BIOCHEMICAL GENETICS OF EYE PIGMENTATION

IN THE AUSTRALIAN SHEEP BLOWFLY,

LUCILIA CUPRINA

by

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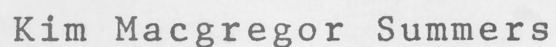

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FRONTISPIECE

From left to right, wild type; grape; tangerine; yellow; white. (The mutants yellowish and topaz^{1} have a similar eye colour phenotype to yellow. Topaz^{2} is similar to tangerine).

The five eye colour phenotypes of *Lucilia cuprina*. (See Chapter 2 for description).

STATEMENT

All the work reported in this thesis was carried out by myself except for the study reported in Chapter 7 where Dr N.A. Pyliotis performed the electron microscopy on material which I provided.

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ABSTRACT

The work in this thesis was undertaken primarily with the aim of elucidating the biochemistry of eye pigmentation in wild type and eye colour mutants of the Australian sheep blowfly, *Lucilia cuprina*. Initially, the characteristics of synthesis of the brown eye pigment, xanthommatin, in wild type, were determined. Levels of xanthommatin and its precursors, tryptophan, kynurenine and 3-hydroxykynurenine, throughout pupal life, were measured and developmental changes in the activities of three enzymes of the xanthommatin biosynthetic pathway were established. This allowed a comparison of xanthommatin biosynthesis in *L. cuprina* with that in other dipteran species.

Xanthommatin formation in eye colour mutants was then considered, to determine the functions of eye pigmentation loci. Three known loci are probably involved in the production of xanthommatin biosynthetic enzymes: yellowish (tryptophan oxygenase), yellow (kynurenine hydroxylase) and tangerine (phenoxazinone synthase). At least two other loci, white and topaz, are apparently concerned with the uptake of xanthommatin precursors into specific tissues and these processes were studied in some detail in wild type and the mutants. The results of this work draw attention to the critical role of the larval malpighian tubules in successful pigmentation of the adult eye.

The production of pteridine screening pigment was also examined. The yellow pigment of *L. cuprina* eyes was shown to be the pteridine sepiapterin and its production during pupal and adult life in wild type was measured. Analysis of levels of pteridine levels in the mutant strains revealed two which were deficient in sepiapterin, white and grape. The roles of these loci in the production of pteridine compounds is not yet known.

The ultrastructure of the eyes of wild type and mutants was also studied. Xanthommatin is probably deposited on round, electron dense granules in the primary and secondary pigment cells of the eye. In the xanthommatin deficient mutants, these granules are absent, but two abnormal forms are seen. There was no evidence that sepiapterin was deposited on granules and it seems likely that this pigment is present in unbound form in the eyes of *L. cuprina*. The eyes of grape insects were found to have an altered pigment cell morphology and those of white contained no pigment granules although there were lysosome-like vesicles in the pigment cells of white. The possible functions of the various granules found in wild type and mutants are considered.

The results presented in this thesis have identified the homologies of *L. cuprina* eye pigment mutants with those of *D. melanogaster*. *L. cuprina* can now be used for future studies of eye pigmentation which might be difficult with *D. melanogaster*, a smaller insect. An example of the

advantages of working with *L. cuprina* was provided by the studies of uptake and storage of xanthommatin precursors, where injection and dissection techniques for the larger insect were perfected. Further experiments using *L. cuprina*, which make use of these advantages, are discussed in the final chapter.

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CHAPTER 1
GENERAL INTRODUCTION

* A basic protein with properties somewhat similar to histone H2B has been found associated with the DNA in *Escherichia coli* (See Rouviere-Yaniv (1977). Localization of the HU protein on the *Escherichia coli* nucleoid. Cold Spring Harbour Symposium on Quantitative Biology XLII, 439-447). It has been postulated that this protein does play a role in condensing the bacterial chromosomal DNA, like the histone proteins in eukaryotes. However, the stability of the protein-DNA structure does not appear to be nearly as great as the histone-DNA structure.

** Defined messenger RNA's have been found in the cytoplasm of eukaryotic cells as free messenger ribonucleoprotein particles (m-RNP), non-associated with polyribosomes (See Revel and Groner (1978). Post-transcriptional and translational control of gene expression in eukaryotes. Ann. Rev. Biochem. 47, 1079-1126). The proteins of these m-RNP probably control the availability of the mRNA for translation.

1.1 GENE EXPRESSION DURING TISSUE DIFFERENTIATION

The mechanisms which control the selective expression of genes during differentiation in higher eukaryotes remain largely unknown. As with prokaryotes, control of gene expression may occur at any stage in the sequence DNA → RNA → final product. In eukaryotes, this sequence has many features not found in prokaryotes. These include:

a) greater sequence complexity of DNA;

b) binding of basic nuclear proteins to DNA;*

c) processing of RNA: capping, polyadenylation, cleaving and splicing;

d) interaction of RNA with proteins;**

e) the nuclear membrane;

f) variable stability of mRNA.

At the molecular level, a number of structural and functional features of eukaryotic chromosomes are beginning to emerge. For example, the interaction between DNA and histone proteins, the location and possible roles of highly repeated sequence DNA, the nucleotide sequence of genes (as revealed by molecular cloning techniques) and the mechanisms of hormone action have all been clarified in recent years. At the level of organisation of differentiated tissues, very little is known concerning the factors which regulate the expression of specific genes to bring about

the onset and maintenance of the differentiated state.

One model system which has often been used for studies of gene expression during development and differentiation in higher eukaryotes is the process of eye pigmentation in insects. The deposition of accessory pigments in insect eyes is developmentally regulated and the steps involved in the production of pigment are biochemically relatively simple. *Drosophila melanogaster* has frequently been used for such studies because of its well-characterised genetic system. The study of eye colour mutants in this species in the 1930's led to early ideas on the action of genes and the relationship between genes and enzymes (summarised in Ephrussi, 1942, and Butenandt, 1952). Since then, studies of gene-enzyme systems in *D. melanogaster* have accumulated much information concerning eye pigmentation in this species. At least 100 loci are known to affect eye morphology and/or eye colour and much work remains to be done to determine the role of each of these loci. Unfortunately, the small size of *D. melanogaster* makes some manipulations (for example, injection, transplantation and isolation of specific tissues for *in vitro* study) rather difficult. The approaches which have been used with *D. melanogaster* could be applied to another, larger, species if detailed biochemical and genetic information were available.

One species of fly about which there is now considerable genetic information is the Australian sheep blowfly, *Lucilia cuprina* (Whitten *et al.*, 1975). The genome is not so well marked as that of *D. melanogaster* but many mutations are known. These flies are much larger than the fruit fly. For example, pupae of *L. cuprina* are about 6 mm long, while those of *D. melanogaster* are only 2-3 mm long, and adult *L. cuprina* flies weigh about 25 mg compared with a weight of 1 mg for *D. melanogaster* adults. There is also considerable biochemical and morphological information concerning the events of metamorphosis in *L. cuprina* (Section 1.2). The events of eye pigmentation have not yet been studied in *L. cuprina*, and some knowledge of the process is necessary before the homologies of *L. cuprina* mutants with those of *D. melanogaster* can be made. The work reported in this thesis is a study of the biochemical genetics of eye pigmentation in *L. cuprina*, which establishes these basic homologies.

In this introduction, a brief review of the events of metamorphosis in flies is made (Section 1.2). This is followed by a detailed consideration of the process of eye pigmentation in insects, particularly flies, drawing mainly on studies of *D. melanogaster*. The biochemical and genetic features which make eye pigmentation a useful system in the study of differentiation are outlined.

1.2 *TISSUE DIFFERENTIATION DURING INSECT METAMORPHOSIS*

Metamorphosis is the term used to describe the process of change which occurs when insects pass from the sexually immature larval form to the reproductive form. The extent of change accompanying metamorphosis varies among insects and three metamorphic states have been described (Wigglesworth, 1972; Rockstein, 1973):

a) ametaboly, where there is essentially no change from larva to adult (silver fish, aphids);

b) hemimetaboly, where the active immature larva is gradually transformed through a number of stages to the adult which it resembles morphologically (termites, grasshoppers, cockroaches);

c) holometaboly, where the larval and adult forms are very different and the change occurs in one distinct pupal stage in which the insect is usually immobile and incapable of feeding (bees, beetles, butterflies, flies). Wigglesworth (1972) points out that these divisions are somewhat artificial, since the physiological changes which accompany the morphological changes are essentially the same. There are differences in the extent of change rather than sharply defined classes of change (Agrell & Lundquist, 1973). The nomenclature above will, however, be used occasionally in this thesis since it is a convenient way of expressing gross morphological differences. Metamorphosis in the order Diptera (flies) represents one end of this

continuum, where the changes are most pronounced (Agrell & Lundquist, 1973) and this will therefore largely be the subject of the following discussion.

Hormonal factors determine the developmental state of the insect (Gilmour, 1961; Wigglesworth, 1972; Gilbert & King, 1973; Willis, 1974; Emmerich, 1977; for reviews). The juvenile hormone, a terpenoid compound secreted by the corpus allatum (found in the ring gland in Diptera) may be a DNA synthesis inhibitor (Willis, 1974). It acts on the epidermal cells in larvae to ensure that they maintain their larval form. The cells are, however, only sensitive to the juvenile hormone in the presence of ecdysone, a steroid hormone produced by the thoracic glands (also found in the dipteran ring gland). An increase in the ecdysone titre leads to the initiation of a moulting cycle and the level of juvenile hormone directs the nature of the moult (larval → larval or larval → pupal or pupal → adult). Little is known about the action of the insect hormones or their transfer from site of synthesis to site of action. Ecdysone binding proteins have recently been found with the characteristics of mammalian steroid hormone receptors (see Ashburner & Lawrence, 1978), suggesting that ecdysone may act in the same way as the mammalian hormones. Possible effects of ecdysone at the gene level and its effect on chromosome puffing are discussed by Ashburner & Richards (1976).

In holometabolous insects following pupariation and the onset of adult development in the puparium, three morphological processes can be observed (Agrell & Lundquist, 1973). These are histolysis, histogenesis and differentiation. For Diptera, these changes have been reviewed by Perez (1910), Snodgrass (1924) and Robertson (1936). Bodenstein (1965) reviews the changes which occur in *D. melanogaster*, which will be the subject of much of the discussion in Section 1.3. General reviews of the morphological changes during insect metamorphosis are found in Crossley (1965) and in the volume edited by Etkin & Gilbert (1968).

During histolysis programmed cell death occurs. It involves the larval tissues, beginning late in larval life and continuing after puparium formation. It is hormonally induced and neurologically activated. Most larval tissues are histolysed during metamorphosis, particularly the fat body, salivary glands and to some extent the muscles.

The process of histogenesis builds the adult tissues to replace histolysed larval tissues. In Diptera, a few tissues (malpighian tubules, nervous tissue, blood vessels) survive from the larva, undergoing some reconstruction during metamorphosis. The muscles develop from imaginal myoblasts and reorganised larval nuclei. However, most adult tissues are derived from imaginal discs, clusters of cells set apart in the embryo and destined to form the basis of adult structures (Agrell & Lundquist,

1973; Kauffman *et al.*, 1978).

There are eleven pairs of imaginal discs in Diptera. The functions of the discs and their locations in *D. melanogaster* are indicated in Figure 1.1. The developmental fate of each disc is determined early in embryonic life. Discs located in a particular region of the embryo always develop into a predetermined structure even after transplantation into another area of the larva (Slack, 1978) except in rare cases of transdetermination (Agrell & Lundquist, 1973). During larval life, the imaginal discs undergo cell division (unlike the larval tissues which increase in size by cell growth; Bodenstein, 1965). The discs remain virtually undifferentiated until pupariation when there is rapid proliferation of the disc cells. From then their programme of development is irreversible. This irreversibility has been shown for the eye discs of *D. melanogaster* (see review by Ephrussi, 1942). An eye disc from a third instar larva, just before pupariation, transplanted into the abdomen of a young larva develops into an eye in exact synchrony with eye development in the host. If the donor is a few hours older, at least two hours after pupariation, the implant develops into an eye at the same time as it would have in the donor, regardless of the age of the host. Thus, just after pupariation, eye disc development becomes irreversible.

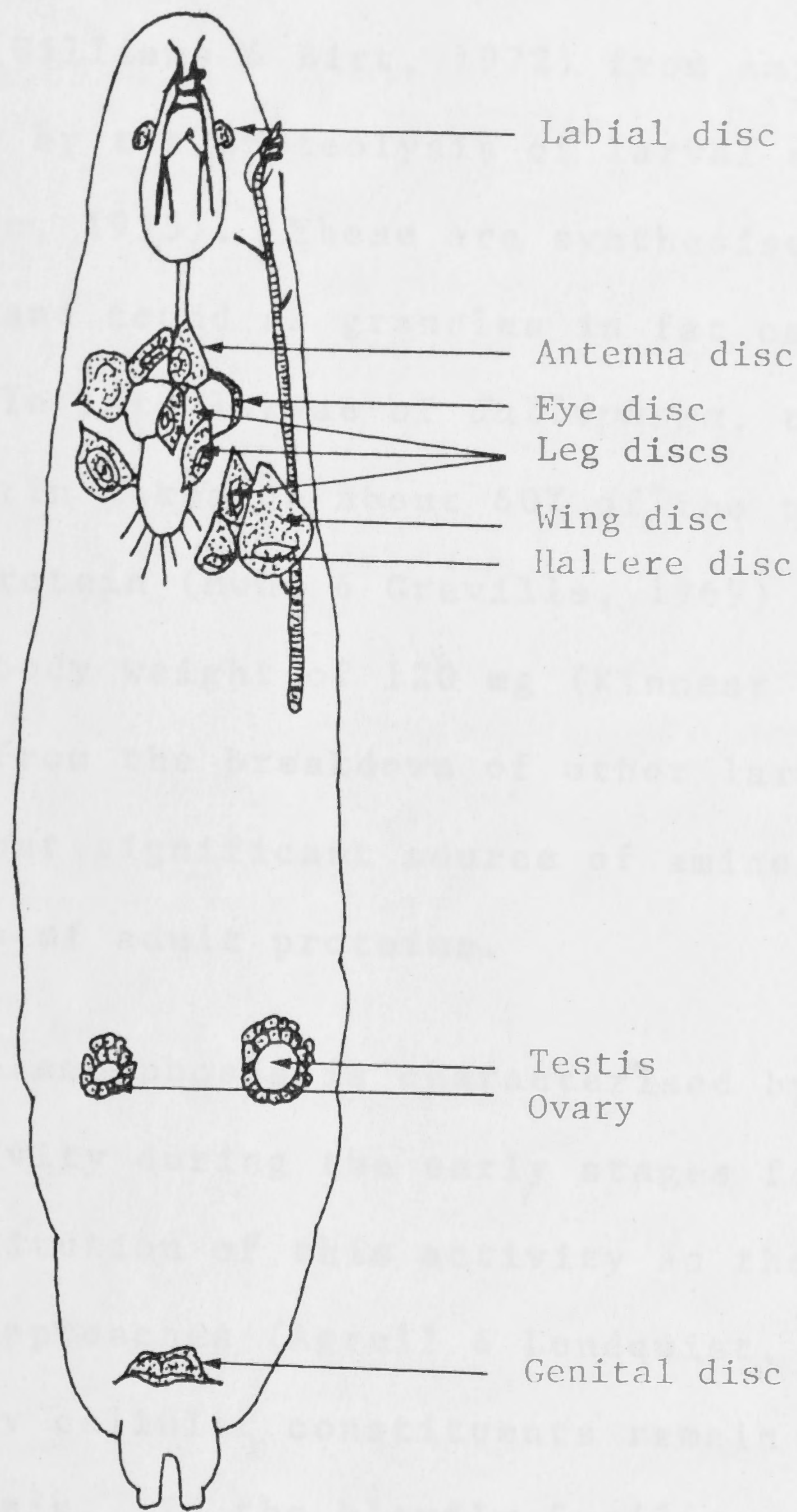


FIGURE 1.1
LOCATION OF IMAGINAL DISCS IN THE MATURE LARVA

The pharynx, brain and ventral ganglia and a portion of one main tracheal truck are shown. Some of the organ discs are shown on one side only, although all are present in pairs, except the fused genital discs.

From Bodenstein (1965)

In Diptera, synthesis of proteins for adult tissues occurs *de novo* (Williams & Birt, 1972) from amino acids provided largely by the proteolysis of larval storage proteins (Thomson, 1975). These are synthesised in the larval fat body and found as granules in fat cells during metamorphosis. In late larvae of *Calliphora*, the storage protein calliphorin makes up about 60% of the total buffer soluble protein (Munn & Greville, 1969) or about 6% of the total body weight of 120 mg (Kinnear & Thomson, 1975). Protein from the breakdown of other larval tissues is a subsidiary but significant source of amino acids for the synthesis of adult proteins.

Insect metamorphosis is characterised by a decrease in metabolic activity during the early stages followed by a successive restitution of this activity as the time of adult emergence approaches (Agrell & Lundquist, 1973). Nevertheless, many cellular constituents remain constant during metamorphosis. In the blowfly *Lucilia cuprina* these include total amino acids (Lennie & Birt, 1965; Birt & Christian, 1969), although some individual amino acids vary (Birt & Christian, 1969; Agrell & Lundquist, 1973), total protein (Lennie & Birt, 1967; Williams, 1972; Campbell, 1973), total carbohydrate (Crompton & Birt, 1967) and, to some extent, nucleic acids (Holmes & Birt, 1977). This constancy suggests that metamorphosis proceeds by closely coordinated breakdown and resynthesis of insect components.

The total nitrogen content also remains constant during metamorphosis. In holometabolous insects, excretion of liquid and solid wastes is not possible during the period of enclosure in the pupal case. Nitrogen is not excreted as ammonia at this stage (Birt & Christian, 1969; Agrell & Lundquist, 1973). Nitrogenous wastes accumulated during life in the puparium are collected in an excretory sac which forms from a section of the larval gut (Bodenstein, 1965). The waste material (the meconium) is excreted after emergence. The major nitrogenous compound found in the meconium is uric acid, but some other minor components are also present (Bodenstein, 1965).

The process of insect metamorphosis, like all differentiation processes, involves the activation and repression of sets of genes. The larval set of genes in Diptera includes those coding for storage proteins (at least 12 structural loci in *L. cuprina*; Thomson *et al.*, 1976), structural and enzymic components of larval cells, special larval secretions such as those from the salivary glands and enzymes of histolysis. These genes are active early in the insect's life but gradually give way to the adult set of genes as the development of the imago within the puparium proceeds. The adult genes include those for structural and enzymic proteins of adult tissues, proteins of the gonads and secondary sexual characteristics and other specifically adult structures. Genes apparently active only in pupae (such as the gene for leucine aminopeptidase; Beckman &

Johnson, 1964) are probably late-acting larval genes (Sakai *et al.*, 1969; Thomson, 1975). Many gene activities, in particular those coding for enzymes of intermediary metabolism, are common to both adult and larva.

Metamorphosis in holometabolous insects is thus a highly regulated series of events, in which genes are activated and repressed according to a predetermined programme to transform the immature larva into the adult form of the insect. Because of this controlled activation and inactivation of specific genes, various aspects of insect metamorphosis provide good systems with which to study development and differentiation. The differentiation of the pigment cells and the formation of screening pigments during the development of the adult eye from the larval eye disc is one system which has frequently been chosen in the study of gene control of development and differentiation. This system will be described in detail in the next section of this introduction.

1.3 *BIOCHEMICAL GENETICS OF EYE PIGMENTATION*

The eye colour in Diptera is due to the colour of pigments found in cells surrounding the photoreceptors. These accessory pigments play no part in light detection but are involved in optically isolating each facet

(ommatidium) of the compound eye from its neighbours. They have therefore been termed screening pigments, to distinguish them from the light sensitive visual pigments. The cells in which the screening pigments are found are the pigment cells, while the visual pigments are found in retinular cells. Further details of eye structure in flies are given in Chapter 7.

In all Dipteran species studied one screening pigment is the brown ommochrome xanthommatin which is derived biosynthetically from tryptophan (Wigglesworth, 1972; Linzen, 1974). In *D. melanogaster*, mutants which lack the brown pigment have bright red eyes caused by the presence of the red pteridine pigments called drosopterins. Drosopterins, like other 4-amino-2-hydroxy pteridines, are derived biosynthetically from guanine (Ziegler & Harmsen, 1969). There is a yellow pteridine, sepiapterin, found in small amounts in the eyes of wild type *D. melanogaster* and raised in level in the eyes of the mutant sepia (which fails to make drosopterins). In the blowflies and the housefly, mutants which lack xanthommatin have yellow eyes. This yellow colour is probably due to the presence of sepiapterin (see Chapter 6).

Biosynthesis of these screening pigments is under developmental control. In *D. melanogaster* the immediate precursor of xanthommatin, 3-hydroxykynurenine, is present in young pupae but xanthommatin synthesis does not begin until midway through pupal life (Ryall & Howells, 1974; see also

Chapter 3). A sudden onset of drosopterin synthesis has also been noted for *D. melanogaster* (Fan *et al.*, 1976). This occurs slightly later than the onset of xanthommatin synthesis.

1.31 THE XANTHOMMATIN BIOSYNTHETIC PATHWAY

Xanthommatin biosynthesis in Diptera involves a series of oxidation steps which convert tryptophan to xanthommatin (Figure 1.2). The steps are catalysed by different enzymes, each of which is coded for at a distinct genetic locus. In many insect species, mutations at several loci have been found to cause deficiencies in the synthesis of xanthommatin. Frequently these mutants lack activity of a pathway enzyme and accumulate the substrate of the missing enzyme, especially during pupal life. Intermediates accumulated during this stage are often excreted with the meconium soon after emergence (Gilmour, 1961; Dustmann, 1968; Bonse, 1969; Howells *et al.*, 1977). Some conversion to other minor tryptophan metabolites such as transamination products of kynurenine and 3-hydroxykynurenine (kynurenic acid and xanthurenic acid respectively) also occurs (Danneel & Zimmerman, 1954; Leibenguth, 1970, 1971).

The first enzyme of the xanthommatin biosynthetic pathway is tryptophan oxygenase. Since this catalyses the conversion of tryptophan to formylkynurenine, mutants lacking tryptophan oxygenase activity are characterised by a build-up of tryptophan and an absence of subsequent

Chapter 3). A sudden onset of drosopterin synthesis has also been noted for *D. melanogaster* (Fan *et al.*, 1976). This occurs slightly later than the onset of xanthommatin synthesis.

1.31 THE XANTHOMMATIN BIOSYNTHETIC PATHWAY

Xanthommatin biosynthesis in Diptera involves a series of oxidation steps which convert tryptophan to xanthommatin (Figure 1.2). The steps are catalysed by different enzymes, each of which is coded for at a distinct genetic locus. In many insect species, mutations at several loci have been found to cause deficiencies in the synthesis of xanthommatin. Frequently these mutants lack activity of a pathway enzyme and accumulate the substrate of the missing enzyme, especially during pupal life. Intermediates accumulated during this stage are often excreted with the meconium soon after emergence (Gilmour, 1961; Dustmann, 1968; Bonse, 1969; Howells *et al.*, 1977). Some conversion to other minor tryptophan metabolites such as transamination products of kynurenine and 3-hydroxykynurenine (kynurenic acid and xanthurenic acid respectively) also occurs (Danneel & Zimmerman, 1954; Leibenguth, 1970, 1971).

The first enzyme of the xanthommatin biosynthetic pathway is tryptophan oxygenase. Since this catalyses the conversion of tryptophan to formylkynurenine, mutants lacking tryptophan oxygenase activity are characterised by a build-up of tryptophan and an absence of subsequent

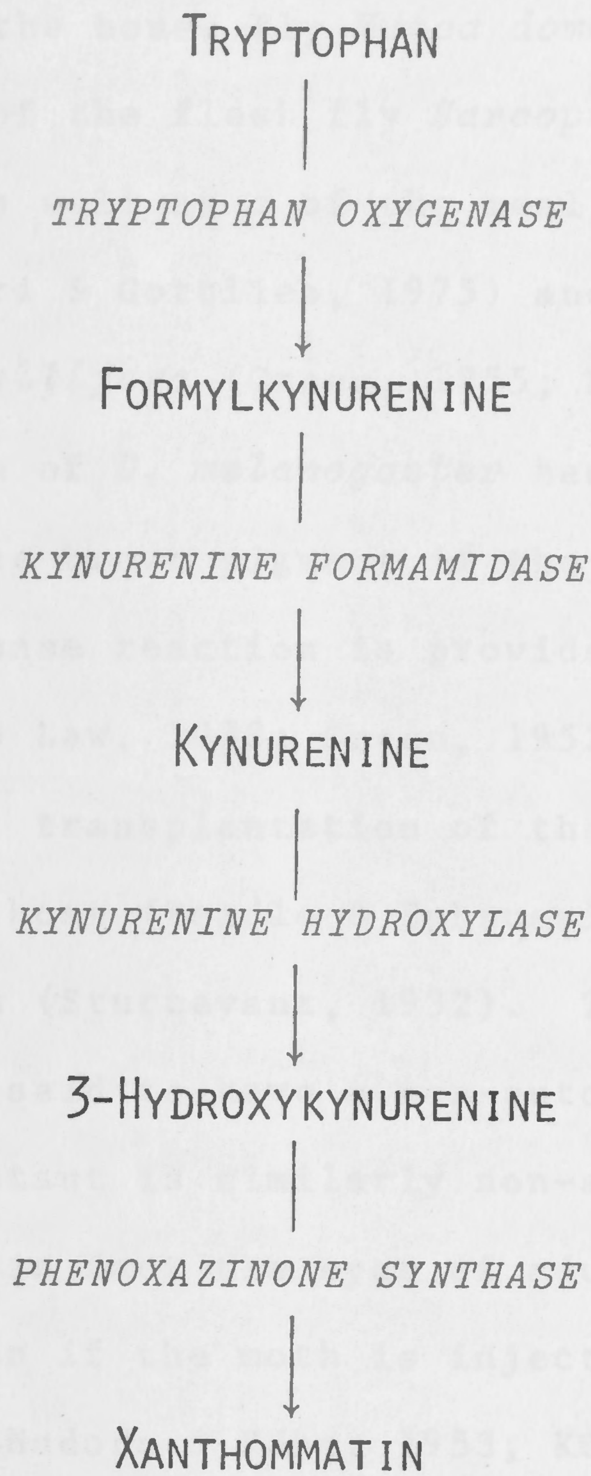


Figure 1.2
THE XANTHOMMATIN BIOSYNTHETIC PATHWAY IN INSECTS
From Linzen (1974).

compounds of the pathway. These mutants include vermilion (*v*) of *D. melanogaster* (Green, 1949; Baglioni, 1959), green of the house fly *Musca domestica* (Milani, 1975) and ivory of the flesh fly *Sarcophaga barbata* (Trepte, 1978) as well as *a* of the meal moth *Ephestia kühniella* (Caspari & Gottlieb, 1975) and snow of the honey bee *Apis mellifera* (Green, 1955; Dustmann, 1968, 1975). Vermilion of *D. melanogaster* has been shown to be able to produce brown pigment if the product of the tryptophan oxygenase reaction is provided to larvae by feeding (Beadle & Law, 1938; Green, 1952), injection (Ephrussi, 1942), transplantation of the developing eye into a wild type host (Beadle & Ephrussi, 1936) or genetic mosaicism (Sturtevant, 1932). The vermilion mutants are thus said to have a non-autonomous phenotype. The *Ephestia a* mutant is similarly non-autonomous (Caspari, 1933); in fact the eyes of adults acquire brown pigment even if the moth is injected with kynurenine after emergence (Hadorn & Kühn, 1953; Kühn & Egelhaaf, 1955). Further evidence that the structural gene for tryptophan oxygenase in *D. melanogaster* is located at the vermilion locus includes the finding that the activity of the enzyme is proportional to the number of v^+ alleles present in the genome (Tobler *et al.*, 1971).

The next enzyme of the pathway is kynurenine formamidase which catalyses the conversion of formylkynurenine to kynurenine. In all dipteran species studied

no mutant has been found which is blocked at this step (Glassman, 1956; Linzen, 1974). Grigolo (1969) found that the tryptophan oxygenase deficient mutant green of *M. domestica* also lacked kynurenine formamidase and that the mutant carnation has much reduced kynurenine formamidase activity. There are a number of possible explanations for the absence of kynurenine formamidase mutants. The spontaneous conversion of formylkynurenine to kynurenine is known to occur (Linzen, 1974) and thus the enzyme activity may not be necessary for pigmentation. Kynurenine formamidase has, however, been found in all species tested. Alternatively, the enzyme may catalyse some other essential reaction so that mutants lacking this activity may not be viable. Recently, Moore & Sullivan (1978) have found that two distinct genetic loci code for different functional kynurenine formamidase enzymes in *D. melanogaster* so that the occurrence of a mutation in both (which is unlikely) would be necessary before a mutant phenotype was expressed. It is interesting to note that in *M. domestica*, where there is apparently only one kynurenine formamidase type (Moore & Sullivan, 1975), mutants with reduced kynurenine formamidase activity have been found, as mentioned above.

The third oxidation step is carried out by kynurenine hydroxylase. The structural gene for this enzyme in *D. melanogaster* is cinnabar (*cn*). Cinnabar mutants accumulate kynurenine (Green, 1949) and fail to

convert kynurenine to 3-hydroxykynurenine *in vitro* (Ghosh & Forrest, 1967; Sullivan *et al.*, 1973). The cinnabar phenotype is non-autonomous when 3-hydroxykynurenine is provided in the diet (Beadle & Law, 1938; Schwabl & Linzen, 1972), by injection (Ephrussi, 1942) or by transplantation of the eye disc into a wild type host (Beadle & Ephrussi, 1936). Dose dependence studies have shown that the activity of kynurenine hydroxylase is proportional to the number of cn^{+} genes present in the genotype (Sullivan *et al.*, 1973). The equivalent gene in *M. domestica* is ocra (Milani, 1975) and in the honey bee is ivory (Dustmann, 1968, 1975).

The final reaction in the xanthommatin biosynthetic pathway is catalysed by a particulate phenoxazinone synthase activity (Phillips & Forrest, 1970; Phillips *et al.*, 1973; Yamamoto *et al.*, 1976). This has proved a difficult enzyme to study, but it now seems likely that in *D. melanogaster* the cardinal locus is involved in its production and that at least one other locus, karmoisin, may also be involved (Phillips *et al.*, 1973; Sullivan & Sullivan, 1975; Howells *et al.*, 1977). The conversion of two molecules of 3-hydroxykynurenine to one of xanthommatin may take more than one step (Ryall, 1973; Bolognese & Scherillo, 1974) and thus there may be more than one polypeptide involved in the production of the phenoxazinone synthase activity. *D. melanogaster* mutants blocked in this final enzymic reaction manifest the block as an accumulation of the immediate precursor of xanthommatin,

3-hydroxykynurenine (Howells *et al.*, 1977). Several honey bee mutants (pearl, cream, chartreuse) also lack the ability to convert 3-hydroxykynurenine into xanthommatin but Dustmann (1968, 1975) has suggested that only chartreuse fails because of the enzymic deficiency, while the other mutants fail to attach 3-hydroxykynurenine to granules prior to enzyme action. Cölln & Klett (1978) have found that mutants at the *alb* locus of *Ephestia kühniella* are also blocked at the final step in xanthommatin formation and are similar in phenotype to chartreuse.

1.32 THE PTERIDINE BIOSYNTHETIC PATHWAY

Pteridine biosynthesis in insects is less well understood than ommochrome biosynthesis. The biosynthetic pathway (Figure 1.3) proposed by Adams (1976), which interrelates various pteridines, appears to have been substantiated by recent findings of Dorsett *et al.* (1979). Many of the enzymes of the pathway have not been studied in detail although those involved in related aspects of purine metabolism are better known (MacIntyre & O'Brien, 1976, and O'Brien & MacIntyre, 1978, for reviews).

