase Activity

The results of the age profile studies of general esterase activity using NA as substrate indicated a variation of enzyme activity corresponding to the fluctuation in hemolymph volume of the female flesh fly and blow fly.

Male flies: In flesh fly the enzyme activity fluctuated considerably with peaks at days 2, 7-10, and 13. Enzyme activity, based on hemolymph protein (Table 1a) also followed this pattern.

Except for a peak in general esterase activity at day 4, the enzyme remained fairly constant throughout the 15-day study of the male blow flies, Table 1b. This was true whether the activity was based on blood volume or blood protein. The activities were about the same as those found in the male flesh flies when expressed on the basis of hemolymph volume but twice that of the flesh fly when expressed on the basis of protein. This is explained by the generally lower level of protein in the blow fly hemolymph.

The Rutgers house fly general esterase activity (Table 1c) fluctuated around the general 13-day average of 8.38 n mol naphthol/ul hemolymph/30 min with what appeared to be a peak at 9 days. Compared to the other two species, this strain of house flies has only 1/7th as much general esterase activity.

Female flies: The general esterase activity of the hemolymph of female flies of various ages is tabulated in Tables 2a-d. The most

Table 1a. Profile of general esterase activity in pooled hemolymph of male flesh flies.

Age, days	General Esterase Activity		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
1	34.6 ± 1.78	1.48 ± 0.07	23.3
2	92.2 ± 0.00	4.88 ± 0.00	18.9
3	42.5 ± 1.55	2.18 ± 0.08	19.5
4	37.15 ± 1.48	0.86 ± 0.01	39.1
5	33.5 ± 0.49	2.08 ± 0.03	16.1
6	38.7 ± 0.07	1.85 ± 0.00	20.9
7	77.2 ± 2.26	2.60 ± 0.08	29.7
8	73.8 ± 1.55	3.75 ± 0.08	19.7
9	66.5 ± 0.49	4.04 ± 0.03	16.5
10	126.1 ± 3.18	5.36 ± 0.13	23.5
11	21.5 ± 0.06	1.24 ± 0.00	17.3
12	22.7 ± 0.42	1.58 ± 0.03	14.4
13	109.4 ± 0.21	4.86 ± 0.01	22.5
14	14.4 ± 0.21	1.38 ± 0.02	10.4
Average	56.45	2.72	20.8

NA as substrate.

Duplicates: mean ± S.D

Table 1b. Age dependent variation in general esterase activity in pooled hemolymph of male blow flies.

Age, days	General Esterase Activity		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
2	49.0 ± 6.64	2.41 ± 0.32	20.3
3	57.13 ± 4.56	4.23 ± 0.33	13.5
4	101.50 ± 10.07	5.05 ± 0.50	20.1
5	54.2 ± 5.94	3.56 ± 0.39	15.2
6	39.25 ± 0.35	3.24 ± 0.03	12.1
7	56.2 ± 0.00	5.73 ± 0.00	9.8
8	47.65 ± 9.40	4.04 ± 0.79	11.8
9	44.75 ± 1.76	5.20 ± 0.20	8.6
10	46.00 ± 2.83	6.05 ± 0.37	7.6
11	47.85 ± 3.32	5.20 ± 0.36	9.2
12	54.35 ± 17.78	6.79 ± 2.22	8.0
13	44.25 ± 1.06	5.98 ± 0.14	7.4
14	46.45 ± 1.34	5.66 ± 0.16	8.2
15	37.05 ± 4.17	6.28 ± 0.71	5.9
Average	50.8	4.9	11.3

NA as substrate.

Duplicates: mean ± S.D.

Table 1c. Age dependent variation in general esterase activity in pooled hemolymph of male Rutgers house flies.

Age, days	General Esterase Activity		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
2	8.0 ± 1.32	0.241 ± 0.040	33.1
3	6.93 ± 0.1	0.232 ± 0.003	29.8
4	7.31 ± 0.83	0.333 ± 0.038	21.9
5	8.88 ± 0.32	0.255 ± 0.009	34.7
6	10.48 ± 0.12	0.209 ± 0.002	50.0
7	6.67 ± 0.11	0.213 ± 0.003	31.2
8	7.87 ± 0.03	0.223 ± 0.001	35.2
9	13.13 ± 0.38	0.299 ± 0.008	43.8
10	7.51 ± 0.27	0.209 ± 0.007	35.8
11	8.09 ± 0.27	0.195 ± 0.006	41.3
12	6.07 ± 0.14	0.253 ± 0.005	26.4
13	9.60 ± 0.56	0.335 ± 0.019	28.6
Average	8.38	0.250	34.3

NA as substrate.

Duplicates: mean ± S.D.

obvious fluctuation in activity in the case of the flesh fly (Table 2a) occurred in the day 8-12 period with a sharp decline at day 13. Another increase might have started by day 15 when the experiment was terminated.

A somewhat similar pattern was seen in the female blow flies, Table 2b, where a three-fold increase in esterase activity per unit volume of blood occurred in the 8-11 day period. This was not confirmed when the assays were based on protein content, however, the increase in this case occurring around day 7.

The results of the assay of Rutgers' house fly hemolymph are tabulated in Table 2c. Only the increase in enzyme activity at day 8 is confirmed by both the blood volume and blood protein calculations and there is a second peak at day 5 judging from the naphthol produced per ul of hemolymph. Aside from these minor fluctuations, the general esterase activity of this strain seemed to be rather constant, at least through the eighth adult day. This result was confirmed in the case of the CSMA house flies, Table 2d. It was noted, however, that the general esterase activity of this strain was considerably higher--approximately 300 percent--than that of the Rutgers strain.

Comparing the three species with respect to general esterase activity in hemolymph, it can be seen that flesh flies and blow flies are approximately equal in activity and about twice (CSMA strain) and ten times (Rutgers strain) that of the house flies.

Table 2a. Age dependent variation in general esterase activity in pooled hemolymph of female flesh flies.

Age, days	General Esterase Activity		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
2	46.85 ± 2.74	1.71 ± 0.10	37.4
3	66.2 ± 16.4	1.58 ± 0.39	41.9
4	37.99 ± 19.52	1.07 ± 0.55	35.5
5	48.89 ± 8.15	1.74 ± 0.29	28.1
6	74.88 ± 6.56	1.94 ± 0.17	38.6
7	47.06 ± 3.96	2.02 ± 0.17	23.3
8	93.39 ± 7.95	5.07 ± 0.43	29.5
9	116.83 ± 11.95	5.03 ± 0.51	18.4
10	108.21 ± 0.23	6.15 ± 0.01	23.2
11	90.55 ± 11.97	5.14 ± 0.68	17.6
12	85.30 ± 5.14	3.98 ± 0.24	17.6
13	45.77 ± 9.77	1.07 ± 0.23	17.6
14	50.54 ± 0.97	1.56 ± 0.03	21.4
15	87.21 ± 1.47	2.37 ± 0.04	36.8
Average	71.4	2.9	26.9

NA as substrate.

Duplicates: mean ± S.D.

Table 2b. Age dependent variation in general esterase activity in pooled hemolymph of female blow flies.

Age, days	General Esterase Activity		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
2	78.71 ± 8.69	4.25 ± 0.47	18.5
3	43.94 ± 4.03	3.38 ± 0.31	13.0
4	72.32 ± 0.44	4.92 ± 0.03	14.7
5	---	---	---
6	55.73 ± 8.11	4.19 ± 0.61	13.3
7	48.30 ± 5.70	7.21 ± 0.85	6.7
8	68.39 ± 1.83	4.12 ± 0.11	16.6
9	101.60 ± 8.00	2.45 ± 0.20	40.0
10	104.96 ± 10.87	3.67 ± 0.38	28.6
11	135.72 ± 18.30	2.60 ± 0.35	52.2
12	55.62 ± 4.59	4.12 ± 0.34	13.5
13	70.60 ± 2.70	5.23 ± 0.20	13.5
14	83.08 ± 3.93	3.64 ± 0.17	23.1
15	129.94 ± 10.29	4.42 ± 0.35	29.4
Average	80.8	4.2	21.8

NA as substrate.

Duplicates: mean ± S.D.

Table 2c. Age dependent variation in general esterase activity in pooled hemolymph of female Rutgers house fly.

Age, days	General Esterase Activity		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
1	8.49 ± 0.51	0.256 ± 0.015	33.1
2	7.00 ± 0.44	0.223 ± 0.015	29.8
3	7.66 ± 0.30	0.349 ± 0.013	21.9
4	8.73 ± 0.52	0.251 ± 0.014	34.7
5	10.00 ± 0.57	0.200 ± 0.013	50.0
6	6.26 ± 0.35	0.200 ± 0.011	31.2
7	8.28 ± 0.58	0.235 ± 0.017	35.2
8	13.58 ± 1.77	0.301 ± 0.005	43.8
Average	8.75	0.252	35.0

NA as substrate.

Duplicates: mean ± S.D.

Table 2d. Age dependent variation in general esterase activity in pooled hemolymph of female CSMA house fly.

Age, days	General Esterase Activity		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
1	30.32 ± 8.99	1.18 ± 0.35	25.7
2	27.01 ± 7.91	0.99 ± 0.29	27.3
3	32.00 ± 9.47	1.25 ± 0.37	25.6
4	30.19 ± 8.36	1.48 ± 0.41	20.4
5	33.12 ± 9.80	1.96 ± 0.58	16.9
6	36.54 ± 10.8	2.03 ± 0.60	18.0
7	23.58 ± 6.49	2.29 ± 0.67	10.3
8	42.74 ± 9.65	1.37 ± 0.31	31.2
9	39.32 ± 2.05	1.53 ± 0.08	25.7
Average	32.8	1.6	22.3

NA as substrate.

Duplicates: mean ± S.D.

Part 2. Characterization of Esterases

Inhibition Studies

In the experiments just described, the fractions contained aryl esterases and choline esterases, both of which will hydrolyze NA. As another approach to the study of these enzymes, it was desired to determine the contribution of these additional esterases in the hemolymph and soluble fraction of the three species. This was done by the use of the inhibitor, paraoxon, which prevents the action of the B esterases. It was also desired to learn more about the fluctuations of the CE-ase aside from the choline and aryl esterases during the life cycles of the insects. This was achieved by the use of the inhibitors eserine and PHMB.

Concentrations of eserine, paraoxon, and PHMB ranging from 10^{-4} to 10^{-7} M, final concentrations, were compared in the inhibition tests. The enzymes were preincubated with inhibitors for 30 minutes prior to the addition of NA to the incubation mixture.

Hemolymph Esterases: Table 3a shows that 10^{-4} M eserine had no effect on the esterases of adult flies, and inhibited only 10 percent of the larval esterases, indicating that there was comparatively little acetylcholinesterase in the hemolymph of the flesh fly. Paraoxon, on the other hand, strongly inhibited both larval and adult esterases. Comparatively, the larval esterases were more susceptible to paraoxon. PHMB was less effective in inhibiting the hemolymph

esterases of the flesh fly. Only 8.0 percent of adult and 11.4 percent of larval esterase activity was inhibited by 10^{-5} M PHMB. These observations indicate that the CE-ases of flesh fly hemolymph are mostly of the B type, sensitive to OP compounds.

The blow fly esterases were slightly more susceptible to eserine than those of the flesh fly. Paraoxon at 10^{-4} M was effective in inhibiting the enzyme activity of both adults and larvae. It is evident (Table 3a) that the hemolymph esterases of blow fly larvae are less susceptible to paraoxon than those of the flesh fly. The hemolymph esterases of blow fly adults were much more susceptible to PHMB than those of flesh flies, but this inhibitor had little effect on larval esterases. These results indicate that adult blow fly hemolymph contains more arylesterase activity than that of flesh flies.

The hemolymph esterases of adult Rutgers house flies was comparatively susceptible to eserine but this inhibitor had no effect on the larval enzymes. In adults, only 16 percent of the esterases were inhibited by 10^{-6} M paraoxon, but in larvae, 77 percent was inhibited. A similar situation was found in tests of PHMB. These results indicate that the enzymes of adult Rutgers house flies are comparatively more resistant to paraoxon than those of the other species.

Soluble Fraction Esterases: Table 3b shows that esterases in the soluble fraction of the flesh fly were resistant to eserine, but were susceptible to paraoxon and PHMB. In this fraction, 59 percent

Table 3a. Selective inhibition of general esterases in hemolymph from two stages of female flesh flies, blow flies, and Rutgers house flies

Inhibitor conc. (Mole)	Percent inhibition ^{a,b}						
	Flesh fly		Blow fly		Rutgers house fly		
	Adult	Larva	Adult	Larva	Adult	Larva	
Eserine	10 ⁻⁴	ND ^c	10.0	33.3	5.9	50.0	ND
	10 ⁻⁵	ND	ND	3.41	ND	36.4	ND
	10 ⁻⁶	ND	ND	ND	ND	ND	ND
	10 ⁻⁷	ND	ND	ND	ND	ND	ND
Paraoxon	10 ⁻⁴	76.9	--	69.5	76.9	38.2	87.4
	10 ⁻⁵	26.7	--	44.5	27.8	25.4	83.2
	10 ⁻⁶	17.8	93.6	12.5	22.9	16.4	77.6
	10 ⁻⁷	ND	39.6	6.5	ND	4.6	42.1
PHMB	10 ⁻⁴	22.8	--	82.4	--	5.8	68.6
	10 ⁻⁵	7.9	11.4	76.8	13.6	3.6	50.9
	10 ⁻⁶	5.5	6.2	32.8	8.6	5.0	39.5
	10 ⁻⁷	ND	ND	7.1	5.8	ND	ND

^a NA as substrate

^b average of duplicates

^c not detectable

Table 3b. Selective inhibition of general esterase in soluble fraction from three stages of female flesh flies.

Inhibitor conc. (Mole)	Percent inhibition ^{a,b}			
	Adult	Pupa	Larva	
Eserine	10 ⁻⁴	12.6	9.0	ND ^c
	10 ⁻⁵	5.3	7.3	ND
	10 ⁻⁶	ND	ND	ND
	10 ⁻⁷	ND	ND	ND
Paraoxon	10 ⁻⁴	72.9	62.1	69.9
	10 ⁻⁵	66.0	27.6	47.5
	10 ⁻⁶	59.4	23.2	34.4
	10 ⁻⁷	7.7	12.9	24.3
PHMB	10 ⁻⁴	58.5	52.7	72.5
	10 ⁻⁵	16.9	45.2	18.1
	10 ⁻⁶	7.2	ND	ND
	10 ⁻⁷	4.6	ND	ND

^aNA as substrate

^baverage of duplicates

^cnot detectable

of adult, 23 percent of pupal, and 34 percent of larval esterase activity was blocked by 10^{-6} M paraoxon. The enzymes were somewhat less affected by PHMB but were still more sensitive to this inhibitor than those of the hemolymph, Table 3a.

The soluble of esterases of the blow flies are also resistant to eserine. Table 3c shows that only 16 percent of adult esterases were inhibited by 10^{-4} M eserine while the larval and pupal esterases were not affected. On the other hand, these esterases are sensitive to paraoxon and PHMB. There were 57 percent of adult, 19 percent of pupal, and 8 percent of larval esterases blocked by 10^{-6} M paraoxon. PHMB was more inhibitory than paraoxon and compared to the case with flesh flies, about 10 times more potent. These results suggest that more arylesterases are present in the soluble fraction of the blow fly than that of the flesh fly.

Table 3d shows that esterases of the cytosol of Rutgers house flies were only slightly more sensitive to eserine than those of the flesh fly and blow fly. Like the other species, the soluble esterase of Rutgers flies were susceptible to 10^{-6} M paraoxon; inhibition was 60 percent, 26 percent, and 34 percent for adult, pupal, and larval esterases, respectively. The esterases were about as resistant to PHMB as those of blow flies, but more susceptible than those of the flesh fly. This is evident from the fact that 10^{-6} M PHMB had virtually no effect on the flesh fly esterase, and inhibited 37-70 percent of the blow fly esterase and 0-18 percent of the house fly esterase.

Table 3c. Selective inhibition of general esterase in soluble fraction from three stages of blow flies.

Inhibitor conc. (Mole)	Percent inhibition ^{a,b}			
	Adult	Pupa	Larva	
Eserine	10 ⁻⁴	16.0 ^{b,c}	ND	ND ^c
	10 ⁻⁵	ND ^c	ND	ND
	10 ⁻⁶	ND	ND	ND
	10 ⁻⁷	ND	ND	ND
Paraoxon	10 ⁻⁴	85.4	61.7	46.0
	10 ⁻⁵	72.4	35.3	18.2
	10 ⁻⁶	57.4	19.4	8.4
	10 ⁻⁷	24.8	20.0	ND
PHMB	10 ⁻⁴	82.1	68.0	72.9
	10 ⁻⁵	76.7	66.0	38.0
	10 ⁻⁶	70.3	38.0	37.1
	10 ⁻⁷	26.3	ND	28.0

^aNA as substrate

^baverage of duplicates

^cnot detectable

Table 3d. Selective inhibition of general esterase in soluble fraction from three stages of Rutgers house flies

Inhibitor conc. (Mole)	Percent inhibition ^{a,b}			
	Adult	Pupa	Larva	
Eserine	10 ⁻⁴	28.0	36.0	5.0
	10 ⁻⁵	25.5	ND ^c	1.0
	10 ⁻⁶	--	ND	ND
	10 ⁻⁷	13.1	ND	ND
Paraoxon	10 ⁻⁴	91.3	--	74.2
	10 ⁻⁵	80.7	39.5	62.1
	10 ⁻⁶	60.7	26.2	34.2
	10 ⁻⁷	23.5	14.4	19.4
PHMB	10 ⁻⁴	91.9	--	78.1
	10 ⁻⁵	61.9	20.0	44.3
	10 ⁻⁶	9.0	14.8	18.3
	10 ⁻⁷	ND	ND	6.8

^aNA as substrate^baverage of duplicates^cnot detectable

Species Differences: The major similarities and differences in esterase characteristics revealed by this study are: eserine has little effect on either hemolymph or soluble enzymes, indicating that the choline esterase content is low in all three species; all fractions are strongly inhibited by 10^{-4} M paraoxon, confirming that the esterases are of the B type; PHMB is a stronger inhibitor of blow fly and house fly enzymes than of the flesh fly enzyme, indicating that the former species contains a higher proportion of arylesterases than the latter.

Age-Dependent Variation in Hemolymph Esterase Activity in the Presence of Inhibitors

It was of interest to measure esterase activity in the presence of the choline esterase and aryl esterase inhibitors to obtain a more refined picture of CE-ase levels at various ages of flies. For this purpose it was assumed that inhibitor concentrations of 10^{-4} M would eliminate or greatly reduce the activity of these enzymes in the hydrolysis of the general substrate, NA.

Table 4a shows that the average enzyme activity of the flesh fly was 69.6 n mole 1-naphthol produced per ul of hemolymph per 30 minutes. The highest enzyme activity was seen at day 9, 136.0 n mol, about three times as high as the activity of esterases of days 2-6. This agrees well with the maximum, 8-10 days, obtained in the corresponding study of general esterase activity, Table 2a.

Table 4b shows that in blow fly hemolymph, the age profile does

Table 4a. Age dependent variation in carboxylesterase activity, characterized by two inhibitors, in pooled hemolymph of female flesh flies.

Age, days	Carboxylesterase Activity ^{a,b}		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
1	58.4 ± 15.3	1.90 ± 0.50	30.7
2	43.9 ± 4.7	1.60 ± 0.17	27.4
3	47.7 ± 4.8	1.14 ± 0.11	41.9
4	52.3 ± 8.7	1.47 ± 0.24	35.5
5	53.2 ± 18.7	1.89 ± 0.66	28.1
6	50.1 ± 14.3	1.29 ± 0.37	38.6
7	46.4 ± 17.2	2.32 ± 0.86	20.0
8	101.6 ± 3.69	6.35 ± 0.23	16.0
9	136.89 ± 16.42	7.44 ± 0.89	18.4
10	87.23 ± 0.75	3.76 ± 0.03	23.2
11	96.9 ± 0.00	5.50 ± 0.00	17.6
12	85.75 ± 4.35	5.87 ± 0.25	17.6
13	44.73 ± 0.45	2.54 ± 0.02	17.6
Average	69.6	3.2	25.6

^aNA as substrate. Enzyme incubated with esterine (10^{-4} M) and PHMB (10^{-4} M).

^bDuplicates: mean ± S.D.

Table 4b. Age dependent variation in carboxylesteras activity, characterized by two inhibitors, in pooled hemolymph of female blow fly.

Age, days	Carboxylesterase Activity ^{a,b}		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
1	38.1 ± 4.9	1.9 ± 0.2	20.0
2	30.2 ± 0.0	2.3 ± 0.0	13.0
3	32.2 ± 2.5	1.4 ± 0.1	22.9
4	29.3 ± 1.8	1.5 ± 0.1	19.0
5	27.33 ± 0.9	1.4 ± 0.0	18.8
6	24.11 ± 4.5	1.2 ± 0.2	20.0
7	24.2 ± 2.9	1.1 ± 0.3	21.7
8	21.9 ± 4.2	1.8 ± 0.3	12.4
9	26.1 ± 0.7	0.9 ± 0.0	28.6
10	28.7 ± 9.6	1.1 ± 0.3	26.2
11	16.4 ± 2.5	0.5 ± 0.0	34.3
12	20.3 ± 3.2	0.7 ± 0.1	27.6
13	18.0 ± 2.8	0.6 ± 0.1	27.6
Average	25.9	1.3	22.5

^aNA₄ as substrate. Enzyme incubated with eserine (10⁻⁴M) and PHMB (10⁻⁴M).

^bDuplicates: mean ± S.D.

not fit that shown in Table 2b for general esterases, probably because of the arylesterase content of blow fly hemolymph, Table 3a. The maximum variation throughout the 13-day period was about two-fold, comparing days 1-3 with day 11.

Table 4c shows that in the presence of eserine and PHMB the CE-ase activity of Rutgers fly hemolymph was low, averaging 5.5 n mol of naphthol produced per ul of hemolymph per 30 minutes. Based on protein, the specific activity of CE-ases was highest at day 3. The average specific activity was .161 u mol which is only 1/10th that of CSMA house fly activity.

Table 4d shows that the hemolymph esterase activity of the CSMA house fly was rather constant with age compared to the other species. The average activity was higher than that of the blow fly: 31.2 n mol of naphthol produced per ul of hemolymph per 30 minutes, compared to 25.9 n mol for the blow fly.

Tables 2 a-d and 4 a-d compare the fluctuations of general esterase activity with and without inhibitors. The major findings in the two studies are as follows: the inhibitors had little effect on the average esterase activity of the flesh fly, 71.4 n mol 1-naphthol without inhibitors (Table 2a) and 69.6 n mol with inhibitors (Table 4a). In blow flies the two treatments resulted in average activities of 80.8 and 25.9, a 68 percent decrease. With CSMA house flies, they were 32.8 and 31.2, essentially unchanged and with Rutgers house flies,

Table 4c. Age dependent variation in carboxylesterase activity, characterized by two inhibitors, in pooled hemolymph of female Rutgers house flies.