The enzyme GTP cyclohydrolase catalyses the first step of the pathway committed to pteridine biosynthesis and has been studied recently by Fan & Brown (1976), Fan *et al.* (1976) and Evans & Howells (1978) in *D. melanogaster*. In the wild type strain there are peaks of

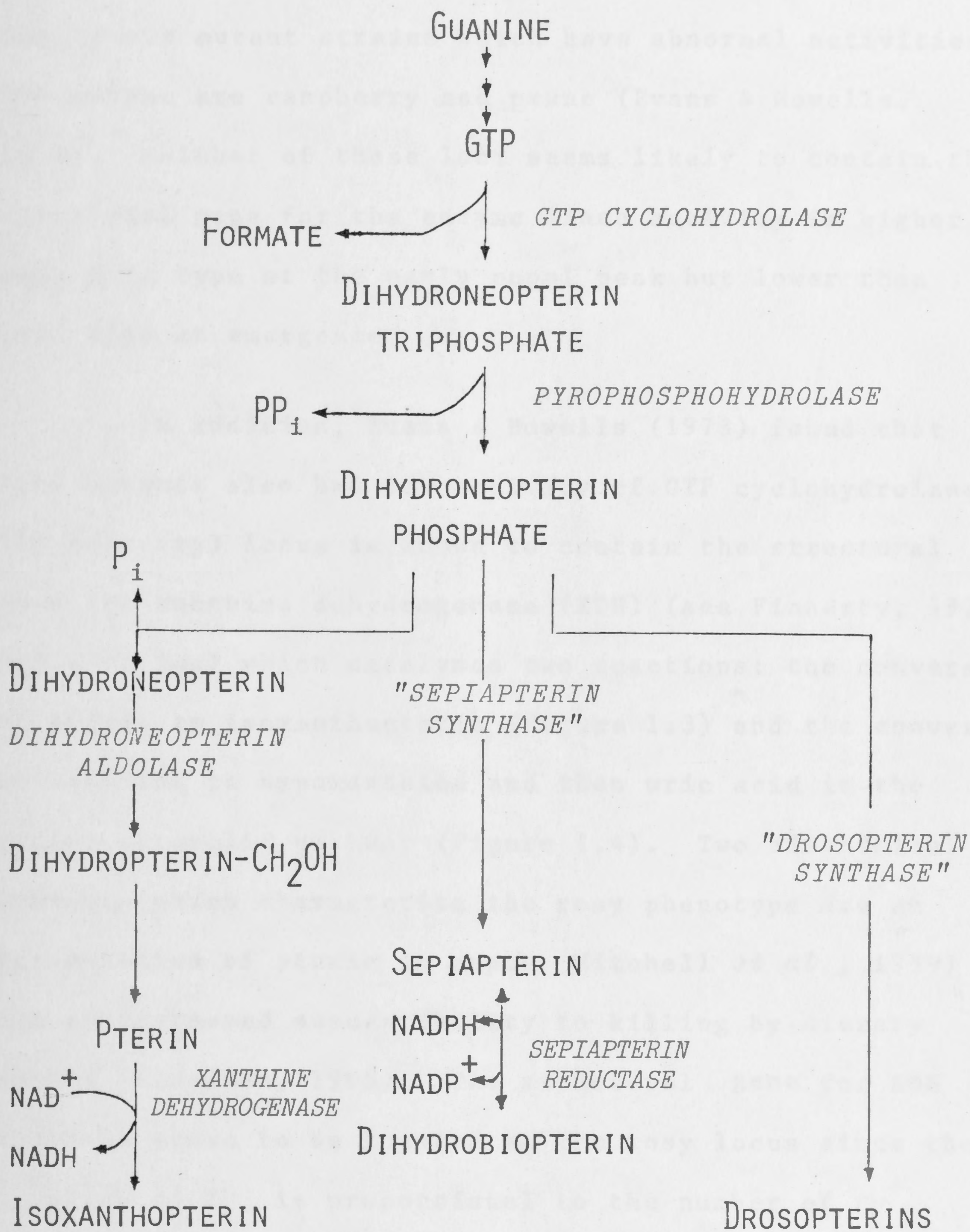


FIGURE 1.3

A PROPOSED PATHWAY FOR THE BIOSYNTHESIS OF PTERIDINES

From Adams (1976).

GTP cyclohydrolase activity at pupariation and at emergence (Fan *et al.*, 1976; Evans & Howells, 1978). Two eye colour mutant strains which have abnormal activities of the enzyme are raspberry and prune (Evans & Howells, 1978). Neither of these loci seems likely to contain the structural gene for the enzyme since activity is higher than wild type at the early pupal peak but lower than wild type at emergence.

In addition, Evans & Howells (1978) found that rosy mutants also had low activity of GTP cyclohydrolase. The rosy (*ry*) locus is known to contain the structural gene for xanthine dehydrogenase (XDH) (see Finnerty, 1976, for a review) which catalyses two reactions: the conversion of pterin to isoxanthopterin (Figure 1.3) and the conversion of xanthine to hypoxanthine and then uric acid in the purine catabolic pathway (Figure 1.4). Two biochemical features which characterise the rosy phenotype are an accumulation of pterin in pupae (Mitchell *et al.*, 1959) and an increased susceptibility to killing by dietary purine (Glassman, 1965). The structural gene for XDH has been shown to be located at the rosy locus since the activity of XDH is proportional to the number of ry^+ alleles present in the genome (Glassman *et al.*, 1962; Grell, 1962), electrophoretic variants of XDH all map at the rosy locus (Yen & Glassman, 1965) and rosy mutants have negligible amounts of material which is immunologically cross-reactive with antibody prepared against purified XDH (Gelbart *et al.*, 1974). The effect of mutation at the

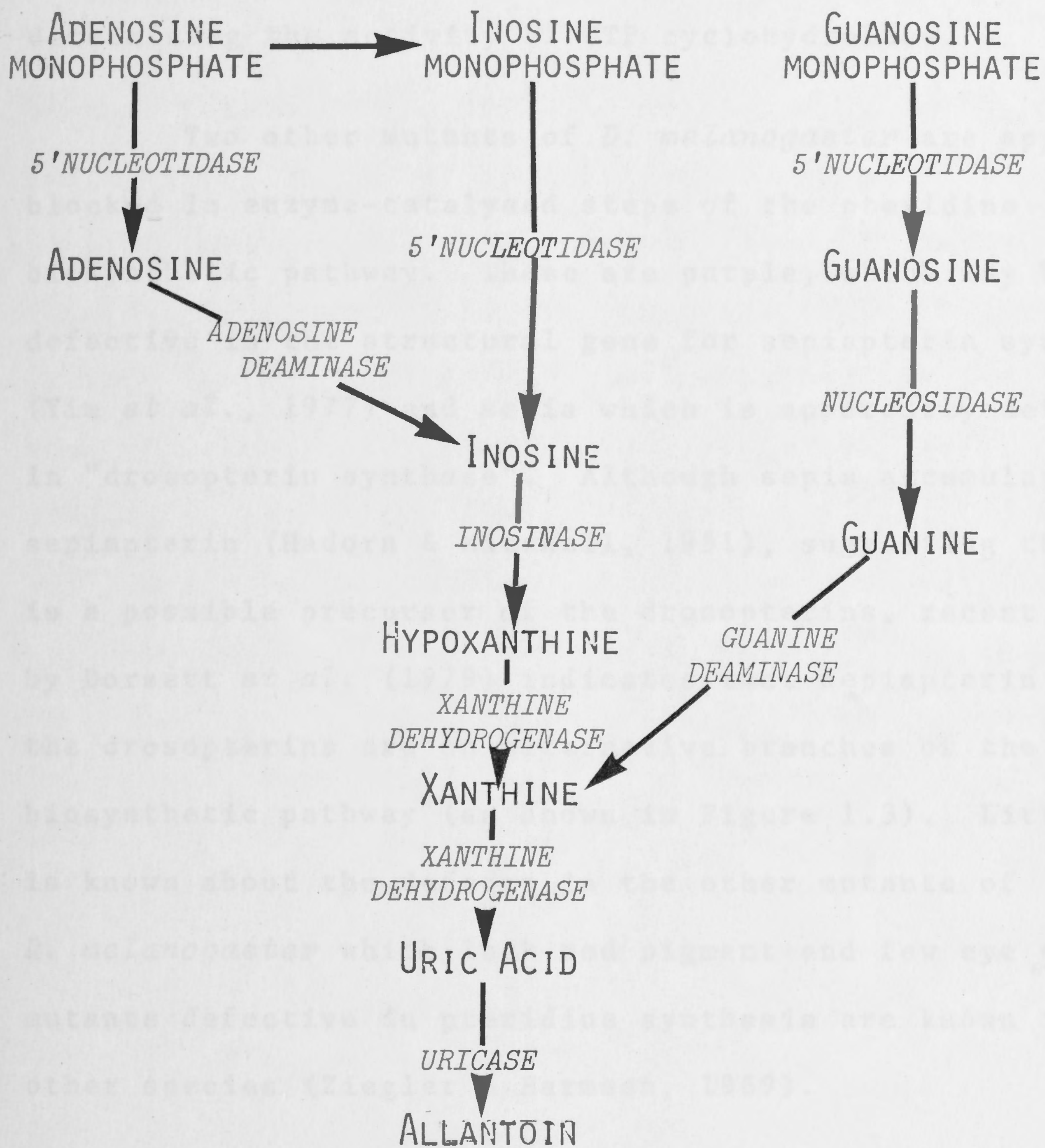


FIGURE 1.4
THE PURINE CATABOLIC PATHWAY

Adapted from O'Brien & MacIntyre (1978)

rosy locus on the activity of GTP cyclohydrolase is therefore a pleiotropic one and suggests an involvement of the products of the XDH-catalysed reactions in determining the activity of GTP cyclohydrolase.

Two other mutants of *D. melanogaster* are apparently blocked in enzyme-catalysed steps of the pteridine biosynthetic pathway. These are purple, which may be defective in the structural gene for sepiapterin synthase (Yim *et al.*, 1977) and sepia which is apparently defective in "drosopterin synthase". Although sepia accumulates sepiapterin (Hadorn & Mitchell, 1951), suggesting that this is a possible precursor of the drosopterins, recent work by Dorsett *et al.* (1979) indicates that sepiapterin and the drosopterins are on alternative branches of the biosynthetic pathway (as shown in Figure 1.3). Little is known about the defects in the other mutants of *D. melanogaster* which lack red pigment and few eye colour mutants defective in pteridine synthesis are known in other species (Ziegler & Harmsen, 1969).

1.33 INTERACTIONS BETWEEN THE XANTHOMMATIN AND PTERIDINE BIOSYNTHETIC PATHWAYS

In many eye colour mutant strains, a single defect alters to a greater or lesser extent the production of both classes of pigment (Hadorn & Kühn, 1953; Kühn & Egelhaaf, 1955; Ghosh & Forrest, 1967; Ziegler & Harmsen, 1969; Parisi, 1971; Parisi *et al.*, 1976a, b). The white eye phenotype which is common to

many species of insect is the extreme example of this phenomenon. This phenotype results from the complete absence of ommochromes and pteridines in adults although body pigmentation is normal. White eye mutants in Diptera include white of *D. melanogaster* (Lindsley & Grell, 1968; Judd, 1976), white of *M. domestica* (Hiraga, 1964; Milani, 1975), chalky of *Calliphora erythrocephala* (Langer, 1967) and white of *L. cuprina* (Whitten *et al.*, 1975). The white locus of *D. melanogaster* has been studied extensively genetically (Judd, 1955, 1976), biochemically (Nolte, 1959a; Howells *et al.*, 1977; Howells, 1979) and electron microscopically (Shoup, 1966).

In addition to white, there are many mutant strains of *D. melanogaster* with abnormalities in levels of both pigment classes. Nolte (1954a, b, 1955, 1959b) assayed red and brown pigment in mutants at 19 different loci. Of these, 14 were different from wild type in levels of both pigments. Two, raspberry and prune, had elevated levels of xanthommatin and reduced drosopterin levels for most alleles tested (Nolte, 1959b). The rest had reductions in levels of both pigments. Where alleles at one locus were assayed (for example the four alleles of garnet reported by Nolte, 1959b), the reduction in each pigment class is not always in constant proportion (Table 1.1a). This is more evident in considering the white allelic series, where both pigments can be completely eliminated (as in white) or reduced to varying percentages of the

TABLE 1.1
LEVELS OF RED AND BROWN PIGMENT
IN EYE COLOUR MUTANT STRAINS

a) The garnet allelic series (from Nolte, 1959b).

ALLELE	RED PIGMENT (% of wild type)	BROWN PIGMENT (% of wild type)
garnet	38	56
garnet^2	16	33
garnet^3	21	44
garnet^4	22	22

b) The white allelic series (from Nolte, 1959a).

ALLELE	RED PIGMENT (% of wild type)	BROWN PIGMENT (% of wild type)
white	0.3	2
white-coloured	17	22
white-eosin^2	7	55
white-apricot^3	6	11
white-coral	6	33
white-satsuma	3	122
white-wine	3	33
white-eosin^3	2	32
white-eosin	1	22
white-apricot	1	11
white-tinged	0.3	3

wild type values (Table 1.1b). Nolte (1954a) notes that cardinal, which has only 15% of the wild type level of brown pigment, has raised red pigment levels. Parisi *et al.* (1976a) also found that at all developmental stages cardinal had raised drosopterins, while other red-eyed mutants (vermilion, cinnabar, scarlet) had lowered levels of drosopterins when compared with wild type. Schwink & Mancini (1973) found that the pteridine fingerprint of cardinal was different from wild type. There is as yet no explanation for this effect of the cardinal mutation.

The interrelationship of production of the two pigment types is also evident in mutant strains of the moth *Ephestia kühniella*. Here the non-autonomous mutant *a* is blocked in the first step of the xanthommatin biosynthetic pathway and pteridine content is also abnormal. Injection of kynurenine into adults restores xanthommatin biosynthesis and also normalises the pteridine levels (Hadorn & Kühn, 1953; Kühn & Egelhaaf, 1955). A white-eyed mutant of *E. kühniella* has been described (Caspari & Gottlieb, 1975) indicating that in Lepidoptera, too, the synthesis of both pigment components of the eyes can be disrupted through mutation at a single locus.

There have been many attempts to explain the interaction between pteridine and ommochrome accumulation, several of which are no longer tenable. The white-eyed

mutants have often been seen as the key to answering this question, because mutation at a single locus effectively eliminates both pathways.

Nolte (1952) suggested that there might be a common substrate for the two pathways so that abnormalities in its production would disrupt both. Variations in efficiency of competition for the common substrate could lead to different effects on the two pathways. As the steps involved in pigment synthesis are now better known (Figures 1.2 and 1.3) this idea can be eliminated. In a similar suggestion, Kühn (1956) proposed that both pigments might be deposited on the same granules so that interdependence of synthesis would be due to competition for sites on the same granules. If the granules were not made (as, perhaps, in the white-eyed mutants) there could be no pigment deposited. The developmental electron microscope studies of Shoup (1966) indicate, for *D. melanogaster* at least, that this is unlikely. Two classes of pigment granule were observed, one for xanthommatin (called type I granules) and the other for drosopterins (type II granules). In addition, it is possible that pteridines are not bound to granules in some insects (Ziegler & Harmsen, 1969; Langer, 1975), whereas ommochromes are apparently always granule-bound.

Other explanations of the interaction between ommochrome and pteridine synthesis have suggested the role of cofactors produced or used by one pathway and involved

in regulating the other. By comparing the hydroxylation of phenylalanine to tyrosine (in rat liver) with the hydroxylation of kynurenine to 3-hydroxykynurenine in *D. melanogaster*, Ghosh & Forrest (1967) postulated that kynurenine hydroxylase has a pteridine cofactor. Failure of the pteridine pathway would reduce the availability of this cofactor and therefore disrupt the ommochrome pathway as well. Sullivan *et al.* (1973) found that kynurenine hydroxylase activity in white and other white alleles was the same as the wild type activity, in spite of the varying levels of pteridines in these mutants. Also, the white mutants have been shown to accumulate significant amounts of 3-hydroxykynurenine during pupal life (Howells *et al.*, 1977) indicating that kynurenine hydroxylase functions *in vivo* in these strains. This proposal thus seems no longer tenable.

Another proposal involving cofactors was made by Parisi *et al.* (1976a) who suggest that xanthommatin acts as a cofactor for the enzyme sepiapterin reductase. This would mean that in *D. melanogaster* this enzyme would have to act in the reverse direction from that found in other organisms, by producing sepiapterin from biopterin (Figure 1.3). The study of Yim *et al.* (1977) which shows that sepiapterin is produced from dihydroneopterin triphosphate does not support this idea. Parisi *et al.* (1976b) have also suggested that xanthine dehydrogenase might control the levels of the reduced nicotinamide

cofactor required for kynurenine hydroxylase activity. This seems unlikely since XDH activity is undetectable in the eyes of insects (Ziegler & Harmsen, 1969) where kynurenine hydroxylase is located (Sullivan *et al.*, 1973; Sullivan & Sullivan, 1975). These hypotheses of Parisi *et al.* (1976a, b) are not based on any enzymological evidence.

A more likely explanation for the interrelationship of the ommochrome and pteridine biosynthetic pathways derives from a consideration of the spacial and temporal separation of the enzymes of pigment synthesis. Pigment precursors must be transported from tissue to tissue and often stored during development pending activation of biosynthetic enzymes. Defects in the processes involved in the movement of pigment precursors and their storage might explain a variety of eye colour phenotypes. The pathways might be interrelated through common use or control of some or all of these processes.

In the course of work described in this thesis, this possibility was explored further, in particular with a view to establishing the nature of the accumulation processes. The possibility that some of the mutants of *L. cuprina* might be defective in these processes was also considered. Therefore, the next sections of this introduction deal with this theory in detail, noting the mechanisms whereby these processes occur and mutant phenotypes which arise when they are defective.

1.34 INTERRELATIONSHIPS OF DIFFERENT TISSUES IN PIGMENT SYNTHESIS

Since the ommochrome biosynthetic pathway is better characterised than that for pteridines, most of the information concerning the roles of different tissues in pigment production is available for xanthommatin synthesis and the discussion will draw largely on this information. Information on pteridine biosynthesis will be included where available. Table 1.2 summarises the different tissues in which pigment biosynthetic enzymes are maximally active and Table 1.3 shows the times of peak activity during development for those dipteran species which have been studied.

From these tables, the following summary can be made. Several tissues are involved in the metabolism of tryptophan to xanthommatin. Tryptophan is probably converted to kynurenine in the larval and adult fat bodies and adult eyes. Since kynurenine hydroxylase appears to be found mainly in the malpighian tubules of larvae and eyes of adults, most of this kynurenine must be transported from the fat body to the tubules or eyes. In addition, tryptophan oxygenase activity peaks well before the onset of adult hydroxylase activity and kynurenine must therefore be stored prior to its oxidation to form 3-hydroxy-kynurenine in the eyes. In *D. melanogaster* the 3-hydroxy-kynurenine stored in the tubules as a result of larval kynurenine hydroxylase activity is of minor importance

TABLE 1.2

TISSUE LOCALISATION OF PIGMENT BIOSYNTHETIC ENZYMES IN DIPTERA

a) Xanthommatin biosynthetic enzymes.

ENZYME	TISSUE	INSECT	REFERENCES
Tryptophan oxygenase	Fat bodies (larval)	*Drosophila melanogaster*	Rizki (1961)
	Malpighian tubules	" "	Kaufman (1962)
	Eye discs	" "	Clancy (1940), Nissani (1975)
	Adult fat body, testes, larval & adult malpighian tubules	*Protophormia terrae-novae*	Linzen & Schartau (1974)
Kynurenine formamidase	Ubiquitous	All species examined	Linzen (1974)
Kynurenine hydroxylase	Larval malpighian tubules, adult eyes	*D. melanogaster*	Sullivan *et al.* (1973), Sullivan & Sullivan (1975)
	Eye discs	"	Danneel (1941), Horikawa (1958)
	Larval malpighian tubules	*Calliphora erythrocephala*	Hendrichs-Hertel & Linzen (1969)
Phenoxazinone synthase	Adult heads, eyes	*D. melanogaster*	Phillips *et al.* (1973) Yamamoto *et al.* (1976)

TABLE 1.2 (continued)

b) Pteridine biosynthetic enzymes.

ENZYME	TISSUE	INSECT	REFERENCES
Xanthine dehydrogenase	Larval fat body, adult haemolymph	*Drosophila melanogaster*	Ursprung & Hadorn (1961)
	Adult malpighian tubules	" "	Munz (1964)
GTP cyclohydrolase	Larval body, adult head	" "	Evans & Howells (1978)
Sepiapterin synthase	Adult head	" "	Dorsett *et al.* (1979)
Drosopterin synthase	Adult head	" "	Dorsett *et al.* (1979)

TABLE 1.3

DEVELOPMENTAL PEAK OF ACTIVITY OF PIGMENT BIOSYNTHETIC ENZYMES

ENZYME	TIME OF PEAK ACTIVITY	INSECT	REFERENCES
Tryptophan oxygenase	Young larvae; after emergence	*Protophormia terrae-novae*	Linzen & Schartau (1974)
	At pupariation; after emergence	*Drosophila melanogaster*	Sullivan & Kitos (1976)
Kynurenine formamidase	Young larvae	*P. terrae-novae*	Linzen & Schartau (1974)
	At pupariation; at emergence	*D. melanogaster*	Sullivan & Kitos (1976)
Kynurenine hydroxylase	At pupariation	*P. terrae-novae*	Linzen & Schartau (1974)
	Early third instar larvae; second half of pupal life	*D. melanogaster*	Sullivan *et al.* (1973), Ryall & Howells (1974)
Phenoxazinone synthase	After emergence	*D. melanogaster*	Yamamoto *et al.* (1976)
Xanthine dehydrogenase	3 days after emergence	*D. melanogaster*	Munz (1964), Altmann (1978)
GTP cyclohydrolase	At pupariation; at emergence	*D. melanogaster*	Evans & Howells (1978)

quantitatively in the production of adult xanthommatin (Ryall & Howells, 1974). This 3-hydroxykynurenine is presumably released from the tubules during the rearrangement which occurs early in metamorphosis (Section 1.2; Gilmour, 1961; Wigglesworth, 1972) and may serve as an activator of phenoxazinone synthase (Muth, 1969; Linzen, 1974; Yamamoto *et al.*, 1976), or be used in the early stages of pigment synthesis before the head kynurenine hydroxylase is fully active. Phenoxazinone synthase has only been found in the head region, probably in the eyes, so that transport of kynurenine (substrate for kynurenine hydroxylase) and 3-hydroxykynurenine (substrate for phenoxazinone synthase) into the eyes may be important. Cell lineage analysis by Nissani (1975) suggests that the eyes of wild type *D. melanogaster* are capable of producing kynurenine from tryptophan but depend on external kynurenine and/or 3-hydroxykynurenine supply to produce normal quantities of xanthommatin. Clearly, the movement of xanthommatin precursors from tissue to tissue via the haemolymph, and storage in specific tissues is vital to the proper functioning of the eye pigment system.

The situation with regard to the interrelationships of tissues in pteridine pigmentation is less clear, due to the lack of information concerning developmental and spatial distribution of pteridine biosynthetic enzymes. In the case of xanthine dehydrogenase, activity is known to be low in the eyes and gonads of many insects, yet

levels of its product isoxanthopterin are high in these tissues (Ziegler & Harmsen, 1969) suggesting that they can take up isoxanthopterin from the haemolymph. GTP cyclohydrolase (Evans & Howells, 1978) and sepiapterin synthase and drosopterin synthase (Dorsett *et al.*,1979) are located in the head of adult *D. melanogaster* which may mean that synthesis of sepiapterin and drosopterin occurs from GTP located in the pigment cells. The origin of the GTP is obscure but its production may depend on the uptake of guanine or guanosine from the haemolymph into the developing eye.

One tissue which is apparently closely linked with eye pigmentation is the malpighian tubule tissue. The mechanism of this link is not clear. The connection between eye and tubule phenotype is however well documented among the mutants of *D. melanogaster*. Many of the eye colour mutants listed by Lindsley & Grell (1968) also have abnormal tubule colour (although there are a few exceptions, for example the eye colour mutants cardinal and Henna^{r3} which have normal tubules). Perhaps the clearest demonstration that genes which act to influence eye pigmentation also affect malpighian tubules are the white-mottled alleles of white. Flies of these strains have blotchy eye colour and mottled malpighian tubules (Lindsley & Grell, 1968). A range of compounds which are either eye pigment precursors or related to eye pigments in structure have been found in malpighian tubules. These are listed in Table 1.4. Many of these are coloured

TABLE 1.4
PIGMENT PRECURSORS AND RELATED COMPOUNDS
FOUND IN DIPTERAN MALPIGHIAN TUBULES (LARVAL)

COMPOUND	REFERENCES
Tryptophan	Wessing & Bonse (1962) Bonse (1969)
Kynurenine	Wessing & Bonse (1962)
3-Hydroxykynurenine	Wessing & Danneel (1961) Wessing & Bonse (1962) Wessing & Eichelberg (1972)
Riboflavin	Gilmour (1961) Wessing & Eichelberg (1968) Nickla (1972) Wigglesworth (1972)
"Pteridines"	Gilmour (1961) Wessing & Eichelberg (1968) Bonse (1969)
Pteridines, including isoxanthopterin, xanthopterin, pterin, sepiapterin, biopterin, riboflavin, 1 unidentified fluorescent compound	Hadorn *et al.* (1958) Handschin (1961) Ziegler & Harmsen (1969)

and/or brightly fluorescent under ultra violet light.

Two other tissues in *D. melanogaster* are frequently affected by mutations which cause abnormal eye colour. These are the adult gonads and the ocelli (which contain xanthommatin). For example, the mutant white has a colourless testis sheath while wild type has yellow testes. The ocelli of many eye colour mutant strains are colourless although wild type have brown ocelli. This indicates that at least some products of pigment biosynthetic pathways are involved in the colouration of organs other than the eyes.

In summary, some tissues synthesise and store pigment precursors which may be released into the insect haemolymph at some later stage during development. Other tissues take up these precursors from the haemolymph and may store them before enzymes are activated. The pigment cells in the eyes appear to be able both to synthesise and to store pigment precursors as well as make the pigments themselves at the appropriate stage during development. These processes occur for both pteridine and ommochrome precursors.

That mechanisms for the uptake and storage of pigment precursors exist in at least some of the larval and adult tissues involved is clear from several studies. Developing eye discs transplanted into larvae are able to take up pigment precursors (Beadle & Ephrussi, 1936). Discs from three non-autonomous strains, vermilion, cinnabar and rosy, were able to form pigment normally in wild type

hosts. This indicates that the products of the missing enzymes were taken up from the haemolymph by the implanted eye discs. Since vermilion and cinnabar are deficient in xanthommatin synthesis and rosy lacks normal drosopterin production, precursors of both pathways must have been taken up. Injection and feeding of the missing intermediates gives the same results, indicating that eye discs in intact mutants are able to take up these compounds.

Uptake and storage of kynurenine and 3-hydroxykynurenine into larval malpighian tubules and adult eyes have been studied in a number of different ways. Eichelberg & Wessing (1971) and Wessing & Eichelberg (1972) observed the storage of 3-hydroxykynurenine in the malpighian tubules of *D. melanogaster* larvae by electron microscopy and proposed that this pigment precursor is stored in the cells of the tubules as granules. Sullivan & Sullivan (1975) have shown that the eyes and malpighian tubules of wild type and some mutants of *D. melanogaster* can take up [^{3}H]-kynurenine *in vitro*.

With regard to the pteridine precursors, little is known of the uptake and storage mechanisms. In wild type *D. melanogaster*, riboflavin (which is related to pteridine pigments in structure; Wigglesworth, 1972) is stored in large amounts in vacuoles in the malpighian tubules (Wigglesworth, 1972). Nickla (1972) used two mutant strains (light and clot) with abnormal pteridine levels to show that the amount of riboflavin stored in

the tubules is reduced when pteridine synthesis is reduced. The results using transplantation of eye discs from rosy suggest that mechanisms exist for the transport of pteridines or related compounds into the eye. These mechanisms remain to be characterised.

1.35 GENETIC LOCI INVOLVED IN THE UPTAKE AND STORAGE OF PIGMENT PRECURSORS

Several authors have made the suggestion that some eye colour mutants might be defective in processes of uptake and storage of pigment precursors. Bonse (1969) noted the lack of fluorescent substances in the malpighian tubules of larvae of the white mutant of *D. melanogaster* and suggested that this was due to a failure to accumulate tryptophan, kynurenine and some pteridines in the tubules. More recently, Sullivan & Sullivan (1975) showed that eyes and malpighian tubules dissected from white and scarlet strains of *D. melanogaster* failed to take up [^{3}H]-kynurenine *in vitro*. Howells *et al*. (1977) showed for these mutants that this inability of the larval malpighian tubules to take up kynurenine was concomitant with a raised level of larval excretion of kynurenine and 3-hydroxykynurenine. In addition, eye discs of white and scarlet fail to pigment when transplanted into a wild type host (Beadle & Ephrussi, 1936). Thus in both white and scarlet of *D. melanogaster*, the absence of xanthommatin in the eyes appears to be due to the inability of the pigment cells to take up

xanthommatin precursors (Sullivan & Sullivan, 1975; Howells & Ryall, 1975; Howells *et al.*, 1977). A similar defect may be responsible for the phenotype of several other xanthommatin mutants. The red-eyed mutant lightoid was shown by Sullivan & Sullivan (1975) to be unable to take up [^{3}H]- kynurenine *in vitro*. Howells *et al.* (1977) found several mutants (claret, light, lightoid and pink in addition to white and scarlet) which failed to accumulate 3-hydroxykynurenine during larval life. This probably means that they also fail to take up and store xanthommatin precursors in the larval malpighian tubules.

Several pteridines (and also riboflavin) are reported to be stored in the larval malpighian tubules (Table 1.4). With regard to riboflavin, Lovelock (1977) showed that excretion of this compound was elevated in larvae of the pteridine-deficient white and brown strains of *D. melanogaster*. By analogy with the rapid excretion of 3-hydroxykynurenine by white and scarlet (Howells *et al.*, 1977) this may mean that white and brown fail to take up and store riboflavin in the larval malpighian tubules. This defect may extend to pteridines and pteridine precursors since Nickla (1972) has demonstrated the relationship between low levels of drosopterins in the eyes and low storage of riboflavin in the tubules.

In other species mutants have been found which fail to bind 3-hydroxykynurenine to granules in the eyes prior to the formation of xanthommatin. These include the

wa mutant of the moth *Ephestia kühniella* (Caspari & Gottlieb, 1975) and the cream and pearl mutants of the honey bee *Apis mellifera* (Dustmann, 1968, 1975). This formation of granular 3-hydroxykynurenine is suggested to be distinct from the enzymological conversion of 3-hydroxykynurenine to xanthommatin and may be part of the same essential uptake and storage process as described for *D. melanogaster* above. As discussed earlier, granules containing 3-hydroxykynurenine have been observed in the malpighian tubules of wild type *D. melanogaster* larvae (Wessing & Danneel, 1961; Wessing & Eichelberg, 1972).

From the preceding discussion it can be seen that mutations at the white and scarlet loci of *D. melanogaster* certainly prevent accumulation of xanthommatin precursors in at least two tissues, the larval malpighian tubules and the adult eyes, while mutations at the white and brown loci may prevent the accumulation of pteridines and their precursors in these two tissues. Work by Howells (1979) suggests that the white and scarlet gene products are necessary at the same time during development. Temperature sensitive mutants at both loci have the same temperature sensitive period (TSP) during pupal life, which begins some time before the onset of pigment production and ends before its completion. It seems that the products of these two loci are needed during the period over which pigment synthesis is initiated but that their presence is not necessary over the whole period of pigment accumulation. This could be interpreted as the period during which the

uptake of kynurenine and 3-hydroxykynurenine by the pigment cells is occurring. The TSP for a temperature sensitive allele of brown (Lovelock, 1977)begins later than that for white and scarlet but well before the onset of pteridine pigmentation. In contrast, temperature sensitive alleles of prune (Orevi & Falk, 1975) have a very short TSP covering the last tenth of pupal life and coinciding with the appearance of GTP cyclohydrolase and the onset of drosopterin synthesis. Thus the prune locus may be involved in triggering the onset of drosopterin synthesis while the brown locus may function during the period of uptake and storage of pteridine percursors.