Age, days	Carboxylesterase Activity ^{a,b}		ug protein/ ul hemolymph
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	
1	5.47 ± 0.1	0.165 ± 0.003	33.1
2	4.47 ± 0.19	0.150 ± 0.006	29.8
3	4.87 ± 0.09	0.223 ± 0.004	21.9
4	5.25 ± 0.21	0.151 ± 0.006	34.7
5	6.66 ± 0.35	0.133 ± 0.007	50.0
6	4.02 ± 0.09	0.130 ± 0.002	31.2
7	5.08 ± 0.24	0.141 ± 0.008	35.2
8	8.41 ± 0.28	0.192 ± 0.006	43.8
Average	5.5	0.161	34.9

^aNA₄ as substrate. Enzymes incubated with eserine (10⁻⁴M) and PHMB (10⁻⁴M).

^bDuplicates: mean ± S.D.

Table 4d. Age dependent variation in carboxylesterase activity, characterized by two inhibitors, in pooled hemolymph of female CSMA house flies.

Age, days	Carboxylesterase Activity ^{a,b}		
	n mols naphthol/ ul hemolymph/ 30 min	u mols naphthol/ mg blood protein/ 30 min	ug protein/ ul hemolymph
1	39.3 ± 11.6	1.22 ± 0.36	32.2
2	33.0 ± 9.8	1.37 ± 0.40	24.1
3	27.3 ± 8.0	1.16 ± 0.34	23.4
4	23.8 ± 7.3	1.50 ± 0.44	16.5
5	33.2 ± 9.6	2.21 ± 0.61	15.0
6	26.5 ± 7.7	2.21 ± 0.61	12.5
7	30.6 ± 0.0	2.5 ± 0.73	12.2
8	34.7 ± 10.2	1.55 ± 0.45	22.3
Average	31.2	1.7	19.8

^aNA as substrate. Enzymes incubated with eserine (10^{-4} M) and PHMB (10^{-4} M).

^bDuplicates: mean ± S.D.

8.8 and 5.5, a 31 percent decrease. These results indicate that aryl-esterases are of importance only in the blow fly and Rutgers house flies.

Estimate of total carboxylesterase activity: In order to estimate the total CE-ase activity in the hemolymph of these flies, two parameters were used: Enzyme activity per μ l of hemolymph, Tables 4a-d, and the total volume of hemolymph per fly, Figures 2a-b. These estimates were based on the averages shown in the two sets of data and are tabulated for the three species in Table 5.

In the case of the female flesh fly, it is evident that esterase activity is high on the day of emergence and that a second peak in activity occurs around days 8-12 with the peak at day 9. At this point the activity is about five-fold that of the lowest values, day 3 and day 13.

The total CE-ase activity of female blow flies is surprisingly constant, there being less than a two-fold variation and no evidence of any significant peak of activity. It is also evident that blow flies have only 1/5th the capacity of flesh flies in respect to these enzymes.

The house fly results are like those for the blow fly with little evidence of any major peak in enzyme activity. One very interesting aspect of the strain comparisons is the much larger enzyme activity, about seven-fold, of the susceptible CSMA strain compared to the insecticide resistant Rutgers strain. On the average, the CSMA strain

Table 5. Estimate of total carboxylesterase activity in the hemolymph of female flies.

Age, days	Total carboxylesterase activity (n mol ⁻¹ of naphthol produced/fly/30 min) ^a			
	SPECIES OF FLY			
	Flesh fly	Blow fly	CSMA house fly	Rutgers house fly
1	1358.9	269.4	145.4	19.2
2	816.5	187.8	136.9	17.9
3	604.3	216.1	139.2	22.3
4	771.9	202.2	100.4	15.8
5	792.1	184.5	124.5	18.6
6	736.9	214.3	98.0	17.6
7	746.5	221.2	127.0	17.5
8	1816.6	200.4	152.7	13.4
9	2921.2	284.7		
10	1140.1	278.1		
11	1187.9	169.6		
12	1428.6	201.2		
13	575.6	201.8		
Average	1145.9	217.8	127.9	17.8

^aData obtained from Figure 2 and Table 4.

contains about half as much CE-ase activity as the blow fly and about 1/9th as much activity as the flesh fly.

Carboxylesterases in the Soluble Fraction

In the profile study of the soluble CE-ases of the three species, the same concentration of inhibitors were used as in the characterization of the hemolymph esterases. The results (Tables 6a, 6b) show that minor peaks of enzyme activity appeared at day 9 for flesh fly, days 5-6 for the blow fly, and day 1 for the house fly. In the case of the flesh fly the peak corresponds to a peak in the hemolymph study (Table 4a).

The "per fly" values shown in Table 6b are probably more accurate than those based on hemolymph esterase, Table 5. This is because the calculations for Table 6b are from the direct assay of a centrifugal fraction (100,000 x g supernatant) of a known weight and number of flies. In the calculations based on hemolymph esterase activity, however, it was necessary to rely on the average hemolymph volume which, according to Figure 2, is subject to considerable variation.

It was of interest to compare the esterase activity levels of hemolymph and soluble fraction on a "whole fly" basis as has been calculated in Tables 5 and 6b. As mentioned in the Methods, the soluble fraction was obtained from headless flies from which the hemolymph samples had been taken and thus includes a small, but unknown quantity of hemolymph. In the case of the flesh fly the average daily level of CE-ase in the hemolymph was equivalent to 1146 n mols

Table 6a. Age dependent variation of carboxylesterase activity, in soluble fraction of female flies of three species.^a

Age, days	n mols 1-naphthol/mg protein/30 min ^b		
	Flesh fly	Blow fly	CSMA house fly
1	401.8 ± 11.1	226.0 ± 6.9	138.4 ± 0.9
2	---	---	---
3	231.6 ± 11.8	262.1 ± 13.4	120.1 ± 0.0
4	294.5 ± 21.7	227.1 ± 16.7	69.9 ± 9.4
5	308.2 ± 24.0	303.0 ± 2.1	64.9 ± 5.2
6	221.4 ± 17.7	304.5 ± 22.5	72.2 ± 0.6
7	224.4 ± 1.6	255.2 ± 7.3	69.8 ± 6.9
8	176.9 ± 2.5	261.0 ± 12.3	62.4 ± 0.0
9	417.4 ± 8.7	251.4 ± 4.6	
10	128.5 ± 2.8	245.0 ± 16.5	
11	207.0 ± 38.3	245.4 ± 8.8	
12	180.2 ± 23.1	221.2 ± 8.8	
13	312.1 ± 9.9	285.3 ± 17.2	
Average	258.6	257.2	85.4

^aNA as substrate, enzyme incubated with Eserine (10^{-4} M) and PHMB (10^{-4} M).

^bMean ± S.D of duplicates.

^cRutgers house fly: average specific activity 77.3 n mols.

Table 6b. Total carboxylesterase activity in soluble fraction of female flies of three species.^a

Age, days	n mols 1-naphthol/fly/30 min ^b		
	Flesh fly	Blow fly	CSMA house fly
1	1211.4 ± 33.5	379.0 ± 11.6	129.4 ± 0.8
2	---	---	---
3	785.6 ± 40.0	459.2 ± 21.1	169.2 ± 0.0
4	747.4 ± 55.1	386.7 ± 28.4	93.1 ± 12.5
5	853.4 ± 66.4	452.1 ± 3.2	93.6 ± 7.5
6	660.6 ± 52.8	552.1 ± 40.8	110.0 ± 0.9
7	659.3 ± 4.7	470.4 ± 13.4	104.8 ± 10.4
8	539.2 ± 7.6	479.2 ± 22.6	
9	2131.7 ± 44.4	405.0 ± 7.4	
10	788.7 ± 17.2	394.2 ± 26.5	
11	1044.5 ± 193.3	441.0 ± 15.8	
12	895.4 ± 114.8	418.5 ± 16.6	
13	1234.0 ± 39.1	707.3 ± 42.6	
Average	962.6	462.1	116.7

^aNA as substrate enzyme incubated with eserine (10^{-4} M) and PHMB (10^{-4} M).

^bMean ± S.D of duplicate.

^cTotal enzyme activity per Rutgers house fly: 70 n mols.

1-naphthol per fly, whereas the amount found in the soluble fraction averaged 963 n mols. This could indicate that both fractions contained about the same relative amount of CE-ases.

In the case of the blow fly there was considerably more CE-ase activity per fly in the soluble fraction (462 n mols) than in the hemolymph fraction (218 n mols) while the opposite was true of the house fly, about twice as much esterase activity in the hemolymph as in the soluble fraction. The blow fly results can be explained as due to a relatively large amount of hemolymph in the soluble fraction but it is more difficult to explain the results with the house fly. There was probably less hemolymph in the soluble fraction of the house fly and this fraction may have been more susceptible to the inhibitor PHMB.

A comparison of the CE-ase activities listed in Tables 6a and 6b shows that the flesh fly and the blow fly have about the same enzyme activity when based on the protein content of the soluble fraction and that these two species contain about three times the enzyme content of the house fly. When the enzyme activity is calculated on a whole insect basis, however, as in Table 6b, the flesh fly soluble fraction contains about twice as much enzyme activity as the blow fly and about eight times that of the CSMA house fly.

The protein content of the soluble fraction of the three species of flies is tabulated in Table 7. Except for a peak in protein level from day 9 to day 12 in the case of the flesh fly, the values are rather constant. The protein level peak in the flesh fly soluble

Table 7. Protein content of soluble fraction of headless flies of three dipteran species.

Age, days	Protein content (mg protein/fly equivalent) ^a		
	Flesh fly	Blow fly	CSMA house fly
1		1.677	0.935
2	3.015		1.045
3	3.292	1.752	1.409
4	2.538	1.703	1.332
5	2.769	1.492	1.442
6	2.984	1.813	1.523
7	2.938	1.841	1.501
8	3.048	1.836	
9	5.107	1.611	
10	6.138	1.609	
11	5.046	1.797	
12	4.969	1.892	
13	3.954	2.479	
Average	3.816	1.792	1.312

^a105,000 g x supernatant as soluble fraction.

fraction corresponds with the CE-ase activity peak seen earlier (Table 6a).

Substrate Specificity

Hydroprene: The result of the CE-ase studies described in the previous paragraphs are incomplete as to the substrates attacked. Hydroprene, a synthetic juvenile hormone, was used to further characterize the CE-ases.

Flesh fly hemolymph contained enzyme activity as high as 117.4 p mol hydroprene cleaved per ul at day 9, and as low as 27.9 p mol the next day in the first fluctuation (Table 8). A second peak occurred at day 16. The table also shows that the peaks of hydroprene esterase activity corresponded with the profile obtained with NA as substrate.

Hydroprene esterases were low in activity in the blow fly, Table 8, with minor increases appearing at days 5-6 and day 10. There was less than a two-fold difference between the highest and the lowest enzyme activity of the hemolymph hydroprene esterases. This low activity indicates that the blow fly has relatively less ability to metabolize hydroprene than its capability to metabolize NA. This agrees with the earlier finding of a high arylesterase content compared to its aliesterase content.

Peaks of hydroprene esterase activity occurred in the hemolymph of day 3 and day 8 Rutgers flies, Table 8. The activity of these peaks was 30.0 p mol at day 3, and 35.4 p mol at day 8, more than seven-fold that of day 4. This sharp change in hydroprene esterase

Table 8. Age dependent variation of hydroperene esterases in hemolymph of female flies of three dipteran species.

Age, days	Hydroperene esterases activity ^a (p mols hydroperene acid produced/ul of hemolymph/3 hr.)			
	Flesh fly ^{b,c}	Blow fly ^b	Rutgers house fly ^{b,d}	CSMA house fly
1	---	6.7 ± 1.5	4.7 ± 0.3	---
2	18.3 ± 0.6	8.8 ± 3.2	27.6 ± 10.7	18.97 ± 0.7
3	19.3 ± 0.3	8.0 ± 1.9	30.0 ± 11.1	35.1 ± 4.3
4	16.5 ± 0.6	8.7 ± 1.8	6.3 ± 2.5	40.8 ± 9.2
5	22.0 ± 0.2	11.1 ± 3.8	7.9 ± 1.0	25.4 ± 3.0
6	23.5 ± 0.4	11.3 ± 3.8	ND ^c	8.3 ± 1.8
7	79.15 ± 29.5	8.1 ± 1.3	18.2 ± 3.3	
8	95.5 ± 1.1	7.2 ± 1.5	35.4 ± 5.9	
9	117.4 ± 3.7	8.0 ± 1.2		
10	27.9 ± 0.3	11.8 ± 4.3		
11	19.3 ± 3.4	8.2 ± 2.3		
12	20.4 ± 4.7	7.6 ± 1.3		
13	22.8 ± 1.5			
14	23.0 ± 1.1			
15	18.4 ± 0.4			
16	124.1 ± 3.2			
17	97.2 ± 11.4			

^aBased on triplicate assays for Rutgers house flies and blow flies and duplicates for flesh flies and CSMA house flies, mean ± S.E.

^bTwo different colonies of flesh fly connected at day 10 and day 11.

^cNot detectable.

activity within 24 hours indicates that production of this enzyme was restricted to a certain period during the life cycle. This also indicates that the composition of the hydroprene esterase varies considerably from that of the esterase that hydrolyzes NA.

Only a few assays of hydroprene esterase activity were made on CSMA hemolymph. These showed a five-fold variation and a peak in the days 3-5 region.

The hydroprene hydrolyzing action of enzymes in the soluble fraction of the three species of flies was given limited attention, Table 9. Based on the flesh fly and blow fly tests, day 10 to 13, the enzymes of the latter were only half as active as those of the former species. Comparisons between the flesh fly and house fly indicate that the soluble enzymes of these species have approximately the same activity toward hydroprene.

Assays of hydroprene esterase activity in the hemolymph and soluble fraction of immature stages of the three species are summarized in Table 10a. The soluble fraction of three-day larvae of flesh flies (L_1) contained four times as much hydroprene esterase activity as clear gut larvae (L_2). On a blood protein basis the difference was only two-fold. In the case of the blow fly, both larval stages examined were low in hydroprene esterase activity compared to the flesh fly. The soluble fraction of flesh fly larvae contains considerably more hydroprene esterase activity than that of blow fly larvae.

In comparing the hydroprene cleaving activity of soluble fraction

Table 9. Activity of hydroperene esterases in soluble fraction of female flies.

Age, days	Percentage of hydroperene hydrolyzed		
	Flesh fly	Blow fly	CSMA house fly
1	42.5		
2	47.0		37.1
3	52.0		
4	51.0		48.3
5	51.0		
6	50.0		
7	50.0		
8	62.4		37.8
9	74.7		37.8
10	48.4	25.0	56.4
11		26.0	
12	46.5	21.5	
13		23.6	
14	86.7		

^aOnly labeled hydroperene was used as substrate.

^bAverage of duplicates.

^cEach incubation mixture contains 14,000 dpm or 110 p mole of hydroperene.

Table 10a. Hydroprene esterase activity in larval and pupal stages of three dipteran species.^b

Species and stage ^a		p mols acid produced/mg protein/3 hr. ^c	
		Soluble fraction	Hemolymph
Flesh fly	L ₁	32.38 ± 0.25	46.88 ± 0.39
	L ₂	8.23 ± 1.43	24.95 ± 0.92
	P	2.76 ± 0.51	
Blow fly	L ₁	2.94 ± 0.07	5.66 ± 0.06
	L ₂	4.40 ± 0.85	2.57 ± 0.88
	P	2.30 ± 0.00	
CSMA house fly	L ₁	7.53 ± 0.14	15.34 ± 3.22
	L ₂	7.31 ± 0.28	4.00 ± 0.73
	P		
Rutgers house fly	L ₁	7.40 ± 1.13	15.79 ± 1.52
	L ₂	7.84 ± 1.55	2.44 ± 0.73
	P	2.20 ± 0.32	

^aL₁ = 3-day-old larval; L₂ = clean-gut larval; P = pupal stage.

^bOnly labeled hydroprene as substrate.

^cDuplicate assays for pupae and triplicate assays for larvae: mean ± S.D.

and hemolymph esterases of the three species (Table 10a) it can be seen that the flesh fly fractions are the most active of the three species and that blow fly, especially the soluble fraction, is the least active.

Similar comparisons can be made of CE-ase activity in larvae and pupae of the three species, Table 10b. As far as the soluble fraction is concerned, the esterases of the flesh fly larvae and pupae is two to five times more active than that of the other species. Hemolymph activity, expressed on a basis of protein content, is even more in favor of the flesh fly, up to 20 times more active than that of the Rutgers house fly.

Juvenile Hormone-I

A second substrate of interest in this study of the esterases of the three diptera was juvenile hormone-I. Carbon-14 labeled hormone was used and the analytical method involved resolution of products by thin layer chromatography, location of radioactive regions by radioisotope scanning, and measurement of products by liquid scintillation counting (see Methods for additional details). The results of the experiments are tabulated in Table 11. In the case of flesh fly hemolymph, it is seen that there was a peak in the JH-I hydrolysis rate at day 3, a second peak at day 5, and a third sustained peak at days 9-13. The latter period of high activity corresponds to peaks seen previously in the NA and hydroprene studies. The maximum change in hydrolytic activity was twenty-fold (day 6 vs. day 11).

Table 10b. Carboxylesterase activity in larval and pupal stages of three dipteran species.^b

Species and stage ^a		n mols l-naphthol/mg protein/30 min. ^c	
		Soluble fraction	Hemolymph
Flesh fly	L ₁ ^c	49.66 ± 3.33	509.2 ± 6.07
	L ₂	39.20 ± 0.84	493.55 ± 10.67
	P	68.72 ± 9.23	
Blow fly	L ₁	20.95 ± 2.33	80.00 ± 11.17
	L ₂	20.68 ± 0.45	67.2 ± 8.74
	P	35.3 ± 0.49	
CSMA house fly	L ₁	15.72 ± 1.44	245.30 ± 13.38
	L ₂	7.62 ± 0.45	27.38 ± 2.14
	P		
Rutgers house fly	L ₁	15.69 ± 2.00	25.16 ± 1.73
	L ₂	7.80 ± 0.00	6.33 ± 1.04
	P	23.64 ± 2.17	

^aL₁ = 3-day-old larval; L₂ = clean-gut larval; P = pupal stage.

^bNA as substrate, enzyme incubated with eserine (10⁻⁴M) and PHMB (10⁻⁴M).

^cDuplicate assays for pupae and triplicate assays for larvae: mean ± S.D.

Table 11. Activity of juvenile hormone-1 esterases in hemolymph of female flies.^{a,b}

Age, days	p mols acid/ml. hemolymph		
	Flesh fly	Blow fly	Rutgers house fly
1	---	66.28 ± 4.86	0.67 ± 0.23
2	11.0 ± 1.88	---	ND
3	24.9 ± 3.19	44.92 ± 0.82	2.31 ± 0.11
4	12.5 ± 1.33	32.54 ± 4.11	1.82 ± 0.09
5	22.1 ± 0.27	37.88 ± 2.96	53.42 ± 12.16
6	2.9 ± 0.37	ND ^c	ND
7	12.5 ± 2.76	8.26 ± 1.55	1.58 ± 0.12
8	13.6 ± 1.90	30.71 ± 4.59	
9	33.3 ± 11.9	49.65 ± 10.06	
10	37.2 ± 2.18	ND	
11	59.5 ± 12.17	8.98 ± 2.11	
12	41.1 ± 6.07	21.73 ± 3.49	
13	57.3 ± 5.55	50.99 ± 5.77	

^aDuplicates: mean ± S.D.

^bLabeled juvenile hormone-1 as substrate.

^cNot detectable.

In general, blow fly hemolymph appeared to be more active than that of the flesh fly. Peaks were observed at day 1 and at day 9 and no activity could be detected on days 6 or 10. Thus, the fluctuations in JH-1-ase activity were even greater in the blow fly than in the flesh fly.

Only a few assays were conducted with house flies (Rutgers strain) but these indicated that this species also has rather specific times for JH-1-ase activity. The peak which occurred at day 5 represents activity about 30 times greater than either the day before or the day after (Table 11).

Part 3. Electrophoretic Studies

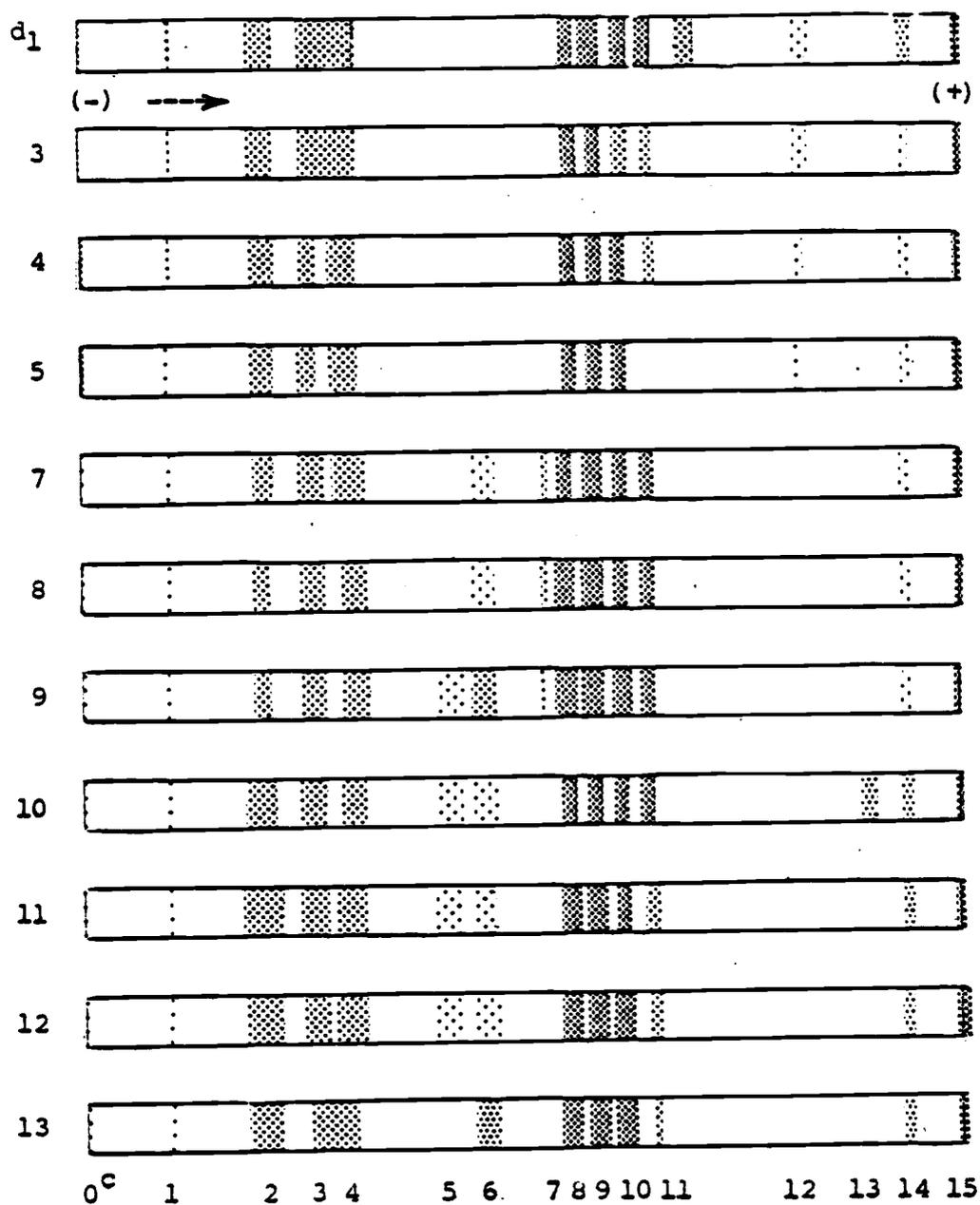
This study revealed that the usual method of comparing electrophoretic bands by their Rf value was not dependable. I found the electrophoretic pattern of a mixture of proteins to vary depending on the number of proteins contained in the sample and the amount of protein applied to each electrophoretic tube. These two factors influenced the mobility of the protein in a gel. Identification of a given protein band was made even more difficult because the content of a sample varied with stage, age, and fraction.