The role of these genes in the uptake and storage processes remains to be clarified. The white protein (product of the white gene) might be an activator of both the brown and scarlet genes or gene products, influencing the production of both pigment types through this common control. Since the products of the white and scarlet genes seem to be necessary at the same developmental stage (Howells, 1979), this is perhaps unlikely. An alternative model suggested by Sullivan (personal communication) is that all three gene products are membrane components with the scarlet and brown proteins being the "permeases" providing passage for the pigment precursors, and the white protein necessary in the membrane for their proper functioning. Alternatively, the gene products could be components of storage granules such as those seen in the malpighian

tubules of *D. melanogaster* larvae (Wessing & Danneel, 1961) and the eyes of *Apis mellifera* (Dustmann, 1968) for 3-hydroxykynurenine. The white protein might provide a matrix for the granules and the scarlet and brown proteins the specificity of binding for the precursors. There are many ways in which other genes might interact with the basic permease or storage granule structure, either through direct or indirect effects.

1.4 *SCOPE OF THIS THESIS*

As outlined in the previous sections of this Introduction, there are a range of unanswered questions concerning the biochemistry, cytology and genetics of eye pigment synthesis in Diptera. Despite its obvious advantages from a genetic viewpoint, the small size of *D. melanogaster* puts certain limitations on the sorts of experiments which can be performed easily. This has led to an interest in the larger blowfly, *Lucilia cuprina*, as an insect to use in the investigation of these problems. Aspects which might be more easily studied in *L. cuprina* are, for example:

- a) precursor transport and uptake;
- b) enzyme localisation within the organism;
- c) tissue transplantation;
- d) aspects of non-autonomy (dose dependence and

the critical time for administration of the compound which induces non-autonomy).
The study of these problems would be facilitated by the larger size of *L. cuprina*, which means that injection of compounds into larvae, adults and (to a lesser extent) pupae is relatively straightforward and that quantities of specific tissues can be obtained by simple dissections. The need to treat or dissect fewer animals means that there is less time involved in the procedures, minimising tissue deterioration.

Before such studies can commence, the basic information about eye pigmentation in *L. cuprina* must be available. The work reported in this thesis enables the characterisation of eye pigment synthesis in wild type *L. cuprina* and seven eye colour mutant strains. Prior to studying the formation of pigment in the mutants, the biochemical details of eye pigment synthesis in wild type were considered and compared with available information about pigmentation in other insects, to clarify the taxonomic specificity of these characteristics. This involved establishing developmental profiles for eye pigment precursors and enzymes of the xanthommatin pathway. Xanthommatin formation was then examined in each of the mutants to give an insight into the possible nature of the defect in each. The nature and amount of the pteridine eye pigment was also examined in wild type and all the mutant strains.

An important part of the thesis concerns the problem of uptake and transport of pigment precursors between tissues (Sections 1.34, 1.35). In the course of these studies, techniques were perfected for injecting radioactively-labelled pigment precursors and for dissecting out relevant tissues such as larval malpighian tubules and adult eyes.

To gain further insights into the phenotype of the various mutants, the ultrastructure of eyes from wild type and mutant strains was examined. Since there have been few studies of pigment granule morphology in the eyes of Diptera, it was uncertain how the biochemical deficiencies would be manifested at the ultrastructural level. The work described on this aspect reveals interesting parallels between some of the *Lucilia* and *Drosophila* mutants and provides a foundation for extensive ultrastructural studies on the development of the pigment cells.

In the course of this study, the nature of the eye pigment deficiencies in the eye colour mutants of *L. cuprina* has been revealed. Homologies of several of the mutants with the mutants of other species, especially *D. melanogaster*, have been established. In the last chapter, these results are discussed in detail and their contribution to our present understanding of the genetics and biochemistry of eye pigmentation in Diptera is critically assessed. Possible future studies using *L. cuprina* and aimed at solving some of the major outstanding problems of eye pigmentation are also considered in this final discussion.

CHAPTER 2
BIOLOGY OF *LUCILIA CUPRINA*

2.1 *TAXONOMY AND ECOLOGY OF* LUCILIA CUPRINA

The blowfly *Lucilia cuprina* (Wiedemann) is a member of the order Diptera. This order is characterised by the presence of a single pair of membranous wings on the mesothorax with the metathoracic pair reduced to small club-like halteres. Diptera undergo holometabolous metamorphosis (Section 1.2) with four morphological stages: egg, larva (with three instars in *L. cuprina*), pupa and adult (Figure 2.1).

Blowflies (superfamily Calliphoridae) feed on carrion or parasitise other animals. Of major economic significance are those species whose larvae are responsible for the cutaneous myiasis of sheep, blowfly strike, which is an extension of the carrion feeding habit. Females of the primary myiasis flies (notably *L. cuprina*) oviposit on areas of the sheep's body where there is superficial bacterial infection due to dampening of the skin by rain, sweat or urine. Young larvae feed on fluid exuding from this superficial wound which is aggravated by their rasping mouthparts. Older larvae attack living tissues causing extensive lesions around which secondary myiasis blowflies (mainly species of *Calliphora* and *Chrysomya*) may lay eggs. The wound may be spread by repeated layings until the animal dies if not treated. If the lesion begins to heal, the larvae of tertiary myiasis blowflies may be found in the scabs. Colless &

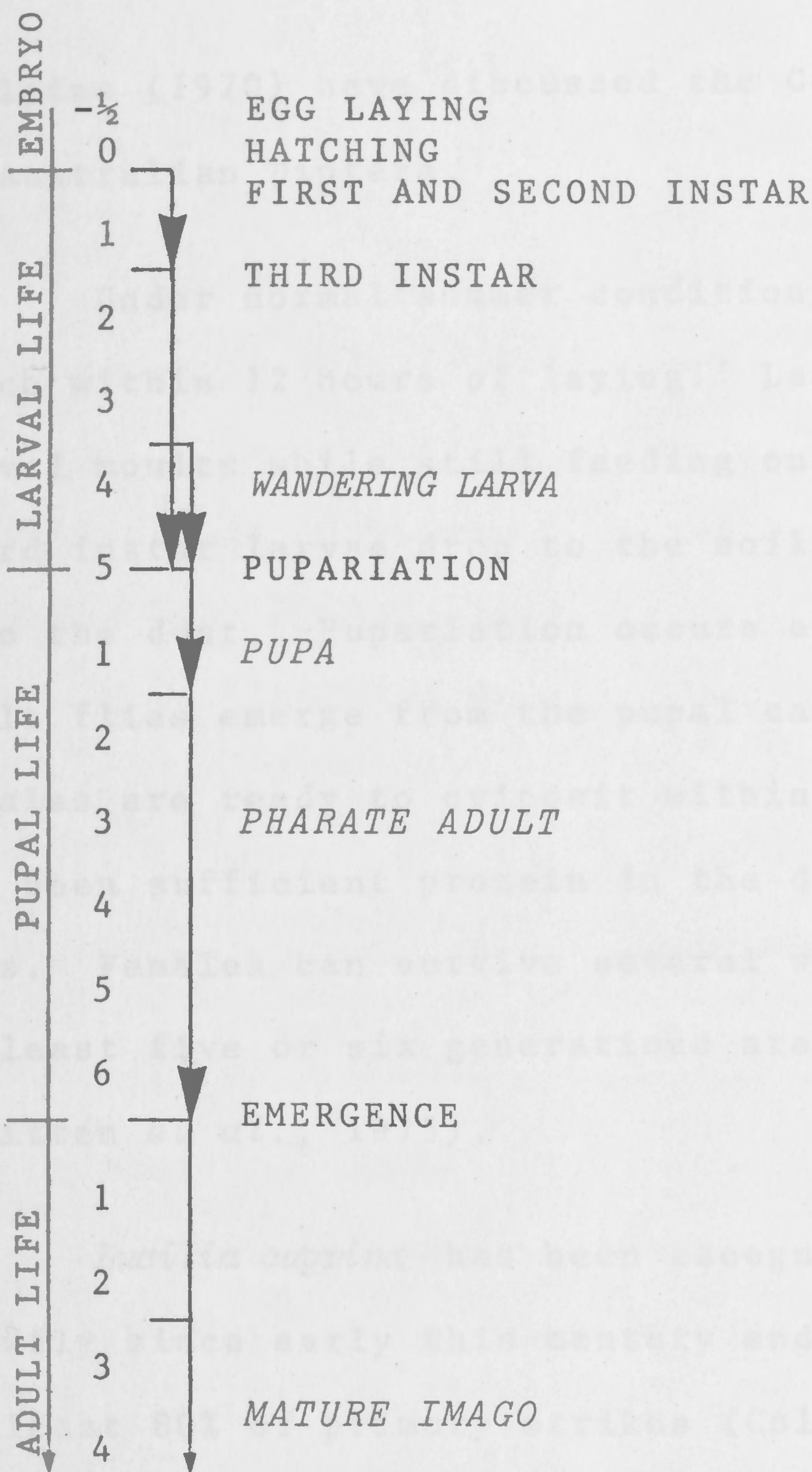


FIGURE 2.1
LIFE CYCLE OF *LUCILIA CUPRINA*

Development of *L. cuprina* at approximately 27°C. The lengths of the four morphological stages (egg, larva, pupa/pharate adult, adult) are indicated.

McAlpine (1970) have discussed the Calliphoridae in a review of Australian Diptera.

Under normal summer conditions the eggs of *L. cuprina* hatch within 12 hours of laying. Larvae undergo two larval moults while still feeding on the sheep. Late third instar larvae drop to the soil where they burrow into the dust. Pupariation occurs about one day later and adult flies emerge from the pupal case within 1 - 2 weeks. Females are ready to oviposit within four days if there has been sufficient protein in the diet to mature their eggs. Females can survive several weeks in the field and at least five or six generations are possible in a season (Whitten *et al.*, 1975).

Lucilia cuprina has been recognised as a major myiasis blowfly since early this century and is now known to cause at least 80% of primary strikes (Colless & McAlpine, 1970; Whitten *et al.*, 1976). It is thought to have been introduced from South Africa or India in the 19th century (Waterhouse & Paramanov, 1950) and has probably only recently abandoned the carrion breeding habit (Whitten *et al.*, 1975). In northern Australia and New Guinea where there are no sheep *L. cuprina* can breed in carrion. In most of Australia competition from native blowflies greatly reduces the chance of successful development in carrion (Waterhouse, 1947) and the evolution of the parasitic myiasis behaviour has allowed the survival of the species.

2.2 CONTROL MEASURES

Since it is a primary myiasis blowfly and initiates wounds in previously healthy sheep allowing strike by other blowflies, *L. cuprina* is a major economic cost to the Australian sheep industry. In the year 1974 - 1975 an estimated cost of $40 million to the industry was caused by fly strike (Whitten *et al.*, 1976) and this figure has probably increased since. Time consuming and expensive control measures contribute to this cost. These have been reviewed by Shanahan (1965).

Traditional farm management practices have been used to reduce blowfly strike. The Mules operation and crutching lower the chance of moisture building up in particular areas of the sheep's body so that the sheep are less susceptible to strike. Insecticides such as dieldrin and organophosphates provide some protection but today the duration of this protection is less than three weeks. Resistance to insecticides has evolved rapidly. Arnold & Whitten (1976) have reviewed the genetics and implications of insecticide resistance.

Froggatt (1918) attempted to reduce blowfly numbers using a hymenopterous parasite but this attempt at biological control was unsuccessful and effective parasites or predators of blowflies have not been found since then (Whitten *et al.*, 1975). Attempts recently have therefore been concentrated on the use of the insect itself (autocidal control) in

biological control.

Autocidal control involves the release of insects which are sterile or impose a high genetic load on subsequent generations, in numbers much greater than the field population, so that the majority of matings will be with released animals and will therefore be infertile or will transmit detrimental characteristics. The sterile insect release method (SIRM) was used with initial success to control the screw-worm fly in southern United States and the mosquito in Florida (Bushland, 1971; Whitten & Foster, 1975). Genetic loads considerably higher, involving the release in the long term of fewer insects, can be initiated by the release of semi-fertile insects whose descendants manifest sterility. This concept has been the basis for a study by CSIRO Division of Entomology in Canberra of the feasibility of using compound chromosome or Y;autosome translocation strains in the genetic control of *L. cuprina*. Due to the complicated genetics required for the development of compound chromosome strains, recent research has concentrated on the second alternative, the use of Y;autosome translocation strains carrying conditional lethal mutations. These have low fertility when crossed with field populations (due to the translocated chromosomes) and their descendants have low viability (due to the conditional lethal mutations).

The conditional lethal mutations chosen for pilot studies result in eye colour abnormalities. The eye colour

mutant strains were originally isolated for routine genetic analysis (Whitten *et al.*, 1976). White, topaz and yellow are all recessive mutations. When released in the field, these mutants cannot be retrapped, indicating that they probably survive poorly under field conditions, although there is adequate viability and fertility under laboratory conditions (Whitten *et al.*, 1976).

Of the control mechanisms discussed above, the method of genetic control using Y;autosome translocations seems the most promising although it is as yet only in the early stages of development. To optimise this strategy, detailed genetic and biochemical knowledge of the strains involved is essential. Genetics of the eye pigment mutants has been established in studies carried out at CSIRO Division of Entomology, Canberra. Biochemical details of eye pigmentation in *L. cuprina* are the subject of the study reported in this thesis.

2.3 *EYE PIGMENT MUTANTS OF* L. CUPRINA

Whitten *et al.* (1975) give details of the genetics of *L. cuprina*. Eye pigment mutants mentioned in Section 2.2 are three of seven eye colour strains (see Frontispiece) occurring at six loci within four of the six linkage groups. A survey of the phenotypes characteristic of the mutants is presented in Table 2.1. This includes

TABLE 2.1

CHARACTERISTICS OF THE EYE COLOUR MUTANTS OF *LUCILIA CUPRINA*

MUTANT	SYMBOL	CHROMOSOME[a]	EYE COLOUR	[b]COLOUR OF LARVAL MALPIGHIAN TUBULES	[c]COLOUR OF ADULT TESTES
Wild type	+		Mid brown, reddens with age	Dark yellow/brown	Orange
Yellowish	*yw*	3	Yellow, darkens with age	Yellow	Yellow
Yellow	*y*	6	Yellow	Yellow	Yellow
Tangerine	*tg*	4	Orange - scarlet	Dark yellow/brown	Light orange
Topaz[1]	to^1	5	Yellow - light orange	Yellow	Orange
Topaz[2]	to^2	5	Orange - scarlet (Allele of topaz[1])	Yellow	Orange
White	*w*	3	White	White	Orange
Grape	*gp*	4	Dark purple - brown	Dark yellow/brown	Light orange

a From Whitten et al (1975), in which further genetic information such as map positions for the different genes is given.

b Late third instar larvae were dissected in insect Ringer's solution. The malpighian tubules are attached to the gut about halfway down the length of the larva. The tubules could also be seen through the cuticle when larvae crawled on the walls of glass vials (see Bodenstein, 1965).

c Testes were dissected from one day old flies. They are easily removed if the lower part of the abdomen is pulled from the upper part. The testes are attached to the ejaculatory duct which opens below the rectum between the 9th and 10th abdominal segments.

the phenotypes of malpighian tubules in larvae and testes in adults, since these two tissues have often been reported to be abnormal in the eye colour mutants of *Drosophila melanogaster* (Lindsley & Grell, 1968; see also Section 1.34).

The white eye colour phenotype has epistatic dominance over all others. The epistatic series of eye colour dominance is:

white > yellow, yellowish > topaz^1, topaz^2 > grape > tangerine (Whitten *et al.*, 1976). Yellow and yellowish are indistinguishable from each other. Topaz^1 is sometimes darker, but in some cultures indistinguishable from yellow and yellowish, especially in young flies. The combination $\frac{\text{topaz}^1}{\text{topaz}^2}$ has orange eyes intermediate between the topaz^1 and topaz^2 phenotypes. The epistatic dominance of grape over tangerine (darker over lighter phenotype) is somewhat anomalous.

In some of the eye pigment mutant strains there are pleiotropic effects which impinge on laboratory fitness, tending for example to slow development or produce smaller flies. The dark-eyed mutant grape is much more susceptible to overcrowding than wild type and other mutants, although at low population density grape flies are normal. Tangerine insects seem to take slightly longer to complete the developmental cycle. In the presence of the white gene these pleiotropic effects are removed (Whitten *et al.*, 1976).

2.4 *LABORATORY CULTURE OF* LUCILIA CUPRINA

In the laboratory *L. cuprina* survives well on protein provided in fresh sheep's liver. The livers used are those unfit for human consumption. The presence of parasites such as hydatids and liver fluke provide a biological test for the absence of toxic compounds which might be detrimental to fly development (Whitten *et al.*, 1975). Such livers, either whole or homogenised in a Waring blender for one minute were used as the food source for all studies described in this thesis.

Routine culture of *L. cuprina* stocks was carried out at CSIRO Division of Entomology, Canberra. Eggs were laid on small pieces of whole liver. After 24 hours the newly hatched first instar larvae were transferred to a larger piece of liver resting on dry, clean vermiculite in a shallow plastic box. Larval feeding continues over the next 3 - 4 days at 27°C. The two larval moults occur during this time. After the second moult, growth occurs very rapidly, to yield a final weight of about 40mg/larva. After four days larvae leave the food and burrow into the vermiculite in preparation for pupariation, which occurs about one day later.

At pupariation the larval cuticle contracts to form the puparium which is initially white and soft. It hardens and darkens after 2 - 4 hours. To obtain synchronous cultures for the developmental studies of pupae reported in this thesis, selection of pupae was made at this short "white pupa" stage. About 24 hours after

pupariation, pupation occurs. This involves a moult so that the insect cuticle becomes distinct from the pupal case (the hardened larval cuticle). From this time the insect, strictly speaking, is a pharate adult but, for convenience, the term "pupa" will be used in this thesis to refer to the insect within the pupal case (that is, from pupariation to adult eclosion).

After six days within the pupal case the adult emerges by expanding and contracting its ptilinum to break the anterior end of the pupal case. The newly emerged fly is dull grey with folded wings but over the next few hours the wings expand and the exoskeleton hardens and takes on the shiny green colour of the mature imago. Cultures used for studies of adults were synchronised at emergence. Flight is possible within a few hours but females require two protein feeds 24 hours apart in the first few days after emergence for maturation of the ovaries. Adults can survive on a diet of sugar and water for up to a month, although females will not produce fertile eggs unless fed protein as well. In the studies of adults reported in this thesis, flies 12 - 24 hours after emergence were frequently used. This was found to be the most suitable age for the injection and dissection techniques used because the cuticle has hardened. The mortality rate among insects kept for longer periods tended to be high and it was felt that the survivors might not be representative of the whole population.

CHAPTER 3

XANTHOMMATIN BIOSYNTHESIS IN WILD TYPE *LUCILIA CUPRINA*

3.1 *INTRODUCTION*

Xanthommatin biosynthesis in Diptera involves a series of oxidation steps which convert the amino acid tryptophan to the phenoxazinone xanthommatin, as was shown in Figure 1.2 (Chapter 1). This pathway will now be discussed in greater detail. Figure 3.1 shows the chemical structures of the compounds involved in the xanthommatin biosynthetic pathway.

The enzymatic conversion of tryptophan to kynurenine is a two step process involving first the splitting by tryptophan oxygenase of the pyrrole ring of tryptophan to form formylkynurenine and then the hydrolysis by kynurenine formamidase of this compound to form kynurenine (Knox & Mehler, 1950; Mehler & Knox, 1950). Tryptophan oxygenase from vertebrate and microbial sources has been well studied. It has also been detected in a number of insects (Linzen, 1974, for a review). For Diptera, Baglioni (1959) first reported the enzyme activity in *D. melanogaster* and others have since reported characteristics of the enzyme from this species (for example, Tartof, 1969; Baillie & Chovnick, 1971; Tobler *et al.*, 1971; Sullivan & Kitos, 1976). Felton (1976) has compared the enzymes from *D. melanogaster* and *D. virilis*. Linzen & coworkers have characterised and partially purified the enzyme from the blowfly *Protophormia terrae-novae* (Linzen & Schartau, 1974; Schartau & Linzen, 1976; Schartau, 1978). Grigolo & Cima (1969) have

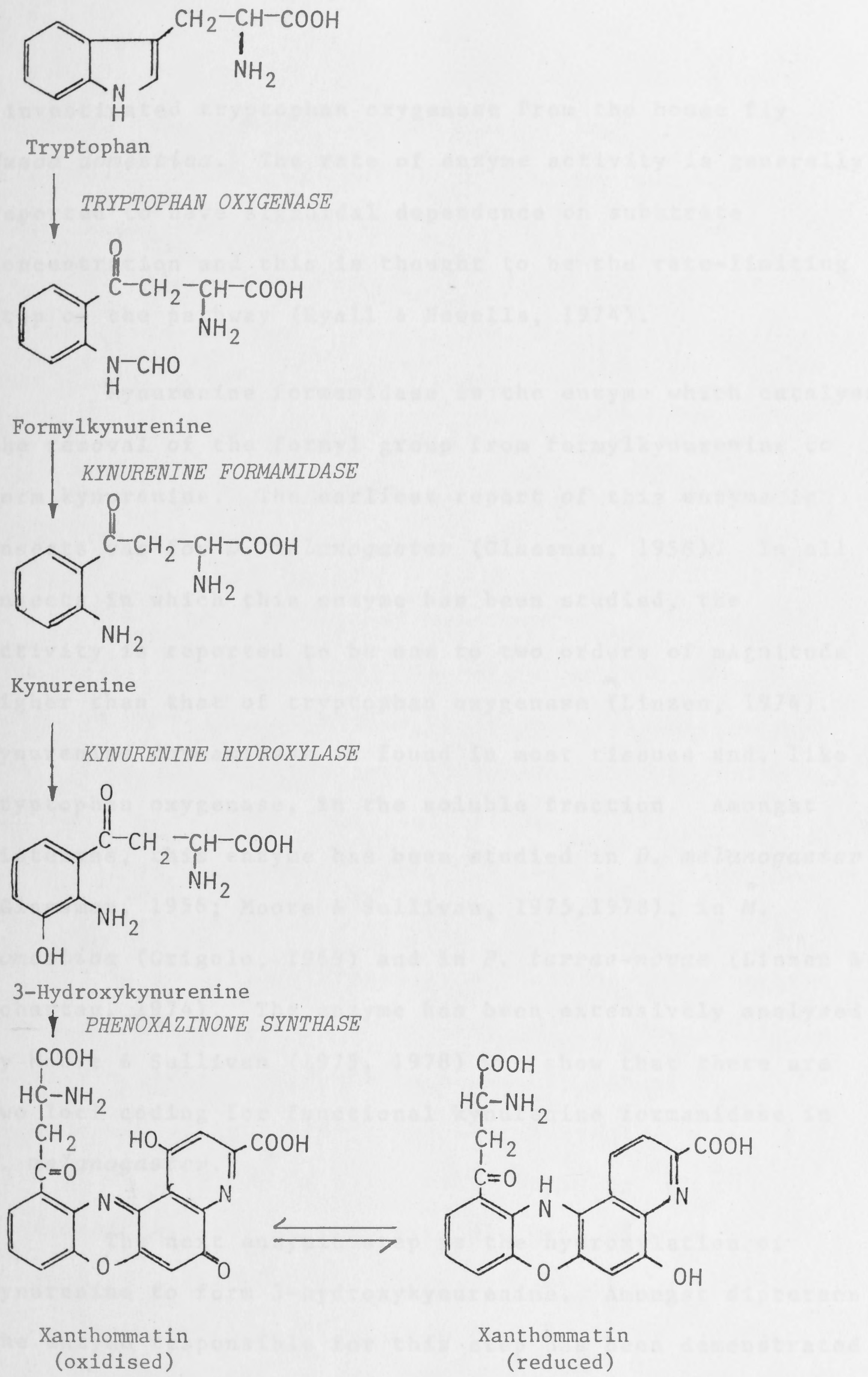


FIGURE 3.1
THE XANTHOMMATIN BIOSYNTHETIC PATHWAY

From Ryall & Howells (1974)

investigated tryptophan oxygenase from the house fly *Musca domestica*. The rate of enzyme activity is generally reported to have sigmoidal dependence on substrate concentration and this is thought to be the rate-limiting step of the pathway (Ryall & Howells, 1974).

Kynurenine formamidase is the enzyme which catalyses the removal of the formyl group from formylkynurenine to form kynurenine. The earliest report of this enzyme in insects was for *D. melanogaster* (Glassman, 1956). In all insects in which this enzyme has been studied, the activity is reported to be one to two orders of magnitude higher than that of tryptophan oxygenase (Linzen, 1974). Kynurenine formamidase is found in most tissues and, like tryptophan oxygenase, in the soluble fraction. Amongst dipterans, this enzyme has been studied in *D. melanogaster* (Glassman, 1956; Moore & Sullivan, 1975,1978), in *M. domestica* (Grigolo, 1969) and in *P. terrae-novae* (Linzen & Schartau, 1974). The enzyme has been extensively analysed by Moore & Sullivan (1975, 1978) who show that there are two loci coding for functional kynurenine formamidase in *D. melanogaster*.

The next enzymic step is the hydroxylation of kynurenine to form 3-hydroxykynurenine. Amongst dipterans the enzyme responsible for this step has been demonstrated in *D. melanogaster* (Ghosh & Forrest, 1967; Sullivan *et al.*, 1973; Ryall & Howells, 1974), in *Calliphora erythrocephala* (Linzen & Hertel, 1967; Hendrichs-Hertel & Linzen, 1969)

and in *P. terrae-novae* (Linzen & Schartau, 1974) although the optimal conditions reported for enzyme activity in these three species of fly are very different. Mayer *et al.* (1968) and Linzen's group (see Linzen & Schartau, 1974) claim that the enzyme is inactivated by light, a factor not mentioned by Sullivan *et al.* (1973). Difficulties have also been experienced in quantifying the formation of the product, 3-hydroxykynurenine, and a number of different techniques have been used.

The final bimolecular condensation of 3-hydroxykynurenine to form xanthommatin was originally thought to be non-enzymic, controlled by the activity of a dopa - dopa quinone redox system (part of the melanin biosynthetic pathway) (Butenandt *et al.*, 1956). Phillips *et al.* (1970) showed that the formation of xanthommatin is not dependent on the presence of this system. This reaction is in fact catalysed by a phenoxazinone synthase enzyme complex (Phillips & Forrest, 1970). Phenoxazinone synthase activity was found to be highly dependent on the concentration of free manganese (Yamamoto *et al.*, 1976; Ryall *et al.*, 1976). A particulate activity, which cosediments on sucrose gradients with the pigment granules of the eyes, appears to be the enzyme involved with xanthommatin synthesis (Phillips *et al.*, 1973; Yamamoto *et al.*, 1976). The soluble activity (Phillips & Forrest, 1970), found predominantly in the body, probably has no role in xanthommatin synthesis (Ryall *et al.*, 1976).

The developmental control of the xanthommatin biosynthetic pathway appears to occur in this final reaction. Yamamoto *et al.* (1976) found that activity of the particulate phenoxazinone synthase appears midway through pupal life. This onset of activity correlates with the time of onset of xanthommatin deposition in *D. melanogaster*, reported by various authors to begin 48 - 50 hours after pupariation (Schultz, 1935; Shoup, 1966; Phillips & Forrest, 1970; Ryall & Howells, 1974). Phenoxazinone synthase has not been studied in other species but xanthommatin has a similar developmental onset in the three species of fly in which it has been measured (Table 3.1), that is, at about mid-pupal life. The onset of xanthommatin synthesis is followed by a rapid build-up of brown pigment until some time after eclosion. Even in a hemimetabolous insect, the stick insect *Carausius morosis*, there is a sudden appearance of xanthommatin followed by the rapid deposition of pigment (Stratakis, 1976).

Clearly, the critical time for pigment synthesis is midway through pupal life in flies. At this stage the regulated histolysis of larval tissues is virtually complete and histogenesis and differentiation are well underway (Section 1.2). There is thus a store of raw materials, including the amino acid tryptophan, provided by the break-down of larval proteins, to be channelled into the production of adult structures. Ryall & Howells (1974) estimate that 30% of the total tryptophan pool present in

TABLE 3.1

ONSET OF XANTHOMMATIN BIOSYNTHESIS IN DIPTERAN SPECIES

SPECIES	[a] ONSET OF XANTHOMMATIN SYNTHESIS	[b] LENGTH OF PUPAL LIFE	REFERENCES
Drosophila melanogaster	48-50 hrs	96 hrs	Schultz, 1935 Ephrussi, 1942 Shoup, 1966 Phillips & Forrest, 1970 Ryall & Howells, 1974
Calliphora erythrocephala	4-5 days	9 days	Linzen, 1963
Protophormia terrae-novae	2-3 days	5 days	Linzen & Schartau, 1974

a Time after pupariation.

b Time from pupariation to adult eclosion.

D. melanogaster at pupariation has been converted to xanthommatin by emergence. Xanthommatin biosynthesis is thus of interest in the study of gene regulation during development inasmuch as it involves the flux of an essential amino acid, tryptophan (Wigglesworth, 1972), through a pathway which is certainly controlled developmentally and probably also controlled allosterically.

In this chapter an assessment is made of the operation of the xanthommatin biosynthetic pathway in the wild type strain of *L. cuprina* during pupal life. As mentioned above, eye pigmentation begins during pupal life and so this is the most important stage of the life cycle to study. In addition, since the insect within the puparium is isolated from the external environment, waste products and excess metabolic intermediates generated by the metamorphic processes, including some tryptophan metabolites, are accumulated in the body fluids and in the meconium. It is possible, therefore, to detect during pupal life the accumulation of compounds which would normally be rapidly excreted by larvae or adults.

The operation of the pathway has been examined, firstly, by estimating the levels of the pathway intermediates during pupal life. This gives a rough estimate of the relative changes in activity of the various enzymes. To substantiate these results, *in vitro* assays of the activities of the enzymes were then made, where possible. The information obtained not only provides an insight into

tryptophan metabolism in this insect, but also makes a basis for examining the operation of the pathway in the various eye colour mutants.

3.2 MATERIALS AND METHODS

3.21 EXPERIMENTAL ANIMALS

The insects used in these assays were reared as described in Section 2.4. Cultures were synchronised as white pupae and the same culture used for a complete series of determinations (that is, all developmental stages). Fresh insects were used for all experiments reported in this chapter.

3.22 CHEMICAL ESTIMATIONS

The values given for the results of each of these determinations are expressed as nmoles/insect. They were also calculated as nmoles/g fresh weight and the trends were found to be the same. Values expressed as nmoles/g fresh weight are used in some comparisons with other species (see Section 3.4; Table 3.3).

a) *Xanthommatin*

This was determined in acid-butanol extracts of ten whole insects or ten heads as described by Ryall & Howells (1974). Xanthommatin in the reduced (dihydro) form was measured at its absorption maximum of 492nm and the level

calculated using a standard curve prepared with pure synthetic xanthommatin (Ryall & Howells, 1974).

b) *3-Hydroxykynurenine and Kynurenine*

These were determined in extracts made from forty insects homogenised in 9 ml 5% trichloroacetic acid. Estimations were made using the colourimetric method of Inagami (1954) for 3-hydroxykynurenine and the method of Bratton & Marshall (1939) for kynurenine, as described by Ryall & Howells (1974). Standard 3-hydroxy-DL-kynurenine and DL-kynurenine were purchased from Sigma Chemical Co., St Louis, U.S.A.

c) *Tryptophan*

Determinations of tryptophan were carried out in 5% trichloroacetic acid extracts by the colourimetric method of Dalby & Tsai (1975). Standard tryptophan was purchased from Sigma Chemical Co., St Louis, U.S.A.

d) *Protein*

Determinations of protein were made using the Biuret method with bovine serum albumin as a standard.