Two enzyme-containing fractions were studied, the hemolymph and the cytosol, i.e., the soluble fraction as free as possible of hemolymph (see "Tissue Fractions" in Methods). The procedure for detecting the enzymatically active bands after electrophoretic resolution of a mixture was to first treat the gel with the two inhibitors, eserine and PHMB to block the action of the choline and aryl esterases. The gels were then treated with NA after which the liberated 1-naphthol was detected with the DBLS dye. A brownish stain indicated CE-ase activity. Since it was difficult to show faint CE-ase bands by photography, the density of each band was estimated visually and recorded in a diagram. Each electrophoresis was repeated at least one time and the results shown (Figures 3-5) represent the total number of bands observed in the replications. In some cases the developed gels were scanned with a densitometer. Additional details can be found in "Methods."

A preliminary electrophoretic study of the hemolymph of the three fly species indicated that their CE-ase isozymes could be divided into five groups according to their mobilities: the cathode group (negative charged), the fastest moving group present at the anode end of the gel; the fast-moving groups, next in mobility to the cathode group; the median-moving group, mostly distributed in the middle area of the gel; the slow-moving group, occupying the area near the cathode end of the gel; and the anode group (positive charged), which remained at the cathode end of the gel. The anode band, median bands, and some slow bands were observed mostly in the hemolymph (Figures 3a,c,e,f). While, fast bands, cathode bands, and some of other slow bands were found mostly in the cytosol (Figure 3h) or soluble fraction (Figures 3b,d,g).

Flesh Fly

Hemolymph. Since the previous work showed that CE-ase activity varied during the life span of the flesh fly, electrophoretic samples were taken throughout the adult stage. The zymogram of day 1 hemolymph was used as a reference standard. Figure 3a shows that 11 bands were detected. The most dominant group of isozymes was the median-moving group, composed of five bands highly active against NA. Two slow bands were light and one slow band was densely colored after staining. The cathode band was dim as were two fast bands. The composition of these bands changed during the life span of the insect. The major changes were:



a : See electrophoresis in method.

b : 5 ul. of haemolymph per gel, duplicates.

c : Code for isozymes : 0, anode band; 1-4, slow bands;
5-11, median bands; 12-14, fast bands; 15, cathode band

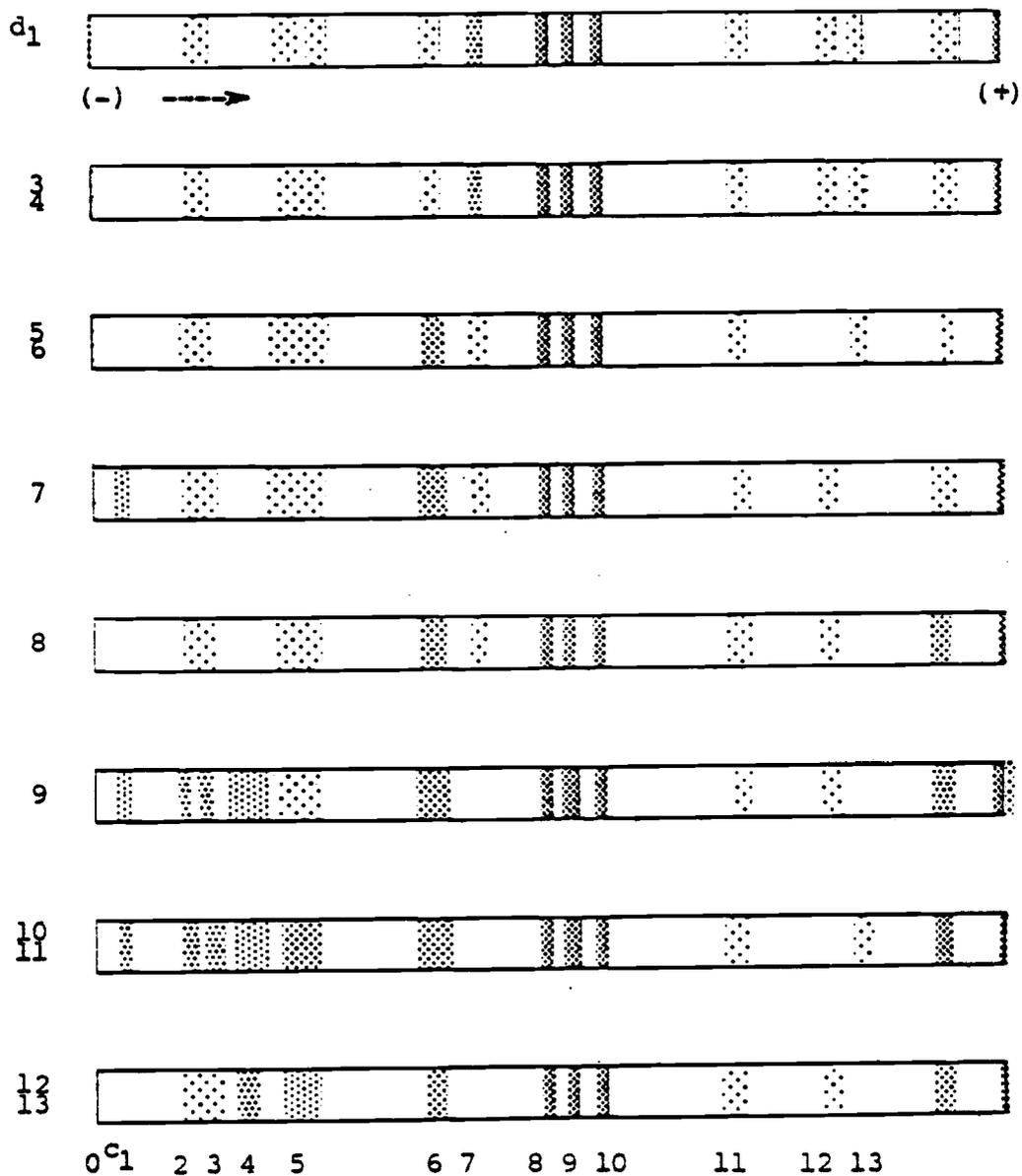
d : Age of fly (day after emergence).

Figure 3a

The Zymograms of Haemolymph Carboxylesterases of
Female Flesh Fly^{a, b}

1. Band 14 was dense at day 1, day 10 to day 13, and dim the other days.
2. Band 13 appeared only at day 10.
3. Band 12 was found in the day 1 zymogram, decreased in density the next couple of days and disappeared by day 5.
4. Median bands (band 7 to 11) were dense in color at day 1, less dense later on, then increased to maximum density at days 9 and 13.
5. Band 6 appeared from day 7 and was at its maximum by days 9 and 13. Band 5 appeared on day 9 through day 12.
6. Bands 3 and 4 gradually increased their density from day 4 and reached their maximum by day 11, then decreased afterwards.
7. Band 2 decreased in width from day 1 to a minimum at day 9, and increased the next day. Its width was twice that at day 9 and by day 11, 12 and 13.

Soluble fraction. Figure 3b shows that 13 bands were detected in the day 1 zymogram of the soluble fraction of the flesh fly. There were two slow-moving bands, five median-moving bands, four fast-moving bands, one cathode band and one anode band. The density of the band, as indicated by the 1-naphthol stain, varied from time to time. In general, median-moving bands were denser in color, while fast-moving and slow-moving bands were light in color. The variation in the pattern of the soluble fraction zymogram of each age fly can be described as follows:



a : See electrophoresis in method.

b : One fourth of a headless fly (equivalent) per gel.

c : Code for isozymes: 0, anode band; 1-5, slow bands; 6-10, median bands; 11-14, fast bands; 15, cathode band

d : Age of fly (day after emergence).

Figure 3b

The Zymograms of Soluble Carboxylesterases of
 Female Flesh Fly^{a, b}

1. Band 15 (the cathode band) became very dense at day 9.
2. Band 14 was densest in color at day 10 and 11.
3. Band 7 decreased in density after day 1, and disappeared after day 8.
4. The width of band 6 increased after day 1 and the densest appearance showed during day 9, 10, and day 11.
5. Band 5 increased in density to the maximum during day 10 through day 13. There was one extra slow band (band 4) which appeared during day 9 through day 13. Band 2 and 3 were densest during day 9, 10, and 11.
6. Band 1 was detected only at days 7, 9, 10, and 11.

Flesh fly larvae. Two larval stages were used in the electrophoresis study, fourth day larvae and clear gut larvae. Figure 5a shows that the major components of the three groups of isozyme in the larval hemolymph were similar to those of the adult stage. Slow-moving bands in the larval stages, however, were diffused into each other, making separation difficult.

The major difference in the zymograms between the two stages were that in the day 4 sample there was an extra median-moving band. Also, there was more enzyme activity (more dense color) in other slow-moving isozymes next to the anode band. These two bands are believed to be related to the higher CE-ase activity of the larval stage of the flesh fly.

The soluble fraction of flesh fly larvae contained a very limited

amount of esterases making the identification and separation of isozymes difficult, Figure 5b. It is possible that the soluble fraction has the same esterase as the hemolymph. For the zymogram study of the esterases in the hemolymph, 65 ug of protein was sufficient. However, more than 3.0 mg of larval soluble protein was required. Still, the result was not satisfactory. The four isozymes of the five median-moving groups, the three slow-moving isozymes, and the two fast-moving isozymes were present in the larval hemolymph at all times of examination, indicating that they may comprise the major portion of esterases that metabolize NA and hydroperene. A fast-moving band which was dense in color after staining was found only in soluble fraction of larval stage of all three fly species examined. Function of this band was unknown.

Correlation of enzyme assays with zymograms. In the age profile studies of NA hydrolysis by the esterases (Tables 3, 6, and 12), there were activity peaks at day 9 and day 13 and enzyme activity was at its lowest at day 10. On examining the hemolymph zymogram of flesh flies of these ages, it is seen that both the slow and the fast-moving groups either increased in 1-naphthol staining density or increased in number of bands, except that the last fast-moving band decreased in density. The only group that was decreased in density was the median-moving group. A light band which was present in the zymogram of days 7, 8, and 9, disappeared. This fact indicates that the median-moving groups are most likely responsible for the observed decrease in

CE-ase activity. A similar pattern was found in the zymogram of the soluble fraction. It confirms that the weak CE-ase activity of day 10 hemolymph was mostly due to the low concentration of enzymes represented by median-moving bands.

On the other hand, the zymogram of the day 9 soluble fraction showed the densest median-moving and cathode band. The concentration of enzyme in this cathode band from the soluble fraction was so high that it became divided into two parts.

Besides this, the two extra slow-moving bands also had their highest 1-naphthol density at day 9. In hemolymph the median-moving band exhibited minimum enzyme activity at day 11, and increased afterward. These changes in density correspond to the decreasing enzyme activity of CE-ase observed in the profile studies through days 9 to 11. A close look at the zymograms of the soluble fraction reveals that band 6 was missing from its original location.

In the study of juvenile hormone-1 esterase activity of the flesh fly (Table 11), peaks were found in the hemolymph at days 7, 10, and 11. The lowest activity occurred at days 2, 3, and 13. In the electrophoretic study of these esterases no special band except an extra slow band (band 1) was found near the cathode end of the soluble fraction zymogram of day 7 and days 9 to 11. This band was not seen after day 11. The number of bands in the slow-moving group increased from 2 to 4. This change in number of bands did not occur in the hemolymph but their width increased to twice that of the original bands. These bands increased in enzyme activity to a maximum

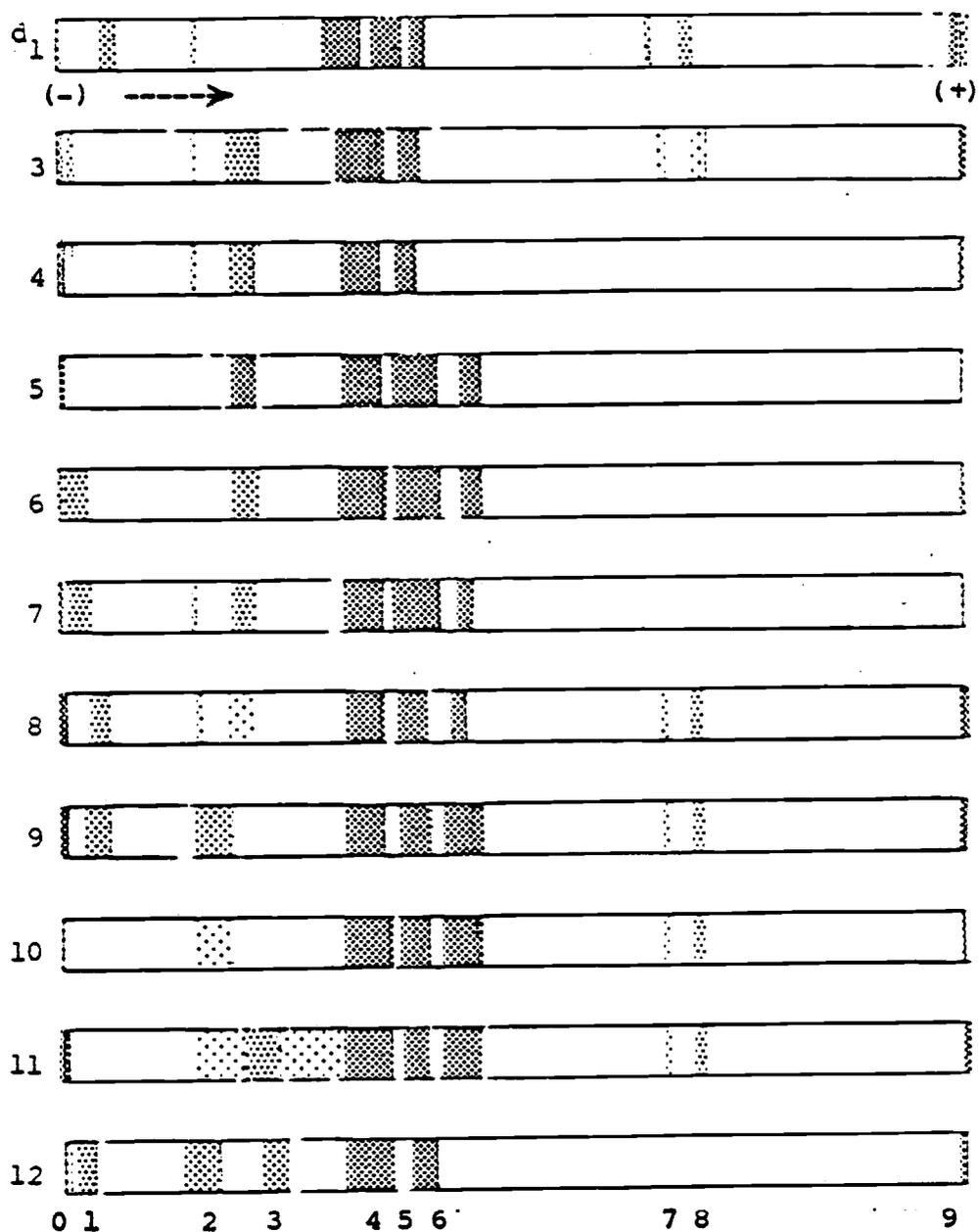
by day 11 declining at day 12. The corresponding band was faint in the zymogram of days 1, 3, and 13 and is assumed to be responsible for the hydrolytic activity against juvenile hormone-1.

The extra slow band found at the cathode end in the soluble fraction zymogram was not present in the hemolymph. This might be due to its lower concentration in the hemolymph, or to its lower sensitivity to the NA staining. Anyhow, the appearance of this band in the zymogram of the soluble fraction at least reflected that it may be related to the high enzyme activity to JH-1. One of the fast-moving bands in the hemolymph also increased its density by day 10 when the corresponding bands in the soluble fraction had their highest density. These bands may also be involved with the esterase activity to JH-1. More direct evidence of these relationships is presented in the gel-extraction experiments to be discussed later.

Some other characteristics of the zymograms are that the extra esterases seemed limited to the cytosol, and were never found in the hemolymph. Other CE-ases, like the one that gradually disappeared from the hemolymph after adult emergence are possibly related to the functioning of emergence. These enzymes will not be discussed further.

Blow Fly

Hemolymph. Figure 3c shows that nine bands were detected in the electrophoresis of day 1 adult blow fly hemolymph: one anode band, two slow-moving bands, three median-moving bands, two fast-moving



- a : See electrophoresis in method
 b : 12 ul. of haemolymph per gel, duplicates.
 c : Code for isozymes: 1, anode band; 2-4, slow bands;
 5-7, median bands; 8-9, fast bands; and 10, cathode
 band.
 d : Age of fly (day after emergence).

Figure 3c
 The Zymograms of Haemolymph Carboxylesterases of
 Female Blow Fly^{a, b}

bands, and one cathode band. Changes in these isozymes as flies aged were as follows:

1. The cathode band was dense at day 1 and days 8 to 10.
2. Band 7 and 8 did not appear during day 4 to day 7 and days 12 to 13.
3. The median and slow band decreased in density from day 5 and were least dense by day 8. Their density increased after day 9.
4. The slow band became obscure by day 10 and day 11, but was dense at day 12.
5. The band (band 1) near the cathode area was observed at days 6, 7, 12, and 13, but was absent during days 3 to 5, and days 10 and 11.

Soluble Fraction. Figure 3d shows 7 bands were detected in the day 1 blow fly soluble fraction. Some of these bands divided into two closely associated bands in some cases. In general, the fast and the median band were very active against NA. This can be seen from zymogram of days 5, 6, or day 10. The major changes in these zymograms as flies aged were:

1. The cathode band had double appearance at days 9 to 11.
2. Two fast bands (bands 10, 11) varied with highest enzyme activity at days 5, 6, 7, 8, and 9.
3. Median bands (bands 4-7) were light only at day 8.
4. Slow bands (bands 2, 3) become densest at days 10, 11. One

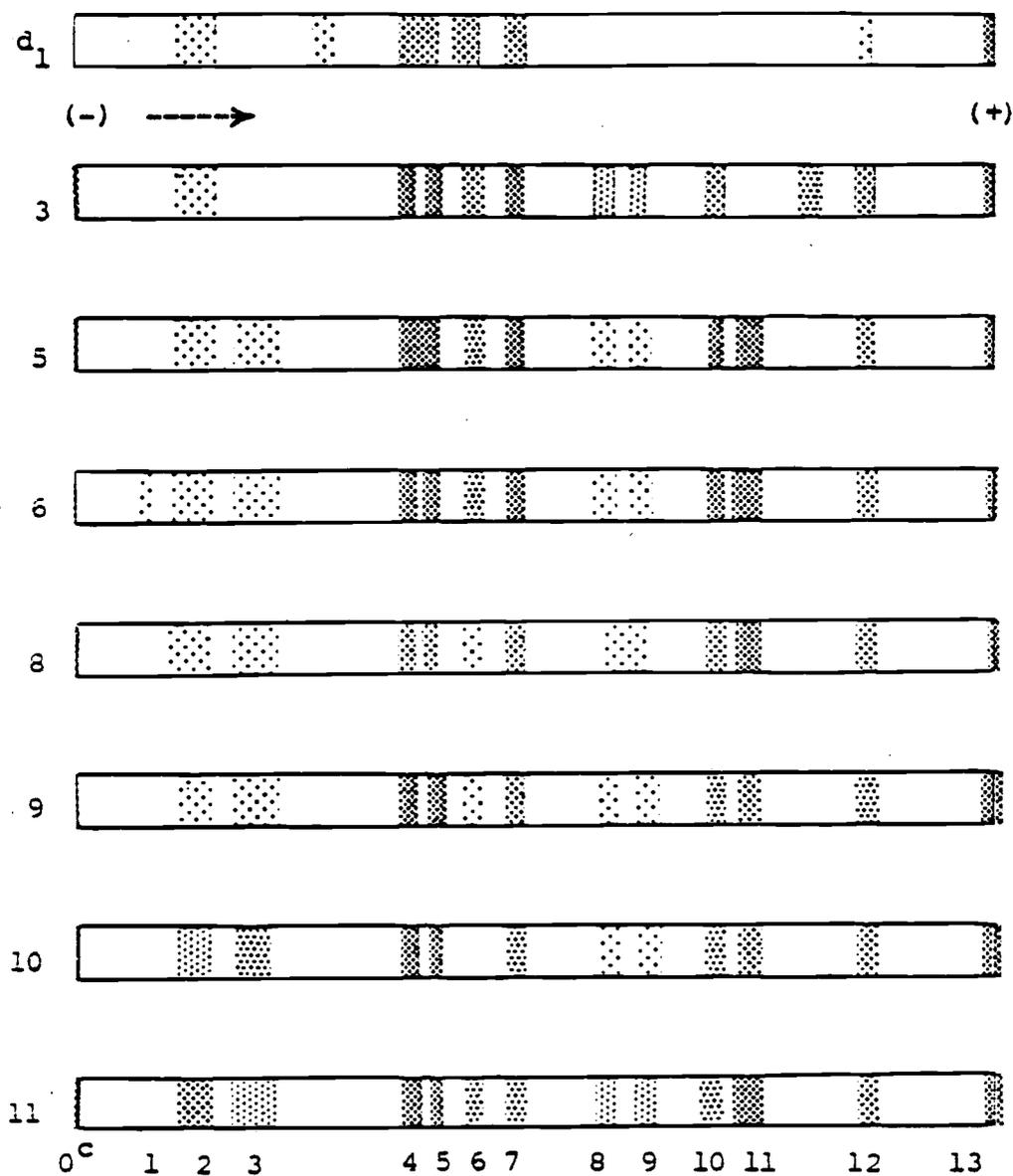
extra slow band (band 1) was detected on day 6.

Blow fly larvae. At least 2.0 mg of soluble protein had to be used per zymogram for detection of larval CE-ases. This was about four times the amount of protein required for hemolymph electrophoresis. The basic structure of the CE-ase zymogram of larval hemolymph (Figure 5a) was similar to that of adult hemolymph. The number of isozymes in the soluble fraction of larva was about the same as with adults, but slow-moving bands were all enzymatically more active. Possibly, there was an extra slow-moving band associated with the top end of the cathode position. The enzyme activity of the CE-ase in the soluble fraction was low (Figure 5b).

The specific fast-moving band found in larval soluble fraction of flesh fly was also found in this species.

Correlation of Enzyme Assays with Zymograms. It is rather difficult to relate the CE-ase zymogram with the CE-ase activity in blow fly. However, by comparing the hemolymph zymogram (Figure 3c) and the hemolymph CE-ase activity (Table 4c) with the soluble fraction zymogram (Figure 3d) and the soluble fraction CE-ase activity (Table 6a), it can be supposed that the median bands are responsible for the CE-ase activity in the hemolymph. In the case of the soluble fraction, some fast bands (bands 10 and 11) are possibly related to soluble fraction CE-ase activity of days 5 and 6 fly, but to a less extent.

The hydrolysis of hydroperene in blow fly hemolymph was very low.



a : See electrophoresis in method.

b : One third to one half of a headless fly (equivalent) per gel; duplicates.

c : Code of isozymes: 0, anode band; 1-3, slow bands; 4-7, median bands; 8-12, fast bands; 13, band

d : Age of fly (day after emergence).

Figure 3d

The Zymograms of Soluble Carboxylesterases of

Female Blow Fly^{a,b}

No clear-cut relationship can be drawn between the zymogram pattern and the enzyme activity. However, it is reasoned that the enzyme activity against hydroprene was due to the anode band, cathode band, or slow bands. Band 1 and band 9 which were dense at days 1, 8, and 9 could be related to the hydrolytic activity against JH-1.

House fly

Hemolymph. Figure 3e shows that ten bands were detected in day 1 CSMA house fly hemolymph: one anode band, one slow-moving band, five median-moving bands, two fast-moving bands, and one cathode band. One of the fast-moving bands was actually composed of four strips associated together in a zone. This zone disappeared 48 hr after emergence. The hemolymph zymogram of day 7 flies showed that fast-moving bands were undetectable and median bands were dim. At days 5 and 6 the zymogram had its densest median bands and some dense slow bands (bands 2, and 4) which were not found in days 1 to 4, or in day 7 zymogram. Band 3 and 4 were also dense during days 5 and 6.

Figure 3f shows that nine CE-ase bands could be detected in the zymogram of the hemolymph of day 1 Rutgers flies: one anode band, one slow band, four median bands, four fast bands and two cathode bands. The major changes in these zymograms with fly age were as follows:

1. Both anode and cathode bands were dense in color response at the day of emergence. The density of these bands dropped

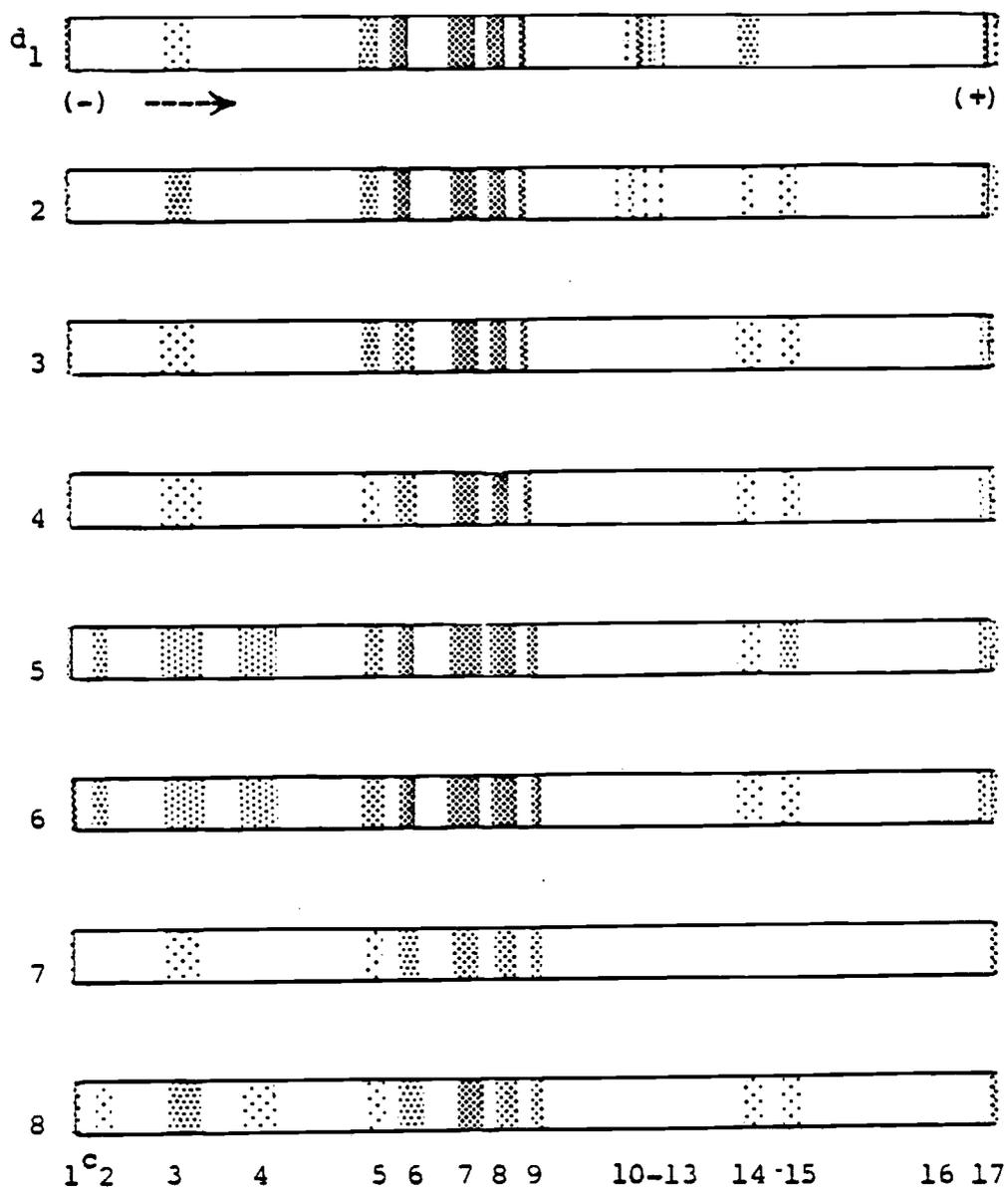
- to a minimum at day 5 and then increased to maximum at day 8.
2. No fast-moving bands except a broad zone was found in the day 1 zymogram. the zone with four narrow strips disappeared 48 hr after emergence.
 3. Median-moving bands were dense at day 1, decreasing in density later on.
 4. One extra median band (band 4) appeared only at days 3, 4, and days 7, 8.
 5. The zymogram of day 5 and day 6 had the densest slow band. These bands were dim at day 1.
 6. A band near the cathode end (band 1) appeared on days 3 to 7 and had a double appearance by day 5. It was absent on day 8.

A comparison of the zymograms of hemolymph and cytosol of the two house fly strains (Figures 3e, f, g) shows that the basic CE-ase composition is similar but with the following differences:

CSMA

Rutgers

- | | |
|---|---|
| 1. Strong staining in all median bands. | 1. Median bands comparatively weak. |
| 2. Cathode bands were less dense compared to the other bands. | 2. Cathode bands were denser. |
| 3. Slow bands were light and narrow in appearance. | 3. Slow bands were dense and broad in appearance. |



a : See electrophoresis in method.

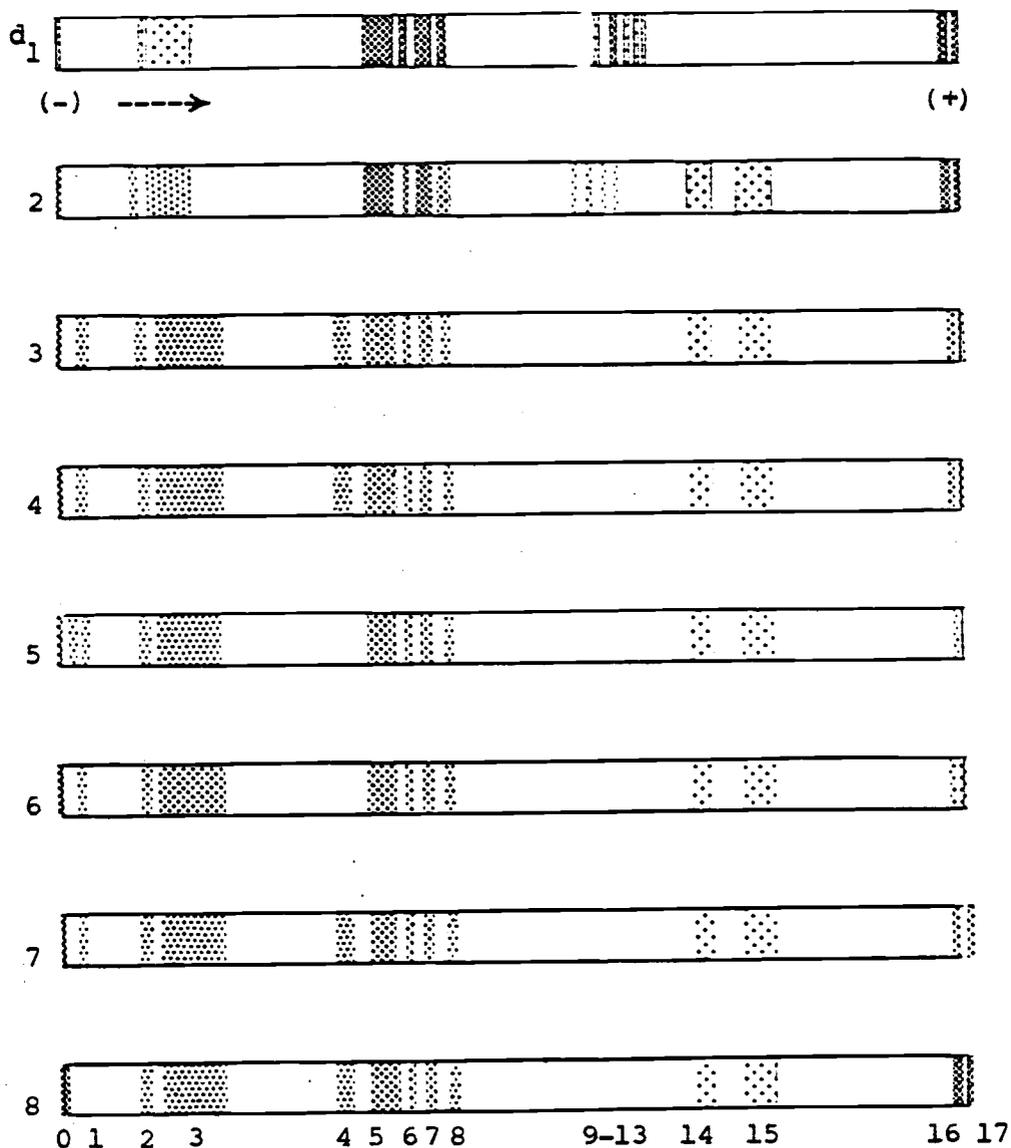
b : 12 ul. of haemolymph per gel; duplicates.

c : Code for isozymes : 1, anode band; 2-4, slow bands;
5-9, median bands; 10-15, fast bands; 16, 17,
cathode band(s).

d : Age of fly (day after emergence).

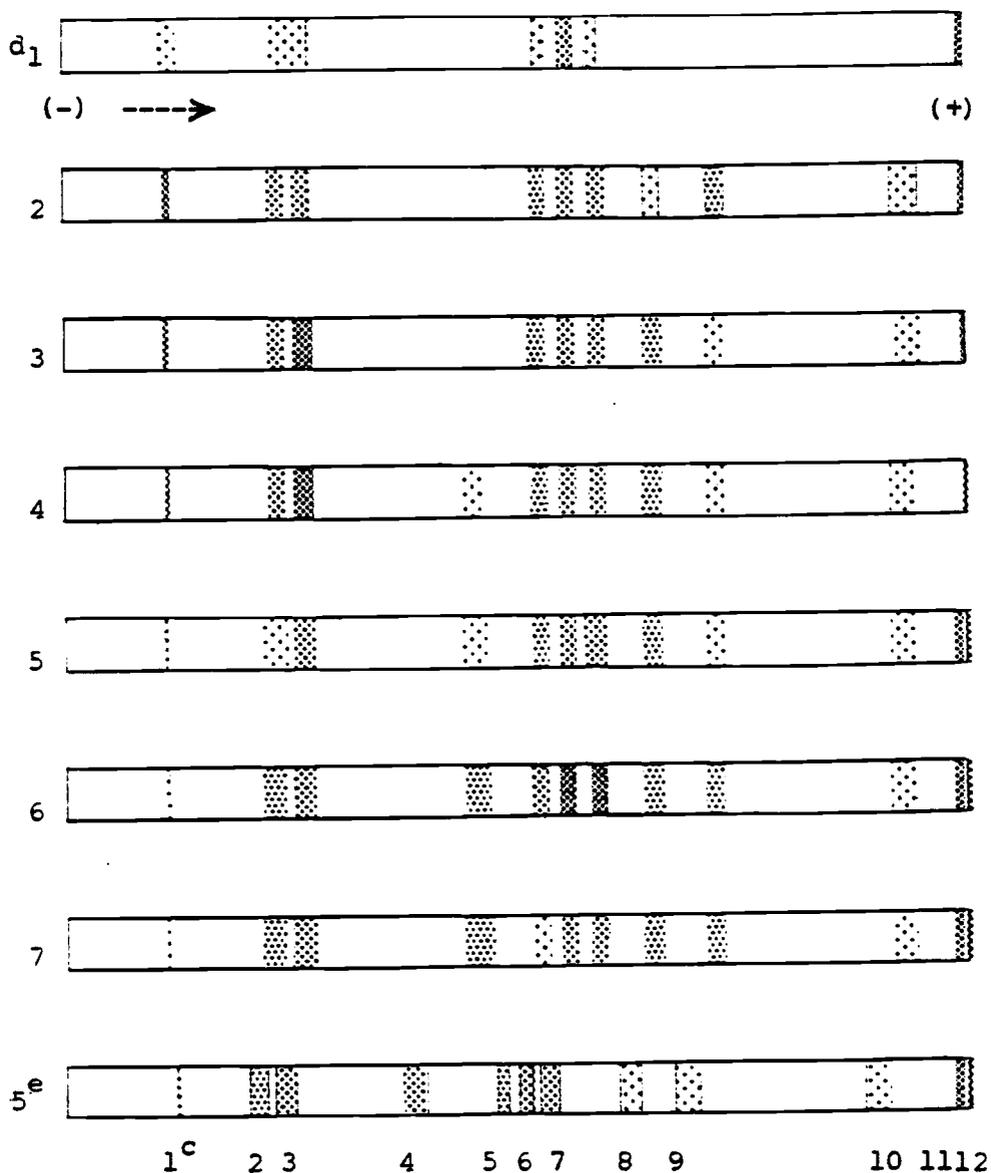
Figure 3e

The Zymograms of Haemolymph Carboxylesterases of
Female CSMA House Fly^{a, b}



- a : See electrophoresis in method.
 b : 12 ul. of haemolymph per gel, duplicates.
 c : Code for isozymes : 1, anode band; 2-5, slow bands;
 6-10, median bands; 11-17, fast bands; 18-19,
 cathode bands.
 d : Age of fly (day after emergence).

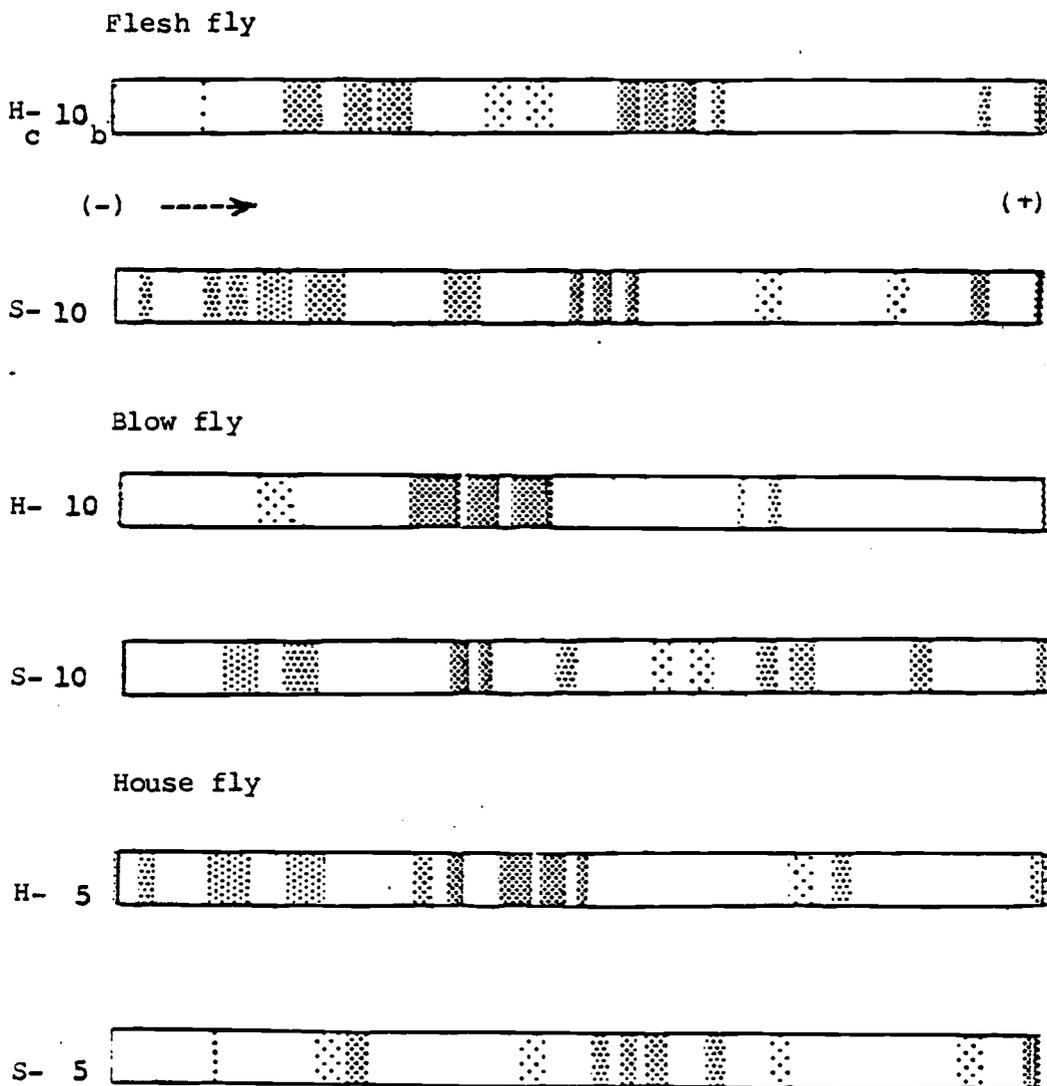
Figure 3f
 The Zymograms of Haemolymph Carboxylesterases of
 Female Rutgers House Fly^{a, b}



- a : See electrophoresis in method.
 b : Half of a headless fly (in equivalent) per gel;
 duplicates.
 c : Code of isozymes: 1-3, slow bands; 4-7, median
 bands; 8-10, fast bands; 11, 12, cathode band(s).
 d : Age of adult CSMA house fly (day after emergence).
 e : 5 day old Rutgers house fly.

Figure 3g

The Zymograms of Soluble Carboxylesterases of
 Female CSMA and Rutgers House Flies^{a,b}



a : Detail of isozymes distribution see Figure Figure 3-5.
 b : Age of adult flies(day after emergence)
 c : H, hemolymph; S, Cytosol.

Figure 3h

The zymograms of hemolymph and cytosol carboxylesterases of female flies of three dipteran species a.

House fly larvae. Figure 5a shows that 12 bands were found in the hemolymph of third-day CSMA house fly larvae: three fast-moving bands, one cathode band, five median-moving bands, and three slow-moving bands. Enzyme activity in the hemolymph of the third day larvae was much stronger than that of clear gut larvae since 70 ug protein of hemolymph from third day larvae gave a strong color response to NA, about one-fourth the amount required for detection of hemolymph esterases from clear gut larvae. Two fast-moving bands and one median-moving band which were present in adult hemolymph were not seen in the zymogram of hemolymph of clear gut larvae. The difference in concentration of median-moving isozymes in clear gut larvae and third day larvae, and the extra band found in the third day larvae indicate that these bands were related to the hydroperene metabolizing esterases in larval hemolymph. The concentration of esterases in the larval soluble fraction was low, Figure 5b. The fast-moving isozyme, which is to be cytosol in nature, was also present in this species.

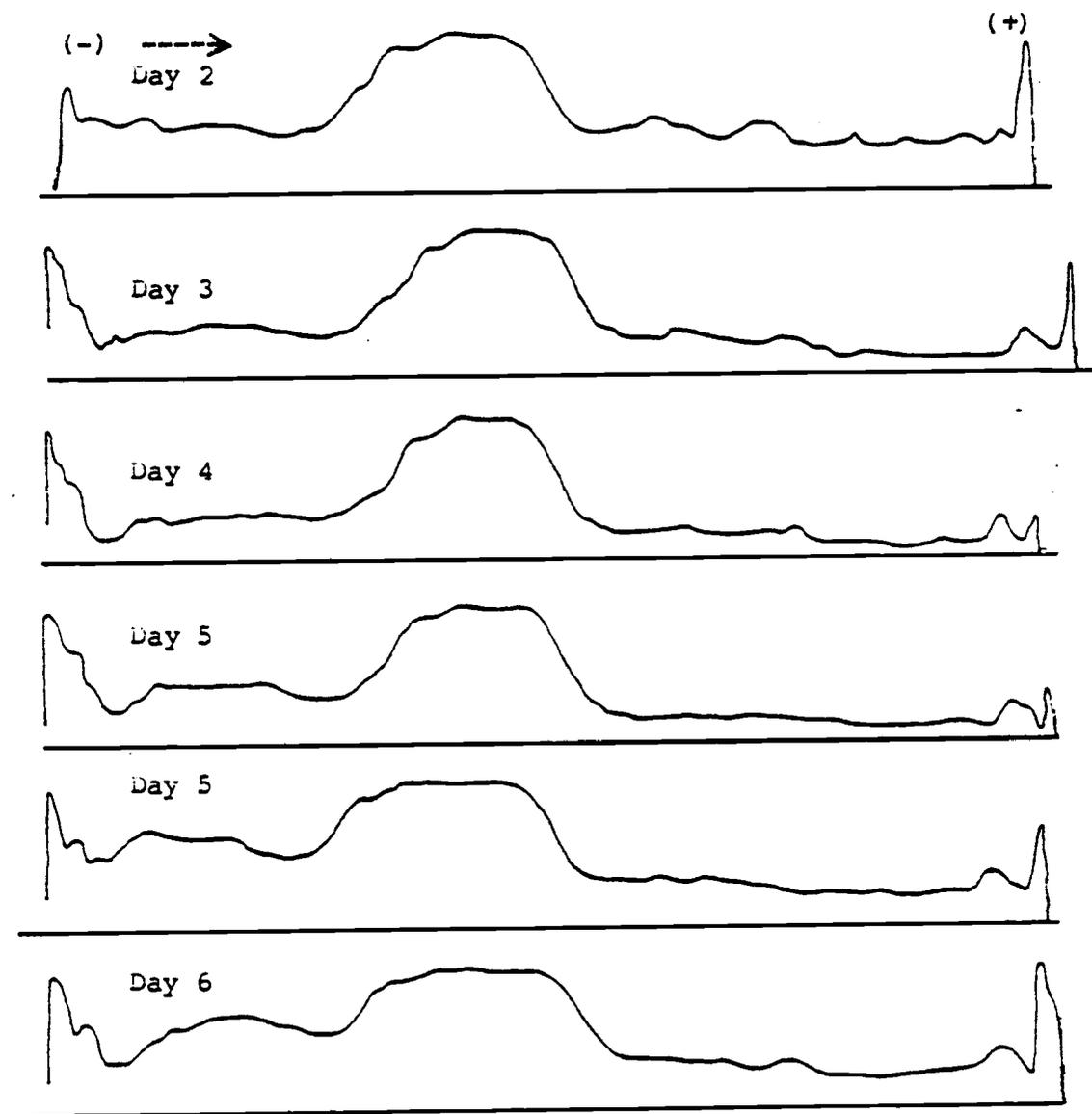
Correlation of enzyme assays with zymograms. The absence of major change in median bands of CSMA hemolymph zymogram (Figure 3e), agrees with the CE-ase activity to NA, Table 4c. A close look at CE-ase activity of the soluble fraction and the hemolymph reveals that median-moving bands are mainly responsible for the CE-ase activity (also see Figure 4a). A higher CE-ase activity in day 3 soluble fraction (with dense slow bands 2, 3) may indicate that these

bands contributed to the hydrolytic activity to NA in the soluble fraction.