3.23
ENZYME ASSAYS

All enzyme activities are given as specific activities. As discussed by O'Brien & MacIntyre (1978) this value is often misleading in developmental studies where the baseline may change, so values were calculated on a per insect basis as well. The trends were not found to

differ significantly in the two cases.

a) *Activity of tryptophan oxygenase*

Forty insects were homogenised in 5 ml of a homogenising buffer (modified from Linzen & Schartau, 1974) containing 0.1M sucrose; 0.1M Tris-HCl (pH 8.25); 0.045% phenylthiourea and 2mM 2-mercaptoethanol. The homogenate was treated with 100 mg activated charcoal for one hour at $0^{o}C$ (Evans & Howells, 1978) and was then centrifuged for 30 minutes at 31,000g in a Sorvall RC-2B refrigerated centrifuge. The clear supernatant fluid was dialysed overnight against 2 l homogenising buffer and either assayed immediately or stored at $-20^{o}C$. Determinations of enzyme activity were carried out at $37^{o}C$ in a Varian-635 spectrophotometer. Cuvettes contained 1.5 ml 0.25M Tris-HCl (pH 8.25); 0.6 ml 30mM tryptophan; 75 µl 0.33M 2-mercaptoethanol and 75 µl 1.5M KCl. The reaction was started by the addition of 0.75 ml of the enzyme extract and the production of kynurenine monitored at its absorption maximum of 365nm over 3 hours against a control without tryptophan. At this wavelength the molar extinction coefficient of kynurenine is 4,530. This method of assay of tryptophan oxygenase activity depends on the presence of excess kynurenine formamidase activity in the extract to convert formylkynurenine into kynurenine. Kynurenine formamidase activity was measured in each extract (Section 3.23b).

b) *Activity of kynurenine formamidase*

Activity of this enzyme was determined in the extracts made for the assay of tryptophan oxygenase activity (above). The assays were carried out in the Varian-635 spectrophotometer at 37°C. Cuvettes contained 1.5 ml 0.1M potassium phosphate buffer (pH 7.4) and 0.5 ml 7mM formylkynurenine (purchased from Calbiochem, San Diego, U.S.A.). Reactions were started by the addition of 0.5 ml of the enzyme extract and the formation of kynurenine followed over 15 minutes against a cuvette containing water. There was negligible formation of kynurenine in the absence of substrate.

c) *Activity of phenoxazinone synthase*

Ten insects were homogenised in 5 ml cold 0.05M potassium phosphate buffer (pH 6.7) and the extracts prepared and enzyme activity assayed as described by Yamamoto *et al.* (1976). The assay of this enzyme depends on the use of a manganese buffer as developed by Ryall *et al.* (1976). Conversion of the synthetic substrate 3-hydroxyanthranilic acid (obtained from Sigma Chemical Co., St Louis, U.S.A.) to the phenoxazinone, cinnabarinic acid, is measured. Synthetic cinnabarinic acid (Ryall *et al.*, 1976) was used as a standard.

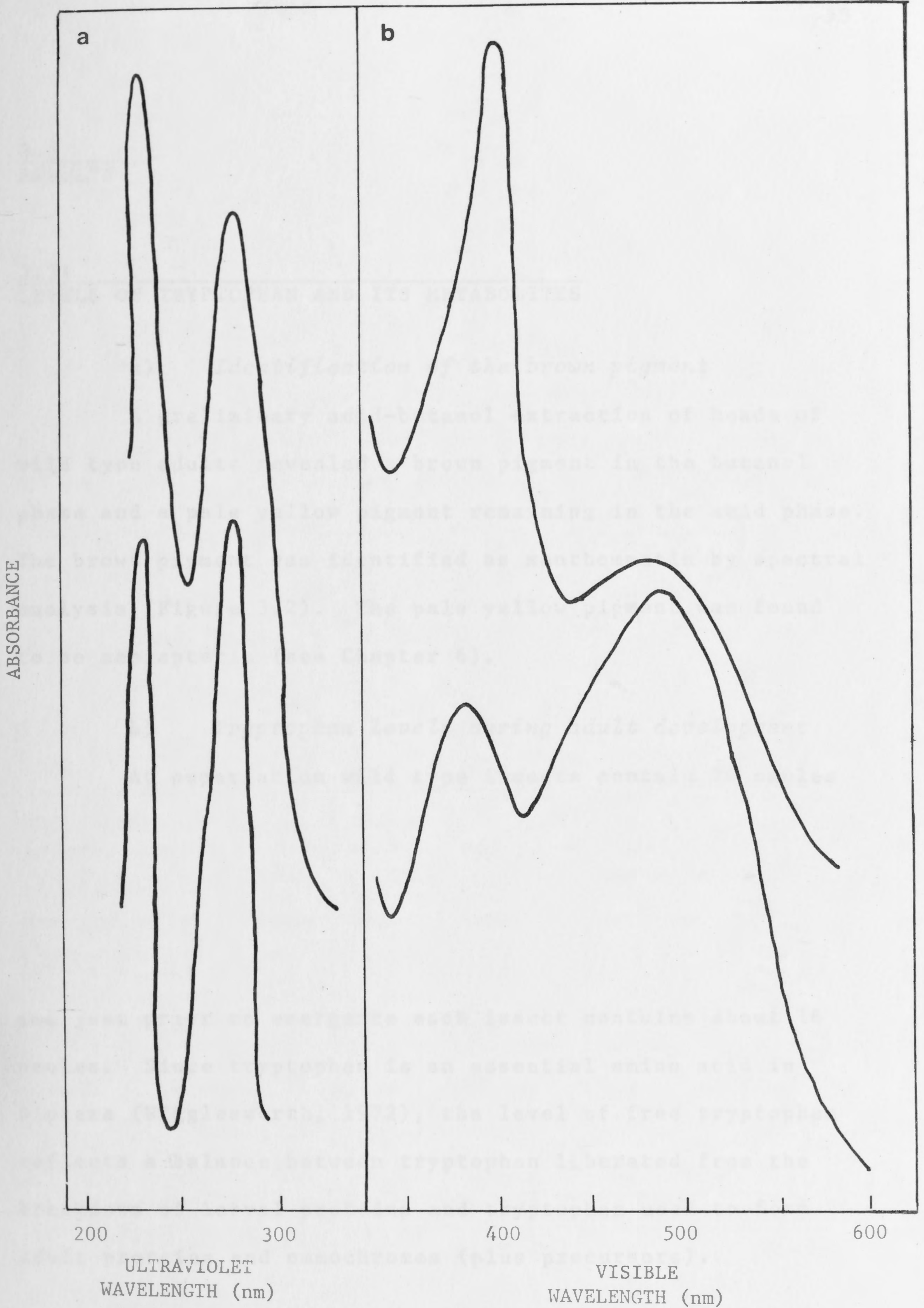
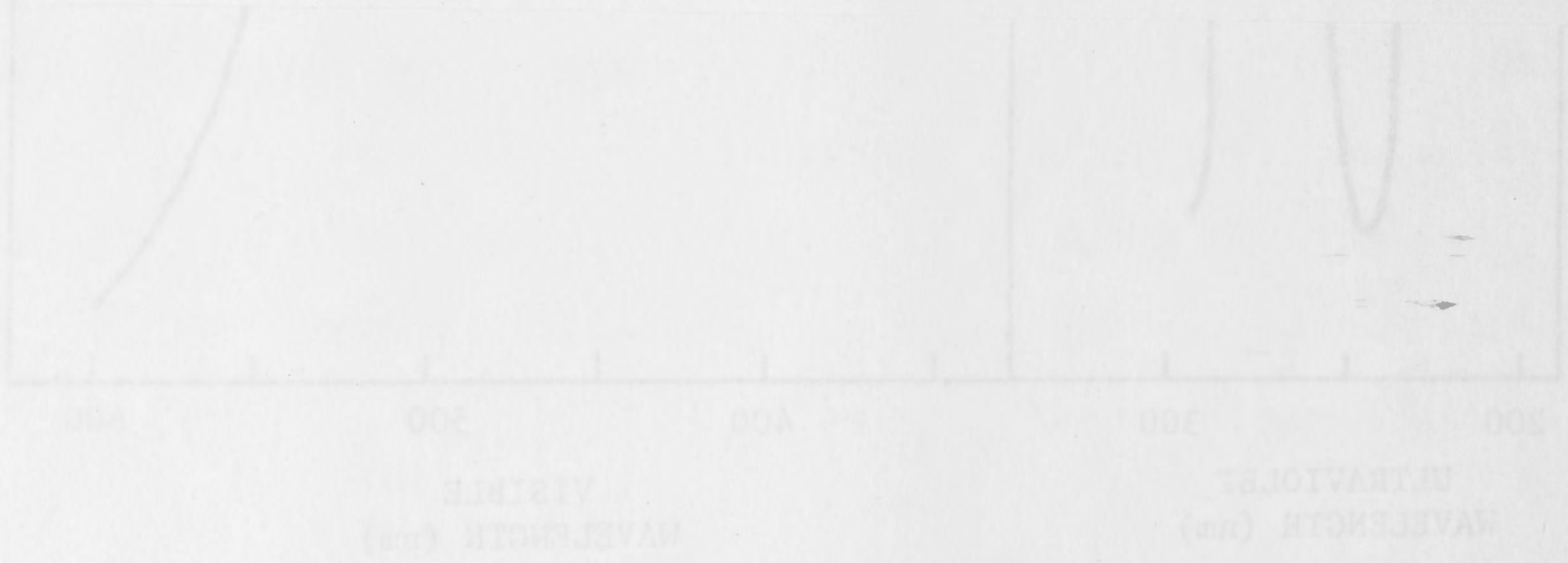


FIGURE 3.2

ABSORPTION SPECTRA OF BROWN PIGMENT FROM *L. CUPRINA* AND *D. MELANOGASTER*

Brown pigment was extracted into butanol as described in Section 3.22a. The extract was diluted 1 in 20 for ultraviolet spectra (a) and used undiluted for the visible spectra (b). Heads of wild type *L. cuprina* (lower curves) and the mutant brown of *D. melanogaster* (upper curves) were used.

*   The drop in the level at day 3 was consistently found. The second peak at day 4 may mean that there is increased breakdown of tryptophan-rich proteins between days 3 and 4, co-inciding with the onset of xanthommatin synthesis.



3.3 *RESULTS*

3.31 LEVELS OF TRYPTOPHAN AND ITS METABOLITES

a) *Identification of the brown pigment*

A preliminary acid-butanol extraction of heads of wild type adults revealed a brown pigment in the butanol phase and a pale yellow pigment remaining in the acid phase. The brown pigment was identified as xanthommatin by spectral analysis (Figure 3.2). The pale yellow pigment was found to be sepiapterin (see Chapter 6).

b) *Tryptophan levels during adult development*

At pupariation wild type insects contain 20 nmoles of tryptophan (Figure 3.3a). The level rises to 27 nmoles two days later, falls after this,* but peaks again at the same level at about the 4th day after pupariation. After this time there is a steady decline in free tryptophan, and just prior to emergence each insect contains about 16 nmoles. Since tryptophan is an essential amino acid in Diptera (Wigglesworth, 1972), the level of free tryptophan reflects a balance between tryptophan liberated from the breakdown of larval proteins and tryptophan used to form adult proteins and ommochromes (plus precursors).

c) *Kynurenine levels during adult development*

Young wild type pupae have about 8 nmoles of kynurenine per insect (Figure 3.3b). By about one day after pupariation the level has fallen to 3.5 nmoles and

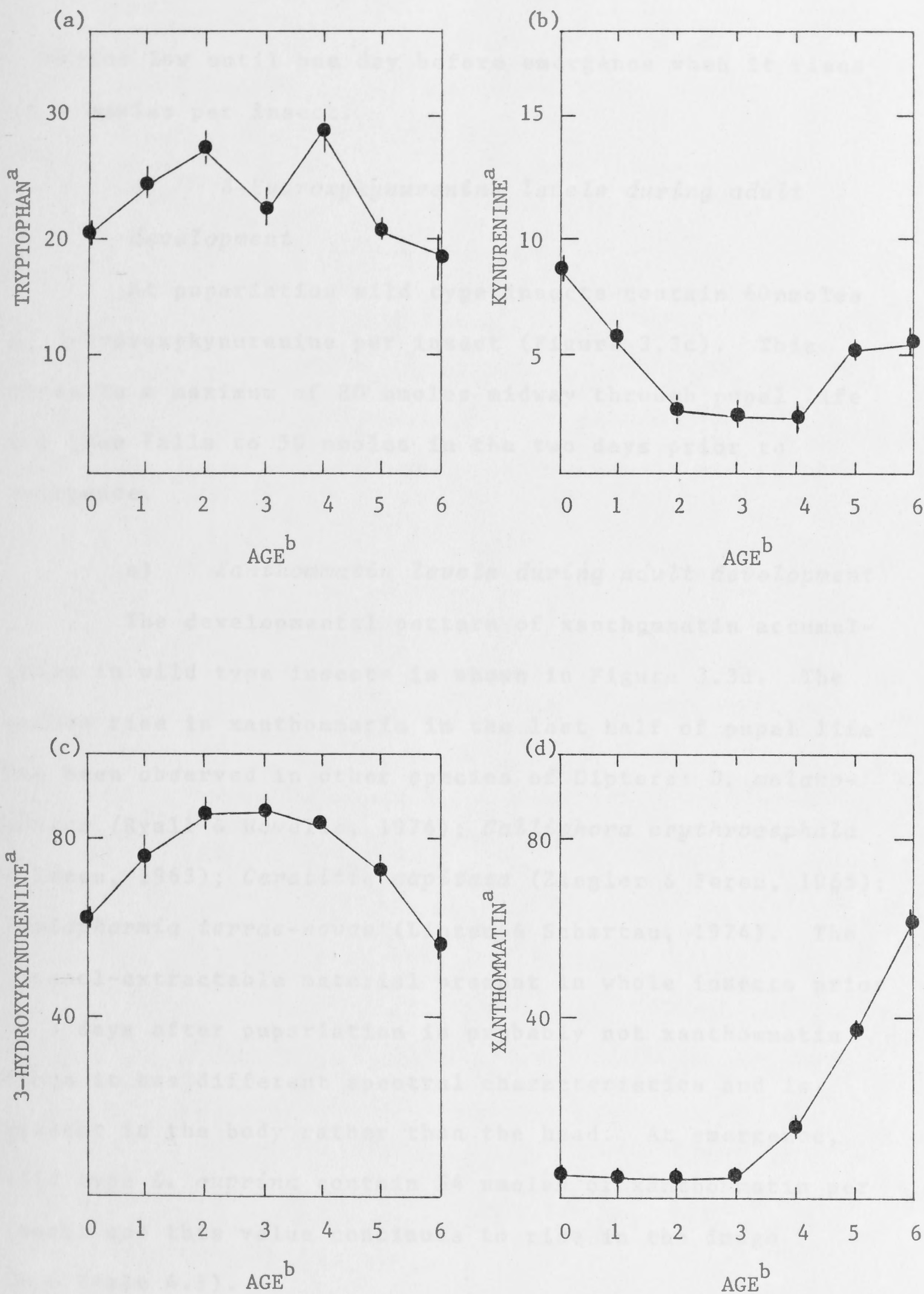


FIGURE 3.3

LEVELS OF TRYPTOPHAN AND ITS METABOLITES IN WILD TYPE *L. CUPRINA* DURING DEVELOPMENT

a Values (expressed as nmoles/insect) are the means of 4 separate determinations and vertical bars represent 1 SD either side of the mean.

b Age is expressed as days after pupariation which occurs at day 0. Flies emerge between days 6 and 7.

remains low until one day before emergence when it rises to 5 nmoles per insect.

d) *3-Hydroxykynurenine levels during adult development*

At pupariation wild type insects contain 60 nmoles of 3-hydroxykynurenine per insect (Figure 3.3c). This rises to a maximum of 80 nmoles midway through pupal life and then falls to 50 nmoles in the two days prior to emergence.

e) *Xanthommatin levels during adult development*

The developmental pattern of xanthommatin accumulation in wild type insects is shown in Figure 3.3d. The sudden rise in xanthommatin in the last half of pupal life has been observed in other species of Diptera: *D. melanogaster* (Ryall & Howells, 1974); *Calliphora erythrocephala* (Linzen, 1963); *Ceratitis capitata* (Ziegler & Feron, 1965); *Protophormia terrae-novae* (Linzen & Schartau, 1974). The butanol-extractable material present in whole insects prior to 3 days after pupariation is probably not xanthommatin since it has different spectral characteristics and is present in the body rather than the head. At emergence, wild type *L. cuprina* contain 54 nmoles of xanthommatin per insect and this value continues to rise in the imago (see Table 4.1).

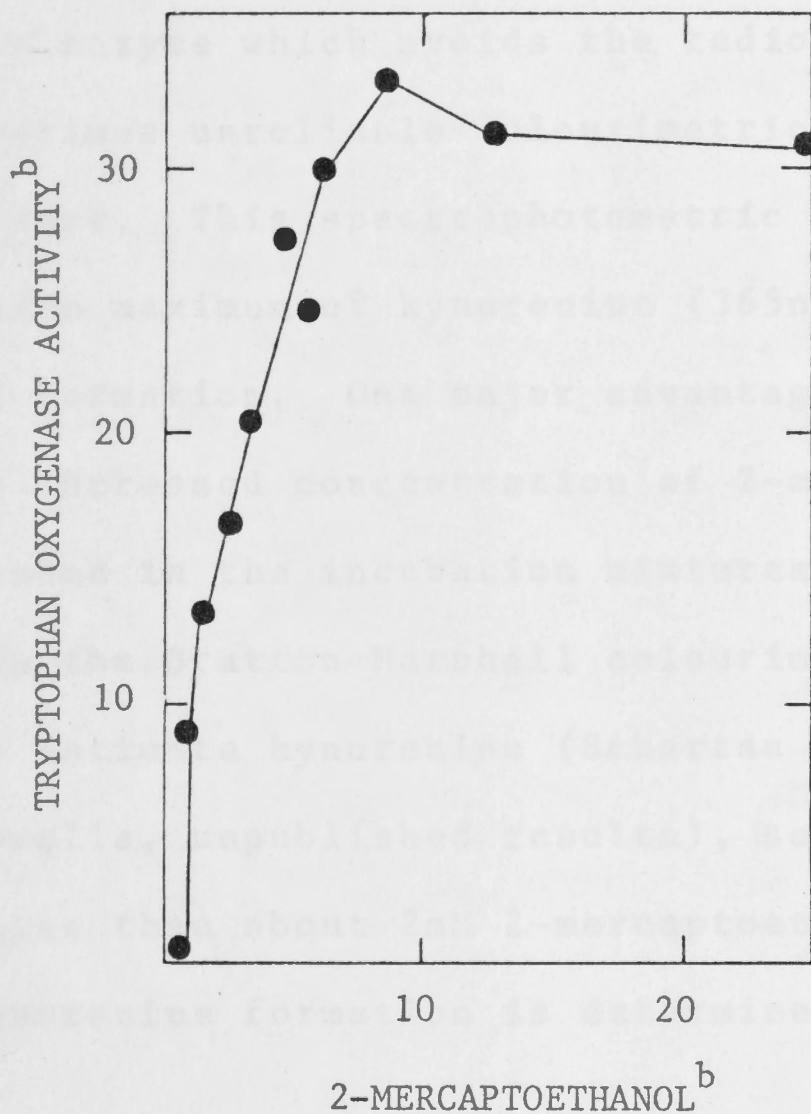


FIGURE 3.4

DEPENDENCE OF TRYPTOPHAN OXYGENASE FROM *LUCILIA CUPRINA* ON CONCENTRATION OF 2-MERCAPTOETHANOL

a Unit of enzyme activity: nmoles kynurenine produced/mg protein/hour.

b Concentration of 2-mercaptoethanol in final incubation (mM).

3.32 ENZYMES OF XANTHOMMATIN BIOSYNTHESIS

a) *Activities of tryptophan oxygenase*

A direct spectrophotometric assay was developed for this enzyme which avoids the tedious, time-consuming and sometimes unreliable colourimetric determination of the product. This spectrophotometric assay uses the absorption maximum of kynurenine (365nm) to determine product formation. One major advantage of this assay is the greatly increased concentration of 2-mercaptoethanol which can be used in the incubation mixtures. This reagent inhibits the Bratton-Marshall colourimetric reaction usually used to estimate kynurenine (Schartau & Linzen, 1976; A.J. Howells, unpublished results), so that concentrations of greater than about 2mM 2-mercaptoethanol cannot be used when kynurenine formation is determined in this manner.

Two characteristics of the *L. cuprina* tryptophan oxygenase are similar to those reported in the literature. There was a marked stimulation of enzyme activity by 2-mercaptoethanol. Increasing stimulation was observed up to a final concentration of 8.5 mM 2-mercaptoethanol (Figure 3.4) with no increased stimulation observed in response to tripling this concentration. The 2-mercapto-ethanol optimum concentration of 8.5mM was used in subsequent experiments. A second feature of the *L. cuprina* enzyme is the sigmoidal dependence of initial rate of reaction on substrate concentration (Figure 3.5). The apparent K_m for tryptophan oxygenase from *L. cuprina*

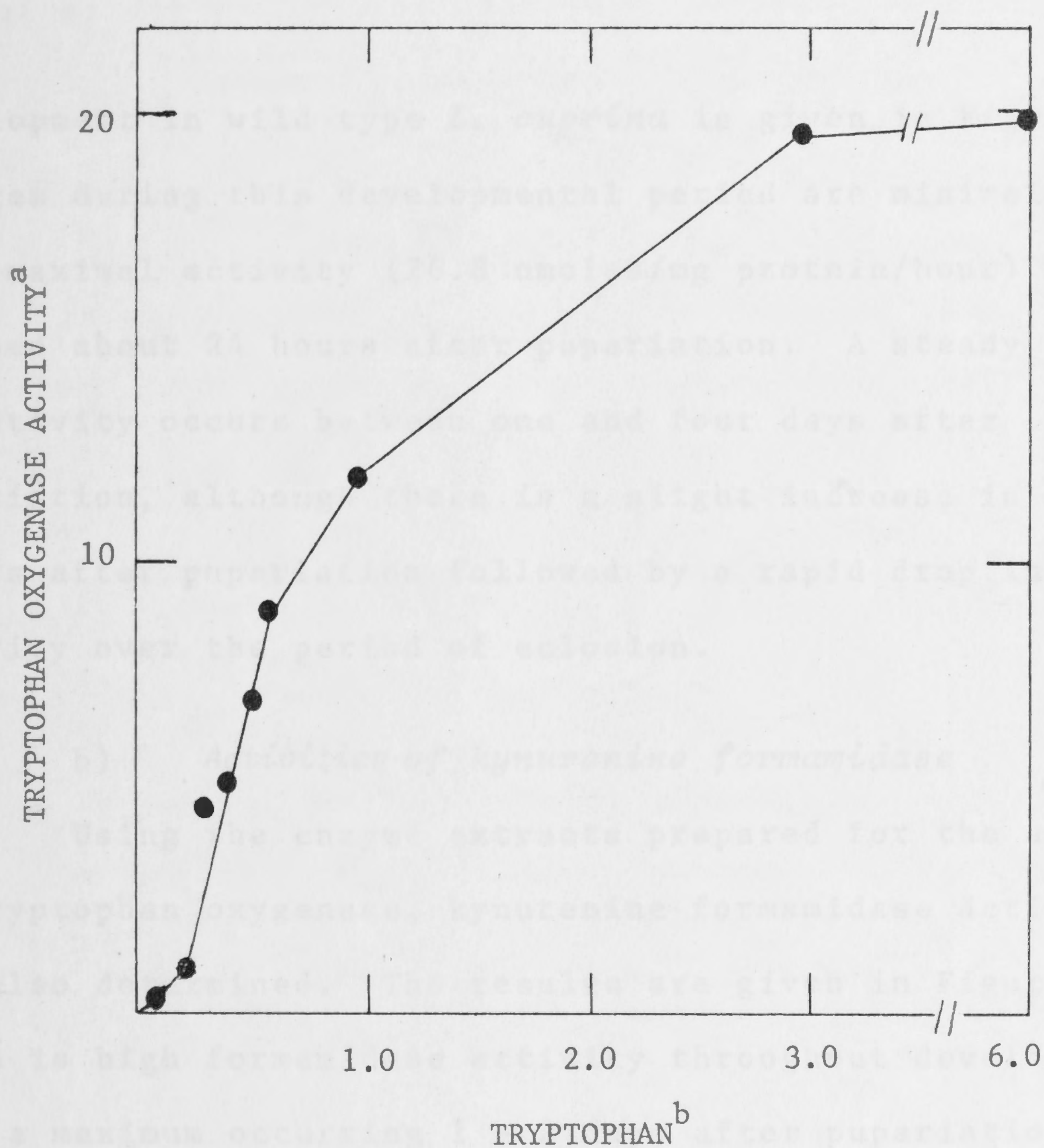


FIGURE 3.5

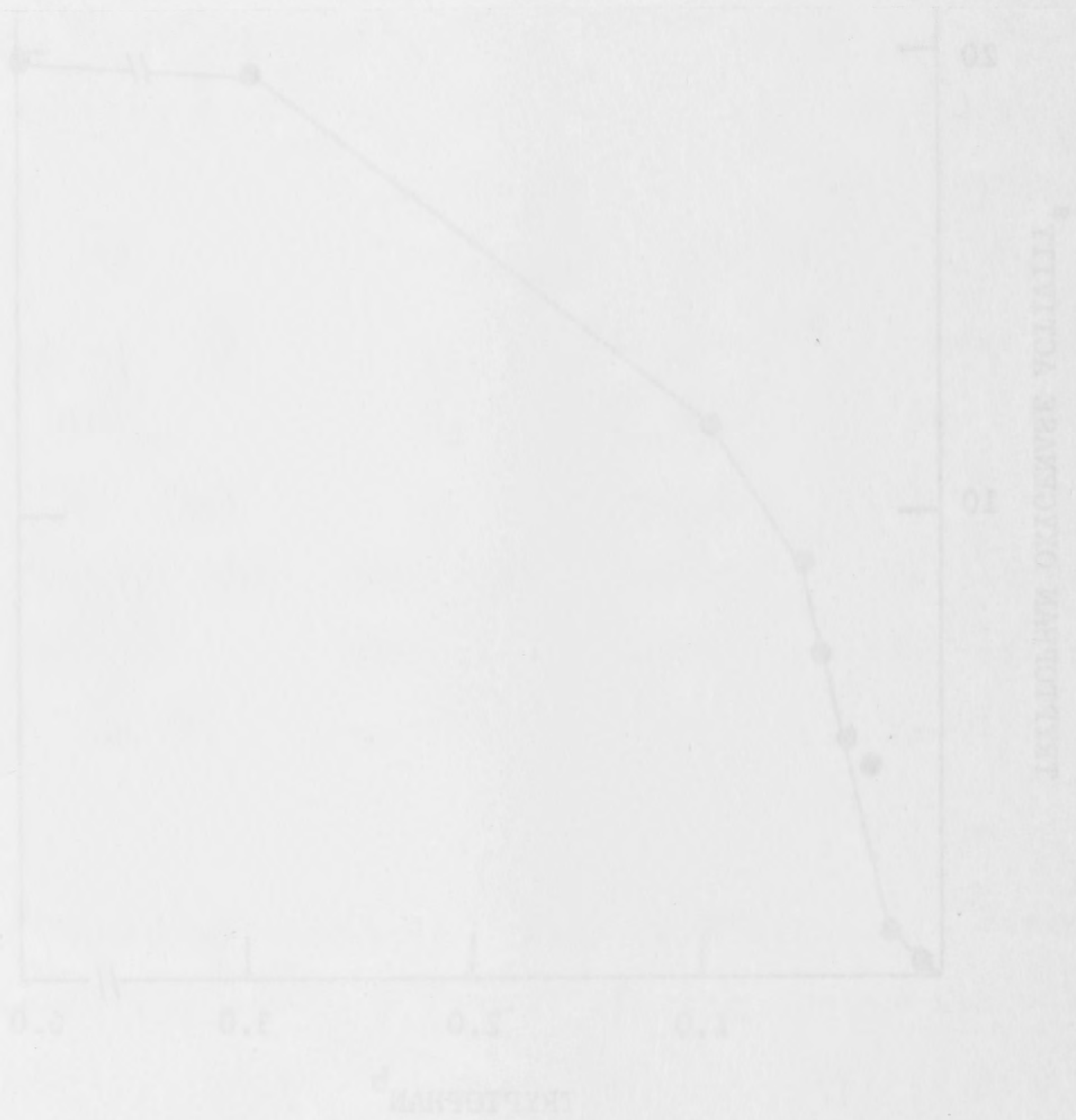
DEPENDENCE OF TRYPTOPHAN OXYGENASE FROM *L. CUPRINA* ON CONCENTRATION OF THE SUBSTRATE, TRYPTOPHAN

A typical experiment showing sigmoidal dependence of *L. cuprina* tryptophan oxygenase on the concentration of the substrate. Hill coefficients were calculated from five such experiments. The mean of these five values was 1.64 ± 0.19

a Unit of enzyme activity: as for Figure 3.4.

b Concentration of tryptophan in final incubation (mM).

* The value for the Hill co-efficient (1.64), and the small standard deviation (0.19), shows clearly that the curve is sigmoidal. However, a Hill co-efficient of less than 2 means that the degree of sigmoidicity is not great. Ideally, more data is needed at low tryptophan concentrations, but this is the most difficult to obtain because activities are so low.



was about 0.7 mM and the Hill coefficient was calculated to be 1.64 ± 0.19 (standard deviation) (Figure 3.5)*. All routine assays were performed using a final tryptophan concentration of 6mM which is higher than the saturation concentration for the enzyme.

The activity of tryptophan oxygenase throughout development in wild type *L. cuprina* is given in Figure 3.6. Changes during this developmental period are minimal, with maximal activity (26.8 nmoles/mg protein/hour) being reached about 24 hours after pupariation. A steady decline in activity occurs between one and four days after pupariation, although there is a slight increase in activity 5 days after pupariation followed by a rapid drop in activity over the period of eclosion.

b) *Activities of kynurenine formamidase*

Using the enzyme extracts prepared for the assay of tryptophan oxygenase, kynurenine formamidase activity was also determined. The results are given in Figure 3.6. There is high formamidase activity throughout development with a maximum occurring 1 - 2 days after pupariation, at the same time as tryptophan oxygenase activity peaks. The levels of activity of kynurenine formamidase are about fifty times greater than those of tryptophan oxygenase in these extracts. In similar extracts, Sullivan & Kitos (1976) found that *D. melanogaster* kynurenine formamidase was about 100 times more active than its tryptophan oxygenase. Since the *L. cuprina* extracts were prepared to optimise tryptophan oxygenase activity rather than that

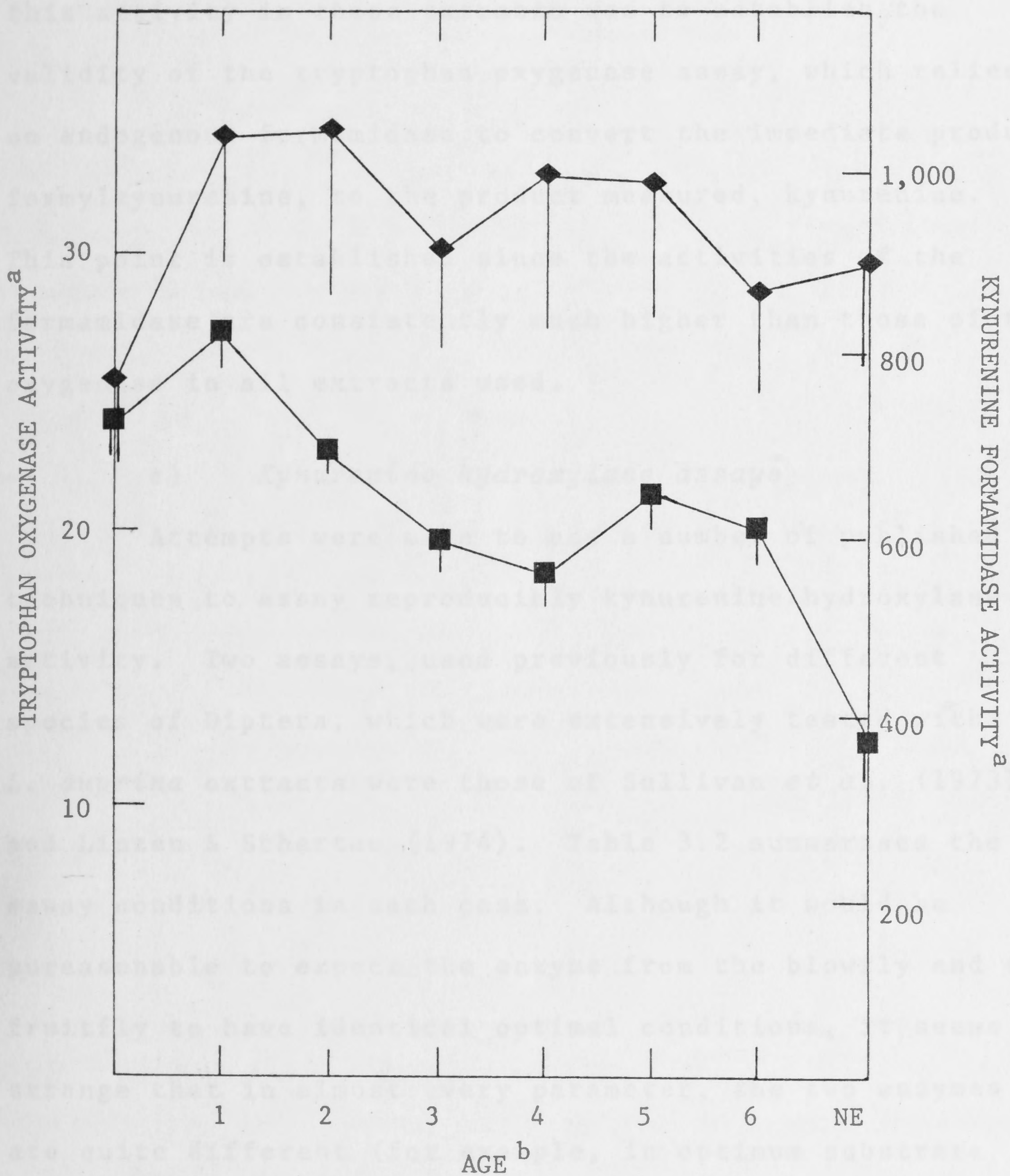


FIGURE 3.6
ACTIVITY OF TRYPTOPHAN OXYGENASE AND KYNURENINE FORMAMIDASE THROUGH DEVELOPMENT IN *L. CUPRINA*

Tryptophan oxygenase ■ Kynurenine formamidase ◆

a Units of activity: as for Figure 3.4. Values are the means of 3 separate determinations and vertical bars represent 1 SD below the mean.

b Age: as for Figure 3.3. NE: newly emerged flies.

of kynurenine formamidase, this might explain both the greater variability in kynurenine formamidase determinations and the lower relative activity when compared with the *D. melanogaster* system. The main reason for measuring this activity in these extracts was to establish the validity of the tryptophan oxygenase assay, which relies on endogenous formamidase to convert the immediate product, formylkynurenine, to the product measured, kynurenine. This point is established since the activities of the formamidase are consistently much higher than those of the oxygenase in all extracts used.

c) *Kynurenine hydroxylase assays*

Attempts were made to use a number of published techniques to assay reproducibly kynurenine hydroxylase activity. Two assays, used previously for different species of Diptera, which were extensively tested with *L. cuprina* extracts were those of Sullivan *et al.* (1973) and Linzen & Schartau (1974). Table 3.2 summarises the assay conditions in each case. Although it would be unreasonable to expect the enzyme from the blowfly and the fruitfly to have identical optimal conditions, it seems strange that in almost every parameter, the two enzymes are quite different (for example, in optimum substrate concentration and pH, response to KCN, light sensitivity and developmental stage of maximum activity). Neither of these published assays gave reproducible results when used with *L. cuprina* extracts; nor did the method of Ryall & Howells (1974), which is similar to that of

TABLE 3.2

ASSAYS FOR KYNURENINE HYDROXYLASE

REFERENCE	Sullivan *et al.* (1973)	Linzen & Schartau (1974)
CONDITION		
Species	*D. melanogaster*	*P. terrae-novae*
Substrate optimum	1.0 - 3.0 mM	0.33mM
pH Optimum	8.1	6.8
NADPH Optimum	0.8 - 2.0 mM	1.0mM
Monovalent cations	Inhibit 50% at 0.1M	No effect
Divalent cations	Inhibit over 0.02M	No effect
Phenylthiourea	Absent from assay	"A little" in assay
KCN	5mM in assay	Inhibits
2-mercaptoethanol	1mM in assay	Absent
Sodium azide	Absent	10mM in assay
Cysteine	Absent	1.6mM in assay
Lighting	Not mentioned	Red light/dark
Temperature	37°C	37°C
3-Hydroxykynurenine determination	Inagami (1954) or radiometric	Linzen (1973)
Peak activity	During second half of pupal life	In wandering larvae

Sullivan *et al.* (1973). An attempt was made to develop a direct spectrophotometric assay based on that used by Bandlow (1972) with the yeast *Saccharomyces cerevisiae*. The difficulty with such an assay is that the enzyme is reported to be in the outer mitochondrial membrane, and thus a cloudy extract containing mitochondria must be present in the cuvette. Even when a substrate blank containing the extract was used to correct for this problem reproducible values could not be obtained. Mitochondrial preparations from *L. cuprina* were tested by all these methods but showed negligible activity. In Chapter 5 an assay of the conversion of kynurenine to 3-hydroxykynurenine *in vivo* is described. This involves the injection of [^{3}H]-kynurenine and the detection of the radioactive product of kynurenine hydroxylase activity, [^{3}H]-3-hydroxykynurenine.

d) *Activities of phenoxazinone synthase*

The activity of this enzyme throughout development in extracts of wild type pupae is shown in Figure 3.7. Some activity is detected in extracts of young pupae, although this may be a spurious result caused by the extremely active phenol oxidases which produce tanning in these extracts. The use of phenylthiourea (present in the assays of tryptophan oxygenase to inhibit this class of enzymes) was not possible. Phenylthiourea was found to interfere with the phenoxazinone synthase reaction in extracts where phenol oxidase activity was low as well as in those with an appreciable degree of tanning. Phenyl-

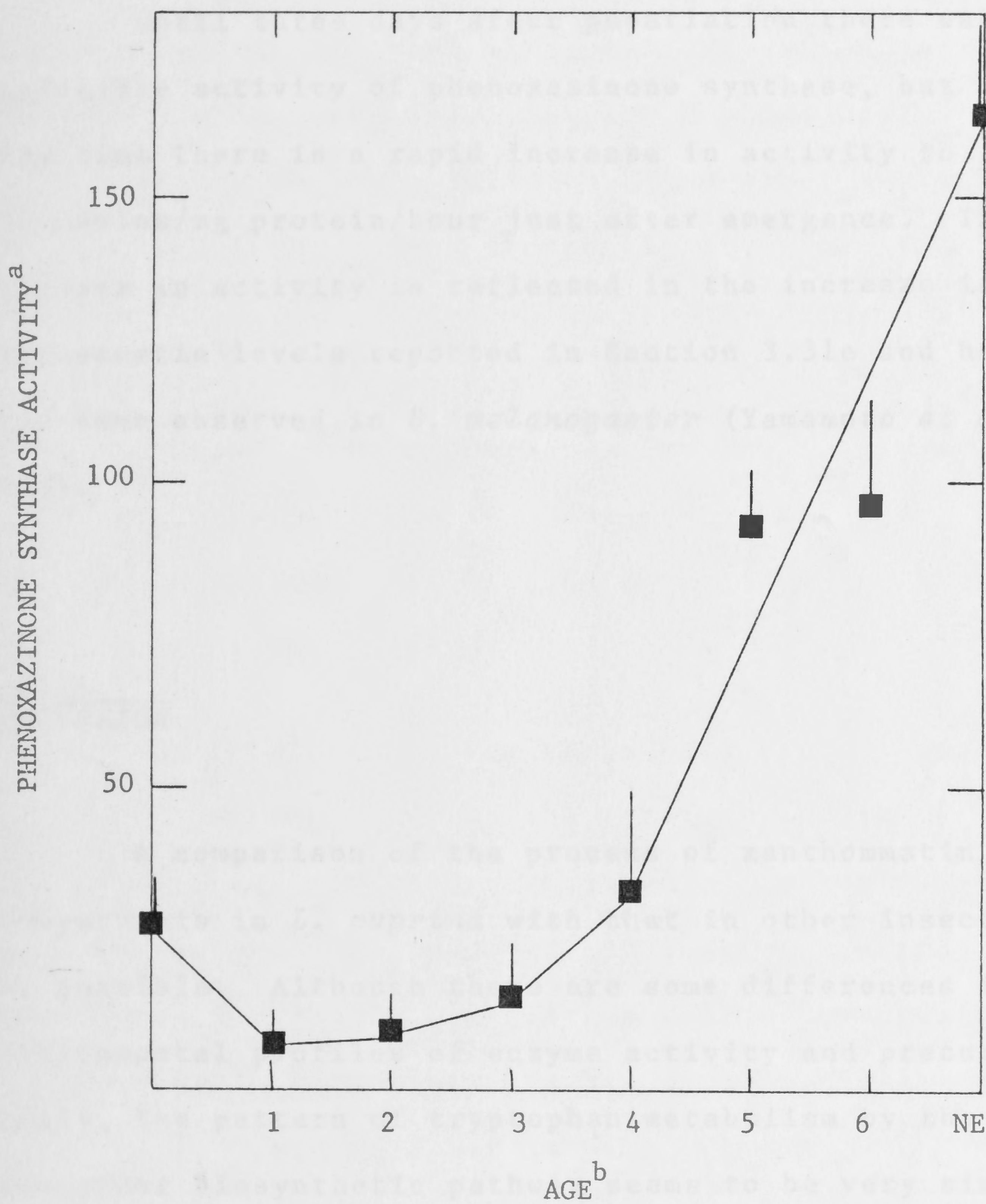


FIGURE 3.7

ACTIVITY OF PHENOXAZINONE SYNTHASE THROUGH DEVELOPMENT IN *L. CUPRINA*

a Unit of activity : nmole phenoxazinone produced/mg protein/hour. Values are the means of 3 separate determinations done in duplicate and vertical bars represent I SD above the mean.

b Age : as for Figure 3.3. NE : newly emerged flies.

thiourea is an agent which complexes copper ions. It probably also binds manganese ions which are essential for the phenoxazinone synthase reaction.

Until three days after pupariation there was negligible activity of phenoxazinone synthase, but after this time there is a rapid increase in activity to 156 nmoles/mg protein/hour just after emergence. This increase in activity is reflected in the increase in xanthommatin levels reported in Section 3.31e and has also been observed in *D. melanogaster* (Yamamoto *et al.*, 1976).

3.4 *DISCUSSION*

A comparison of the process of xanthommatin biosynthesis in *L. cuprina* with that in other insects is now possible. Although there are some differences in developmental profiles of enzyme activity and precursor levels, the pattern of tryptophan metabolism by the ommochrome biosynthetic pathway seems to be very similar to that in *D. melanogaster* and to a lesser extent that in *P. terrae-novae* which have both been studied in detail (*D. melanogaster* by Sullivan *et al.*, 1973; Sullivan *et al.*, 1974; Ryall & Howells, 1974; Sullivan & Kitos, 1976 and *P. terrae-novae* by Linzen & Schartau, 1974; Schartau & Linzen, 1976; Schartau, 1978).

The fluctuations in tryptophan levels during development are not great. *L. cuprina*, like *D. melanogaster*, begins and ends pupal life with about the same level of free tryptophan. There is a build-up of tryptophan early in pupal life prior to xanthommatin synthesis in both species. *P. terrae-novae* begins to accumulate tryptophan earlier than the other two species, but the peak level occurs at roughly the same time. The relative constancy of tryptophan levels conforms with the observations of Williams & Birt (1972) that the total free amino acid pool in *L. cuprina* remains remarkably constant during adult development in the puparium, in spite of the extensive biochemical and morphological changes which occur over this period.

The level of kynurenine in wild type *L. cuprina* is always low compared with the other metabolites, suggesting that flux through kynurenine is rapid. In *D. melanogaster* and *L. cuprina* the level of 3-hydroxykynurenine is markedly higher than that of kynurenine at all stages of development (Ryall & Howells, 1974; Section 3.31c & d). Linzen & Schartau (1974) observed the reverse trend throughout development in *P. terrae-novae*. This difference in the 3-hydroxykynurenine/kynurenine ratio may relate to methodology. The Inagami (1954) reaction with nitrite was used to determine 3-hydroxykynurenine in the present study and in that by Ryall & Howells (1974), whereas Linzen & Schartau (1974) used a different method, in which 3-hydroxykynurenine in the presence of ferricyanide is converted to

xanthommatin which is then reduced with ascorbic acid. In my hands, the latter method produced only 10% of the expected conversion of 3-hydroxykynurenine to xanthommatin and so estimates made using this method may be low.

As already discussed (Section 3.31e) the accumulation of xanthommatin in *L. cuprina* follows a similar developmental pattern to that in the other dipteran species which have been studied. Interestingly, the level reached at emergence by four different species, when expressed as μmoles/g fresh weight, is the same, about 1.5 μmoles (Table 3.3).

Table 3.3 also shows the maximum amounts of xanthommatin precursors which accumulate at any time during pupal life in these four species. It is noted that *L. cuprina* and *P. terrae-novae* reach a maximum tryptophan level about twice that of *D. melanogaster* and *C. erythrocephala*, while the maximum kynurenine level in *L. cuprina* is about half that in *D. melanogaster* and *P. terrae-novae*. 3-Hydroxykynurenine levels are also very variable, with the maximum level in *L. cuprina* being seven times that in *P. terrae-novae*, 2.5 times that in *D. melanogaster* and 1.5 times that in *C. erythrocephala*.

In spite of these variations, the proportion of total tryptophan which is channelled into the ommochrome pathway is similar in *D. melanogaster*, *P. terrae-novae* and *L. cuprina*. Ryall & Howells (1974) calculate that approximately 30% of the total tryptophan pool present in

TABLE 3.3

MAXIMUM LEVELS OF TRYPTOPHAN METABOLITES DURING PUPAL LIFE IN DIPTERAN SPECIES

	SPECIES			
	D. melanogaster	*C. erythrocephala*	*P. terrae-novae*	*L. cuprina*
COMPOUND[a]				
Tryptophan	0.39	0.39	0.85	0.68
Kynurenine	0.43	-	0.45	0.22
3-Hydroxykynurenine	0.89	1.38	0.35	2.13
Xanthommatin (at emergence)	1.40	1.49	1.67	1.49
WEIGHT[b]	1	120	40	25
REFERENCE	Ryall & Howells (1974)	Langer & Grassmäder (1965) Linzen (1963)	Linzen & Schartau (1974)	Section 3.31

a Expressed as μmoles/g fresh weight; calculated from figures given in references where necessary.

b Approximate fresh weight of an adult insect, expressed in mg.

D. melanogaster at pupariation appears as xanthommatin at emergence. Similar calculations were made for *L. cuprina* and *P. terrae-novae* (Table 3.4). For *P. terrae-novae* this gives a value for total tryptophan plus kynurenine and 3-hydroxykynurenine of about 11 μmoles/g fresh weight at pupariation, of which 3.0 μmoles/g fresh weight has been converted to xanthommatin at emergence. A further 0.5 μmoles/g fresh weight is present at emergence in the form of kynurenine and 3-hydroxykynurenine. In other words, for *P. terrae-novae* (as for *D. melanogaster*) between 30% and 40% of the total tryptophan present at pupariation has been converted to xanthommatin and its immediate precursors by emergence. Of this figure, most is in the form of xanthommatin. Although a figure for protein-bound tryptophan at pupariation is not available for *L. cuprina*, the combined level of kynurenine, 3-hydroxykynurenine and xanthommatin at emergence (4.6 μmoles/g fresh weight) is very similar to that in the other two species. This strongly suggests that a similar proportion of total tryptophan is channelled into these metabolites.

It is not possible to compare the levels of activity of the enzyme tryptophan oxygenase directly in *L. cuprina*, *P. terrae-novae* and *D. melanogaster*. Not only are the insects of different size and life style, but also each author has expressed results ona different basis. Looking at patterns of activity during development, several similarities are seen. Both the *D. melanogaster* enzyme (Sullivan & Kitos, 1976) and the *L. cuprina* enzyme

TABLE 3.4

TRYPTOPHAN AND ITS METABOLITES AT PUPARIATION AND EMERGENCE IN DIPTERAN SPECIES

	SPECIES		
	D. melanogaster	*P. terrae-novae*	*L. cuprina*
COMPOUND[a]			
At pupariation			
Protein-bound tryptophan	7.0	10.0	-
Free Tryptophan	0.2	0.7	0.6
Kynurenine	0.9	0.1	0.2
3-Hydroxykynurenine	0.7	0.2	1.6
TOTAL	8.8	11.0	-
At emergence			
Free tryptophan	0.4	0.3	0.4
Kynurenine	0.4	0.1	0.2
3-Hydroxykynurenine	0.9	0.4	1.4
Xanthommatin[b]	2.8	3.0	3.0
TOTAL	4.5	3.8	5.0

a Expressed as μmoles/g fresh weight; calculated for *D. melanogaster* and *P. terrae-novae* from values given in Ryall & Howells (1974) and Linzen & Schartau (1974). Values for *L. cuprina* obtained in the present study.

b Value for xanthommatin multiplied by 2, since 2 molecules tryptophan → 1 molecule xanthommatin.

(Section 3.32a) have their peak activity soon after pupariation, which, as Sullivan & Kitos (1976) point out, is a feature common to many general metabolic enzymes. At the time of this rise in activity the tryptophan level is also rising, indicating that tryptophan is being released by protein breakdown more rapidly than it is being used for protein synthesis and kynurenine formation. Although the enzyme activity after eclosion was not followed in the present study, Sullivan & Kitos (1976) found a relatively constant level of activity in *D. melanogaster* after this time. In contrast, the tryptophan oxygenase activity measured in *P. terrae-novae* by Linzen & Schartau (1974) has its maximum very early in larval life and peaks again long (six days) after emergence. This means that for this species, when the free tryptophan level is falling, midway through pupal life, there is negligible tryptophan oxygenase activity. Since excretion of tryptophan is not possible at this stage, this would suggest that there is an increase in protein-bound tryptophan. However, no such increase is evident in the results of Linzen & Schartau (1974). These anomalous results cast doubts on the accuracy of the profile obtained by these workers. In addition, at the times of maximal tryptophan oxygenase activity found in *P. terrae-novae* there is negligible kynurenine formamidase activity and no accumulation of the other metabolites of the pathway, kynurenine, 3-hydroxykynurenine and xanthommatin (Linzen & Schartau, 1974).

Assuming that free tryptophan is distributed uniformly throughout the insect, the concentration of free tryptophan in *L. cuprina* can be calculated to be about 0.6mM at the time of maximal tryptophan oxygenase activity. Since the half-saturating substrate concentration for the *L. cuprina* enzyme is about 0.7mM (Figure 3.4) the enzyme probably responds sigmoidally to tryptophan concentration *in vivo*. This lends weight to the idea that this is the rate limiting step of the pathway (Ryall & Howells, 1974). Further support for this idea comes from the uniformly low levels of kynurenine throughout development, suggesting that kynurenine hydroxylase is much more active than tryptophan oxygenase and so kynurenine is rapidly converted to 3-hydroxykynurenine (without accumulating) whenever kynurenine hydroxylase is active. Such allosteric control at the beginning of the pathway is important because tryptophan is an essential amino acid in Diptera, and the insect must ensure that sufficient free tryptophan is available for protein synthesis but that the level does not become too high, by channelling excess free tryptophan into ommochrome production.

Some authors have investigated the tissue localisation of tryptophan oxygenase. The activity of this enzyme has been reported to be high in the fat body and malpighian tubules of larvae and adults and in the developing eye discs (see Table 1.2 for references). The cell lineage analyses of Nissani (1975) indicate that the developing eye disc of *D. melanogaster* is capable of some kynurenine

production although tryptophan oxygenase activity elsewhere in the body is also important. Kaufman (1962) reports that 35% of the *D. melanogaster* tryptophan oxygenase activity is located in the head region. In a preliminary experiment using the enzyme from *L. cuprina* only about 10% of the activity was found in the head region, indicating that the localisation of the enzyme may be different from that in *D. melanogaster*. This may mean that less xanthommatin is produced directly from tryptophan within the pigment cells in *L. cuprina* compared with *D. melanogaster*, and that the pigment cells of the former species depend more on the uptake of kynurenine and 3-hydroxykynurenine from the haemolymph for production of this pigment. This feature will be discussed further in Chapter 5.

Kynurenine formamidase shows little developmental variation in *L. cuprina* (Figure 3.6) although the *D. melanogaster* and *P. terrae-novae* enzymes both have marked fluctuations of activity during adult development in the puparium (Sullivan & Kitos, 1976; Linzen & Schartau, 1974). In *D. melanogaster* the peak activities occur before pupariation and late in adult life (Table 1.3); in *P. terrae-novae* the activity gradually decreases as the insect passes from the larval stage through pupal life and is low from one day after emergence. These two profiles are very different and the *L. cuprina* profile is different again. However, the enzyme in all cases is so much more active than tryptophan oxygenase that even the lowest levels of activity of kynurenine formamidase are meaningless in

terms of flux of tryptophan through the pathway.

The activity of kynurenine hydroxylase in *L. cuprina* can only be inferred from the presence of 3-hydroxykynurenine in extracts of pupae at various stages (Figure 3.3c). In *D. melanogaster*, the activity peaks in third instar larvae (Sullivan *et al.*, 1973) and again in the second half of pupal life (Ghosh & Forrest, 1967; Sullivan *et al.*, 1973; Ryall & Howells, 1974). According to Linzen & Schartau (1974) the *P. terrae-novae* enzyme has peak activity at pupariation. The level then falls to negligible activity for the rest of the life cycle. It is strange that kynurenine, tryptophan and particularly 3-hydroxykynurenine are relatively low at this time of maximum activity and also that 3-hydroxykynurenine and later xanthommatin appear in the insect at the time when the observed activity of kynurenine hydroxylase is negligible. These conflicting results for *P. terrae-novae* are difficult to understand, except in terms of the problems which have traditionally been experienced in assaying this enzyme (Linzen, 1974) and the different methods used to measure the product, 3-hydroxykynurenine, as discussed earlier in this section.

The activity of kynurenine hydroxylase is probably present in *L. cuprina* larvae since the level of 3-hydroxy-kynurenine is quite high compared with that of the other xanthommatin precursors just after pupariation (see Table 3.4). Similarly, activity is probably present in the first half of pupal life when the kynurenine level is

falling (even though activity of tryptophan oxygenase is high) and the level of 3-hydroxykynurenine is rising (Figure 3.3). In the last half of pupal life, the 3-hydroxykynurenine level falls, due to the onset of xanthommatin synthesis. The kynurenine level does not rise until just before emergence. This would suggest that the kynurenine hydroxylase activity peaks over the period of onset of xanthommatin synthesis, similar to the *D. melanogaster* enzyme (Ghosh & Forrest, 1967; Sullivan *et al.*, 1973; Ryall & Howells, 1974).

It is difficult to see why this activity could not be detected *in vitro* when extracts of *L. cuprina* pupae at the appropriate developmental stage were made. Since crude extracts were used, there may be inhibitors of the enzyme present in the incubations. However, dialysed extracts and mitochondrial preparations also had no activity, so such inhibitors would have to be tightly bound to the enzyme. The enzyme may be unstable, particularly if processing removes it from the mitochondrial membrane. An additional problem found in attempting to assay this enzyme was the high background of endogenous 3-hydroxykynurenine (compare *L. cuprina* with the other species in Tables 3.3 and 3.4). This makes the use of colourimetric methods for the determination of the enzyme activity difficult. Activity was not detected, however, when a radiometric method (Sullivan *et al.*, 1973) was used to measure the product of enzyme activity.

An insect phenoxazinone synthase has previously only been detected in *D. melanogaster* (Phillips *et al.*, 1973; Yamamoto *et al.*, 1976). The particulate enzyme activity measured for *L. cuprina* has similar developmental characteristics to this *D. melanogaster* enzyme (Yamamoto *et al.*, 1976) and it seems likely that such an enzyme is responsible for the terminal reaction of xanthommatin biosynthesis in flies in particular and in insects in general.

At emergence, the activity of the *D. melanogaster* phenoxazinone synthase in wild type flies is 76.8 nmoles/g fresh weight/hour (Yamamoto *et al.*, 1976). Calculated on this basis, the activity of the *L. cuprina* enzyme at the same developmental stage is about 160 nmoles/g fresh weight/hour. This 2-fold difference in activity might be explained by the fact that the synthetic *o*-aminophenol, 3-hydroxyanthranilic acid, is used as substrate for the enzyme instead of the biological substrate, 3-hydroxy-kynurenine, in the *in vitro* assays. This is necessary because of the instability of xanthommatin under the conditions of incubation (Ryall, 1973) which makes it difficult to monitor the conversion of 3-hydroxykynurenine to xanthommatin. Ryall (1973) reports that the conversion of 3-hydroxykynurenine to xanthommatin by the *D. melanogaster* enzyme occurs at about 10% of the rate of the conversion of 3-hydroxyanthranilic acid to cinnabarinic acid. The difference in activities seen between *D. melanogaster* and *L. cuprina* may reflect a difference in the affinity of

the enzymes from the two species for the synthetic substrate.

The events which result in xanthommatin formation in the eye pigment cells of wild type *L. cuprina* thus appear to take the following ordered sequence. Larval histolysis releases tryptophan so that early in pupal life there is an accumulation of this amino acid. Since the first two enzymes of the pathway, tryptophan oxygenase and kynurenine formamidase, are active at this time, some of this tryptophan is converted to kynurenine and the levels of this metabolite remain relatively high. Kynurenine hydroxylase is probably activated early in pupal life so that the kynurenine levels fall and 3-hydroxykynurenine accumulates after the first day following pupariation. After the onset of phenoxazinone synthase activity and consequent xanthommatin formation between 3 and 4 days after pupariation, the level of 3-hydroxykynurenine begins to fall. Flux through the pathway is probably rapid over the last two days of pupal life, since kynurenine levels remain low and 3-hydroxykynurenine levels are decreasing. At around the time of emergence, tryptophan oxygenase activity also falls, in spite of the fact that the kynurenine level is rising, indicating that flux through the pathway is diminishing. A steady rate of tryptophan conversion to xanthommatin probably continues for a few days after emergence until maximum pigmentation is reached, as has been observed for other dipteran species.

CHAPTER 4

XANTHOMMATIN BIOSYNTHESIS IN EYE COLOUR MUTANTS OF *LUCILIA CUPRINA*

4.1 *INTRODUCTION*

The results discussed in Chapter 3 have established for wild type *L. cuprina* the levels of the enzymes and intermediates of the tryptophan → xanthommatin pathway. In this chapter these results are compared with those for the seven eye pigment mutants of *L. cuprina* (Section 2.3) in an attempt to establish the nature of the defect in these strains.

One common biochemical result of a genetic defect is disruption of an enzyme activity. This happens where the mutation occurs in the structural gene coding for the enzyme, or in a gene coding for a protein involved in the regulation of enzyme activity at any stage between RNA transcription and final catalytic function. Thus rosy and maroon-like mutants of *D. melanogaster* both lack xanthine dehydrogenase activity. Rosy is the structural locus and maroon-like is involved in regulating the activity of the product of the rosy locus, probably at the post-translational or catalytic level (O'Brien & MacIntyre, 1978).

The biochemical phenotype of a mutant defective in an enzyme often includes the accumulation of the substrate of the enzyme and a low level of its product. Negligible activity of the enzyme is detected *in vivo* and *in vitro*. In some cases the phenotypic effect of the mutation can

be overcome by supplying the mutant organism with the product of the missing enzyme (a non-autonomous mutation). In the case of eye pigmentation, pigment production occurs when the product of the missing enzyme is provided exogenously, for example in the diet, by transplantation of the relevant tissue into a host which has enzyme activity, by injection or through genetic mosaicism (Section 1.31).

These parameters have been studied among ommochrome-deficient mutants of many insect species. There is characteristically a class of mutants which lack tryptophan oxygenase and a second group lacking kynurenine hydroxylase (Linzen, 1974; see also Section 1.31) but only one report of a mutant lacking kynurenine formamidase activity exists and this is in a mutant which is primarily defective in the production of tryptophan oxygenase (green of *Musca domestica*; Grigolo, 1969). Phenoxazinone synthase has been insufficiently studied among eye colour mutants, although at least one *D. melanogaster* mutant (cardinal) and one *Apis mellifera* mutant (chartreuse) seem likely to be defective in this enzyme.

Nevertheless, the majority of eye colour mutants lack one or both pigments and have no definitive enzyme deficiency to which the lack of pigment production might be attributed. In Section 1.34 the importance to eye pigmentation of successful uptake and storage of pigment precursors by specific tissues was noted. *D. melanogaster* mutants which are

defective in these processes have been studied by several authors (Phillips *et al.*, 1973; Sullivan & Sullivan, 1975; Howells & Ryall, 1975; Howells *et al.*, 1977). Howells *et al.* (1977) used a simple diagnostic test for xanthommatin mutants of this type. They showed that white and scarlet of *D. melanogaster* excrete more kynurenine and 3-hydroxykynurenine than wild type as larvae. Consequently, the 3-hydroxy-kynurenine level in early pupae was very low and this metabolite could only accumulate during the pupal stage in these mutants (when excretion is not possible; Section 1.2). The level of 3-hydroxykynurenine in early pupae is a measure of larval accumulation and the level in late pupae of pupal accumulation of the compound. The level in adults soon after emergence gives some idea of the location of the compound within the developing adult. If the level falls rapidly soon after emergence (as observed in white and scarlet; Howells *et al.*, 1977) the compound has presumably been excreted with the meconium, while if it falls only slowly it has probably been stored in specific tissues connected with eye pigmentation.

The levels of tryptophan metabolites in mutants, then, may reveal an enzymatic defect (accumulation of enzyme substrate and low level of enzyme product) or indicate a storage or uptake defect (significant accumulation of 3-hydroxykynurenine only in pupae; low levels in larvae and adults). Studies of enzyme activities and an assessment of the effect of providing pigment precursors in the diet

can be used to substantiate the conclusions drawn from the levels of intermediates. These aspects were examined in the eye colour mutants of *L. cuprina* and the results are reported in this chapter.

4.2 *MATERIALS AND METHODS*

4.21 EXPERIMENTAL ANIMALS

These were cultured and staged as in Section 3.21. Adults were collected at emergence and maintained on a diet of sugar and water for 18 - 20 hours at 28°C until used.

4.22 CHEMICAL ESTIMATIONS

These were made as described in Section 3.22

4.23 ENZYMES OF XANTHOMMATIN BIOSYNTHESIS

a) *Activity of tryptophan oxygenase.*

The 31,000g supernatant liquid was obtained as described in Section 3.23a. This was brought to 60% ammonium sulphate by addition of saturated ammonium sulphate solution; this was done at 0°C over one hour with constant stirring. The precipitated material was spun down at 31,000g for 30 minutes in the Sorvall centrifuge and

dissolved in 1 ml homogenising buffer. The extracts were stored at $-20^{o}C$ and made up to 3 ml before assay.

Incubation conditions were as described in Section 3.23a with cuvettes containing 1.5 ml 0.25M Tris-HCl, pH 8.25; 0.6 ml 30mM tryptophan; 75 µl 50mM 2-mercaptoethanol and 75 µl 1.5M KCl.

b) *Activity of kynurenine formamidase*

This was measured as described in Section 3.23b.

c) *Activity of phenoxazinone synthase*

This was measured as described in Section 3.23c.

4.24 FEEDING EXPERIMENTS WITH KYNURENINE AND 3-HYDROXYKYNURENINE

Vials containing 4 ml homogenised sheep's liver (Section 2.4) and 1% or 2% DL-kynurenine or 3-hydroxy-DL-kynurenine were prepared. Ten to fifteen first instar larvae (12 - 18 hours after hatching) were placed in the vials and allowed to develop to late third instar. At this stage the liver was covered with dry vermiculite. Four to six days later the vermiculite was sieved and the pupae recovered. Xanthommatin determinations were performed on the adults which emerged from these pupae.

4.3 *RESULTS*

4.31 LEVELS OF TRYPTOPHAN AND ITS METABOLITES

a) *Xanthommatin*

This was determined in the heads of adult flies 48 hours after emergence (Table 4.1). Wild type insects had 65 nmoles xanthommatin/insect while the mutants ranged from 1% of this value in the white- and yellow-eyed mutants (white, yellow, yellowish and topaz[1]) to 35% of the wild type level in the dark-eyed mutant grape. The variations correlate with the visual phenotypic differences in eye colour (Section 2.3; Table 2.1).

b) *Tryptophan levels during development*

The levels of tryptophan in early pupae (at pupariation) and late pupae (144 hours after pupariation) of the mutants (Table 4.2) show that only one strain has a pattern different from the wild type. The yellowish mutant begins pupal life with 60 nmoles free tryptophan/insect, 3 times the wild type level, and has accumulated 85 nmoles by emergence. Assuming that the tryptophan is uniformly distributed throughout the insect, this represents a concentration of 2.1mM in the whole insect. In *D. melanogaster* the mutant vermilion shows a similar accumulation (Green, 1949; Ryall & Howells, 1974) and has been demonstrated to be defective in tryptophan oxygenase (see, for example,

TABLE 4.1

XANTHOMMATIN LEVELS IN HEADS OF WILD TYPE AND MUTANTS

STRAIN	XANTHOMMATIN (nmoles/head)[a]
Wild Type	64.9 ± 4.7
Yellowish	0.6 ± 0.2
Yellow	0.6 ± 0.1
Tangerine	8.2 ± 0.5
Topaz[1]	2.1 ± 0.5
Topaz[2]	12.0 ± 2.1
White	1.2 ± 0.5
Grape	23.8 ± 2.1

a Values ± 1 standard deviation are the mean of at least 3 separate determinations done in duplicate. The heads of adult flies 48 hours after eclosion were used.