Similar results were seen in the Rutgers hemolymph CE-ase activity. In reference to the weak CE-ase activity of Rutgers hemolymph (Table 4d), it is seen that a rather constant CE-ase activity was present. All the bands shown in the zymogram (Figure 3f) might be responsible for the CE-ase activity. In Figure 4b median bands decreased their response to NA staining after emergence. The slow band, on the contrary, increased in density to maximum at days 5 and 6. A constant CE-ase activity (Table 4d) and a changed CE-ase density (Figure 4b) indicate that slow and median bands were mostly responsible for the CE-ase activity in Rutgers house fly hemolymph.

Hydroprene esterase activity was high on days 2, 3, 7, and 8 (Table 8). It was found that an extra median band in Rutgers house fly hemolymph appeared on days 3, 4, 7, and 8 (Figure 3f) zymograms. It is assumed that this extra median band (band 6) was related to the high hydrolytic activity to hydroprene. There is disagreement, however, between the zymogram of day 4 with low enzyme activity and day 2 with high enzyme activity with absence of this extra band.

The double appearance of band 2 (slow band) in Rutgers house fly hemolymph of day 5 (Figure 3f) is believed to be related to the high JHE-ase activity (Table 11). The increased density of band 2 and 3 in zymogram and the correspondingly increased JHE-ase activity will be discussed later.



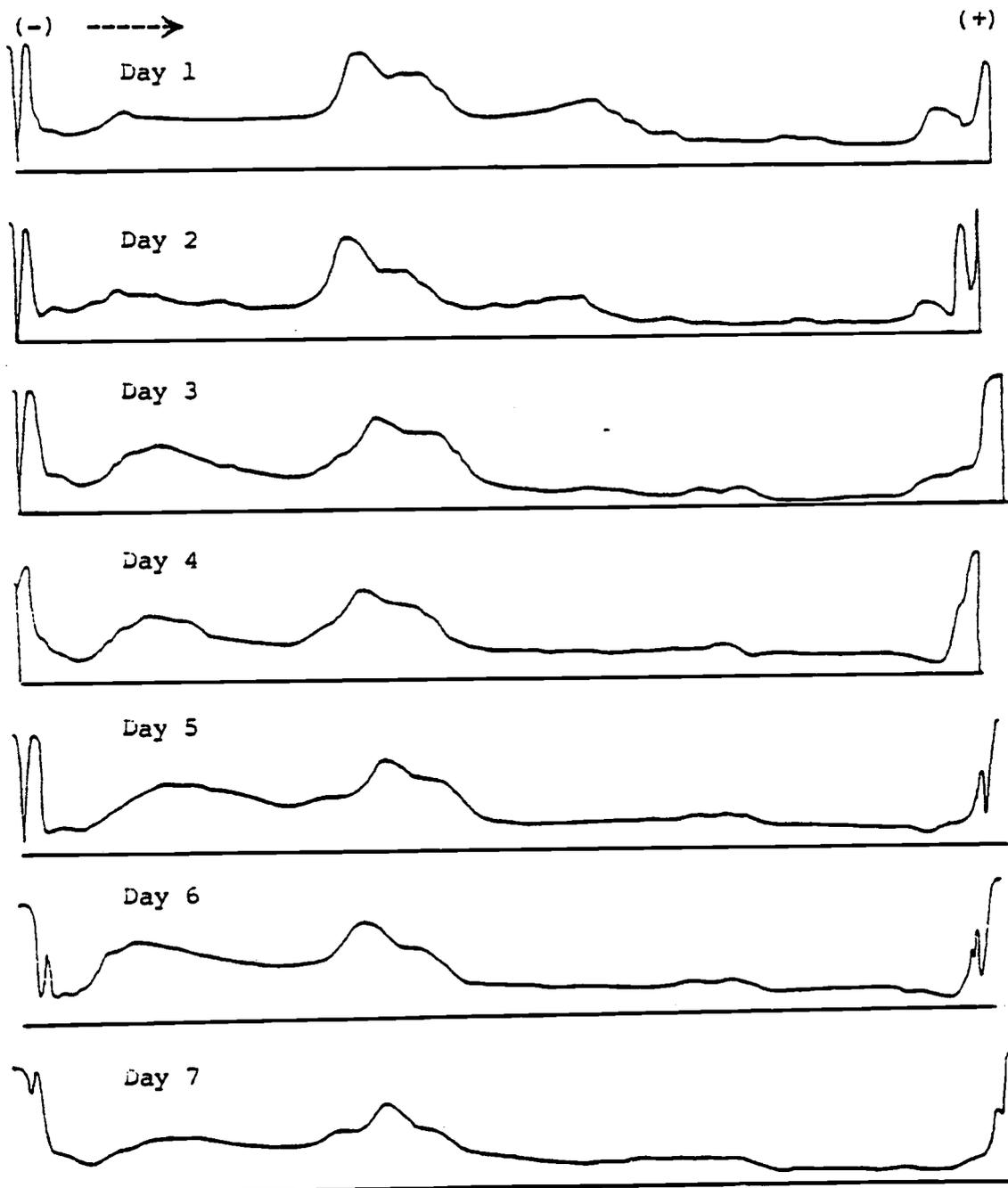
^a $10^{-4}M$ of eserine and PHMB in final concentration for preincubation.

^b 0.2 of the original size.

^c NA and DBLS for staining, 10 ul. of hemolymph per gel

Figure 4a

Densitometric electropherograms showing temporal change of the carboxylesterase patterns of CSMA hemolymph a,b,c.



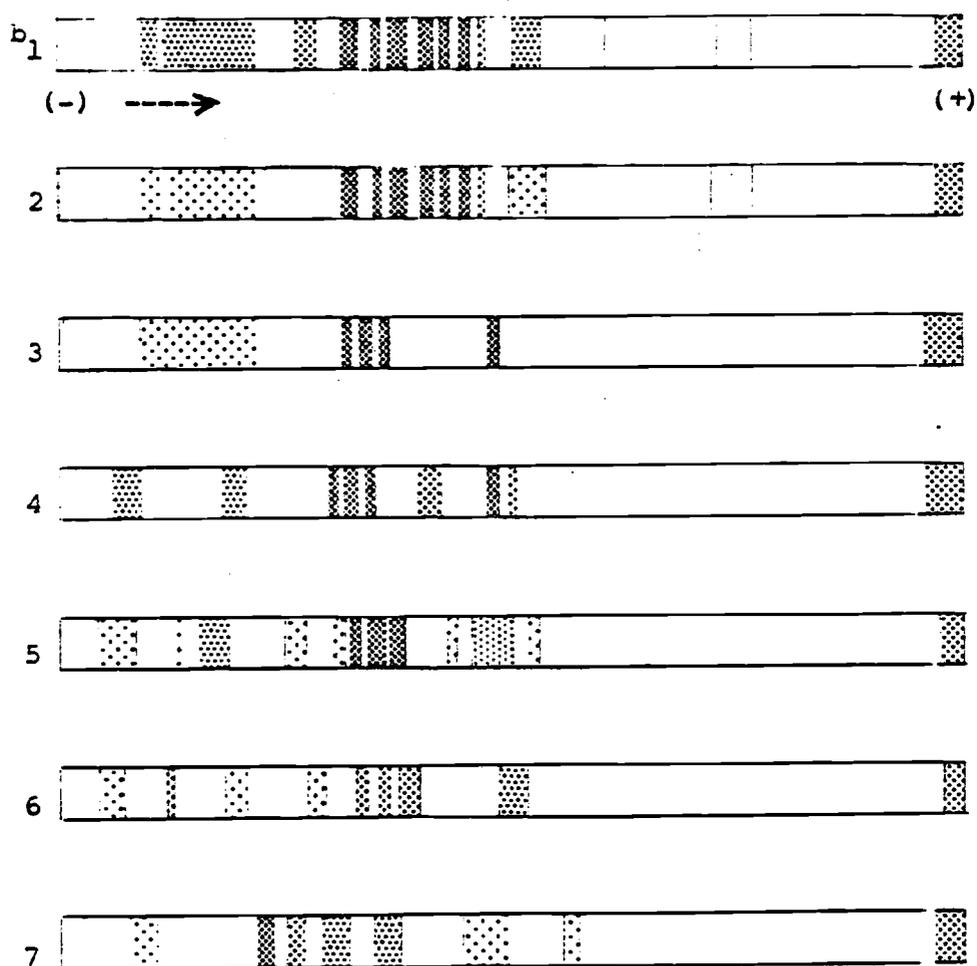
^a 10^{-4} M of eserine and PHMB in final concentration for preincubation.

^b 0.2 of the original size.

^c NA and DBLS for staining, 10 ul. of hemolymph per gel.

Figure 4b

Densitometric electropherograms showing temporal changes of the carboxylesterase patterns of Rugters' hemolymph ^{a,b,c}.



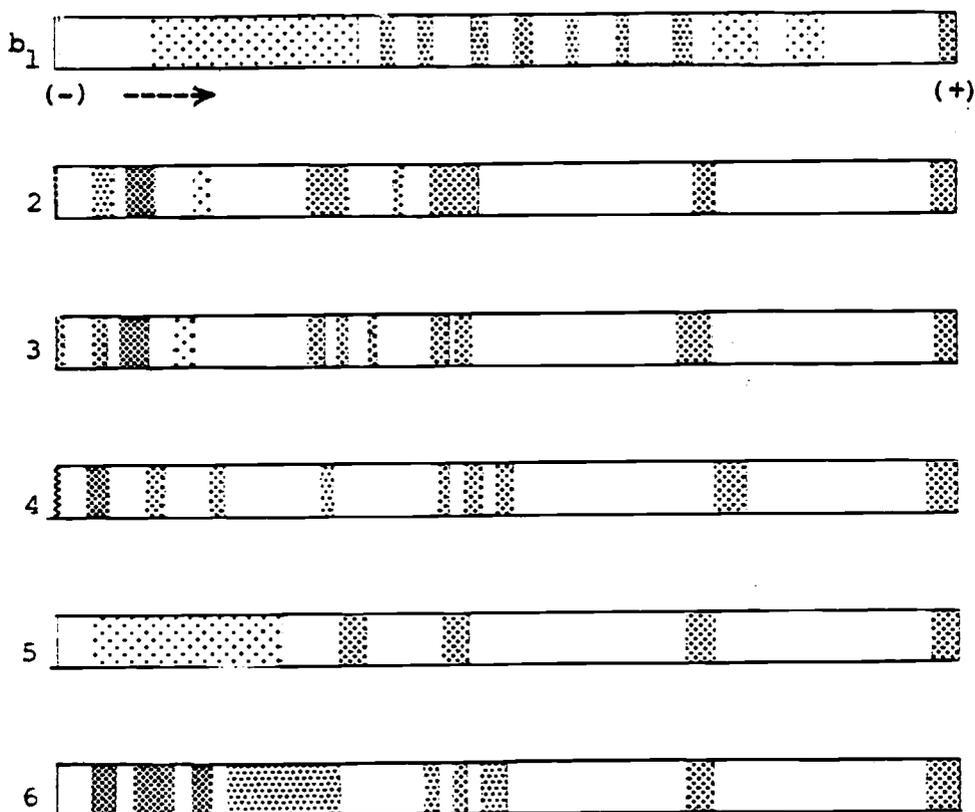
a : See electrophoresis in method.

b : Age of insect (and protein content applied to the gel).

1. 4 day old flesh fly larva (0.06 mg).
2. Clean-gut larva of flesh fly larva (0.06 mg).
3. 3.5 day old blow fly larva (0.25 mg).
4. Clean-gut larva of blow fly (0.25 mg).
5. 3 day old CSMA house fly (0.14 mg).
6. Clean-gut larva of CSMA house fly (0.50 mg).
7. Rutgers house fly larva (0.25 mg).

Figure 5a

The Zymograms of Haemolymph Carboxylesterase of
Larvae of the Three Fly Species^a



a : See electrophoresis in method.

b : Age of insect (and protein content applied to the gel).

1. 4 day old flesh fly larva (2.8 mg).
2. 3.5 day old blow fly larva (not certain).
3. Clean-gut larva of blow fly (not certain).
4. 3 day old CSMA house fly larva (2.0 mg).
5. Clean-gut larva of CSMA house fly (0.4 mg).
6. 3 day old Rutgers house fly larva (not certain).

Figure 5b

The Zymograms of Soluble Carboxylesterases of
Larvae of the Three Fly Species^{a,b}

Part 4. Induction of Esterases

Flesh Fly

Hydroprene was the inducing chemical in these experiments and the induction was measured in terms of hydroprene and NA hydrolysis. Some induction of hydroprene esterase was noted in the hemolymph 12 hours after treatment of the flies with 10.0 ug hydroprene (Table 12). By 24 hours there was evidence of induction by the lower doses of hydroprene. The increase in hydroprene esterase activity continued to 48 and 72 hours after treatment, the maximum being a 4-fold increase after the high dose and a 2-fold increase after the .1 and 1.0 ug dose. The flies in these experiments were 1.5 days old at the time of the hydroprene treatments.

Hydroprene treatment had little effect on the hydroprene esterase activity of the soluble fraction of 1.5-day-old flesh flies (Table 13a). There was a 32 percent increase 72 hours after the application of 10.0 ug hydroprene. At shorter periods after treatment the effect was negligible.

The effect of the inducer on the general esterase of the hemolymph of 3-day-old females was erratic (Table 13b). There appeared to be some inhibition of esterase activity at 2 days and at 4 days after treatment with 10.0 ug hydroprene but at 1 day the effect was slight if at all. However, a 66.5 percent induction was shown at day 3 after treatment. The general esterases of the soluble fraction were induced to a considerable extent, however, when 3-day-old flesh flies

Table 12. Dose Dependence of Hydroprene Induction of Hemolymph Esterases in 1.5 day Female Flesh Flies^a

Treatment, ug/fly	Hours after treatment	n mols hydroprene acid/mg protein/ 3 hr.	Esterase activity, % of control
0.10		1.1	64.7
1.00		1.9	111.8
10.0	12 hr	2.7	158.8
Control		1.7	
0.10		1.7	121.4
1.00		1.8	128.6
10.0	24 hr	3.4	242.9
Control		1.4	
0.10		2.4	200.0
1.00		2.8	233.3
10.0	48 hr	4.2	350.0
Control		1.2	
0.10		1.3	162.5
1.00		2.0	250.0
10.0	72 hr	3.3	412.5
Control		0.8	

^aAverage of duplicates.

Table 13a. Hydroprene esterase in the soluble fraction of flesh flies treated with hydroprene^a

Treatment, ug/fly	Hours after treatment	n mols hydroprene acid/mg protein/ 3 hr.	Esterase activity, % of control
10.0	12	8.5	82.5
Control		10.3	
10.0	24	8.3	117.0
Control		7.1	
10.0	48	7.5	107.1
Control		7.0	
10.0	72	12.1	131.5
Control		9.2	

^a1.5 day female adults average of duplicates.

Table 13b. Carboxylesterase in hemolymph and soluble fraction of flesh flies treated with hydroprene^a

Treatment ug/fly	Hours after treatment	n mols naphthol/mg protein/30 min		Enzyme activity % of control	
		soluble fraction	hemolymph	soluble fraction	hemolymph
10 ug	12	67.4	1485	50.2	97.7
Control		134.3	1516	100.0	100.0
10 ug	24	240.6	1044	144.0	78.2
Control		166.8	1331	100.0	100.0
10 ug	48	298.9	1222	180.9	43.2
Control		165.2	2655	100.0	100.0
10 ug	72	496.2	2345	440.3	166.5
Control		112.7	1408	100.0	100.0
10 ug	96	217.0	971	141.8	69.9
Control		153.0	1390	100.0	100.0

^aNA as substrate, PHMB and eserine as inhibitors, 3.0-day-old female adults, average of duplicates.

were treated (Table 13 c). By the third day after treatment NA hydrolysis was up more than 300 percent in the hydroprene treated flies compared to the controls.

In order to prove that protein synthesis was involved in hydroprene induction, actinomycin D. was used to block the transcription of mRNA in this experiment, Table 13c. The inhibition effect was checked at 36 hours after treatment since the inductive effect of hydroprene is most efficient around this period of time, Table 13a. The inhibition experiment was completed by injecting the aqueous actinomycin D (with water as control) into the insect and, sequentially, by topically treating hydroprene (with acetone as control) on the abdomen of the insect (see "Method" for detail). It is evident that either acetone or actinomycin D have an enhancing effect. However, all combinations of actinomycin D and hydroprene resulted in less esterase activity than in the corresponding actinomycin D--acetone controls. Although inconclusive, these results provide some evidence that the induction involves a synthesis of new protein.

Blow Fly

The enzyme activity of hemolymph hydroprene esterase was unusually high in the blow fly induction experiments. Therefore, it was necessary to express the results in percents rather than absolute values, Table 14a. Data obtained in experiments on soluble fraction are expressed more directly in Table 14b.

Table 14a shows that a biphasic effect of hydroprene on enzyme

Table 13c. Actinomycin D inhibition of hydroprene esterase in the soluble fraction of the flesh fly^{a,b}

Treatment	n mols hydroprene acid/mg protein/ 3 hr.	Enzyme activity % of control	Enzyme activity in % of control	
			induction	inhibition
H ₂ O 1 ul + Acetone	1.4	100.0		
H ₂ O 1 ul + Hydroprene 5 ug	2.0	146.5	146.5	
Act. D. 0.1 ug + Acetone 1 ul	2.0	100.0		
Act. D. 0.1 ug + Hydroprene 5 ug	1.5	73.0		100.0
Act. D. 0.05 ug + Acetone 1 ul	1.8	100.0		
Act D. 0.05 ug + Acetone 1 ul	1.6	92.1		100.0
Act D. 0.02 ug + Acetone 1 ul	2.1	100.0		
Act D. 0.02 ug Hydroprene 5 ug	1.8	83.7		100.0

^aAqueous Act. D. given by injection, water alone as control; hydroprene given by topical treatment on abdomen, acetone alone as control.

^bFlies were examined 36 hours after treatment; average of duplicates, mortality of treated flies was 10 ± 2 per hundred flies.

Table 14a. Dose dependence of hydroprene induction of soluble esterases in 6-day-old blow flies^a

treatment, ug/fly	hours after treatment	Esterases activity % of control	
		soluble fraction	hemolymph
1.0		91.0	101.3
5.0	12	113.4	92.7
Control		100.0	100.0
1.0		112.1	105.0
5.0	24	214.5	98.0
Control		100.0	100.0
1.0		137.2	105.6
5.0	36	141.3	97.2
Control		100.0	100.0
1.0		87.0	122.6
5.0	48	62.7	136.2
Control		100.0	100.0
1.0		73.4	91.4
5.0	72	124.8	120.0
Control		100.0	100.0

^aAverage of duplicates, hydroprene as substrate.

Table 14b. Hydroprene esterase in the soluble fraction of blow flies treated with hydroprene^{a,b}

Treatment ug/fly	Hour after treatment	n mols hydroprene acid/mg protein/ 3 hr.	Esterase activity, % of control
5.0	12	2.366	113.4
Control		2.087	100.0
5.0	24	3.896	214.5
Control		1.818	100.0
5.0	36	3.641	141.3
Control		2.877	100.0
5.0	48	1.008	38.4
Control		2.704	100.0
5.0	72	2.216	124.8
Control		1.776	100.0

^a Average of duplicates.

^b Same data as 5 ug on soluble fraction, Table 14a, indicated by absolute values.

activity was found in the blow fly. The inductive effect was also dose dependent. A 215 percent increase in hydroperene esterase activity was observed in the soluble fraction of the fly 24 hours after treatment. However, almost 40 percent of the enzyme activity was repressed by the second day after treatment. The activity increased again the next day. Hydroperene had only a slight effect on hydroperene esterase in blow fly hemolymph, and this did not appear until the second day after treatment.

The effect of hydroperene on the general esterases of the soluble fraction was also studied in the blow fly, Table 14c. The increase in enzyme activity amounted to 65 percent 12 hours after treatment, and to 86 percent in 24 hours and dropped below the control at 36 hours. The CE-ase activity increased again to 46 percent of the control in the 72-hour assay. In hemolymph, not shown, the induction of general esterase was as high as 180 percent of control activity. The repressive effect was also seen but to a lower extent. The induction of hydroperene esterase in the soluble fraction of blow flies injected with 5 ug of hydroperene was too low (maximum 128 percent of control) to permit a reliable test with Actinomycin D as a blocking agent.

Rutgers House Fly

Table 15a shows that 36 hours after the treatment of 6-day-old flies, the hydroperene esterase activity of soluble esterase increased to 138 percent of the control. The effect remained until at least 60 hours after treatment with a 76 percent increase in enzyme activity.

Table 14c. Carboxyl esterase in soluble fraction of blow flies treated with hydroprene^a

Treatment ug/fly	Hour after Treatment	n mols naphthol/ mg protein/ 30 min.	Enzyme activity % of controls
5.0	12	481.1	165.4
Control		290.9	
5.0	24	456.3	185.9
Control		245.4	
5.0	36	248.0	74.4
Control		333.3	
5.0	48	118.3	42.8
Control		276.6	
5.0	72	202.2	146.3
Control		138.2	

^a6.0 day female adults, average of duplicates.