TABLE 4.2

TRYPTOPHAN LEVELS IN WILD TYPE AND MUTANTS

STRAIN	TRYPTOPHAN (nmoles/insect)[a]	
	Young pupae[b]	Old pupae[b]
Wild Type	19.7 ± 4.3	15.4 ± 2.1
Yellowish	57.8 ± 10.0	84.6 ± 11.8
Yellow	21.1 ± 4.6	15.3 ± 3.2
Tangerine	22.1 ± 2.3	14.7 ± 1.6
Topaz[1]	17.2 ± 1.4	16.2 ± 2.9
Topaz[2]	22.1 ± 2.1	15.2 ± 3.0
White	20.6 ± 3.0	18.1 ± 2.4
Grape	21.1 ± 2.9	11.8 ± 2.2

a Values ± 1 standard deviation are the mean of at least 4 separate determinations done in duplicate.

b Development was at 27°C. Young pupae were those which still had the soft white cuticle just after pupariation; old pupae were 144 hours after pupariation.

Baglioni, 1959; Tartof, 1969; Baillie & Chovnick, 1971; Sullivan & Kitos, 1976), the enzyme responsible for the conversion of tryptophan into formylkynurenine.

c) *Kynurenine levels during development*

One mutant strain has an abnormal level of kynurenine (Table 4.3). This is yellow which has 2½ times the wild type level of kynurenine at pupariation and has doubled this level just prior to emergence. This is approximately seven times the level in wild type. Cinnabar of *D. melanogaster* also accumulates kynurenine (Green, 1949) and has been shown to be defective in the activity of kynurenine hydroxylase (Ghosh & Forrest, 1967; Sullivan *et al.*, 1973), which catalyses the conversion of kynurenine to 3-hydroxykynurenine. Note that yellowish, which appears to be blocked in the first step of the pathway, has negligible kynurenine at both stages assayed.

d) *3-Hydroxykynurenine levels during development*

3-Hydroxykynurenine levels for young and old pupae and adults 18 - 20 hours after emergence are given in Table 4.4. None of the mutant strains conforms to the wild type pattern. Tangerine has 41 nmoles of 3-hydroxykynurenine per insect at pupariation (75% of the wild type level) and has accumulated twice this amount (1½ times the wild type level) by emergence. After emergence the level falls only slowly, indicating that the excess 3-hydroxykynurenine accumulated during pupal life is not stored in the meconium. This pattern of 3-hydroxykynurenine levels is very similar

TABLE 4.3
KYNURENINE LEVELS IN WILD TYPE AND MUTANTS

STRAIN	KYNURENINE (nmoles/insect)[a]	
	Young pupae[b]	Old pupae[b]
Wild type	8.0 ± 0.9	5.6 ± 1.0
Yellowish	1.0 ± 0.2	1.4 ± 0.8
Yellow	20.2 ± 2.7	41.3 ± 9.1
Tangerine	4.8 ± 1.5	6.3 ± 0.8
Topaz[1]	6.1 ± 2.7	6.9 ± 2.3
Topaz[2]	5.6 ± 1.1	6.1 ± 0.7
White	5.5 ± 2.0	7.0 ± 0.9
Grape	2.8 ± 0.4	3.0 ± 0.3

a Values ± 1 standard deviation are the mean of at least 4 separate determinations done in duplicate.

b As in Table 4.2

TABLE 4.4
3-HYDROXYKYNURENINE LEVELS IN WILD TYPE AND MUTANTS

STRAIN	3-HYDROXYKYNURENINE (nmoles/insect)[a]		
	Young pupae[b]	Old pupae[b]	Adults[c]
Wild Type	57.8 ± 12.4	50.8 ± 8.0	27.9 ± 4.0
Yellowish	9.2 ± 0.9	11.5 ± 2.3	0
Yellow	6.5 ± 3.8	9.6 ± 3.7	1.5 ± 2.5
Tangerine	41.2 ± 11.1	77.5 ± 14.2	51.0 ± 4.1
Topaz[1]	29.7 ± 9.9	60.1 ± 17.5	12.4 ± 2.5
Topaz[2]	21.4 ± 7.2	48.2 ± 16.7	15.0 ± 2.6
White	24.6 ± 9.8	60.4 ± 12.5	16.6 ± 3.0
Grape	24.7 ± 4.5	36.2 ± 4.3	17.4 ± 2.6

a Values ± 1 standard deviation are the mean of at least 4 separate determinations done in duplicate.

b As in Table 4.2

c Development was at 27°C. Adults were 18 - 20 hours after emergence.

to that found in cardinal of *D. melanogaster* (Summers, 1974) which is probably defective in the enzyme which catalyses the conversion of 3-hydroxykynurenine to xanthommatin, phenoxazinone synthase (Phillips *et al.*, 1973; Yamamoto *et al.*, 1976; Howells *et al.*, 1977). Cardinal has the wild type pattern of accumulation of 3-hydroxykynurenine until the time of onset of xanthommatin synthesis in wild type. After this time excessive accumulation of 3-hydroxykynurenine begins in cardinal (Summers, 1974). The similarity between cardinal and tangerine of *L. cuprina* suggests that tangerine, too, may be blocked in the final step of xanthommatin biosynthesis. Further results which support this idea will be presented in Chapter 5.

Yellowish and yellow, which are probably blocked earlier in the pathway, have low 3-hydroxykynurenine at all stages. The other mutants with greatly reduced xanthommatin levels, white, topaz^1 and topaz^2, have about half the wild type level of 3-hydroxykynurenine in young pupae but accumulate the compound so that they are comparable with wild type just before emergence (old pupae). After emergence, the 3-hydroxykynurenine level in these mutants falls faster than it does for wild type or tangerine suggesting that much of the pupal accumulation is excreted with the meconium. Grape, which appears defective primarily in the production of the yellow acid-soluble pigment sepiapterin (Chapter 6),also has reduced levels of 3-hydroxykynurenine in both young pupae (approximately half wild

* However, it is present even in very young white pupae (untanned puparia), so that at this stage (at least) it cannot derive from melanin.

type) and old pupae.

e) *Xanthommatin levels during development*

The mutants with pale eyes (yellowish, yellow, topaz[1] and white) show little accumulation of xanthommatin through pupal life (Table 4.5). As with the wild type developmental profile (Section 3.31; Figure 3.3d), the material contributing to the value for young pupae and most of the value for old pupae in these mutants is probably not xanthommatin, since it is localised in the body and has different spectral characteristics from xanthommatin. This material could not be identified. Its UV-visible spectrum was also different from those of sepiapterin, 3-hydroxy-kynurenine and riboflavin. It may be colour derived from melanin and extracted from the pupal case and integument which are darkly coloured. * In the estimates of xanthommatin in the heads of adult flies (Table 4.1) most of this colour had been removed but the spectra of the extracts from the pale-eyed strains still showed the mysterious peak, possibly from the integument surrounding the eye. In these strains there was no peak at 492 nm, the absorption maximum for xanthommatin (Figure 3.2) and the value obtained is apparently due to this unknown compound. Although the presence of this compound makes the interpretation of the results more difficult, it is clear that the accumulation of xanthommatin over pupal life correlates with the amount of xanthommatin found in the heads 48 hours after emergence (Table 4.1).

TABLE 4.5

XANTHOMMATIN LEVELS IN WILD TYPE AND MUTANTS

STRAIN	XANTHOMMATIN (nmoles/insect)[a]	
	Young pupae[b]	Old pupae[b]
Wild Type	7.6 ± 1.7	54.1 ± 1.8
Yellowish	5.6 ± 2.0	7.1 ± 1.8
Yellow	6.4 ± 0.8	8.5 ± 1.5
Tangerine	6.5 ± 0.3	12.1 ± 2.1
Topaz[1]	5.9 ± 2.4	9.1 ± 3.8
Topaz[2]	6.4 ± 0.4	16.6 ± 2.7
White	8.2 ± 2.4	5.6 ± 2.0
Grape	6.2 ± 0.9	27.8 ± 4.4

a Values ± 1 standard deviation are the mean of at least 4 separate determinations done in duplicate.

b As in Table 4.2.

4.32 ENZYME ACTIVITIES IN WILD TYPE AND MUTANTS

a) *Activity of tryptophan oxygenase*

The activities of tryptophan oxygenase in wild type and the eye colour mutants were determined in extracts prepared from young pupae and the results are presented in Table 4.6. Only yellowish has a level of activity significantly lower than that of wild type. This correlates with the high tryptophan levels found in this mutant (Section 4.31b) and confirms that yellowish is blocked in the conversion of tryptophan to kynurenine.

b) *Activity of kynurenine formamidase*

All the mutant strains showed high kynurenine formamidase activity, about ten times the activity of tryptophan oxygenase, in the ammonium sulphate extracts prepared for assay of tryptophan oxygenase activity, although there were marked variations among the mutants (Table 4.6). Similarly, in *D. melanogaster*, no mutants lacking in kynurenine formamidase have been found (Glassman, 1956). Unlike green of *Musca domestica*, which is reported to lack kynurenine formamidase activity as well as tryptophan oxygenase activity (Grigolo, 1969), yellowish of *L. cuprina* only lacks tryptophan oxygenase activity and is within the wild type range of activity for kynurenine formamidase. It must be noted that the ammonium sulphate extracts were prepared to optimise tryptophan oxygenase activity and thus

TABLE 4.6
LEVELS OF ACTIVITY OF TRYPTOPHAN OXYGENASE AND KYNURENINE FORMAMIDASE IN WILD TYPE AND MUTANTS

STRAIN	ENZYME ACTIVITIES[a]	
	Tryptophan oxygenase	Kynurenine formamidase
Wild Type	41.8 ± 4.0	377 ± 30
Yellowish	1.5 ± 0.3	240 ± 8
Yellow	28.5 ± 6.0	220 ± 28
Tangerine	39.4 ± 8.0	405 ± 49
Topaz[1]	33.9 ± 0.8	276 ± 59
Topaz[2]	37.5 ± 2.0	431 ± 27
White	36.4 ± 2.9	291 ± 53
Grape	36.3 ± 2.5	250 ± 40

a Values ± 1 standard deviation are the results of at least three separate determinations. Extracts were prepared from young pupae. Unit of enzyme activity: nmoles kynurenine produced/mg protein/hour.

kynurenine formamidase activities may be lower than would be obtained under optimal conditions. However, this does not alter the conclusion that kynurenine formamidase is present, at roughly wild type level, in all the mutants. When dialysed extracts (Section 3.23a & b) of young pupae were used, yellowish still lacked tryptophan oxygenase activity and had wild type level of activity of kynurenine formamidase. In this case, as noted in Section 3.32, the wild type formamidase activity was 50 times the tryptophan oxygenase activity.

c) *Activity of phenoxazinone synthase*

Phenoxazinone synthase activity was determined in wild type and the mutants in extracts prepared from pupae 120 hours after pupariation. The results are presented in Table 4.7. Only grape among the mutants has the wild type level of activity. All the other mutants have only 20% or less of the wild type activity. Since two mutants (yellow and yellowish) have already been shown to lack completely the activity of specific enzymes earlier in the pathway, this effect on phenoxazinone synthase must be a pleiotropic one in these strains. A similar pleiotropic effect of defects early in the xanthommatin biosynthetic pathway on phenoxazinone synthase activity was also found for *D. melanogaster* mutants (Phillips *et al.*, 1973; Yamamoto *et al.*, 1976). From these phenoxazinone synthase assays it is not possible to say which of the other mutants are affected primarily in this step. Since tangerine is the strain with

TABLE 4.7

ACTIVITY OF PHENOXAZINONE SYNTHASE IN WILD TYPE AND MUTANTS

STRAIN	PHENOXAZINONE SYNTHASE ACTIVITY [a]
Wild Type	93.9 ± 18.5
Yellowish	15.0 ± 12.5
Yellow	23.4 ± 15.5
Tangerine	21.3 ± 9.7
Topaz^1	12.2 ± 7.3
Topaz^2	25.3 ± 14.6
White	17.9 ± 4.7
Grape	88.3 ± 25.4

[a] Values ± 1 standard deviation are the mean of at least 3 separate determinations done in duplicate. Pupae 120 hours after pupariation were used.
Unit of enzyme activity: nmoles phenoxazinone produced/mg protein/hour.

3-hydroxykynurenine accumulation most similar to cardinal of *D. melanogaster* it seems likely that tangerine is blocked solely in this step , possibly in the production of phenoxazinone synthase.

4.33 FEEDING EXPERIMENTS WITH KYNURENINE AND 3-HYDROXYKYNURENINE

In *D. melanogaster* and *Ephestia kühniella* it has been found that the phenotypes of some eye pigment mutants can be altered if the insects are provided with the product of the missing enzyme at a suitable developmental stage (see, for example, Beadle & Ephrussi, 1936; Beadle & Law, 1938; Green 1952; Hadorn & Kühn, 1953; Kühn & Egelhaaf, 1955; Schwabl & Linzen, 1972). Accordingly, larvae of three mutant strains of *L. cuprina* (yellowish, yellow and topaz[1]) were fed diets supplemented with kynurenine or 3-hydroxy-kynurenine and xanthommatin determinations were carried out on the heads of flies which developed from these larvae (Table 4.8).

Yellowish insects, which lack kynurenine (Table 4.3) and have low activity of tryptophan oxygenase *in vitro* (Table 4.6), were able to make some pigment (6% of the wild type level) when supplied with kynurenine in the diet. This is about 5 times the level in unsupplemented flies. Even though the amount of pigment is quite small the eye was much darker than that in the controls (similar to tangerine). When supplied with 3-hydroxykynurenine, the level of pigment produced by yellowish insects increased

TABLE 4.8

FEEDING EXPERIMENTS WITH KYNURENINE AND 3-HYDROXYKYNURENINE

DIETARY SUPPLEMENT[a]	XANTHOMMATIN (nmoles/head)[b]		
	Yellowish	Yellow	Topaz[1]
None	0.6	0.8	2.1
0.1% Kynurenine	3.3	0.9	-
0.2% Kynurenine	4.5	1.2	2.3
0.1% 3-Hydroxykynurenine	-	7.8	-
0.2% 3-Hydroxykynurenine	6.8	12.3	2.8

a A solution of kynurenine or 3-hydroxykynurenine was mixed with a measured volume of homogenised sheep's liver to the concentrations indicated. First instar larvae were placed on the liver and development allowed to proceed as usual at 27°C.

b Heads of adult flies 48 hours after emergence were used. Values are the means of at least 2 separate determinations done in duplicate.

to a maximum of 13% of wild type in one experiment. These results support the contention that yellowish is blocked in the step catalysed by tryptophan oxygenase.

Yellow insects, which have very high kynurenine levels (Table 4.3) and low 3-hydroxykynurenine levels (Table 4.4) *in vivo*, failed to produce pigment when fed kynurenine but a dietary supplement of 3-hydroxykynurenine resulted in the production of 12% - 23% of the wild type level of pigment, up to 25 times the level in unsupplemented controls. These results are in line with the suggestion that yellow insects fail to carry out the step catalysed by kynurenine hydroxylase. The pigment produced in both yellow and yellowish has the same spectral characteristics as xanthommatin from wild type and there is no reason to suspect that it is any different.

In contrast with these results, topaz[1] insects failed to produce an appreciable increase in pigment under either feeding regime, showing that the block in these insects cannot be overcome simply by the provision of pigment precursors, in other words that it is not a defect in one of the first three enzymes of the pathway. Thus the topaz[1] phenotype is autonomous, like the majority of the eye colour phenotypes of *D. melanogaster*, while the yellowish and yellow phenotypes are non-autonomous, like vermilion and cinnabar of *D. melanogaster*.

4.4 DISCUSSION

The results presented in this chapter have allowed the identification of the nature of the block in two of the eye colour mutant strains (yellowish and yellow) and possibly in a third (tangerine). By comparing the biochemical phenotypes of three other mutants with those of eye pigment mutants of *D. melanogaster*, tentative identification of possible transport/storage mutants (Section 1.35) is also made.

Vermilion of *D. melanogaster* has accumulated 6 times the wild type level of free tryptophan by emergence (Ryall & Howells, 1974); yellowish of *L. cuprina* also has about 6 times the wild type level at the same stage. Yellowish lacks tryptophan oxygenase activity in both ammonium sulphate and dialysed extracts although kynurenine formamidase activity is within the wild type range. When yellowish is provided with compounds beyond the step catalysed by tryptophan oxygenase in the pathway, xanthommatin is formed. In all these features yellowish is identical with vermilion and so the yellowish locus may well be that coding for the production of tryptophan oxygenase, as is the vermilion locus.

Yellow insects are clearly affected in the reaction which converts kynurenine to 3-hydroxykynurenine. Although kynurenine hydroxylase was not assayed *in vitro* (for the

reasons discussed in Section 3.32c), there are other lines of evidence which support this idea and place yellow in the same class as cinnabar of *D. melanogaster*. There is a massive accumulation of kynurenine in yellow over pupal life, to 8 times the wild type level, as is found in cinnabar (Green, 1949). Yellow displays the same non-autonomous behaviour as seen in cinnabar, that is a dietary supplement of 3-hydroxykynurenine permits some xanthommatin synthesis. In the next chapter, an *in vivo* assay of kynurenine hydroxylase is described, which shows that yellow cannot convert [^{3}H]-kynurenine to [^{3}H]-3-hydroxy-kynurenine, whereas wild type and yellowish have a high rate of conversion. Thus it seems likely that the yellow locus is involved in the production of the enzyme kynurenine hydroxylase.

All the mutants except grape lack appreciable phenoxazinone synthase activity. This pattern was also found for the xanthommatin mutants of *D. melanogaster* (Phillips *et al.*, 1973; Yamamoto *et al.*, 1976). In cases where the defect is clearly localised in a step preceding the final condensation reaction, this deficiency must be seen as a pleiotropic effect of the primary deficiency. Among those mutants which have no block earlier in the pathway, there may be one which has a primary defect in the step catalysed by phenoxazinone synthase. This might be tangerine, which accumulates 3-hydroxykynurenine through pupal life like the *D. melanogaster* mutant cardinal,

thought to be defective only at this step. In the next chapter the roles of the tangerine, topaz and white loci in pigment production will be considered further.

The evidence presented in this chapter for the role of the yellowish, yellow and tangerine loci in the production of enzymes of the xanthommatin biosynthetic pathway does not indicate whether these are the structural genes coding for the proteins involved or regulatory genes controlling the expression of these structural genes. In *D. melanogaster* evidence has been provided that vermilion is the structural gene for tryptophan oxygenase and cinnabar the structural gene for kynurenine hydroxylase by looking at the relationship between enzyme activity and the dose of the wild type allele at the locus in question (Tobler *et al.*, 1971; Sullivan *et al.*, 1973). Similarly, the rosy locus of *D. melanogaster* has been shown to be the structural gene for xanthine dehydrogenase using three different approaches:

a) Xanthine dehydrogenase activity is proportional to the number of ry^+ alleles present in the genome;

b) Isozyme variants of xanthine dehydrogenase have been mapped to the rosy locus;

c) Rosy mutants contain little material which cross-reacts with antibody to purified xanthine dehydrogenase. Finnerty (1976) and O'Brien & MacIntyre (1978) have summarised these experiments. Similar research will be necessary to establish the role of the *L. cuprina* genes yellowish, yellow and tangerine in the production of

tryptophan oxygenase, kynurenine hydroxylase and phenoxazinone synthase respectively.

In topaz^1, topaz^2, white and grape, 3-hydroxykynurenine accumulates during pupal life and the level falls rapidly after emergence. These mutants are somewhat similar to white and scarlet of *D. melanogaster* (Howells & Ryall, 1975; Howells *et al.*, 1977). The results presented in the next chapter investigate further the similarities between these *D. melanogaster* and *L. cuprina* mutants.

CHAPTER 5

UPTAKE AND STORAGE OF XANTHOMMATIN PRECURSORS IN *LUCILIA CUPRINA*

5.1 INTRODUCTION

In the previous chapter, the functions of two loci involved in xanthommatin biosynthesis (yellowish and yellow) were described. One other locus, tangerine, is probably involved in the production of the final enzyme of the xanthommatin biosynthetic pathway. The functions of the white and topaz loci in xanthommatin biosynthesis could not be determined. The grape locus, which is primarily involved in the production of sepiapterin (Chapter 6), also has some role in xanthommatin biosynthesis. In this chapter further experiments aimed at clarifying the functions of the white, topaz and grape loci in xanthommatin production are described.

If a gene affecting xanthommatin formation is not involved in the production of an enzyme of the pathway, it may be concerned with the accumulation of pigment precursors into specific tissues (Sections 1.34, 1.35). There is now good evidence that the white and scarlet loci of *D. melanogaster* code for proteins involved in xanthommatin precursor uptake or storage. Howells & Ryall (1975) and Howells *et al.* (1977) examined 3-hydroxy-kynurenine accumulation. They showed that while larvae and adults of white and scarlet had only about 20% of the wild type level of 3-hydroxykynurenine, this compound was accumulated substantially over pupal life. The low larval level of this metabolite correlated with a high rate of excretion of kynurenine and 3-hydroxykynurenine by third

instar larvae (Howells *et al.*, 1977). Sullivan & Sullivan (1975) were able to assay uptake of kynurenine into larval malpighian tubules and adult eyes of *D. melanogaster* strains *in vitro* and found that these tissues from white and scarlet were unable to take up the pigment precursor. Clearly the defect in these strains prevents accumulation of pigment precursors in these tissues at critical times.

When a gene involved in accumulation of xanthommatin precursors is mutated, the phenotype which results may have some or all of the following characteristics:

a) Low levels of 3-hydroxykynurenine in larvae and adults although pupal accumulation of 3-hydroxykynurenine may be seen. This developmental profile of 3-hydroxykynurenine levels probably results from the excretion of pigment precursors by larvae and adults. The pupal accumulation is probably in the meconium rather than in the eyes and the rapid fall after emergence would coincide with the excretion of the meconium. Data concerning 3-hydroxykynurenine profiles in white, topaz[1], topaz[2] and grape was presented in the previous chapter.

b) Raised levels of excretion of pigment precursors by larvae, as measured after feeding radioactively labelled kynurenine or 3-hydroxykynurenine.

c) The inability to accumulate pigment precursors into larval malpighian tubules or adult eyes *in vivo* or *in vitro*.

For mutants of this type, the mutant phenotype results from the inability to accumulate pigment precursors into

specific tissues at a critical time (see Section 1.35 for a detailed discussion and references). The raised level of excretion and concomitant low levels of pigment precursors whenever their excretion is possible, probably represents a mechanism utilized by the mutant insects to overcome the raised haemolymph levels of 3-hydroxykynurenine resulting from this failure of precursor accumulation.

In Chapter 4 it was seen that levels of 3-hydroxykynurenine were much lower than wild type in white, $topaz^1$, $topaz^2$ and grape as early pupae and adults (Table 4.4) although pupal accumulation of 3-hydroxykynurenine was seen to occur, in white, $topaz^1$ and $topaz^2$ at least. Hence these mutant strains are possibly defective in precursor accumulation. In this chapter, an investigation of the accumulation and excretion of pigment precursors by these strains is reported and the possibility that white and topaz in *L. cuprina* might be analogous in properties with white and scarlet of *D. melanogaster* is considered in detail. In addition, a method for assaying conversion of kynurenine to 3-hydroxykynurenine *in vivo* is described. The results of these assays provide further information about the nature of the abnormality in white, $topaz^1$ and $topaz^2$.

5.2 MATERIALS AND METHODS

5.21 EXPERIMENTAL ANIMALS

Larvae were obtained as described in Section 2.4. Adults were reared as described in Section 4.21.

5.22 RADIOISOTOPES

Tritiated water (approximately 5 Ci/ml) was obtained from the Radiochemical Centre, Amersham, England, and diluted with distilled water to the required activity. Tritiated L-kynurenine and 3-hydroxy-L-kynurenine were prepared and purified as described by Howells *et al.* (1977).

5.23 LARVAL EXCRETION STUDIES

The procedure was essentially that of Howells *et al.* (1977) adapted to suit *L. cuprina*. Early third instar larvae were placed in 30 ml glass vials (15/vial) containing 4 g homogenised sheep's liver plus approximately 5 µCi purified [^{3}H]-3-hydroxykynurenine. After 24 hours feeding (at 28°C) the larvae were recovered from the vial and washed thoroughly with insect Ringer's solution.

Excretion tests were carried out in bench centrifuge tubes. Each tube contained one radioactively

labelled larva in approximately 0.4 g dry vermiculite. After a suitable excretion time the larva was moved from the vermiculite. The level of excretion was estimated by first extracting the vermiculite in each tube with a total of 4 ml water and then determining the radioactivity in 0.4 ml of the extract in a Beckman liquid scintillation counter using Butyl-PBD scintillation fluid (Howells & Ryall, 1975). The radioactivity remaining in the larva was estimated by homogenising it in 0.2 ml 2N perchloric acid, centrifuging the homogenate at 12,000 rpm for 3 minutes in an Eppendorf microcentrifuge and counting 0.1 ml of the supernatant liquid. Levels of excretion are expressed as percentages of the total radioactivity per larva which was released into the vermiculite.

5.24 ACCUMULATION OF [^{3}H]-3-HYDROXYKYNURENINE BY LARVAL MALPIGHIAN TUBULES

a) *Accumulation by larval malpighian tubules*

Late first instar larvae were placed in vials containing [^{3}H]-3-hydroxykynurenine (5μCi) in homogenised liver and left until the end of the feeding period, about three days later. The larvae were then washed thoroughly with insect Ringer's solution. The set of malpighian tubules dissected from each labelled larva was homogenised in 0.1 ml 2N perchloric acid and 50 μl of the supernatant liquid obtained after centrifugation was counted. Total radioactivity per whole larva was estimated as described in Section 5.23.

b) *Nature of the radioactivity in these larvae*

Larvae were fed for three days on homogenised liver containing [^{3}H]-3-hydroxykynurenine as described in Section 5.24a and then 10 larvae were homogenised in 0.5 ml 2N perchloric acid. The homogenate was centrifuged (12,000 rpm for 3 minutes in an Eppendorf microcentrifuge) and the supernatant liquid neutralised with 10N potassium hydroxide. The precipitated potassium perchlorate was spun down and 50 μl of the final supernatant liquid was spotted on Whatman 3MM paper for descending chromatography in butanol : acetic acid : water (4:1:5; organic phase). Chromatograms were developed for 6 - 8 hours. After chromatography, the paper was dried, cut into strips from the origin to the solvent front and the strips cut into 1cm slices. The radioactivity in each slice was determined. Peaks of radioactivity were identified by reference to marker compounds (Howells *et al.*, 1977).

5.25 UPTAKE OF [^{3}H]-WATER, [^{3}H]-3-HYDROXYKYNURENINE AND [^{3}H]-KYNURENINE INTO ADULT EYES

Adult flies 18 - 20 hours after emergence were injected with 0.5 μCi tritiated water or 2.0 μCi of either [^{3}H]-3-hydroxykynurenine or [^{3}H]-kynurenine in 0.5 μl using a 25 μl S.G.E. microsyringe attached to a Hamilton PB600-1 repeating dispenser. This volume of liquid can be injected into the insect with no ill effects (Barton Browne, 1968; Williams & Birt, 1972). After appropriate times the eyes were dissected out and rinsed through

* The term "Ringers" is used loosely. It is the "insect saline" solution described by Bodenstein (1946) ("Investigations of the locus of action of DDT in flies (Drosophila)". Biol. Bull. Marine Biology Laboratory, Woods Hole, 90, 148-157), adjusted to pH 7.5 with 0.01M morpholinopropane sulphonic acid (MOPS) buffer.

four washes of insect "Ringer's" solution.* To estimate the radioactivity in the eyes, each set was homogenised in 0.2 ml 2N perchloric acid, centrifuged and the whole supernatant liquid was counted as described above. To estimate the total radioactivity per insect, the eyeless bodies were homogenised twice in a total of 0.3 ml 2N perchloric acid, centrifuged, and the whole supernatant liquid counted. Total radioactivity equals counts in eyes plus counts in eyeless bodies.

5.26 CONVERSION OF [^{3}H]-KYNURENINE TO [^{3}H]-3-HYDROXYKYNURENINE *IN VIVO*

Adult flies 18 - 20 hours after emergence (or mid-third instar larvae) were injected with 1.5 μCi [^{3}H]-kynurenine and the eyes of adults dissected out two hours later as described in Section 5.25. Five pairs of eyes (or five whole larvae) were homogenised in 0.5 ml 2N perchloric acid and the supernatant liquid after centrifugation prepared for chromatography and liquid scintillation counting as described in Section 5.24b. Counts in kynurenine and 3-hydroxykynurenine were calculated.

5.3 *RESULTS*

5.31 EXCRETION OF [^{3}H]-3-HYDROXYKYNURENINE BY LARVAE

Since the low larval accumulation of 3-hydroxykynurenine by white, topaz^1, topaz^2 and grape (Section 4.31d)

could result from an increased rate of excretion of this metabolite, excretion rates of [^{3}H]-3-hydroxykynurenine were determined for the different strains. A time course of excretion over 24 hours was established for wild type and showed that the larvae excrete only a small proportion (approximately 5%) of the radioactivity they accumulate during the feeding period. The rate of excretion by the wild type larvae was reasonably constant. Thus 3.6% of the radioactivity had been excreted by 12 hours and 5.0% by 24 hours (Table 5.1).

Levels of excretion at 12 and 24 hours were determined for the mutant strains and are also shown in Table 5.1. In most the rate of excretion of [^{3}H]-3-hydroxykynurenine is higher than that for wild type; for white the rate is more than four times greater over the first 12 hour period although excretion during the second 12 hours seems limited. For topaz^1 and topaz^2 the rate during the first 12 hours appears little different from wild type, although by 24 hours the levels of excretion are somewhat higher. The mutants yellow and yellowish also have higher excretion rates than wild type, possibly merely a reflection of the smaller endogenous pool of 3-hydroxykynurenine into which the labelled material equilibrates (see Table 4.4). Tangerine and grape also have excretion rates which are approximately twice that of wild type. Nevertheless, the differences in excretion rates between wild type and the mutants are relatively small and the total level of excretion at

TABLE 5.1
EXCRETION OF $[^3H]$-3-HYDROXYKYNURENINE BY LARVAE

STRAIN	$[^3H]$-3-HYDROXYKYNURENINE EXCRETED[a] (% total cpm in larva)	
	12 hours[b]	24 hours[b]
Wild type	3.6	5.0
Yellowish	5.4	13.4
Yellow	6.6	13.4
Tangerine	10.1	15.9
Topaz [1]	3.4	7.6
Topaz [2]	5.0	10.5
White	16.1	14.2
Grape	8.4	11.8

a Values are the means of at least two separate experiments.

b Excretion time.

24 hours is only a small proportion (much less than 20%) of the accumulated radioactive material in all cases.

5.32 ACCUMULATION OF [3H]-3-HYDROXYKYNURENINE BY LARVAL MALPIGHIAN TUBULES

Since 3-hydroxykynurenine is accumulated in the cells of the malpighian tubules during larval life in *D. melanogaster* (Wessing & Danneel, 1961), accumulation of this compound into the larval malpighian tubules in each of the strains of *L. cuprina* was measured. After a three day feeding period, the proportion of total radioactivity which was found in the tubules was determined (Table 5.2). Wild type, yellowish, yellow, tangerine and grape accumulate at least 30% of the total radioactivity in the tubules over this time, but the tubules of white, topaz^1 and topaz^2 are markedly abnormal and only contain about 2% of the total radioactivity after the three day feeding period. The reduced larval accumulation of 3-hydroxykynurenine (Table 4.4) is therefore due largely to a failure of the malpighian tubules to store this metabolite.

The majority of the radioactivity found in larvae after the three day feeding period co-chromatographed with the 3-hydroxykynurenine marker spot and there were no other large peaks of radioactivity in any of the strains. This indicates that, in the absence of conversion to xanthommatin, there is no other significant metabolism

TABLE 5.2

ACCUMULATION OF [^{3}H]-3-HYDROXYKYNURENINE IN LARVAL MALPIGHIAN TUBULES

STRAIN	[^{3}H]-3-HYDROXYKYNURENINE IN MALPIGHIAN TUBULES (% total cpm in larva)[a]
Wild type	42.6
Yellowish	31.5
Yellow	32.0
Tangerine	34.4
Topaz[1]	1.8
Topaz[2]	2.3
White	1.7
Grape	32.0

[a] Values are the means of at least two separate experiments with at least five larvae sampled per experiment.

of 3-hydroxykynurenine. The radioactivity measured in the tubules, therefore, represents 3-hydroxykynurenine accumulation.

5.33 UPTAKE OF [^{3}H]-WATER, [^{3}H]-3-HYDROXYKYNURENINE AND [^{3}H]-KYNURENINE INTO ADULT EYES

Time courses of uptake of [^{3}H]-water and [^{3}H]-3-hydroxykynurenine by adult eyes of wild type and white strains of *L. cuprina* are shown in Figure 5.1. The time course for water uptake is essentially the same for both strains. The water is rapidly equilibrated into the cells of the eye, within 30 minutes of injection, and this process was found to be temperature-independent. The level of uptake (≈6%) presumably indicates that about 6% of the total body water is located in the region dissected as eyes. The accumulation of [^{3}H]-3-hydroxykynurenine in both strains is temperature-dependent. In wild type, uptake of [^{3}H]-3-hydroxykynurenine appears to occur in two phases, a rapid initial phase over the first 30 minutes, followed by a slower, relatively constant rate of accumulation which continues until at least 2 hours after injection. In this period, wild type has accumulated about 15% of the injected radioactivity into the eyes. In white, the rate of uptake in the initial phase is reduced to about 70% of that of wild type and the slower phase after 30 minutes is virtually abolished, so that the level of accumulation after 2 hours is about 7% of the total radioactivity injected, half the wild

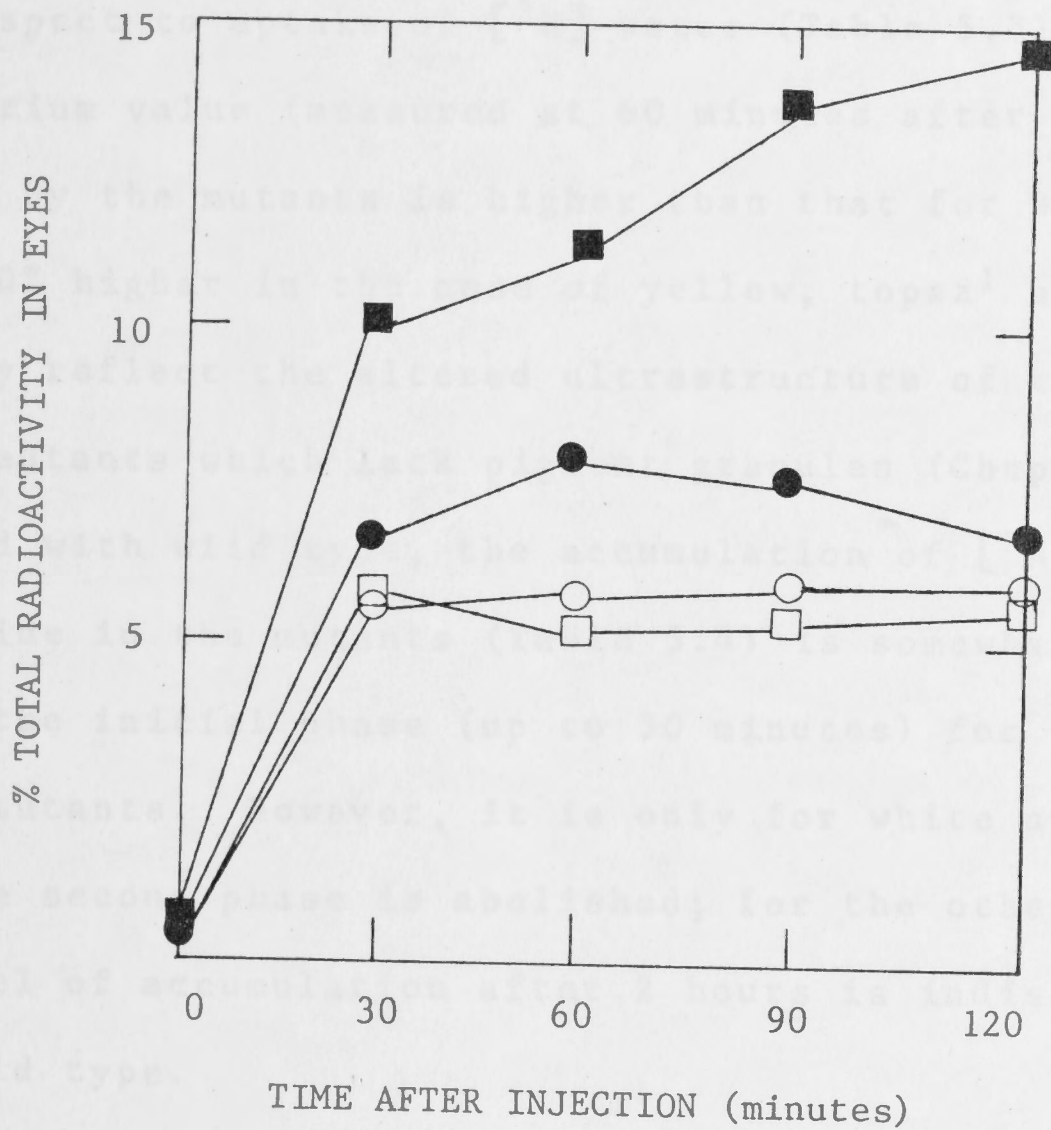


FIGURE 5.1

UPTAKE OF $[^3H]$-WATER AND $[^3H]$-3-HYDROXYKYNURENINE INTO ADULT EYES OF WHITE AND WILD TYPE *L. CUPRINA*

Rate of uptake of $[^3H]$-water (open symbols) and $[^3H]$-3-hydroxykynurenine (closed symbols) by eyes of wild type (squares) and white (circles). Each point is the average of at least two (water) or five (3-hydroxykynurenine) separate determinations with five flies assayed per determination. Standard deviations were too small to be included on the graph.

type value.

Estimations were also made for the other mutants, and Tables 5.3 and 5.4 show the total radioactivity measured in the eyes at different times after injection. With respect to uptake of [^{3}H]-water (Table 5.3), the equilibrium value (measured at 60 minutes after injection) reached by the mutants is higher than that for wild type, up to 40% higher in the case of yellow, topaz^1 and topaz^2. This may reflect the altered ultrastructure of the eyes of the mutants which lack pigment granules (Chapter 7). Compared with wild type, the accumulation of [^{3}H]-3-hydroxykynurenine in the mutants (Table 5.4) is somewhat lower during the initial phase (up to 30 minutes) for several of the mutants. However, it is only for white and grape that the second phase is abolished; for the other mutants the level of accumulation after 2 hours is indistinguishable from wild type.

An estimation of [^{3}H]-kynurenine uptake into adult eyes was also made and the same trends were found as for [^{3}H]-3-hydroxykynurenine uptake (Table 5.5). At 30 minutes the level of uptake was similar in all strains but both white and grape failed to accumulate as much radioactivity in the eyes as wild type over the next 90 minutes, similar to the situation with [^{3}H]-3-hydroxykynurenine. In the case of topaz^1 the situation is not so clear as with [^{3}H]-3-hydroxykynurenine and uptake by topaz^1 of [^{3}H]-kynurenine may be lower than wild type although still

TABLE 5.3

UPTAKE OF $[^3H]$-WATER BY ADULT EYES

STRAIN	$[^3H]$-WATER IN EYES (cpm x 10^{-3})[a]
Wild Type	6.8 ± 0.4
Yellowish	9.1 ± 1.5
Yellow	9.6 ± 1.0
Tangerine	8.2 ± 1.2
Topaz[1]	9.2 ± 0.8
Topaz[2]	9.5 ± 0.2
White	8.5 ± 1.0
Grape	8.3 ± 1.3

a Values ± 1 standard deviation are the means of at least 3 separate determinations, made 60 minutes after injection of $[^3H]$-water, with eyes from 5 flies assayed in each.

Total radioactivity recovered per whole insect: $1.30 \pm 0.09 \times 10^5$ cpm.

TABLE 5.4
UPTAKE OF $[^3H]$-3-HYDROXYKYNURENINE BY ADULT EYES

STRAIN	$[^3H]$-3-HYDROXYKYNURENINE IN EYES $(cpm \times 10^{-4})$[a]	
	30 minutes[b]	120 minutes[b]
Wild type	2.7 ± 0.2	4.6 ± 0.2
Yellowish	3.3 ± 0.4	5.3 ± 0.1
Yellow	1.9 ± 0.3	3.8 ± 0.4
Tangerine	2.6 ± 0.3	4.2 ± 0.2
Topaz[1]	1.9 ± 0.2	3.9 ± 0.3
Topaz[2]	2.9 ± 0.3	4.9 ± 0.4
White	2.2 ± 0.1	1.9 ± 0.1
Grape	2.0 ± 0.2	2.2 ± 0.2

a Values ± SEM are the means of 5 - 8 separate determinations with the eyes from 5 flies assayed in each determination. Total radioactivity recovered per whole insect: $2.93 \pm 0.09 \times 10^5$ cpm.

b Time after injection of $[^3H]$-3-hydroxykynurenine at which eyes were dissected and assayed.

TABLE 5.5
UPTAKE OF [^{3}H]-KYNURENINE BY ADULT EYES

STRAIN	[^{3}H]-KYNURENINE IN EYES (cpm x 10^{-4})[a]	
	30 minutes[b]	120 minutes[b]
Wild type	1.5	2.5
Yellowish	2.2	2.9
Yellow	2.8	3.3
Tangerine	1.6	2.5
Topaz[1]	1.3	1.8
Topaz[2]	1.4	2.5
White	1.6	0.6
Grape	1.5	1.7

a Values are the means of at least two separate experiments with the eyes from 5 flies assayed in each experiment. Total radioactivity recovered per whole insect: $2.1 \pm 0.1 \times 10^5$ cpm.

b Time after injection of [^{3}H]-kynurenine at which eyes were dissected and assayed.

far higher than white. The other mutants were indistinguishable from wild type in [^{3}H]-kynurenine uptake into eyes.

5.34
CONVERSION OF [^{3}H]-KYNURENINE TO [^{3}H]-3-HYDROXYKYNURENINE *IN VIVO*

The results of studies assaying the conversion of [^{3}H]-kynurenine to [^{3}H]-3-hydroxykynurenine *in vivo* are presented in Table 5.6. When [^{3}H]-kynurenine was injected into the adults, eyes of wild type and yellowish after 2 hours contained about 70% of the radioactivity as [^{3}H]-3-hydroxykynurenine. In whole larvae of these strains 2 hours after injection, 30 - 40% of the radioactivity was found as 3-hydroxykynurenine. In contrast, both larvae and adults of yellow (which is thought to be defective in kynurenine hydroxylase; Chapter 4) retained all the radioactivity as kynurenine. They had the same proportion of radioactivity in 3-hydroxykynurenine as zero time samples from wild type. Sullivan & Sullivan (1975) found that eyes of cinnabar flies of *D. melanogaster* failed to convert [^{3}H]-kynurenine to [^{3}H]-3-hydroxykynurenine *in vitro*. This result further strengthens the comparison between cinnabar and yellow suggesting that yellow is involved in the production of kynurenine hydroxylase.

Of great interest in the results of Table 5.6 is the low level of conversion of [^{3}H]-kynurenine to

TABLE 5.6
CONVERSION OF [^{3}H]-KYNURENINE TO [^{3}H]-3-HYDROXYKYNURENINE *IN VIVO*

STRAIN	% CONVERSION[a]		
	Larvae[b]	Adult eyes[c]	Adult bodies[c]
Wild type	32	65	43
Yellowish	48	69	37
Yellow	7	2	8
Tangerine	29	49	31
Topaz[1]	15	20	39
Topaz[2]	12	24	39
White	13	31	35
Grape	21	37	29

a % of total radioactivity recovered 2 hours after injection which was found as [^{3}H]-3-hydroxykynurenine.

b Whole larvae (mid-third instar) were injected. Cpm recovered per chromatogram strip approximately 7,000. Means of two experiments done in duplicate.

c Adults 18 - 20 hours after emergence were injected. Cpm recovered per chromatogram strip, approximately 4,000 for eyes and 16,000 for bodies. Means of 2 (bodies) or three (eyes) experiments assayed in duplicate.

[^{3}H]-3-hydroxykynurenine in larvae of topaz^{1}, topaz^{2} and white. These are the three strains which failed to take [^{3}H]-3-hydroxykynurenine into the larval malpighian tubules. It was also found (Table 5.6) that the value for conversion in adult eyes was much lower in these strains than in wild type, although the proportion of [^{3}H]-3-hydroxykynurenine in the bodies of these strains was roughly the same as in wild type. The values for grape in Table 5.6 are also somewhat lower than those seen for wild type, but these were very variable and the real level of conversion in grape is not clear.

5.4 *DISCUSSION*

The experiments reported in this chapter enable a comparison to be made of white, topaz^{1} and topaz^{2} of *L. cuprina* and white and scarlet of *D. melanogaster* with respect to a number of aspects of tryptophan metabolism. As listed in Section 5.1, there are three characteristics which distinguish white and scarlet of *D. melanogaster* from wild type: low 3-hydroxykynurenine levels in larvae and adults but accumulation during pupal life, elevated levels of excretion of 3-hydroxykynurenine and kynurenine by larvae, and failure by adult eyes and larval malpighian tubules to take up [^{3}H]-kynurenine *in vivo* and *in vitro*. The first of these characteristics, 3-hydroxykynurenine levels, was considered for white, topaz^{1} and topaz^{2} in the previous chapter and it was noted that these strains are somewhat similar in this respect to white and scarlet of *D. melanogaster*. Although the larval

and adult levels are not as low as those of white and scarlet, there is a similar accumulation over pupal life.

An explanation for the higher early pupal level (that is, higher larval accumulation) of 3-hydroxykynurenine in these *L. cuprina* strains (compared with white and scarlet of *D. melanogaster*) can be found in the results of assays of larval excretion of this compound. For *L. cuprina*, no strain excreted more than 16% of the radioactivity over the 24 hour period (2 - 3 times the wild type level), while for white and scarlet of *D. melanogaster* more than 70% of the radioactivity was excreted within 24 hours (about 10 times the wild type level in this species) (Howells *et al.*, 1977).

The results presented in Table 5.2 show that the larval malpighian tubules of white, topaz^1 and topaz^2 fail to accumulate 3-hydroxykynurenine. This could be due to a failure to take it up from the haemolymph or to store it within the tubules. This is a striking point of similarity with white and scarlet of *D. melanogaster* in which the tubules fail to take up and store pigment precursors *in vivo* (Beadle, 1937; Wessing & Eichelberg, 1968; Bonse, 1969) or *in vitro* (Sullivan & Sullivan, 1975). In these *D. melanogaster* mutants, the failure of the tubules to accumulate 3-hydroxykynurenine is apparently compensated for by the activation of a rapid excretion process, so that the level of this metabolite in the haemolymph and other tissues is kept low. With the white

and topaz mutants of *L. cuprina* no such compensatory excretion mechanism is activated.

In the case of white of *L. cuprina* the defect in the capacity of the malpighian tubules to accumulate 3-hydroxykynurenine extends to the eyes as well. In the studies of the uptake of 3-hydroxykynurenine by the eyes, a rapid initial phase is seen for wild type and to a lesser extent for white (Figure 5.1). This may reflect a rapid uptake of 3-hydroxykynurenine into the cells of the eye. This uptake process might involve either active transport or facilitated diffusion since it is temperature-dependent. Since accumulation in white is virtually complete by 30 minutes after injection, the process is probably reversible, with equilibrium between uptake and efflux being established within that time. The second slower phase (30-120 minutes) may involve the storage (sequestering) of the labelled material in the cells. Alternatively, the rapid initial phase may represent the accumulation of 3-hydroxykynurenine into the intercellular space and the slower phase the accumulation of 3-hydroxykynurenine from this space into the cells. If the first interpretation of these kinetics is correct, then white and grape appear to be defective only in their ability to store the metabolite and not in the uptake process. If the second interpretation is correct, then perhaps white and grape are defective in the uptake process so that 3-hydroxykynurenine is not taken up from the intercellular space into the cells. In either case the process does not depend on the conversion of 3-hydroxykynurenine to xanthommatin since it occurs successfully in yellow and yellowish and these strains make virtually no pigment (Chapter 4).

The results of the uptake experiments with kynurenine

are more difficult to interpret because storage probably requires conversion of the kynurenine to 3-hydroxykynurenine. None of the mutants (including white and grape) showed a decrease relative to wild type in the initial rapid uptake phase (30 minutes), whereas by 120 minutes white (and to a lesser extent topaz[1] and grape) showed much less accumulation of radioactivity. As will be discussed later, white and the topaz strains also show reduced rates of conversion of kynurenine to 3-hydroxykynurenine in the eyes.

No comparable *in vivo* studies have been performed with the mutants of *D. melanogaster*. In the *in vitro* studies with [^{3}H]-kynurenine carried out by Sullivan & Sullivan (1975), eyes from scarlet and white were found to accumulate only 36% and 23% of the radioactivity taken up by the eyes of wild type. Thus it seems likely that for white of both species uptake and storage of xanthommatin precursors by the eyes is defective. However, the topaz strains of *L. cuprina* appear to be different from scarlet of *D. melanogaster* in this respect, with only the latter mutant showing a defect in precursor uptake and storage by the eyes.

The results of studies on the conversion of [^{3}H]-kynurenine to [^{3}H]-3-hydroxykynurenine by larvae are open to a number of interpretations. They may indicate that white, topaz[1] and topaz[2] have a lower larval activity of kynurenine hydroxylase *in vivo* than wild type. An alternative explanation of the low larval

conversion is suggested by the results of the experiments measuring accumulation of 3-hydroxykynurenine into the malpighian tubules. If this is due to a defect in an uptake mechanism for xanthommatin precursors (which seems likely considering the *in vitro* results of Sullivan & Sullivan (1975) for the mutants of *D. melanogaster*) and if the kynurenine hydroxylase is located largely in the tubules in larvae (which has been found in several studies; see Table 1.2), then the results may be explained simply by the inability of the substrate (kynurenine) to get to the enzyme from the haemolymph. Supporting this idea is the observation that larval build-up of kynurenine is low in these strains. (Table 4.3), which is unexpected if the mutants are deficient in kynurenine hydroxylase. In this, these strains are unlike yellow, which has a primary defect in the production of kynurenine hydroxylase. In their *in vitro* studies, Sullivan & Sullivan (1975) looked at the conversion of [^{3}H]-kynurenine taken up by the larval malpighian tubules of *D. melanogaster*. Tubules from scarlet showed about half the conversion of wild type tubules, similar to the reduced larval conversion seen in topaz^1 and topaz^2 of *L. cuprina*. The level of kynurenine hydroxylase *in vitro* in scarlet larvae is similar to that of wild type (Sullivan *et al.*, 1973), emphasising the point that reduced larval conversion is not necessarily caused by a reduced level of enzyme activity. Unfortunately the conversion rate by larval malpighian tubules of white could not be calculated

from the data of Sullivan & Sullivan (1975).

Conversion of [^{3}H]-kynurenine to [^{3}H]-3-hydroxykynurenine was also examined in adults of *L. cuprina*. The amounts of [^{3}H]-3-hydroxykynurenine in the eyes after injection of [^{3}H]-kynurenine were assessed and it was found that white, topaz^1 and topaz^2 had a much lower level than wild type or yellowish. In contrast, the eyeless bodies of all the strains tested had about the same proportion of [^{3}H]-3-hydroxykynurenine. Even though this percentage is much lower in the eyeless bodies compared with the eyes (43% compared with 65% for wild type), it should be noted that approximately 80% of the injected label is in the body rather than the eyes 2 hours post-injection. This means that there is 3 - 4 times more [^{3}H]-3-hydroxykynurenine in the bodies compared with the eyes. This suggests that the conversion of [^{3}H]-kynurenine to [^{3}H]-3-hydroxykynurenine occurs mainly in the body tissues rather than in the eyes. This contrasts with results for *D. melanogaster* (Table 1.2) where most of the adult kynurenine hydroxylase activity has been detected in the eyes. To confirm that body tissues of *L. cuprina* do have kynurenine hydroxylase activity *in vivo*, eyes were removed from wild type flies before the injection of [^{3}H]-kynurenine and the level of [^{3}H]-3-hydroxykynurenine was assessed after 2 hours. The percentage obtained (30%) was similar to the value obtained when intact flies were injected and the eyes removed at the end of the conversion period.

It is thus likely that in *L. cuprina* most of the kynurenine hydroxylase activity is located in the body and that xanthommatin synthesis occurring in the eyes depends largely on the uptake of 3-hydroxykynurenine from the haemolymph. Nevertheless, some kynurenine hydroxylase is probably located in the pigment cells of the eyes, since the proportion of [^{3}H]-3-hydroxykynurenine in the eyes in these experiments (Table 5.6) is much greater than the proportion in bodies for wild type, and since no difference in the accumulation of kynurenine and 3-hydroxykynurenine was noted (Tables 5.4 and 5.5 - about 15% of the total radioactivity was taken up by the eyes of wild type in both cases). Hence, [^{3}H]-3-hydroxy-kynurenine in the eyes 2 hours after injection probably results partly from uptake and partly from conversion within the pigment cells. In the case of white and grape the relatively low percentage in the eyes could be attributed to the reduced uptake or storage mechanism for 3-hydroxykynurenine. However, for the topaz mutants, uptake and storage of 3-hydroxykynurenine in the eyes is apparently normal, so that the reduced percentage of this metabolite must reflect a reduced rate of conversion *in vivo*.

Again in this respect the topaz mutants seem to be different from scarlet of *D. melanogaster*. The results of Sullivan & Sullivan (1975) show that eyes of scarlet, which had taken up a small amount of [^{3}H]-kyn-urenine *in vitro*, converted about the same proportion of this radioactivity to [^{3}H]-3-hydroxykynurenine as

wild type, in accordance with the finding that the activity of kynurenine hydroxylase in adult extracts of scarlet is the same as wild type (Sullivan *et al.*, 1973).

The results of the experiments reported in this chapter provide strong evidence that the white and topaz genes influence the transport and storage of tryptophan metabolites into specific tissues, similar to the scarlet and white genes of *D. melanogaster*. In the case of white of both species, the larval malpighian tubules and the adult eyes are affected and it seems likely that the function of the white genes of *L. cuprina* and *D. melanogaster* are almost identical. However, there does appear to be a major difference between the scarlet gene of *D. melanogaster* and the topaz gene of *L. cuprina*. Although both genes affect the larval malpighian tubules, only scarlet has a major effect on the uptake and storage of xanthommatin precursors in the pigment cells of the eyes. The results obtained with the topaz strains underline the extremely important relationship between larval storage of 3-hydroxykynurenine in the malpighian tubules and adult xanthommatin synthesis in the eyes. In *D. melanogaster* 3-hydroxykynurenine stored in the larval malpighian tubules makes only a minor quantitative contribution to adult xanthommatin production (Howells, 1979) and the same is probably the case in *L. cuprina* (Chapter 3). Nevertheless as can be seen with the topaz mutants, failure to carry out this storage results

in a failure to deposit xanthommatin in the adult eyes, despite the fact that the topaz mutants do have the enzymic capacity to convert tryptophan to 3-hydroxykynurenine and their eyes have the capacity to take up this metabolite from the haemolymph. In the case of grape, which has normal larval storage of 3-hydroxykynurenine, there is only 40% of the wild type xanthommatin in the adult eye (Table 4.1). This may be a consequence of the reduced ability of the pigment cells of grape flies to store 3-hydroxykynurenine. Although this inability is of the same magnitude found in white, grape insects make substantial amounts of xanthommatin, possibly a reflection of the fact that in this mutant larval storage of 3-hydroxykynurenine in the malpighian tubules is normal.

CHAPTER 6

PTERIDINES IN WILD TYPE AND MUTANT *LUCILIA CUPRINA*

6.1 INTRODUCTION

Pteridines are compounds possessing a basic pyrimidine-pyrazine ring and are found in micro-organisms, plants and animals. The largest range of pteridine compounds is found in the Insecta. The general structure of the pterins (2-amino-4-hydroxy pteridines) is shown in Figure 6.1 (pterin) along with the structures of other pteridines discussed in this chapter. The pathways resulting in the biosynthesis of pteridines are less well understood than that for ommochrome biosynthesis, mainly due to uncertainty concerning the metabolic relationships between various pteridines and to the lack of precise knowledge (at least until recently) of chemical structures (Ziegler & Harmsen, 1969). The study of pteridine mutants in *D. melanogaster* is helping to elucidate the pathways.

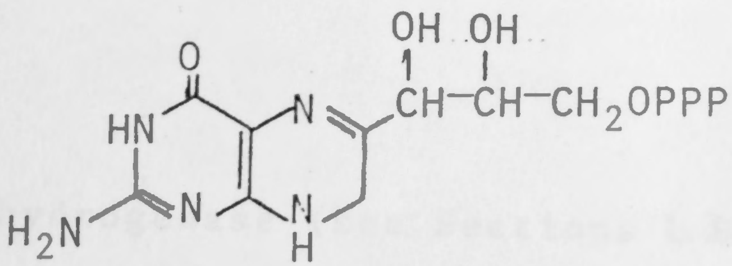
In Figure 1.3 a tentative pathway for the biosynthesis of pteridines was shown. Three steps involved in this pathway have been identified in *D. melanogaster*. These are:

a) GTP → dihydroneopterin triphosphate via GTP cyclohydrolase (Fan & Brown, 1976; Evans & Howells, 1978);

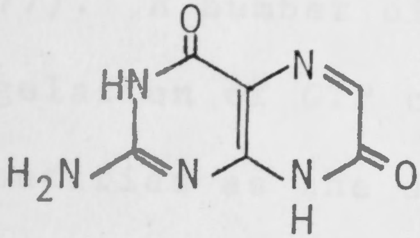
b) dihydroneopterin triphosphate → sepiapterin via sepiapterin synthase (Fan *et al.*, 1975);

c) pterin → isoxanthopterin via xanthine dehydrogenase (Glassman & Mitchell, 1959).

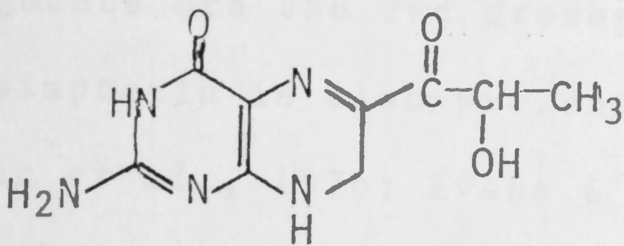
The rosy locus controls the synthesis of xanthine



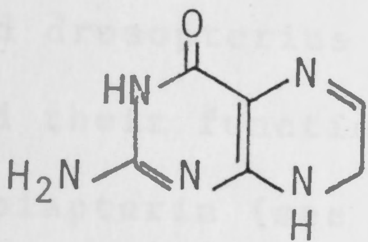
Dihydroneopterin
triphosphate



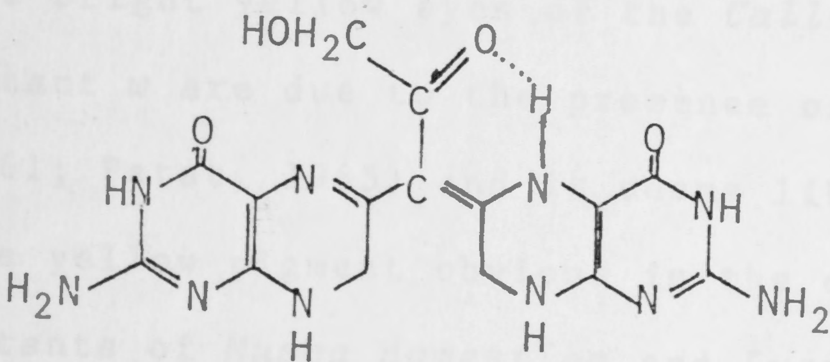
Isoxanthopterin



Sepiapterin



Pterin



Drosopterin and
Isodrosopterin

FIGURE 6.1
THE STRUCTURES OF THE MAIN PTERIDINES OF DIPTERA

From Ziegler & Harmsen (1969); Sugiura & Goto (1973).

dehydrogenase (see Sections 1.32 and 4.4 for further discussion) and the purple locus has been implicated as the structural gene for sepiapterin synthase (Yim *et al.*, 1977). A number of loci are clearly implicated in the regulation of GTP cyclohydrolase but none has yet been identified as the structural locus for the enzyme (Evans & Howells, 1978).

In *Drosophila* species, the main pteridine eye pigments are the red drosopterins. The yellow pteridine sepiapterin is also present in wild type *D. melanogaster* (Fan *et al.*, 1976; Evans & Howells, 1978) but only takes on a major screening role in the drosopterin deficient mutant sepia (Ziegler & Harmsen, 1969) where it is bound to granules (Nolte, 1961) and hence has a brown colour. The bright red drosopterins are lacking from other dipteran families and their function in the eye seems to be taken on by sepiapterin (see Ziegler & Harmsen, 1969, for a summary of the occurrence of the pteridine pigments in insect eyes). The bright yellow eyes of the *Calliphora erythrocephala* mutant *w* are due to the presence of sepiapterin (Ziegler, 1961; Patat, 1965) and it seems likely that this is also the yellow pigment obvious in the ommochrome deficient mutants of *Musca domestica* and *Lucilia cuprina*.

Reported in this chapter are investigations which demonstrate the nature of the yellow pigment in *L. cuprina* and the time of onset of its biosynthesis during development. Comparison of wild type and mutant strains was made and the deficiency of sepiapterin levels in two mutants, white

and grape, has been established. The nature of the defect in sepiapterin synthesis in these mutants has not yet been identified.

6.2
MATERIALS AND METHODS

6.21
EXPERIMENTAL ANIMALS

Insects for the developmental study were collected as described in Section 3.21. Adults for routine assays were selected at emergence and kept for at least 48 hours on a diet of sugar and water.

6.22
PURIFICATION OF SEPIAPTERIN

Sepiapterin was purified from adults of the yellow mutant strain as described by Evans & Howells (1978). Extracts were made from the heads of 10 g of flies and sepiapterin separated from other coloured compounds on a phosphocellulose ion exchange column. Sepiapterin was also extracted from heads of 10 g of adults of the sepia mutant strain of *D. melanogaster* by an identical procedure.

6.23
DETERMINATION OF PTERIDINE LEVELS IN *L. CUPRINA* STRAINS

The heads of adults or the anterior quarters of pupae were cut from the bodies and homogenised in

chloroform : 0.1% NH_4OH (25 heads in 0.25 ml each solvent). The homogenate was centrifuged for 3 minutes at 12,000rpm in an Eppendorf microcentrifuge and the upper aqueous phase removed. 50 µl of this extract was spotted on Whatman 3MM paper for chromatography in n-propanol : 1% NH_4OH (2:1) made 0.2% with respect to 2-mercaptoethanol. Chromatograms were developed in the descending mode for 5 hours. The paper was then dried and sepiapterin located by its bright yellow fluorescence under UV light and by reference to purified sepiapterin (from the heads of flies of the sepia strain of *D. melanogaster*) run as a marker. In this system the R_f value for sepiapterin is about 0.45. Sepiapterin spots, including markers, were cut out of the paper and sepiapterin eluted by boiling for 5 minutes in 3 ml 0.1% NH_4OH. The elution procedure was found to give complete recovery of a known amount of purified sepiapterin. The absorbance of the extracts was read at 440 nm in the Varian-635 spectrophotometer and the amount of sepiapterin calculated by reference to values obtained for the purified sepiapterin markers which had been chromatographed beside the insect extracts and eluted in the same way.