Table 15a. Hydroprene esterase in soluble fraction of Rutgers house flies^a

Treatment ug/fly	Duration of Treatment	n mols hydroprene acid/mg protein/ 3 hr.	Enzyme activity % of controls
5.0		1.507	
Control	36 hr.	0.633	238
5.0		1.416	
Control	48 hr.	0.972	146
5.0		1.337	
Control	60 hr.	0.760	176

^a6.0-day-old female flies, average of duplicates.

Figure 6a shows flies of 3.0, 3.5, 5.0 and 6.0 days were used for the inductive experiment. The maximum repression of enzyme activity was below 10 percent. Induction is around 10 to 15 percent at day 4 to day 6, 37 percent at day 6.5, 4 percent at day 7, 27 percent at day 7.5. The induction was 100 percent in day 8, 55 percent in day 9, and 76 percent in day 10. It is known that the Rutgers flies have a high enzyme activity during day 8. It is apparent that induction in these flies is age-dependent.

Table 15b shows that the inhibition effect of actinomycin D was checked at 36, 48, and 72 hours after treatment. As with the flesh fly, actinomycin D and acetone have an enhancing effect. However, combinations of actinomycin D and hydroprene resulted in less esterase activity than in the corresponding actinomycin D-acetone controls. Table 15b also shows that hydroprene esterases were induced to 27 percent of net increase of activity by 36 hours after treatment, 72 percent by 48 hours, and 64.9 percent by 72 hours. Inconclusively, it is evident that the induction involves a new protein synthesis.

Electrophoresis Experiments

Hemolymph samples taken from hydroprene-treated and control Rutgers flies were applied to the acrylamide gel for electrophoretic separation. After the electrophoresis, the gels were stained for 1-naphthol and scanned with the densitometer. The electrophoregram paper was then weighed according to the range that the bands of each

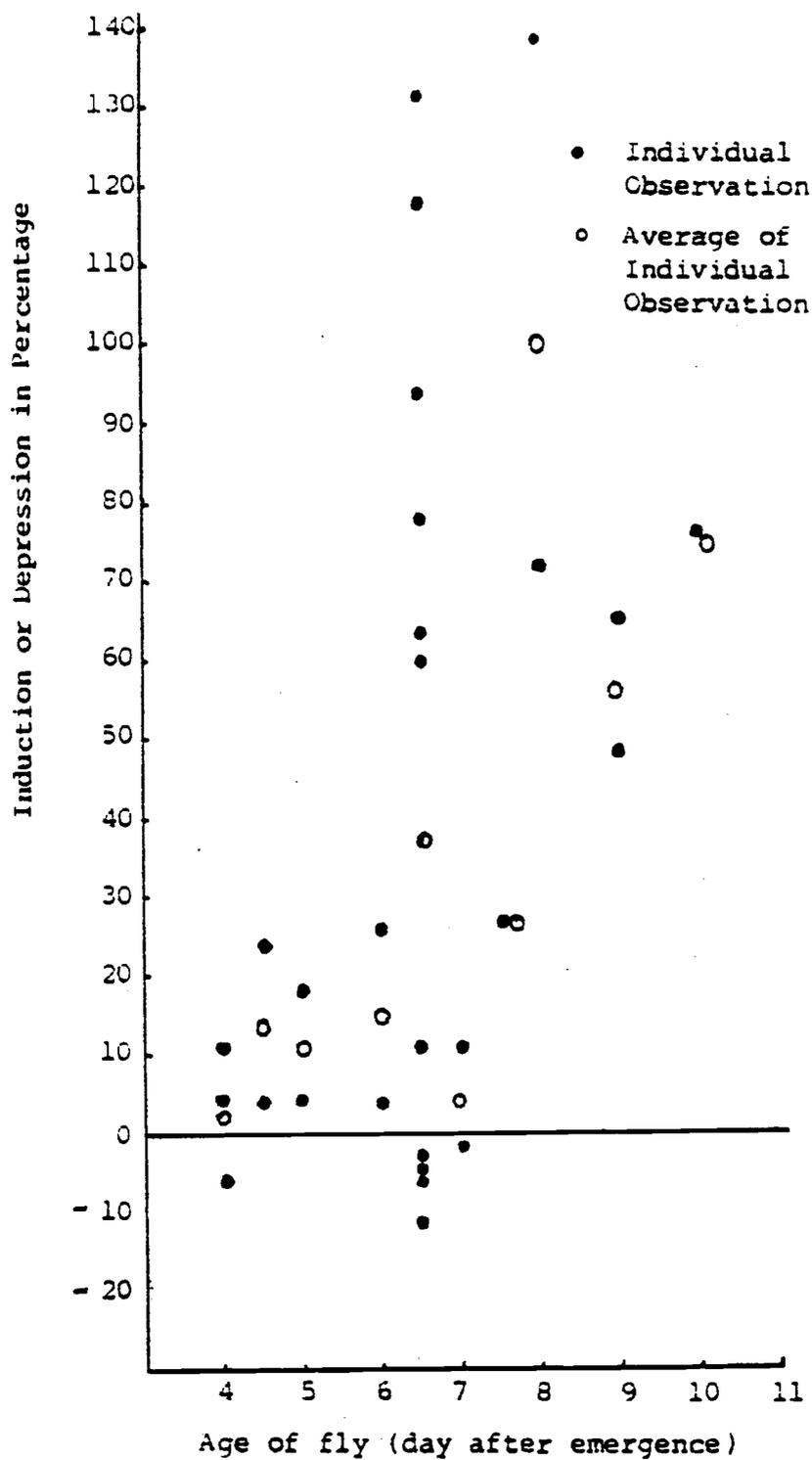


Figure 6

Age-Profile of Hydroprene Induction
of Soluble Carboxylesterase of
Female Rutgers House Fly

Table 15b. Actinomycin D inhibition of hydroprene esterase in the soluble fraction of Rutger house flies^{a,b}

Treatments	Duration of treatment	p mols hydroprene acid/mg protein/3 hr.	Enzyme activity % of controls	
			induction	inhibition
H ₂ O 1ul				
Acetone 1ul	48 hr	295.1	172.0	
	72 hr	272.9	164.85	
H ₂ O 1ul				
Hydroprene 5 ug	48 hr	507.5		
	72 hr	448.1		
Act. D. 0.1 ug	48 hr	346.4		100
Acetone 1ul	72 hr	442.1		100
Act. D. 0.1 ug	48 hr	260.8		
Hydroprene 5 ug	72 hr	392.7		

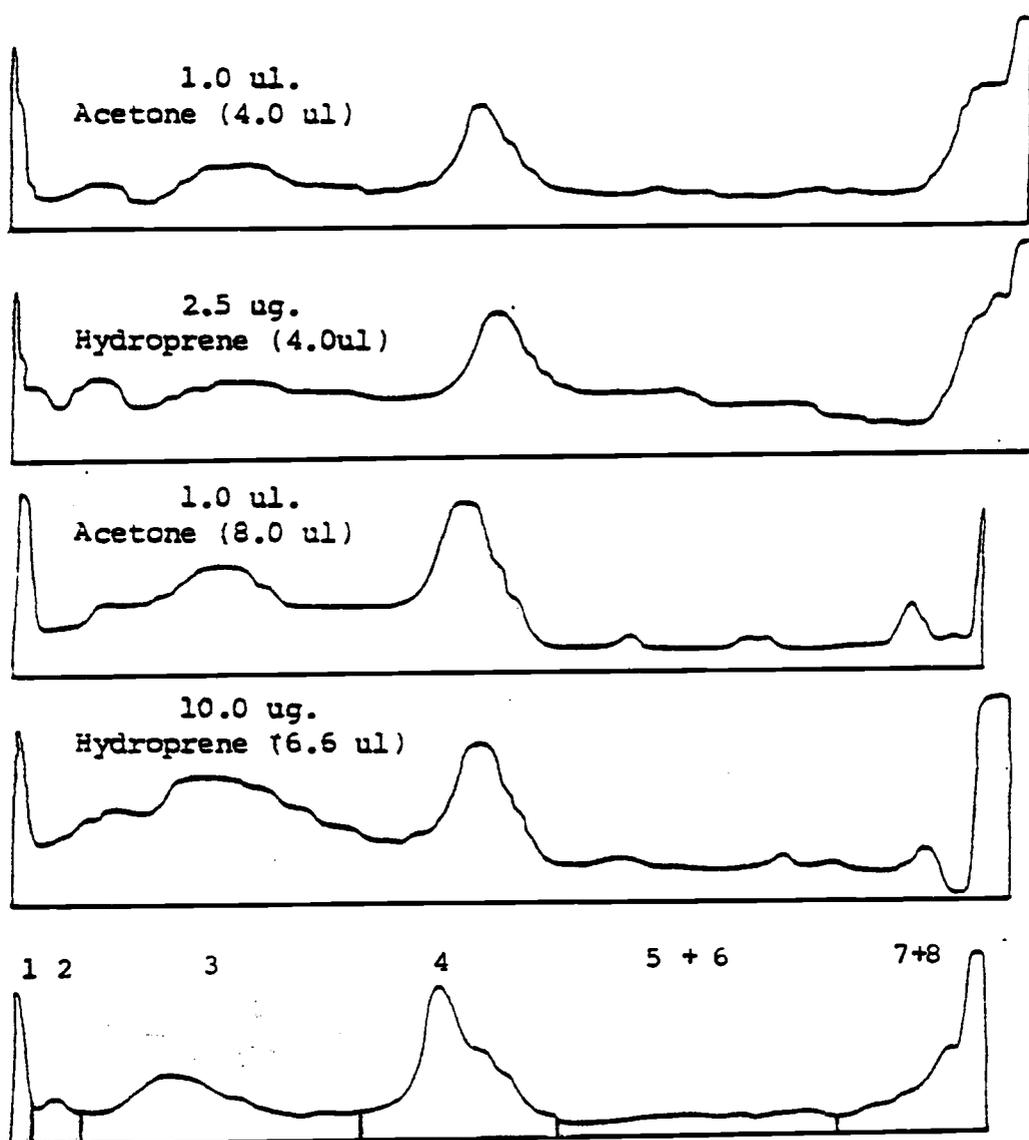
^a6.0-day-old female flies, average of duplicates.

^bAqueous Act D. given by injection with water alone as control; hydroprene given by topical treatment with acetone alone as control.

group of isozymes occupied on the paper. These weights represented the enzyme activity of different CE-ase groups in the sample.

The result shows that most CE-ase bands were induced (see Figure 7), especially the slow bands and one of the median bands. The first case shows (see Table 16a) that there was a 41 percent net increase of enzyme activity. The CE-ase of the band was induced to 90 percent and the second slow band was induced 67 percent, and the third, 26 percent. The median bands were induced to 33 percent while a repression of eight percent of enzyme activity was found in the fast bands and 18 percent in the cathode bands. A band in between the slow and the median bands appeared in the electrophoregram of the induced hemolymph sample but was not seen in the control sample.

In the second case, five-day-old flies treated with 10.0 ug hydroprene and control flies were used for the electrophoregram estimate of CE-ase induction. Hemolymph samples from these flies were assayed, then electrophore-sized, stained, and scanned. The enzyme assay shows that the hydroprene esterases increased 86 percent in the hydroprene treated flies. The electrophoregram study showed (Table 16b) that all slow bands were induced except the anode band. There was no significant change in the median bands except that there was an extra band present between the median bands and the slow bands. The fast bands were induced 58 percent and the cathode band was induced to 145 percent. The induction from this group of insects is different from the result of the younger flies mentioned above in several aspects:



^a 10^{-4} M of eserine and PHMB in final concentration for preincubation.

^b 0.2 of the original size.

^c Volume of hemolymph applied to gel.

Figure 7. Densitometric scans of electropherograms showing changes of carboxylesterase zymogram with different treatments or no treatment on 4.5 day Rutgers hemolymph^{a, b}

Table 16a. Estimation of low dose hydroprene induction of hemolymph isozymes by densitometric electrophoregram

Code for Bands	Enzyme activity (Weight of graph paper)		Enzyme induction % of controls
	Treated 2.5 ug (Hydroprene)	Control (Acetone)	
1	2.17	1.14	190
2	3.07	1.84	167
3	15.83	12.56	126
4	15.60	11.75	133
5 + 6	6.55	7.29	92
7 + 8	6.55	7.96	82

^a5-day-old female Rutgers house fly, enzyme assayed 36 hours after treatment.

^bAverage of duplicates.

^cNA and DBLS for staining, with 10^{-4} M of eserine and PHMB for preincubation.

^dIsozyme distribution, see Figure 6b.

Table 16b. Estimation of high dose hydroprene induction of hemolymph carboxylesterase isozymes by densitometric electrophoregram

Code for Bands	Enzyme Activity (Weight of graph paper)		
	Treated (10.0 ug Hydroprene)	Control (Acetone)	Enzyme activity % of control
1	1.65	2.33	71
2	5.12	3.13	163
3	16.37	13.43	122
4	11.30	11.75	96
5 + 6	7.37	4.67	158
7 + 8	4.75	1.94	245

^a5-day-old female Rutgers house fly, enzyme assayed 36 hours after treatment.

^bAverage of duplicates.

^cNA and DBLS for staining, with 10^{-4} M of eserine and PHMB for preincubation.

^dIsozyme distribution, see Figure 6b.

1. No induction in median bands was detected in this group of insects.
2. All the fast bands were inducible in this group of flies.
3. The similarity of hydroperene induction between these two groups of fly is that the slow band observed in this and the other group are inducible no matter what dose of hydroperene was used.

The CE-ase assay showed that the total CE-ase induction of hemolymph CE-ase was 95.9 percent net increase of enzyme activity. The increased activity of CE-ase in hemolymph somehow corresponds to the hydroperene esterase induction in hemolymph.

Part 5. The Substrate Specificity of Isozymes

The esterases of adult female flies were recovered from acrylamide gel according to the method described in the "Methods" section. All three fly species, including two house fly strains, were used. The soluble fraction was the major esterase source for this study. Because of the shortage of labeled substrates, no preliminary experiments were done to estimate the proper combination of non-labeled and labeled chemicals for the enzyme study. Therefore, only labeled JH-1 and hydroprene were used as substrates in these experiments.

The enzyme activity of each band or group of bands was estimated as a unit in the following way:

$$\text{enzyme activity} = \frac{[\text{percentage of produce (acid) produced by that band(s)}]}{[\text{the total percentage of product produced by all of the recovered esterases}]}$$

The percentage of acid produced by the recovered enzyme is also listed in the tables.

Flesh Fly Soluble Fraction

Nine-day-old female flesh flies were used for this experiment. The esterases recovered from the gel were incubated with labeled JH-1 or hydroprene. Seven regions on the gel were extracted, the anode band, the 1st slow band, the 2nd slow band, the median bands (with several isozymes), the 1st fast band, the 2nd fast band and the cathode band.

Table 17a shows that the esterases extracted from the slow bands and the cathode band were the most active of those examined. The 1st fast band and the cathode band also convert smaller amounts of JH-1 into acid. The median bands, however, showed less activity. No activity could be detected in the 2nd fast band, possibly because of the low concentration of the esterases recovered from the gel.

Against hydroperene, only the cathode band had unusual activity. All the others, except the 2nd fast band, were slightly active. The results strongly suggest that the cathode bands contained the esterases that break down hydroperene in the soluble fraction. On the other hand, slow bands and the anode band are responsible for the hydrolysis of JH-1.

Blow Fly Soluble Fraction

Ten to eleven-day-old female blow flies were used for the experiment with the soluble fraction. According to the staining response of the esterases in the gel, six bands were present and these were extracted. These bands were classified as one anode band, one slow band, median bands with four isozymes, two separated fast bands, and a cathode band.

Table 17a shows that none of the esterases were very active against JH-1 and that the 1st fast and the 2nd fast bands were inactive. The anode band had no enzyme activity for hydroperene and the others were only slightly active. The results of this experiment indicate a similarity in esterase activity between the soluble

Table 17a. Recovered enzyme activity to juvenile hormone-1 and hydroprene from soluble fraction of flesh flies and blow flies^a

Band	Percent JH-1 hydrolyzed ^b		Percent hydroprene hydrolyzed ^b	
	Flesh fly	Blow fly	Flesh fly	Blow fly
Anode	12.64	10.45	9.57	0
Slow ₁	11.12	2.34	7.22	2.73
Slow ₂	26.45	6.40	6.05	1.77
Median	7.26	1.90	8.40	4.22
Fast ₁	10.09	0	8.13	3.33
Fast ₂	ND ^c	0	ND	1.58
Cathode	8.77	7.40	19.04	2.37

^a9-day-old female flesh flies, 2.67 flies equivalent per extraction. 10-11-day-old female blow flies, 4.28 flies equivalent per extraction.

^bPercent of substrate hydrolyzed by isozyme. JH-1 incubated for 1 hour, hydroprene, 3 hours. Average of duplicates.

^cNot detectable after 5-10 min staining.

fraction of the flesh fly and blow fly.

House Fly Soluble Fraction

Five to six-day-old female CSMA house flies were used for the soluble fraction studies. Four sections of gels were examined: one slow-moving group with two bands, median-moving groups with four bands, one fast band, and one cathode band (Table 17b).

The esterases from the slow bands were able to hydrolyze JH-1 producing about 58.73 percent of the total acid produced by all of the extracts from the fel. No metabolism of the substrate was seen in the esterases from the median bands. The esterases from the cathode band were also active. Only the esterase from the cathode band had significant activity against hydroprene.

The hemolymph was examined in the case of the Rutgers flies. Four groups of esterases were obtained from the 3-4-day-old flies; Figure 18b: one anode, two separated slow bands, and one cathode band. The median bands and the fast band could not be detected.

All of the esterases recovered from the gel were active against JH-1, the two slow bands producing slightly more JH-1 acid, Table 17b. The table also shows that the activity of the recovered enzymes to hydroprene was about equal. The result differs from those of the other flies, especially in the capability to degrade hydroprene by the esterase in the cathode band.

Three to four-day-old female flies were used in the study of Rutgers fly soluble fraction. Five groups of esterases were

Table 17b. Recovered enzyme activity to juvenile hormone-1 and hydro-
prene from soluble fraction or hemolymph of house flies^a

Band	Percent JH-1 hydrolyzed ^b			Percent hydroprene hydrolyzed ^b		
	Rutgers soluble fraction	Hemo-lymph	CSMA soluble fraction	Rutgers soluble fraction	Hemo-lymph	CSMA soluble fraction
Anode	7.92	ND ^c	ND	12.30	ND	ND
Slow ₁	46.74	30.50	13.76 ^d	15.80	11.70	1.44 ^d
Slow ₂	21.60	29.20		15.40	16.05	
Median	0.00	24.80	0.00	17.30	11.50	0.00
Fast ₁	ND	ND	2.46 ^d	14.70	ND	0.69 ^d
Fast ₂	ND	ND		0.00	ND	
Cathode	28.01	26.60	7.14	ND	14.70	19.57

^a5-6-day-old female CSMA house fly, 8 flies equivalent per extraction. 3-4-day-old female Rutgers house fly, 8 flies equivalent per extraction for soluble fraction, 10 flies equivalent per extraction for hemolymph.

^bPercent of substrate hydrolyzed by isozyme. JH-1 incubated for 1 hour, hydroprene, 3 hour. Average of duplicates.

^cNot detectable after 10-5 min. staining.

^dTotal enzyme activity of slow bands, or fast bands.

detected: anode band, two separated slow bands, a median band, and a cathode band. Table 17b shows that four of the five bands were active against JH-1, some with considerable activity.

Part 6. Age-Dependent Variation in CE-ases,
Additional Experiments

In all of the experiments described to this point, separate colonies of flies were used for the studies of CE-ase, JHA-ase and JH-1-ase. In some cases, because of shortages of flies of the proper ages, it was necessary to use overlapping colonies of insects. In other words, it was not always possible to complete an age-profile study with the same group of flies. In an attempt to improve the data collected in this way, additional experiments were performed. All three enzymes were assayed in the hemolymph of different ages of flies taken from the same large colony. Using the hemolymph volume data collected earlier (Figure 2) the total quantity of each enzyme was estimated for each of the ages examined. Parallel electrophoretic and enzyme assay experiments were also conducted. In this case, the samples were split with one part being used for the enzyme assays and the other for the electrophoreses.

Figure 8a shows that the activity of the hydroperene esterase and CE-ases of flesh fly hemolymph reached their maxima at days 9 and 13. The JH-1-ase was generally low through days 1 to 8 with peaks at days 10 to 12. This indicates that the activity of this enzyme is not related to the hydroperene esterases, or CE-ases.

The age profile of the esterases in the hemolymph of the blow fly is shown in Figure 8b. There was very high JH-1-ase activity at emergence and this dropped steadily to a nearly undetectable

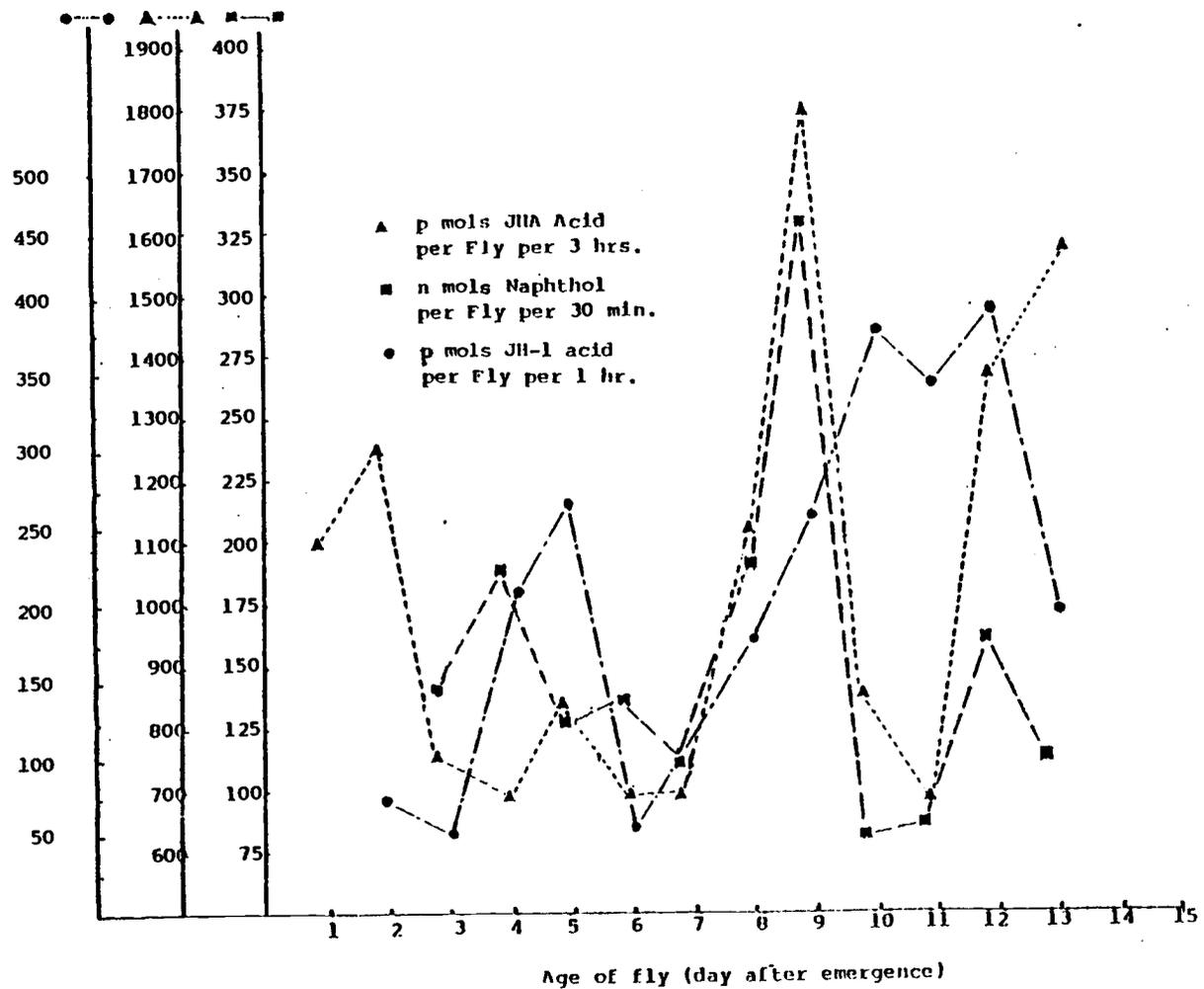


Figure 8a

Age-profile of carboxyl, hydroprone, and juvenile hormone-1 esterases in hemolymph of female flesh flies

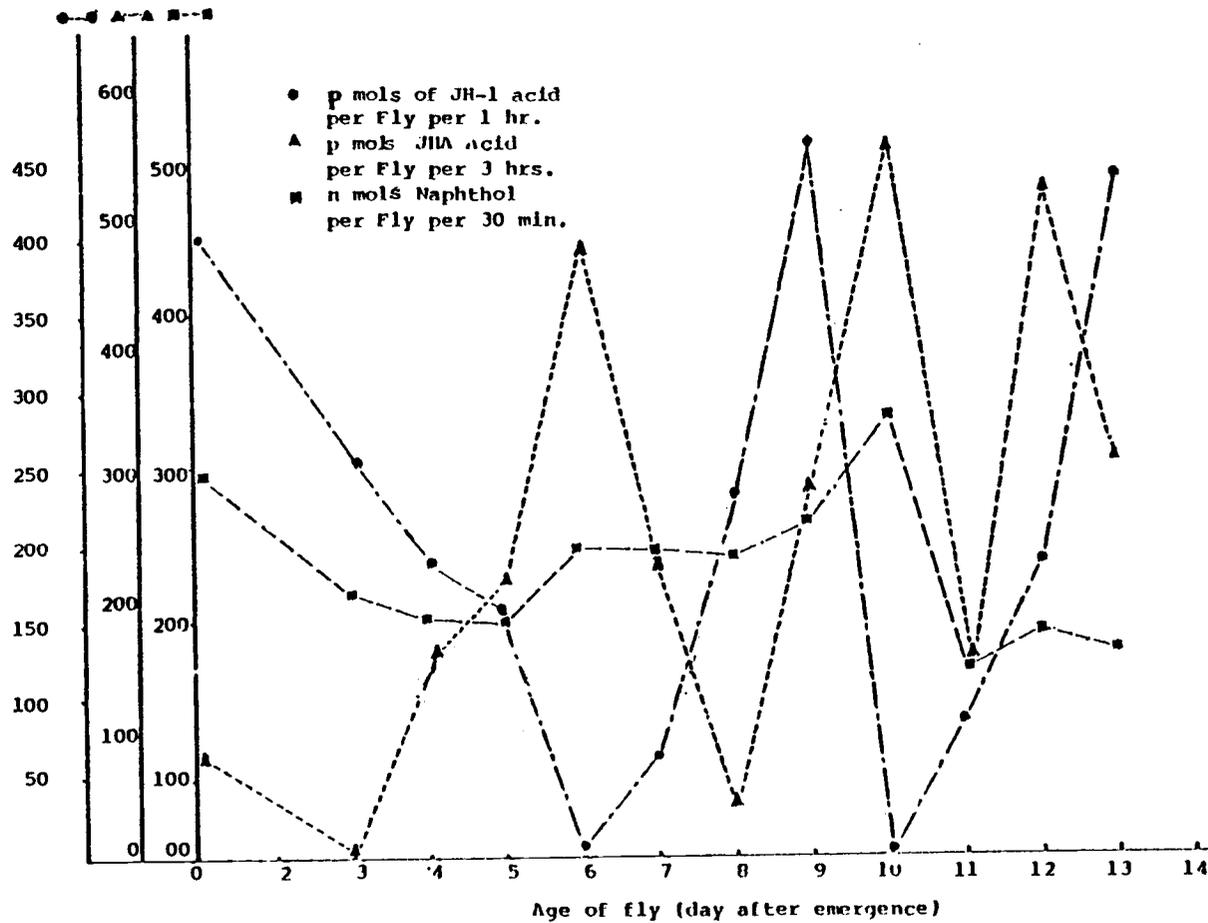


Figure 8b

Age-profile of carboxyl, hydroprene, and juvenile hormone-1 esterases in hemolymph of female blow flies

level at day 6 at a time when the JHA-ase level was at its maximum. JH hydrolyzing activity reached another peak at day 9 and at day 13 whereas the peaks for JHA-ase activity were at days 10 and 12. The CE-ase profile showed peaks at day 10, coinciding with the JHA-ase peak and dropped to a low level at day 11, also in coincidence with JHA-ase. These patterns suggest that the CE-ases and the JHA-ases are different from the JH-1-ases.

In the experiments with Rutgers flies, it was not possible to assay for all three enzymes in the same sample. Therefore, two separate colonies were used, one for CE and JHA-ase and one for JH-1-ase. Figure 8c shows that the activity of the hydroprene esterases peaked at day 3 and day 8. CE-ases activity, with NA as substrate was most active during day 8. JH-1-ases, on the other hand, peaked at days 5, 6 and 8.

There was some overlapping of enzyme activity between hydroprene esterase and the CE-ases especially in the hemolymph of day 8 flies. This suggests that there is a direct relationship between these two esterases in both blow fly and flesh fly. However, there appears to be no direct relationship between the hydroprene esterases and the JH-1-ases.

According to Figure 8d, the JH-1-ase activity of CSMA house fly hemolymph reached its peak at 3 days and declined sharply thereafter. The JHA-ase also peaked about days 3-4 but declined only moderately at day 7 and increased again at day 8. This indicates that two

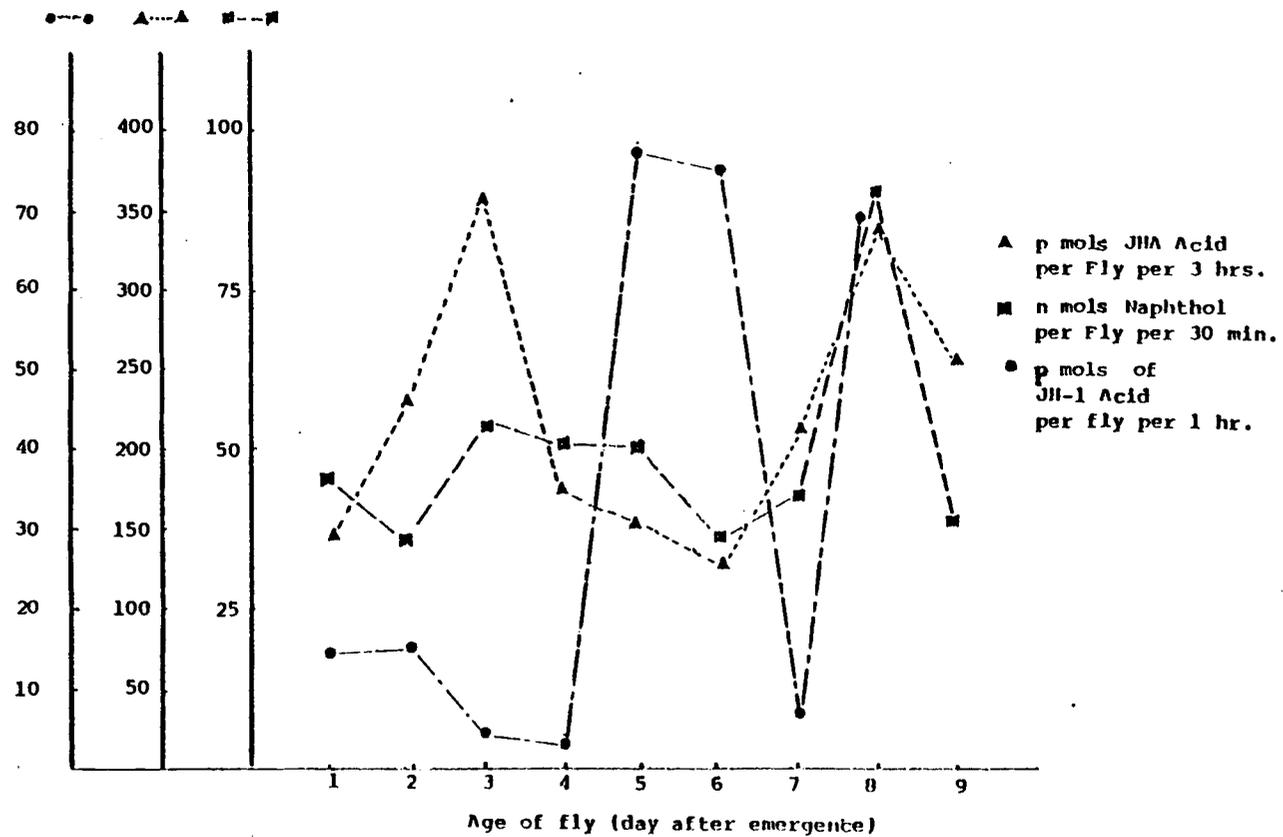
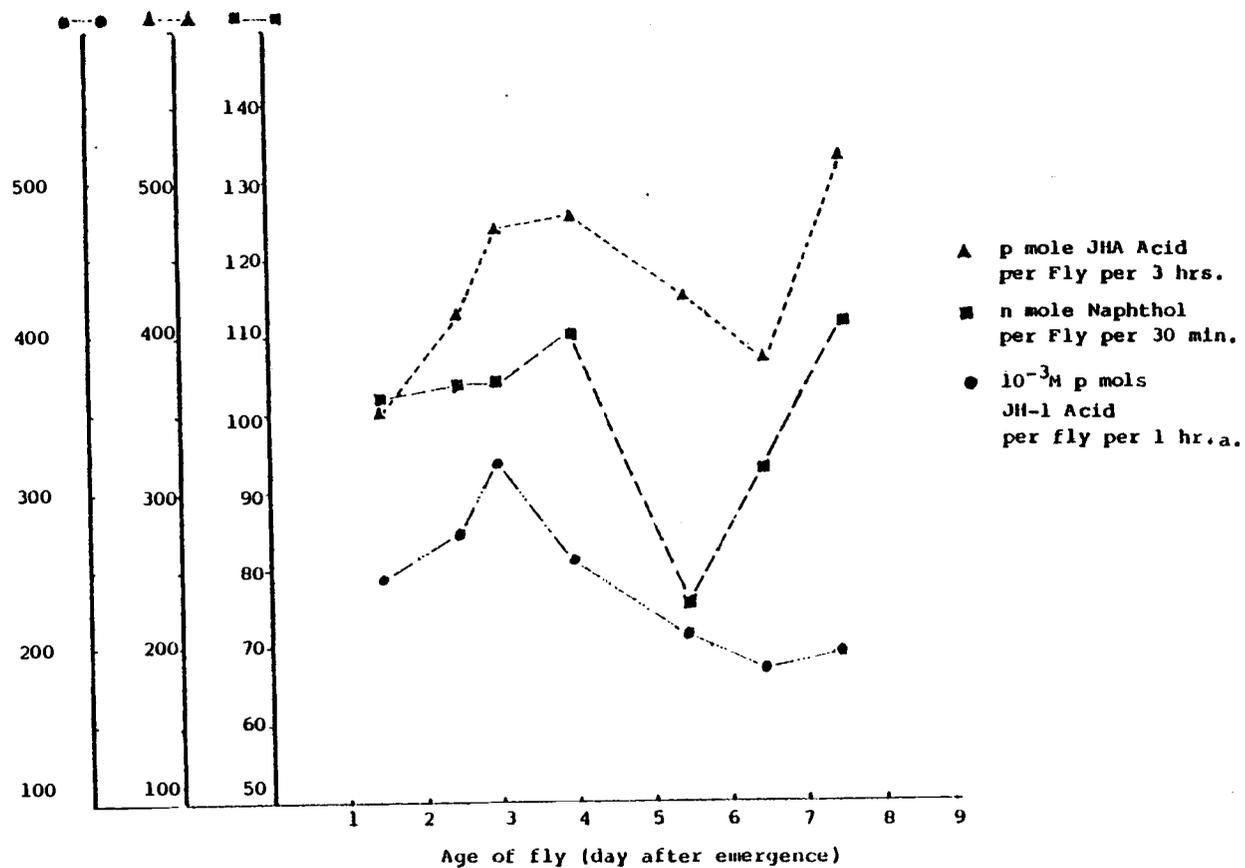


Figure 8c

Age-profile of carboxyl, hydroptene, and juvenile hormone-1 esterases in hemolymph of female Ruggers house flies



^aOnly labeled juvenile hormone-1 was used

Figure 8d

Age-profile of carboxyl, hydroprone, and juvenile hormone-1 esterases in hemolymph of female CSMA house flies

different enzymes are involved in the hydrolyses of these substrates. The pattern of the CE-ase was more or less like that of the JHA-ase with a peak at day 4 and a second peak at day 8. From this it would appear that the hydrolysis of NA and hydroprene is catalyzed by the same group of esterases.

Correlation of Enzyme Activity with Electrophoretic Patterns

An examination of relationships between enzyme activity and flesh fly isozyme distribution patterns is depicted in Table 18a. Selected samples of flesh fly hemolymph and soluble fraction (not cytosol) were assayed for CE-ase and JHA-ase activity and aliquots of the same samples were electrophoresed by the slab gel technique. The zymograms of the five hemolymph samples were identical except in two respects, there was an extra fast band in sample B and an extra slow band in sample A. The extra fast band (sample B) did not appear to coincide with unusual enzyme activity but the extra slow band (sample A) did coincide with the maximum JHA-ase activity, 18.1 percent of hydroprene metabolized per incubation in 3 hours.

The lower half of the table shows corresponding data for the five soluble fractions. All of the zymograms were identical except those for samples A and B where the fast band was missing. There were no obvious relationships between these two zymograms (A and B) and the esterase activities in the same samples. Except for evidence that the isozymes represented by the fast bands were not involved in the hydrolysis of either substrate (no increase in enzyme

Table 18a. Carboxylesterase activity to naphthyl acetate and hydro-
prene with corresponding carboxylesterase zymogram of
flesh fly hemolymph and soluble fraction

Age of fly ^a	CE-ase ^b	Enzyme activity			Slab electrophoretic zymogram ^c		
		Hydroprene ^b esterase ^b	ug protein /gel		Hemolymph		
					—	→	+
A	560.8	18.1	182.2				
B	764.2	10.1	188.7				
C	872.3	4.1	218.6				
D	1440.2	7.2	163.6				
E	755.0	2.7	277.1				
Soluble Fraction							
A	143.1	12.8	403.6				
B	470.0	11.9	264.0				
C	233.6	15.0	272.4				
D	320.9	8.4	247.6				
E	244.8	7.3	321.8				

^a(A) 8-9-day-old fly; (B) 9-10-day-old fly; (C) the day of de-
positing larvae; (D) 1 day after larva-depositing; (E) 2 days after
larva-depositing.

^bn mols naphthol/mg protein/30 min., percentage of hydroprene
metabolized per incubation.

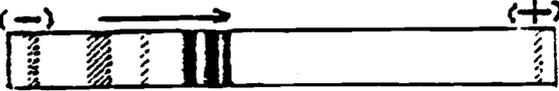
^cZymogram of soluble fraction CE-ase not clear separated in slow
and median moving area.

activity when they were present), the slab gel method did not give sufficient resolution to establish any other relationships.

Experiments with the Rutgers strain of house flies using the tube gel method are summarized in Table 18b. These studies were conducted in the same way as those described above except that the substrate was JH-1. The most obvious association between enzyme activity and isozyme patterns is that seen in the experiments with the five and six-day-old flies. A large increase in JH-1-ase activity on day 5 was associated with the appearance of slow-moving bands in the zymogram. It is also evident that the median bands which were very dense at days 2-3 were less prevalent at days 2-6, which could mean that these isozymes have nothing to do with JH-1 hydrolyses.

The pattern is somewhat the same in the soluble fraction experiments except that the increase in JH-1 activity occurred on day 8. However, this increase was associated with the appearance of a heavy slow-moving band.

Table 18a. Carboxylesterase activity to juvenile hormone with corresponding carboxylesterase zymogram of Rutgers house fly hemolymph and soluble fraction^a

Age of fly (day)	n mols acid/ mg/hr ^b	Disc electrophoretic zymogram
Hemolymph		
2-3	0.056	
4	0.031	
5	2.056	
6	2.056	
8	1.710	
Soluble fraction		
2-2.5	12.67	
8	24.07	
8 ^c	20.53	
15	22.48	

^aAverage of duplicates.

^bJuvenile hormone-1 as substrate with 25 ul hemolymph or 1.25 fly equivalent of 105.000 g supernatant.

^cEnzyme stored in -16 C° for 2 weeks.

DISCUSSION

The simple molecular equation, one gene-one catalytic reaction, was a useful and stimulating generalization in the early days of biochemical genetics. For the past two decades, the concept that every enzyme is encoded by a gene and that every gene may mutate to produce functioning alleles has been widely accepted. This has led to the belief that most enzymes will eventually be found to exist in isozymic forms. Another concept is that organisms synthesize many of their enzymes in several different molecular forms, presumably to fulfill specialized metabolic requirements. Now, it is known that multiple varieties of an enzyme are needed to catalyze the same reaction, but under different metabolic conditions, or in different parts of the same cell, or in the same cell at successive stages of differentiation (Markert, 1975).

In recent years, progress in the understanding of isozymes has provided insight into the genetic and structural interrelationships of multiple enzyme forms, the physiological significance of this multiplicity, and the control of enzyme synthesis during the ontogenesis of insects (Menzel, *et al.*, 1963; Whitemore, *et al.*, 1972; Gunter and Wern, 1973; Weirich, *et al.*, 1973; Eguchi and Iwamoto, 1975; Korochkin, 1975; Sanburg, *et al.*, 1975; Nowock, *et al.*, 1975; Yu and Terriere, 1975, 1977a, 1977b, 1978; Vedbrat and Whitt, 1975; Ahmad, 1976; Kramer, *et al.*, 1979; Klages and Emmerich, 1979; Bigley and Vinson, 1979; Peter, *et al.*, 1979; Sparks and Hammock, 1979; and

Sparks, et al., 1979). Therefore, it has become increasingly evident that a comprehensive approach is necessary in this type of study in order to avoid incorrect interpretations of the data.

These principles apply with particular force to the carboxylesterases of dipteran insects, because of the extensive heterogeneity of these enzymes and their overlapping biochemical characteristics. It is with this background in mind that this intensive treatment of hemolymph carboxylesterases has been presented as a unified study. The desirability and advantages of this procedure are indicated further during the following discussion.

Figure 1 indicates that the hemolymph volume of the flies fluctuated throughout the life-span. Different patterns of blood volume fluctuation were found in different species, Figure 2a,b. In the flesh fly the maximum and minimum volumes occurred during day 9 to day 10. The difference was about two-fold with a fluctuation cycle of 7 days. In the blow fly the volume increased from day 6 forming a plateau until day 14. In house flies, the volume varied less than 1.5-fold with peaks showing at day 3 and day 8, a fluctuation cycle of 6 days.

Slama (1964) noticed that the hemolymph volume of adult *Pyrrhocoris apterus* increased at the beginning of the reproductive cycle as protein built up. In the house fly (Table 2c,d) the blood protein built up in a gradual accumulation which began soon after dietary protein was fed, and vitellogenesis began. This phenomena was already noticed by Adam and Hintz (1969) and Adams and Eide

(1972). In *Glossina austeni* maximum hemolymph volume was associated with maturing oocytes at day 16 and minimum hemolymph volume with larvae deposition at day 20 (Tobe and Davey, 1972). A similar result was found in the flesh fly.

The influence of hemolymph volume on the total esterase activity can be seen in the following estimation of general esterase activity based on different calculations. When the esterase activity was estimated based on per unit of volume of hemolymph, it was found that the high enzyme level in 9-day-old flesh fly was 116.8 μ mole product per μ l of hemolymph, whereas the low enzyme level in 4-day-old flies was 38 μ mole: a difference of three-fold. It was also three-fold in the case of the blow fly, but only two-fold in difference in case of the Rutgers house fly. When the per fly general esterase activity was considered the difference became 4.4-fold for flesh fly, 4.7-fold for blow fly, and 2.6-fold for Rutgers house fly. Therefore, the esterase activity was enhanced by the volume factor.

The enzyme activity of hemolymph on a per fly basis, therefore, amplified the difference between the flies with low esterase level and the flies with high esterase level. An example of the difference can also be found by comparing the results in Table 4a,b and Table 5. In the house fly the variation of enzyme activity between low level and the high level is not so obvious as the flesh fly. In this study the completion of a reproductive cycle was judged by the length of the long diameter of the oocyte. In the

case of the flesh flies and house flies the volume fluctuation of hemolymph was related to the reproductive cycle of the flies. On the other hand, the esterase activity of hemolymph also varied coincidentally with the hemolymph volume in flesh fly (Table 2a).

Simon (1969) noticed that in *Culex pipiens fatigans* the esterase activity increase, with specific fluctuations, during each intermoult period. In this study it was found that cyclically repeating peaks of esterase activity coincided with volume built up in hemolymph, and the growth of oocytes, suggested relationship among these three phenomena.

It is clear that both hemolymph volume and the esterase activity (based on unit of volume of hemolymph) should be considered as basic elements for evaluating the hemolymph esterase capacity when critical comparison study of enzyme activity are made. The estimation of esterase activity per fly eliminated the possible confusion caused by the variation of enzyme concentration in the hemolymph.

On the other hand, the enzyme activity can also be measured based on per unit of hemolymph volume, or based on blood protein content. The values of enzyme activity per unit of volume does not necessarily match that shown per fly. Enzyme activity based on blood protein varied drastically depending upon the concentration of protein. In the hemolymph assays this measurement was not dependable since there was no corresponding relationship between the protein content and the activity of the esterases. In some cases,

however, the high esterase activity based on blood protein did correspond with the activity based on unit of volume.

When the daily average esterase activity (based on volume) was measured (Table 1a-c, 2a-d), the activity of female flies was higher than that of the male flies. A similar result was found in other insect species (Cook and Forgash, 1965). The female flesh fly and blow fly had the highest general esterase activity, nine times that of the Rugter house flies. Esterase activity of the CSMA house flies was as much as four times that of Rugters flies. The lower esterase activity of resistant house flies was noted by Van Asperen (1962). The result from this study confirm this although different strains of house flies were used.

The maximum general esterase activity in the hemolymph of these flies was found at days 8-11 for flesh flies, days 7 and 12 for blow flies, and days 5-6 and day 9 for Rugters house flies. These dates correspond to the longest long diameter of the oocytes. The low hemolymph volume and low esterase activity were found just after egg deposition.

Most previous reports do not provide specific esterase activity during the ontogenetic development of the adult stage of cyclophora insects except to note the increasing esterase activity during adult maturation. The present results, however, make evident a more graduated response. While female flesh fly hemolymph exhibited a sharp fluctuation in volume as well as in general esterase activity during the adult maturation, blow flies and house flies showed

relatively minor changes in both blood volume and enzyme activity. This indicates that esterase activity varies from species to species and that age dependence of enzyme activity is a common phenomenon in the cyclophora insects studied thus far.

When the fly emerges it is expected that esterase activity should be high, according to Nemev (1972), who found high esterase activity in freshly ecdysed adults. In this study a rather high CE-ase was found in one-day-old flies, but not a major peak (see Table 2b,c and 4a-d). A large volume of hemolymph in blow fly and house fly was expected since it was known that reabsorption of pupal fluid into the adults before emergence would drastically increase the volume of hemolymph (Jones, 1977).

A large volume of hemolymph was found in one-day-old flesh flies but not in blow flies or house flies. The reason for this in these species is probably because the one-day-old flies had already excreted the extra body fluid before the assays.

These results show that it is necessary to consider the physiological age of an insect species in a critical comparison of these esterases.

Krueger and Casida (1961), using inhibitors, were able to detect choline esterase and aliphatic and aromatic esterases in the house fly. Inhibition experiments in the present study (Table 4a-d) revealed that the hemolymph esterase of these flies was mostly CE-ase, a B type esterase, the major esterase found in dipteran insects as well as in many other insect species (Townsend and

Bosvine, 1969; De Kort, 1979; Vedbrat and Whitt, 1975; Devonshire, 1975; Whitten and Bull, 1970; Cohen et al., 1977; Cook and Forgash, 1965).

Cholinesterase and arylesterase were minor esterases in the insect species tested by Lord and Potter (1953). Acetylcholinesterase activity was estimated as 1/4th to 1/150th of the total activity found in larvae and adults of many insect species. In agreement with this, I found that PHMB was a strong inhibition of only blow fly hemolymph, indicating that arylesterase was one of the major esterases in this species but not the other two.

The Rutgers house fly, which showed less CE-ase activity than other flies, was more resistant to OP inhibition. On the other hand, higher aliesterase activity was found in the susceptible CSMA house flies. Van Asperen and Oppenoorth (1960) and Townsend and Bosvine (1969) showed that the low CE-ase in soluble esterase activity of resistant house flies and the blow fly, *Chrosomya putoria*, was demonstrated due to a "mutant gene." The results in this experiment confirmed their report, indicating a ratio of esterase activity between the two house fly strains being 7 to 1 in favor of the susceptible strain.

Thus, this study confirms the finding that the composition of esterase varies with the fraction of tissue, stage, strain and species studied, not only as in other species (Vedbrat and Whitt, 1975; Korochkin, 1975; Eguchi and Ewamota, 1975; and Cohen et al., 1977), but also in cyclorrhapha insects (Menzel, 1970).

The variation of esterase activity in an ontogenetic study of insects revealed that the esterase activity was in the order adult > last feeding larvae > empty gut larvae > pupa (Cohen, et al., 1977; Korochkin, 1975; and Eguchi and Iwamoto, 1975). The same order of esterase activity was found in this study (Table 10a,b).

The differential expression of esterase isozymes during ontogenesis of these flies can be seen in the response to inhibitors, and suggests that the esterase activity of hemolymph and soluble fraction depends upon the fraction, age, stage, and the species of the flies.

The soluble CE-ase activity (Table 4a-d and Table 6a,b) also fluctuated throughout the life-span examined. The daily CE-ase activity can be ranked as high in flesh fly, medium in the blow fly and CSMA house fly, and low in the Rutgers house fly. The total activity of hemolymph esterase was rated 64 for flesh fly, 12 for blow fly, 7 for CSMA house fly, and one for Rutgers house fly. The variation in the capacity of the esterase is partially due to the body weight of the flies.

The CE-ase activity of soluble fraction is low compared with that of hemolymph. Brown and Matsumura (1961) also found a rather low esterase activity in the soluble fraction of various tissues or organs. The average enzyme activity of soluble fraction esterase of blow fly and flesh fly is around 250 n mole per mg of protein. It is 80 n mole for house fly strains. The fluctuation of soluble fraction esterase activity in the flesh fly amounted to a

difference within 24 hours.

A correlation of the fluctuation of esterase activity between the hemolymph and the soluble fraction of the flesh fly suggests that the soluble fraction served as the "enzyme pool" for hemolymph. This phenomenon was more evident in the electrophoresis study. No significant difference in soluble fraction esterase activity was observed in adult blow flies of different age. Like the general esterases, no relationship was found between enzyme activity and the protein content of the hemolymph of any fly species examined. The CE-ase activity fluctuated differently when expressed on unit of blood volume and on a per fly basis. The total esterase activity per fly was preferred in order to avoid the confusion caused by the different rate of vitellogenesis which is influenced by hemolymph volume (Bodnaryk and Morrison, 1966).

The variation in the susceptibility of the esterases to inhibitors which was seen in different tissue fractions from different stage of the flies indicated that a succession of gene sets in each fly which control the development of the homometaborus insects (Williams and Kafatos, 1971). On the other hand, the drastic change of hemolymph esterase activity might involve the migration of the esterase from tissue to the hemolymph and from hemolymph to the target organ (Laufer, 1958).

The electrophoretic study of the CE-ases revealed that the number and type of isozymes present in these flies also varied with tissue fraction, age, stage, and species of the flies examined.

Even when only hemolymph was used to evaluate the relationship between esterase isozymes and enzyme activity, the results were complex. The continuously changing composition of the hemolymph esterases resulted in no definite electrophoretic zymogram pattern for a single fly species, or for a strain, or even for a stage of the insect. This has been the case not only in house flies but also in many other dipteran insects, including Drosophila virilis (Korochkin, 1975), Culex pipiens pipiens (Pasteur and Singere, 1975), and Anopheles albuminus (Vedbrat and Whitt, 1975).

In this study, it was found that the hemolymph CE-ase zymogram of four-day-old flesh flies had 11 bands, whereas that of five-day-old flies had 13 bands. Thus there were two different patterns of CE-ase zymogram within 24 hours. This doesn't include the change of band-width and band-density. The variation in quality and quantity in the esterase composition was also observed in the zymograms of the cytosol esterases of flesh flies and the other fly species. Ahmad (1970, 1974) reported eight CE-ase bands with dim staining, in resistant house flies, and ten bands with dense staining, in susceptible house flies.

Both qualitative and quantitative differences in esterase zymograms were observed in resistant and susceptible house flies (Ogita and Kasai, 1965; Van Asperen and Oppenoorth, 1959; Menzal *et al.*, 1963; and Ahmad, 1970, 1974, 1976). Ontogenetic studies of general esterases also revealed a variation in zymogram pattern in various larval, pupal, and adult stages of flies (Vedbrat and Whitt, 1975;

Rauschenbach *et al.*, 1977; and Cohen *et al.*, 1977). However, information on the correlation of esterase activity with zymogram pattern is limited, especially in cyclorrhapha species. Ahmad (1979) indicated that 50% of soluble esterase activity was in the gut, and these were electrophoretically distributed at the cathode and anode bands. In this experiment the cathode and the anode bands were dim in the zymogram of five-day-old flies, and there was a corresponding low level of esterase activity. Instead, there were more dense slow bands. Ahmad (1976) also assumed that the isozymes in cathode band which was active against NA must be active in the cleavage of the JH. The result of the present experiments measuring isozyme patterns and esterase activity of hemolymph show that the hydrolytic activity against JH increased when the slow bands were not dense, days 5-6. In the same zymogram it is seen that the anode and the cathode bands were weakest in NA staining. The result of the enzyme recovery experiments also revealed that more JH was hydrolyzed by the isozymes in slow bands than by the enzymes in the cathode band. Evidence of high hydrolytic activity to JH was also found in the profile zymogram study of the soluble fraction of Rutgers house fly (Table 18b). It revealed that strong activity against the JH was present in one of the slow bands. Indeed, half of the total JH activity of soluble fraction was related to that slow band. The cathode band seems more associated with hydrolytic activity against NA and hydroprene.

The isozymes in the median bands that were strongest in NA

staining are believed to contribute most of the CE-ase activity in the hemolymph of all flies tested. Such median bands have also been found in many other insect esterase zymograms (Sudderuddin, 1973; Whitten and Bull, 1970; Cohen *et al.*, 1977; and Cook and Forgash, 1965).

In the flesh fly, which had numerous median bands with dense staining, there was an association with high hydrolytic activity to NA in both larval and adult stages. One of the median bands present in the zymogram of 8-9-day-old adult hemolymph and four-day-old larval hemolymph was associated with high hydroperene esterase activity. This band was probably being released from cytosol into hemolymph. This released band corresponded with a several-fold enhancement of hydroperene hydrolysis. Another extra fast band from the hemolymph was also released from the cytosol of 10-day-old flies, and that band did not show significant hydrolytic activity to hydroperene. The result of enzyme recovery experiment indicates that the anode band and/or fast bands of the soluble fraction might contain the major portion of the esterase that hydrolysed hydroperene.

The appearance and the disappearance of an isozyme in the hemolymph or in the cytosol is thought to reflect an active enzyme that effects the physiological condition in the fly, like the juvenile hormone esterase in the last instar larva of *Manduca sexta* (Whitmore *et al.*, 1972, 1974), or the prepupal esterase in *Drosophila virilis* (Rauschenbach *et al.*, 1977), or the epicuticular esterase in

Calpedos ethlius (Locke, 1964).

Nowosielski and Patton (1965) noticed the coincidence of the highest lipid concentration in hemolymph and sexual maturation in *Acheta domestica*. Briegel and Freyogel (1973) found that in the maturing ovary of *Aedes aegypti* the non-specific esterase in the median zone of zymogram increased to a maximum after the mosquito took a blood meal. They suggested that the increasing density of these bands was related to digestive function.

In nine-day-old flesh fly with an increasing hemolymph volume, maturing oocytes and maximum esterase activity occurred concurrently with the densest median bands. The esterase activity of the median bands was very low in the soluble fraction in all stages of the fly. This information with that mentioned above would suggest that the hemolymph esterases may function in digestion of lipid or the like for vitellogenesis. This digestive function may be due to the isozymes in the median band.

The age profile study of hemolymph esterases and the corresponding zymograms also reveal an overlapping of esterase activity to NA and hydroprene. It is possible that the same thing would happen in the other fly species. The result of enzyme recovery experiments, the zymograms of the esterases, and the corresponding esterase activities suggest that the median bands, the cathode band, and the anode band contained the major soluble esterase isozymes that hydrolyze NA and hydroprene. The slow band as well as the anode band probably contain the major enzymes that broke down the

JH. More evidence is needed to fully confirm these relationships.

The presence of esterases found in epicuticle of newly emerged *Calopes ethlius* (Locke, 1964), in the moulting fluid of *Antherea pernyi* (Katzellenbogen and Kafates, 1971), and that in ecdysis fluid which was reabsorbed by the newly emerged *Musca domestica* (Jones, 1977) would suggest that the fast bands found in hemolymph of newly emerged flies in these experiments are either related to lipid digestion or to the breakdown of JH in the epicuticle.

Numerous works on characterization of JH-esterase have been done on lepidopteran and coleopteran insect species. These reveal that the strong JH-esterase activity is contributed partially from one slow isozyme but mostly from several fast bands (Whitmore *et al.*, 1972; Sanburg *et al.*, 1975; and Kramer and Childs, 1977). It is also known that limited hydrolytic activity against JH is found in the dipteran species (Wilson and Gilbert, 1979; Slade and Zibitt, 1972; Ajami and Riddiford, 1973; and Weirich and Wren, 1976). Certainly, the JH-esterases implicated in regulating JH titer in the hemolymph of those lepidopteran and coleopteran insects do not seem to play as dominant a role in the dipteran insects examined, thus far.

In this study a dim slow band, corresponding with high JH-esterase activity, was found in hemolymph zymograms of flies at various adult ages. Also, the fluctuation of JH-esterase activity of fly hemolymph was different from that of NA-esterase and hydroprene esterase activity. Such difference in the fluctuation of NA and JH-esterase activity in the hemolymph of insects had been

found in many insect species (Sparks *et al.*, 1979; Nowock and Gilbert, 1976; and Kramer, 1979). In these flies, I found hormone esterase activity peaked on the days the flies emerged, or at the time when the maturing oocytes were formed.

The finding of a hormone esterase activity of 24 n mole acid per ml. of Rutgers house fly hemolymph in this study agrees with the enzyme activity found by De Kort *et al.* (1979). Although, low capacity of soluble hormone esterase was recognized in dipteran insects, the short half-life of exogenous JH in the house fly (De Kort *et al.*, 1979) makes it reasonable to expect that regulation of JH titer must be a functioning of the microsomal esterases (Yu and Terriere, 1978). However, it is interesting to note that there is a higher ratio of hormone esterase activity over the JH titer in the flies examined in this experiment. An explanation is as follows: The enzyme activity of hormone esterase is 44.5-89 n mole per gm for *Manduca sexta* larva (De Kort *et al.*, 1979), and 6 n mole per gam for flesh fly, 1.4-2.8 n mole for blow fly, and 1.2-2.4 for Rutgers house fly. On the other hand, the JH titer of adult *Manduca sexta* was estimated as 300-600 p mole per gm protein (Schooly *et al.*, 1979), which is 65 to 130 times as concentrated as that of the flies. The ratio of JH-ase activity over hormone titer would be around 0.3 to 0.6 for Rutgers house fly, 0.4 to 0.8 for flesh fly, and only 0.2 to 0.3 for *Manduca sexta*. Moreover, it has been found that the JH-ase activity of the 100,000 g supernatant of Rutgers house fly is 10 times as high as that of the hemolymph,

when mature eggs were formed in six-day-old house fly (Adam, 1969, 1974).

The higher ratio of JH-ase activity over JH titer in these flies may indicate that the concentration of JH at the receptor sites of the target organ is more important than the concentration of the JH in the hemolymph. Therefore, it seems that the soluble esterase and the membrane esterases all play different roles in JH regulation in dipteran insects. The hemolymph JH-ases, in this case, may regulate the JH titer in the hemolymph to a less extent. The microsomal esterases, on the other hand, may restrict the JH titer in the cytosol environment.

The mechanism of JH regulation in dipteran insect hemolymph is not yet known. A higher JH-ase activity in newly emerged adult was found not only in blow flies in this study but also in *Manduca sexta* (Nijhout and William, 1974). These esterases might be responsible for clearing the JH, thus insuring a successful adult ecdysis. It has been found that as the oocyte increased in length in *Locusta migratoria*, the JH titer increased (Johnson and Hill, 1973, 1975). It is also known that in some insects the increase in JH-ase activity is in good agreement with the decline in JH titer, and lends support to the hypothesis that specific esterases clear the hemolymph of JH at a critical period during development (Gilbert et al., 1977; Kramer, 1978).

This might explain why there is a JH-ase peak at the day when the oocyte had the longest long diameter. This also helps explain

the fluctuation of JH esterase in the hemolymph of the flies.

In some insects the general CE-ase which are ubiquitous throughout the insect can attack the unbound JH (Riddiford and Truman, 1978; De Kort *et al.*, 1978). In flesh fly the JH hydrolytic activity was found throughout the life-span of the fly indicating a strong general esterase activity to JH. When the critical period is reached, the hydrolytic activity carried out by the JH esterase, together with the general CE-ase, enhanced the JH metabolism.

The hydroprene esterase activity of the hemolymph of the flies showed a differential substrate specificity in the order adult > last feeding larva > wandering larvae > pupae, in all three fly species examined, the same order as the microsomal esterase activity (Yu and Terriere, 1975; and Terriere and Yu, 1977a, 1977b). This is a different order when compared to the esterase activity to NA in the respect that the higher CE-ase activity was present in pupae than in larvae, indicating a stage differentiation of hydroprene esterase to the substrate.

Hydroprene esterase activity was high in flesh fly hemolymph and low in blow fly hemolymph. The ratio of high level activity over the low level activity of hemolymph hydroprene esterase in flesh flies and house flies was estimated at around 6.5-fold. In blow flies it was less than two-fold. The variation was due mainly to the enzyme activity and to the volume fluctuation of the hemolymph. The age-profile study of hydroprene esterase in flesh fly hemolymph revealed that the variation of the enzyme activity was in a range

between 110 to 910 p mole per mg of proteins, a variation of eight-fold. This variation in activity occurred within 24 hours, indicating that the change in the esterase activity was more in quality than in quantity. In fact, the zymogram study did show an extra band in the hemolymph of the nine-day-old flesh fly. The isozymes had high activity to either NA or hydroprene.

Yu and Terriere (1975) and Terriere and Yu (1977a, 1977b) showed that adult house flies had a microsomal esterase activity to hydroprene ranging from 250 to 350 p mole product per mg of protein, with little fluctuation. In addition, they claimed that the microsomal esterase should be classified as a B-type, whereas those of flesh fly and blow flies should be classified as C-type.

This strongly suggest that two different hydroprene esterase systems are present in two different tissue environments, in hemolymph and in membranous structure, to carry out different biological functions in these flies. The average of hydroprene esterase activity in hemolymph is high in the flesh fly, four-fold that of the blow flies, and twice that of house flies. The immature stages of the blow fly, like those of adults, had the lowest esterase activity to hydroprene among the three fly species. This low hydroprene esterase activity of blow fly hemolymph in all mature or immature stages was assumed to be related to the high content of arylesterase in the hemolymph.

The age-profile studies of hydrolytic activity of hemolymph esterases to NA, hydroprene, and JH-1 reveal that the JH-ase activity

does not appear to coincide with a majority of the NA esterase activity in the hemolymph esterase zymogram studies. Also, the NA bands do not coincide with the JH-ase peaks on gel electrophoresis of hemolymph samples from various ages of flies. JH-ase showed weak staining response to NA. These minor bands not only coincide with JH-ase activity, but correlated with the presence of JH-ase activity. The low CE-ase activity coincides with a maximum JH-ase activity in the hemolymph of 5-6-day-old Rutgers house flies, of 1, 9, and 15-day-old blow flies, and of 5, 10 and 12-day-old flesh flies.

The results of the hydroprene induction experiments indicate that this juvenile hormone analogue is, on one hand, capable of stimulating carboxyl and hydroprene esterase synthesis, and, on the other hand, of depressing the enzyme production, depending upon the dose of hydroprene applied, tissue fraction assayed, age of the adults, and the insect species.

In general, the flesh flies and the Rutgers house flies are more responsive to the inducer than the blow fly. It was found that either the young flesh flies or the Rutgers house flies with one reproductive cycle completed showed the greatest response to the inducer, whereas in blow flies the response was least and shortest.

The age-dependence of enzyme stimulation has been observed in several insect species. The effect of an inducer on certain enzyme systems varied with the physiological stage of an insect (Reddy *et al.*, 1979; Kramer, 1978; Sparks and Hammock, 1979; Whitmore

et al., 1972).

Hydroprene has been found to function as synergists, compete with natural juvenile hormone in insects (Slade and Wilkinson, 1974), to inhibit other enzymes (Yu and Terriere, 1973), or to induce either the general CE-ase or JH-ase activity or both (Reddy et al., 1979; Kramer, 1978; Whitmore et al., 1978; Sparks and Hammock, 1979).

As Adams and Edie (1974) indicated, hydroprene has a gonotrophic effect on house fly ovaries inducing vitellogenesis, and a switch-over in the sensitivity of the prothoracic gland to externally applied JH and its analogues (Cymborowski and Stolary, 1979). Therefore, it is reasonable to expect that hydroprene may act as JH for JH-ase induction if JH regulation is involved in a feedback mechanism (Sparks and Hammock, 1979; Whitmore et al., 1972, 1974; and Yu and Terriere, 1971, 1973). However, it was also noted that not all the stages of an insect had the same response to the same inducer. Whitmore et al. (1972) could only induce JH-ase in *Hyalophora* pupae and Kramer (1978) were able to induce the hormone hydrolysis enzyme only in *Leptinotarsa* diapausing adult. Similarly, Sparks and Hammock (1979) could induce the hormone esterase activity in *Trichoplusia* larvae and Reddy et al. (1979) found that JH reduced esterase and NA esterases activity in larvae, but induced the enzyme in one-day-old pupae.

Mumby et al. (1979) found that carbamates had a low JH-like activity on dipteran insect, and had an inhibitory effect on house fly JH-ases. This group of N-alkyl carbamates of 1-naphthol did not show similar effects on other insect species. These results

clearly indicate that the phenomenon of enzyme induction varies from stage to stage, species to species.

A variation of hydroperene induction with various aged flies from different species would be expected in this study. Moreover, the different esterase isozyme species may be like different enzyme systems of the insect, responding differentially to the same inducer (Yu and Terriere, 1973). The biphasic response at variously physiological ages of the flies during the interval of examination should be understandable, although some other mechanisms may also be involved (Yu and Terriere, 1971; and Terriere and Yu, 1971).

In flesh flies the highest induction occurred with the hemolymph esterases, when the female adults initiated an increasing inconstitutive esterase hydrolytic activity, when the insects were five days old or older. In Rutgers house flies the induced high esterase activity was found in 7.5-day-old flies. The peak of the enzyme activity appeared a half day earlier than in control.

These observations lead to the assumption that hydroperene induction involves two mechanisms in flesh flies and house flies, triggering the release of esterase from cytosol into hemolymph, and inducing the synthesis of new esterase. The results of protein synthesis inhibition provide evidence that in both cases the induction involves a synthesis of new protein. In blow flies, the induced enzyme peak occurred one day earlier than in the controls. The Actinomycin D, however, did not show significant inhibitory effect on protein synthesis in blow flies, possibly because of the

dose of the inhibitor applied being less effective. Therefore, it is possible that in blow flies some other mechanism is involved in esterase induction.

It is reasonable to expect that dose-dependence of hydroprene induction of esterase activity occurred in these fly species as a feedback mechanism. The dose-dependence of esterase induction was also observed in the densitometric electrophoregram studies. As the result showed different esterase isozymes responded differentially to the inducer. Also, the response of the esterase to hydroprene could be induced, unaffected, or even depressed depending on the esterase isozyme species. However, it is difficult to explain the inhibitory effect caused by low doses of the inducer.

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