Other pteridine compounds, pterin and isoxanthopterin were identified by R_f values (0.38 and 0.23 respectively) and their characteristic fluorescence under UV light (blue and purple respectively) and by comparison with synthetic compounds (purchased from Sigma Chemical Co., St Louis, U.S.A.) chromatographed in the same way. Pterin and isoxanthopterin were not eluted but their presence or absence was noted for each preparation.

6.24 FEEDING EXPERIMENTS WITH PURINE

These were set up exactly as described for feeding experiments with kynurenine and 3-hydroxykynurenine (Section 4.24) using concentrations of purine (Sigma Chemical Co., St Louis, U.S.A.) ranging from 0 to 100mM in 4 g homogenised liver. Twenty larvae at late first instar were placed in the vials and development allowed to proceed at 28°C. The number of insects surviving was tallied at pupariation and at emergence.

6.3 *RESULTS*

6.31 IDENTIFICATION OF THE YELLOW PIGMENT IN THE EYES OF *LUCILIA CUPRINA*

When the procedure for purification of sepiapterin (Section 6.22) was followed using heads from flies of the yellow mutant strain of *L. cuprina*, yellow material eluted from the phosphocellulose column in the same fractions as sepiapterin from *D. melanogaster* and had a UV-visible absorption spectrum identical with that of sepiapterin (Figure 6.2; Forrest *et al.*, 1959). Chloroform : ammonia extracts (Section 6.23) from heads of the mutant yellow were compared with the purified sepiapterin from the heads of the sepia strain of *D. melanogaster* using descending paper chromatography in three different solvent systems:

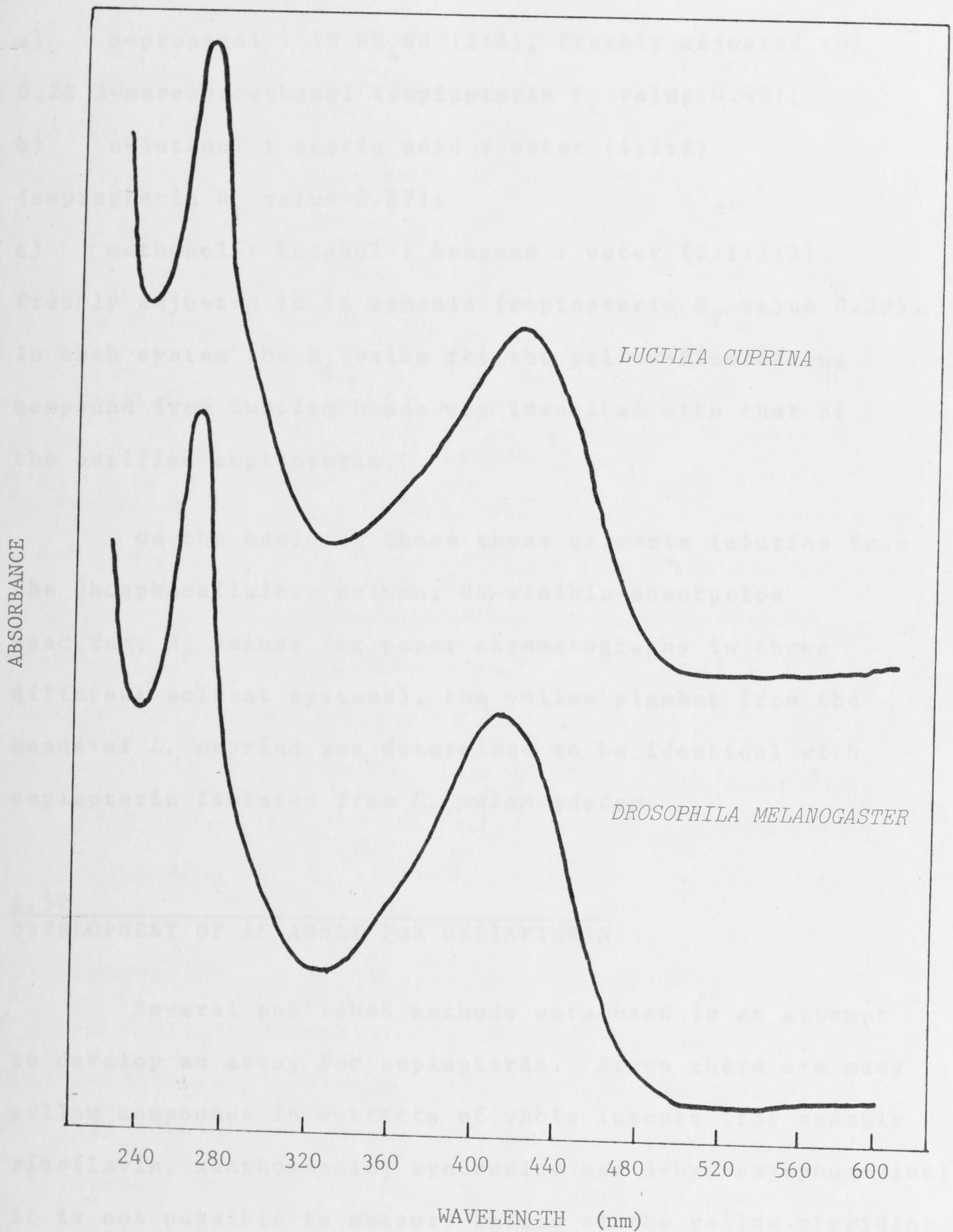


FIGURE 6.2
UV-VISIBLE ABSORPTION SPECTRA OF YELLOW PIGMENT FROM *LUCILIA CUPRINA* AND *DROSOPHILA MELANOGASTER*

Yellow pigment was extracted from the heads of yellow of *L. cuprina* and sepia of *D. melanogaster* as described in Section 6.22.

a) n-propanol : 1% NH_4OH (2:1), freshly adjusted to 0.2% 2-mercaptoethanol (sepiapterin R_f value 0.45);
b) n-butanol : acetic acid : water (4:1:1) (sepiapterin R_f value 0.27);
c) methanol : butanol : benzene : water (2:1:1:1) freshly adjusted to 1% ammonia (sepiapterin R_f value 0.39).

In each system the R_f value for the yellow-fluorescing compound from *Lucilia* heads was identical with that of the purified sepiapterin.

On the basis of these three criteria (elution from the phosphocellulose column; UV-visible absorption spectrum; R_f values for paper chromatography in three different solvent systems), the yellow pigment from the heads of *L. cuprina* was determined to be identical with sepiapterin isolated from *D. melanogaster*.

6.32 DEVELOPMENT OF AN ASSAY FOR SEPIAPTERIN

Several published methods were used in an attempt to develop an assay for sepiapterin. Since there are many yellow compounds in extracts of whole insects (for example riboflavin, xanthommatin, kynurenine and 3-hydroxykynurenine) it is not possible to measure levels of the yellow pteridine directly. Evans & Howells (1978) overcame this difficulty for *D. melanogaster* by using extracts of the pteridine deficient mutant brown as a reference. This was not practicable with *L. cuprina* since no mutant deficient in

sepiapterin has nearly normal levels of xanthommatin (Section 4.31) as has brown. Nickla (1972) reported that sepiapterin is unique among these yellow compounds in that it is decoloured by bromine. Although the yellow pigment from *L. cuprina* was found to be decoloured by bromine (lending further support to the conclusion that it is sepiapterin), it was not possible to use this reaction as the basis for an assay of sepiapterin levels since the brown colour of bromine interfered with the assay and reproducible results could not be obtained. Chromatography separates sepiapterin from xanthommatin, which is the main problem in extracts of adults, but both 3-hydroxykynurenine and riboflavin have R_f values (0.54 and 0.44 respectively) similar to that of sepiapterin (0.45) in the system used. Riboflavin is stored mainly in the malpighian tubules (Wigglesworth, 1972) and the extracts used were made from heads or anterior ends which would not have included the tubules. Riboflavin was not felt to be a great problem. The developmental profile for 3-hydroxykynurenine is known (Figure 3.3c) and there is a relatively constant level of this metabolite throughout development, so that fluctuations in 3-hydroxykynurenine levels could be allowed for when interpreting results. 3-Hydroxykynurenine was found not to interfere with the assay of sepiapterin. Levels of 3-hydroxykynurenine were also known for the mutants. Given these considerations, chromatography in n-propanol : 1% ammonia with 2-mercaptoethanol (Section 6.23) was adopted as the most satisfactory means of separating

sepiapterin from interfering compounds.

In the past, fluorimetric determinations of sepiapterin have been made (see, for example, Nickla, 1972; Fan *et al.*, 1976; Evans & Howells, 1978) but this method was found to be unreliable in the *L. cuprina* extracts because of the high levels of pterin present at some stages. Pterin co-chromatographs with sepiapterin and was found to have identical fluorescence excitation and emission wavelength maxima. The varying amounts of pterin caused irreproducibility of sepiapterin measurements by fluorescence. The two compounds have distinct visible absorption maxima (Figure 6.3) (at 440 nm for sepiapterin and 350 nm for pterin) so that measurement of sepiapterin at 440 nm after elution from the chromatography paper (Section 6.23) was found to give reproducible results, even though the absorbance values were quite low (0.01 - 0.13).

Sepiapterin is very susceptible to oxidation, particularly in light, breaking down to the blue fluorescing pteridine pterin-6-carboxylic acid (Watt, 1967). The presence of pterin-6-carboxylic acid in sepiapterin samples is another source of error when using fluorimetric determination, since this compound fluoresces much more strongly than sepiapterin. The use of 2-mercaptoethanol (0.2%) in the solvent was found to minimise this oxidation during chromatogram development. In addition, to control further for sepiapterin breakdown, a standard amount of purified sepiapterin was spotted on each chromatogram and

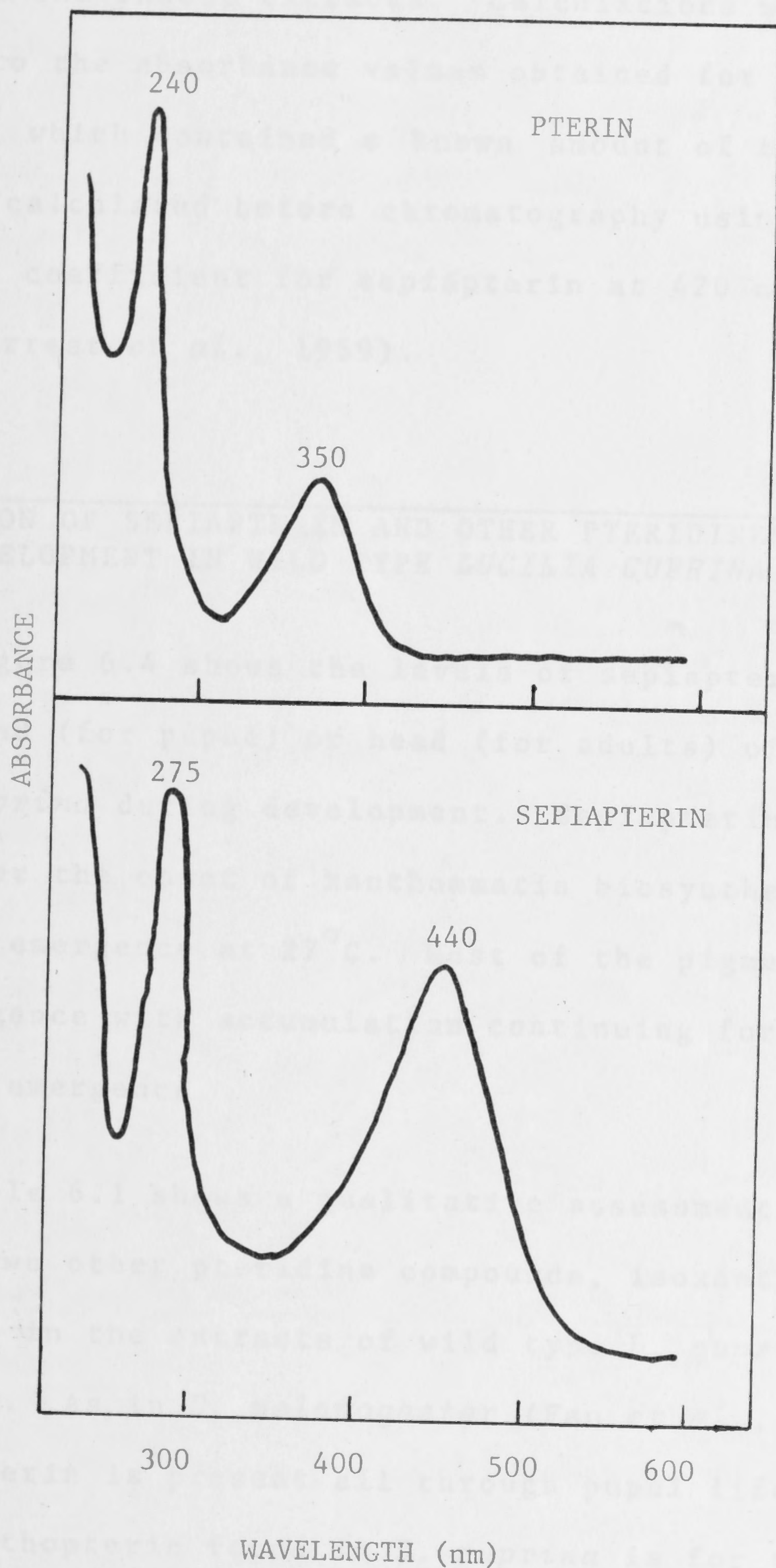


FIGURE 6.3

UV-VISIBLE ABSORPTION SPECTRA OF PTERIN AND SEPIAPTERIN

Sepiapterin was extracted from the heads of yellow of *L. cuprina*: pterin was purchased from Sigma Chemical Co., St Louis, U.S.A. The wavelength at which each peak occurred is indicated in the graphs.

was run and eluted in the same manner as the sepiapterin spots from the insect extracts. Calculations were made relative to the absorbance values obtained for these standards, which contained a known amount of the purified compound, calculated before chromatography using the molar extinction coefficient for sepiapterin at 420 nm of 14,290 (Forrest *et al.*, 1959).

6.33 ACCUMULATION OF SEPIAPTERIN AND OTHER PTERIDINES DURING DEVELOPMENT IN WILD TYPE *LUCILIA CUPRINA*

Figure 6.4 shows the levels of sepiapterin in the anterior end (for pupae) or head (for adults) of wild type *L. cuprina* during development. Sepiapterin biosynthesis begins after the onset of xanthommatin biosynthesis, in the day before emergence at 27°C. Most of the pigment appears after emergence with accumulation continuing for about 3 days after emergence.

Table 6.1 shows a qualitative assessment of the levels of two other pteridine compounds, isoxanthopterin and pterin, in the extracts of wild type *L. cuprina* during development. As in *D. melanogaster* (Fan *et al.*, 1976), isoxanthopterin is present all through pupal life. This isoxanthopterin found in *L. cuprina* is for the head region, although it is not clear whether it is located in the eyes or the haemolymph of the head. When extracts of eyes only were chromatographed (as in Section 5.34) isoxanthopterin was not seen, but this may have been due

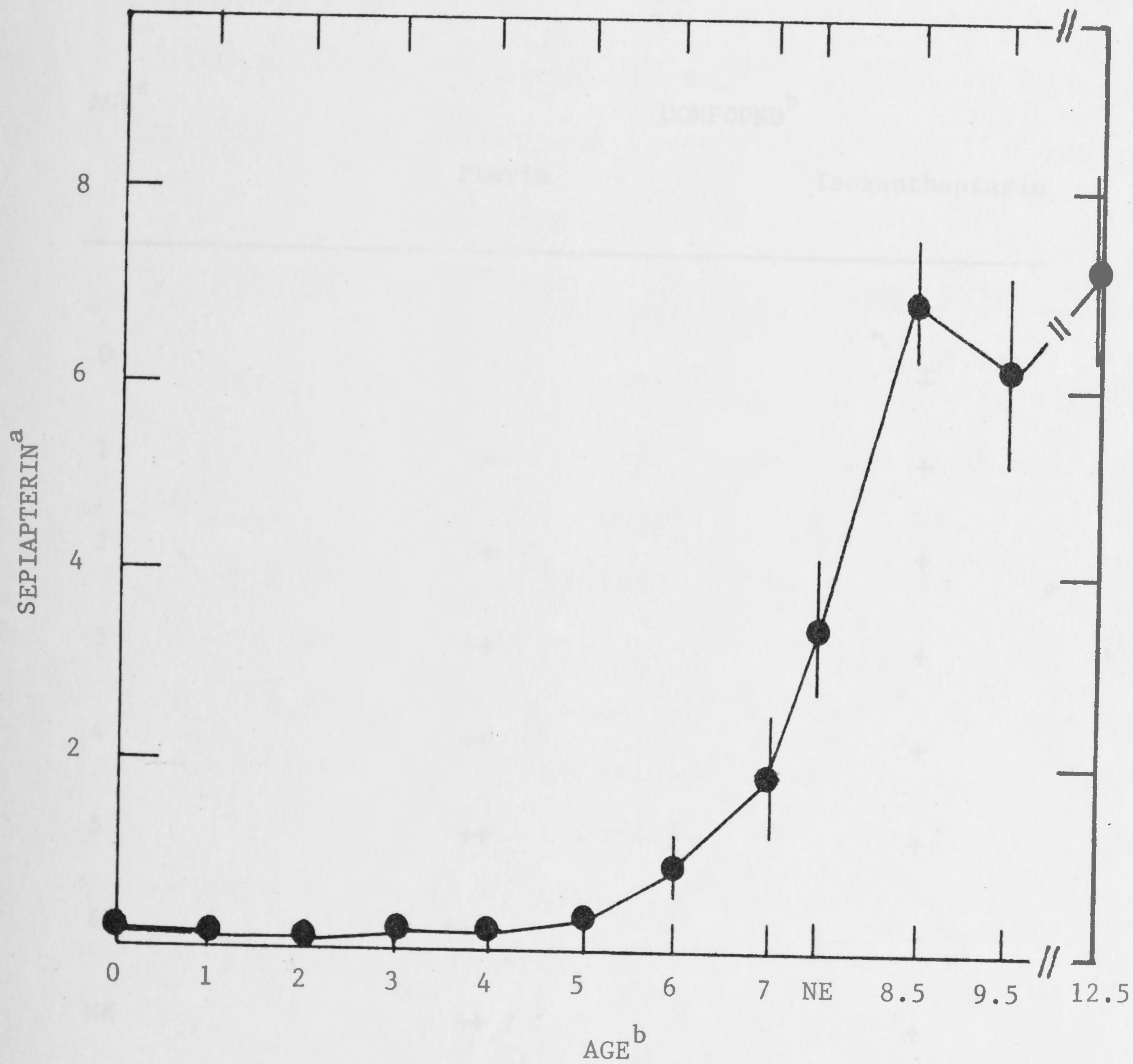


FIGURE 6.4
LEVELS OF SEPIAPTERIN IN WILD TYPE
L. CUPRINA DURING DEVELOPMENT

a

Values (expressed as nmoles/anterior quarter for pupae and as nmoles/head for adults) are the means of 4 separate determinations done in duplicate and vertical bars represent one SD either side of the mean.

b

Age is expressed as days after pupariation which occurs at day 0.
NE: newly emerged flies.

TABLE 6.1

PTERIN AND ISOXANTHOPTERIN IN THE HEAD REGION OF WILD TYPE

AGE[a]	COMPOUND[b]	
	Pterin	Isoxanthopterin
0	-	±
1	-	+
2	+	+
3	++	+
4	++	+
5	++	+
6	+++	+
NE	++ ?	+

a Age in days after pupariation; NE: newly emerged flies.

b A qualitative assessment of the relative levels of the compounds. There is some doubt about the value for pterin in newly emerged flies because of the high fluorescence of sepiapterin at that stage.

to the more dilute nature of the extracts. Pterin first appears in the head region of *L. cuprina* about two days after pupariation and accumulates at least until the onset of sepiapterin biosynthesis when the overlapping fluorescence and increase in sepiapterin levels makes pterin difficult to distinguish. Evans & Howells (1978) found an increase in levels of pterin in extracts of whole *D. melanogaster* pupae, although young pupae had substantial amounts.

6.34 LEVELS OF SEPIAPTERIN IN WILD TYPE AND MUTANT ADULTS

Heads of adults 48 - 72 hours after emergence were used to assay sepiapterin levels in the wild type and eye colour mutant strains of *L. cuprina*. The levels of sepiapterin obtained are given in Table 6.2. Only two mutants are defective in sepiapterin synthesis, grape and white. The yellow- and orange-eyed strains have sepiapterin levels similar to wild type but white and grape have only 2% and 12% respectively of this value. Interestingly, the double mutant yellowish;grape has about twice the level of sepiapterin found in grape.

Both white and grape were found to contain isoxanthopterin and pterin in the anterior portion of pupae five days after pupariation, and the level of these compounds in both strains was judged to be roughly the same as that in wild type. After emergence, no pteridine could be found in the head of white, while pterin but not isoxanthopterin was found in the head region of grape. All the other

TABLE 6.2

SEPIAPTERIN LEVELS IN HEADS OF WILD TYPE AND MUTANTS

STRAIN	SEPIAPTERIN (nmoles/head)[a]
Wild Type	5.9 ± 2.0
Yellowish	5.3 ± 1.0
Yellow	5.9 ± 0.8
Tangerine	5.8 ± 0.6
Topaz^1	5.2 ± 0.6
Topaz^2	6.5 ± 0.6
White	0.2 ± 0.1
Grape	0.8 ± 0.3
Yellowish;Grape	1.7 ± 0.7

a Values ± one standard deviation are the mean of at least three determinations done in duplicate.

mutants, like wild type, contained isoxanthopterin in the head 48 hours after emergence but it was not possible to assess the level of pterin because of the high level of sepiapterin which obscures the blue fluorescence of pterin when the chromatograms are viewed under UV light.

6.35 EFFECT OF DIETARY PURINE ON DEVELOPMENT OF WILD TYPE AND GRAPE

As a preliminary step in investigating the possibility that grape of *L. cuprina* might be the equivalent of rosy of *D. melanogaster* and therefore deficient in xanthine dehydrogenase activity, a study of the effect of dietary purine on development in wild type and grape was undertaken. In rosy, the failure to metabolise purine compounds through to uric acid (Figure 1.4) results in a high susceptibility to dietary purine (Glassman, 1965). It was possible that a similar mutant might be found in *L. cuprina* and the only pteridine deficient strain so far available as a possible candidate was grape. The results of the feeding trials are presented in Table 6.3. It can be seen that there is no real difference in susceptibility to dietary purine between the two strains, since a level of 20mM purine is lethal for both, while 10mM purine causes delayed development and low viability at similar levels for both. Below a concentration of 5mM purine a slight differential is seen, but this may be due more to the experimental conditions than to a real strain-specific difference. In high doses purine killed

TABLE 6.3
SURVIVAL OF WILD TYPE AND GRAPE INSECTS ON A DIET SUPPLEMENTED WITH PURINE

DOSE[a]	% SURVIVAL[b]			
	Wild Type		Grape	
	Pupae[c]	Adults[d]	Pupae[c]	Adults[d]
0	80	70	87	73
0.1	85	60	65	65
0.5	100	90	70	60
2.0	82	62	65	60
5.0	45	10	55	50
10.0	32	22	20	20
20.0	0	0	0	0
100.0	0	0	0	0

a Final concentration of purine (mM).

b Calculated on the basis that 20 first instar larvae were added to the vials. Results of two experiments.

c Proportion of these larvae to pupariate.

d Proportion of these larvae to emerge.

the insects very early in larval life; in lower doses development was frequently slowed.

6.4 *DISCUSSION*

L. cuprina, like *C. erythrocephala* (Ziegler, 1961; Patat, 1965) and *D. melanogaster* (Fan *et al.*, 1976) contains several pteridine compounds. Two colourless pteridines, pterin and isoxanthopterin, are found in all three species as is the yellow sepiapterin. Only in *D. melanogaster* do the eyes contain the red drosopterins which seem to play an important role in screening the retinular cells (see, for example, Broda & Wright, 1978). In other genera, sepiapterin probably plays this screening role, although there is much less sepiapterin in the eyes of adult *L. cuprina* than drosopterins in the eyes of *D. melanogaster*. From the results in Section 6.33, a value of 0.18 μmoles per g flies can be calculated for *L. cuprina* wild type 48 hours after emergence. Using figures presented by Evans & Howells (1978), values for *D. melanogaster* of 1.36 μmoles drosopterins and 0.38 μmoles sepiapterin per g flies can be calculated. Since these workers used a fluorimetric assay to determine sepiapterin levels, the figure of 0.38 μmoles/g flies may be inflated by the presence of pterin and the sepiapterin breakdown product pterin-6-carboxylic acid, so it is likely that the sepiapterin content of *D. melanogaster* and *L. cuprina* expressed on this basis is similar. A much lower level of total pteridines was also

found in *C. erythrocephala* compared with *D. melanogaster* (Harmsen, 1966) which can probably be accounted for by the absence of the drosopterins. It is interesting that Harmsen (1966) found that the meconium of *C. erythrocephala* had a higher pteridine content than that of *D. melanogaster* which suggests that excess guanine nucleotide may be converted to drosopterins and deposited in the eyes in *Drosophila* species, whereas in *C. erythrocephala* the excess nucleotide may be converted to isoxanthopterin and excreted. *L. cuprina* possibly resembles the other blowfly, *C. erythrocephala* in this respect.

In the preparation of purified sepiapterin from the heads of the sepia strain of *D. melanogaster*, it was noted that extracts remained brown (characteristic of the granule-bound form of this pigment) until the neutralisation step (See Evans & Howells, 1978, for details of the method), after which the supernatant liquid became bright yellow. This bright yellow material was applied to the phospho-cellulose column and from it sepiapterin was purified. In contrast, extracts from the heads of the yellow strain of *L. cuprina* (which have bright yellow eyes) were bright yellow throughout preparation. The material applied to the column was identical in colour with that from sepia. This suggests that sepiapterin is not bound to granules in the eyes of *L. cuprina*, although it is quite tightly bound in the eyes of sepia of *D. melanogaster*. The subcellular localisation of sepiapterin in *L. cuprina* eye pigment cells will be discussed more fully in Chapter 7.

The onset of sepiapterin biosynthesis during adult development of *L. cuprina* occurs later in pupal life than that of either drosopterin or sepiapterin in *D. melanogaster* (as measured by Fan *et al.*, 1976). These authors found that sepiapterin synthesis in *D. melanogaster* was almost complete by emergence, while biosynthesis of the main pteridine eye pigments (drosopterins) began about two thirds of the way through pupal life, and half the maximal amount had been deposited by the time of emergence. Since determination of sepiapterin levels in these *D. melanogaster* studies was based on fluorescence measurements, the values quoted as being for sepiapterin may well also include pterin which appears in extracts of *L. cuprina* long before sepiapterin. However, it does seem that the biosynthesis of pteridine screening pigment begins much later in *L. cuprina* than in *D. melanogaster*. This may be related to the lower final amount which is accumulated, as discussed above. Perhaps pteridines play a less important role than xanthommatin in screening the ommatidia of Diptera, so that a greater variation in their concentration, structure and time of deposition has been tolerated during evolution.

Among the mutants, only white has virtually no sepiapterin. During pupal life in this mutant isoxanthopterin and pterin could be detected but these are absent in the adults and may have been excreted with the meconium. In heads of grape adults a small amount of sepiapterin is present, as well as pterin. Pterin and isoxanthopterin are present in apparently wild type quantities in the

anterior portions of five day pupae of grape. The absence of the bright yellow sepiapterin (which has a brightening effect on the eye colour as wild type insects age) may explain the dark eye colour of grape insects (see Frontispiece). An alternative explanation, based on electron microscopic observations, is presented in the next chapter.

The heads of the yellow- and orange-eyed ommochrome deficient mutants contained roughly the same level of sepiapterin as wild type. It is clear that ommochrome deficient mutants do not compensate for the reduced screening capacity by increased sepiapterin synthesis.

Sepiapterin levels in the double mutant yellowish; grape were also measured. The presence of the grape mutation would be expected to reduce pteridine synthesis to the level found in grape. This was not the case, since yellowish;grape insects had about twice the level of sepiapterin found in grape insects. Thus the presence of the yellowish mutation suppresses the grape mutation, permitting more sepiapterin synthesis than found in grape. However, the level of sepiapterin in yellowish;grape adults was only one third of that found in yellowish, so that sepiapterin biosynthesis is not completely restored. Other double mutants such as yellow;grape and topaz[1];grape also have pale yellow eyes (see Section 2.3 for the full series of epistatic dominance of eye colour mutations) and probably have a similar partial restoration of sepiapterin synthesis.

There are as yet few clues as to the nature of the grape mutation. It seems unlikely that it is the equivalent of rosy of *D. melanogaster* since isoxanthopterin and pterin in apparently wild type levels were observed in pupae. Preliminary *in vitro* assay of xanthine dehydrogenase (performed by the method of Glassman & Mitchell, 1959, modified by Seybold, 1974) confirmed that both white and grape strains (assayed as newly emerged adults) had xanthine dehydrogenase activity at the wild type level. In addition, dietary intake of purine affected wild type and grape equally, although it is known to have a differential effect on wild type and rosy of *D. melanogaster* (see Section 6.35). As described in Chapters 4 and 5, grape is somewhat abnormal in xanthommatin biosynthesis as well as in sepiapterin production. Thus it seems likely that grape, like a large number of *D. melanogaster* mutants, is blocked in some as yet unknown way in both biosynthetic pathways. Further studies of the double mutants may help clarify the functions of the grape locus.

The white-eyed mutant of *L. cuprina*, like those of other species, is an extreme case of a mutant blocked in both pathways. Xanthommatin and sepiapterin are virtually absent, and there is an apparent lack of riboflavin, pterin and isoxanthopterin from the heads of adults, as well as abnormalities in the developmental profile of 3-hydroxy-kynurenine (Chapter 4) and its accumulation in larval malpighian tubules and adult eyes (Chapter 5). Since the uptake or storage of xanthommatin precursors is apparently

defective in white, it is possible that this defect extends to pteridine precursors. Future studies on the role of uptake and storage in pteridine production in *L. cuprina* are required.

The lack of pteridine mutants limits the studies of pteridine biosynthesis which can be carried out using *L. cuprina* at the present time. Two new approaches to mutagenesis which might result in the isolation of mutants homologous with brown and rosy of *D. melanogaster* are suggested by this research. Firstly, mutagenesis of yellow-eyed (rather than wild type) insects would make easier the detection of mutants defective in sepiapterin synthesis, since these should have white eyes (as do vermilion;brown and cinnabar;brown double mutants of *D. melanogaster*). Secondly, the use of medium supplemented with purine on which to rear the progeny of mutagenised flies might allow the identification of "rosy" mutants of *L. cuprina* as has been frequently done with *D. melanogaster* (see Finnerty, 1976).

CHAPTER 7

THE MORPHOLOGY OF THE PERIPHERAL RETINA IN *LUCILIA CUPRINA* : PIGMENT GRANULE ULTRASTRUCTURE IN WILD TYPE AND MUTANTS

7.1 *INTRODUCTION*

In the previous chapters, the biochemical manifestations of the disruptions to eye pigment synthesis found in the eye colour mutants of *L. cuprina* were discussed, and the functions of several loci identified. The present chapter presents an investigation of the morphological consequences of these disruptions in pigment synthesis, considering in particular the effects on pigment granule ultrastructure.

The compound eye of Diptera consists of the peripheral retina (made up of ommatidia containing the photosensitive cells), the lamina (consisting of nerve axons leading from the ommatidia and forming the optic nerve at the optic chiasm), the medulla and the optic lobe (which contains the principal and laminar foci). The general anatomy of the compound eye of Diptera is reviewed by Trujillo-Cenóz (1972). The different regions of the eye are shown in Figure 7.1. In the peripheral retina of *D. melanogaster* there are reported to be about 800 facets or ommatidia (see Shoup, 1966), while there may be as many as 3,200 in the larger fly *Musca domestica* (Braitenberg & Strausfeld, 1973). Each ommatidium has the following parts (from Stavenga, 1975):

a) The corneal lens, a modified transparent part of the chitinous exoskeleton;

b) The pseudocone, a cup-shaped cavity below the lens, containing a semi-fluid substance;

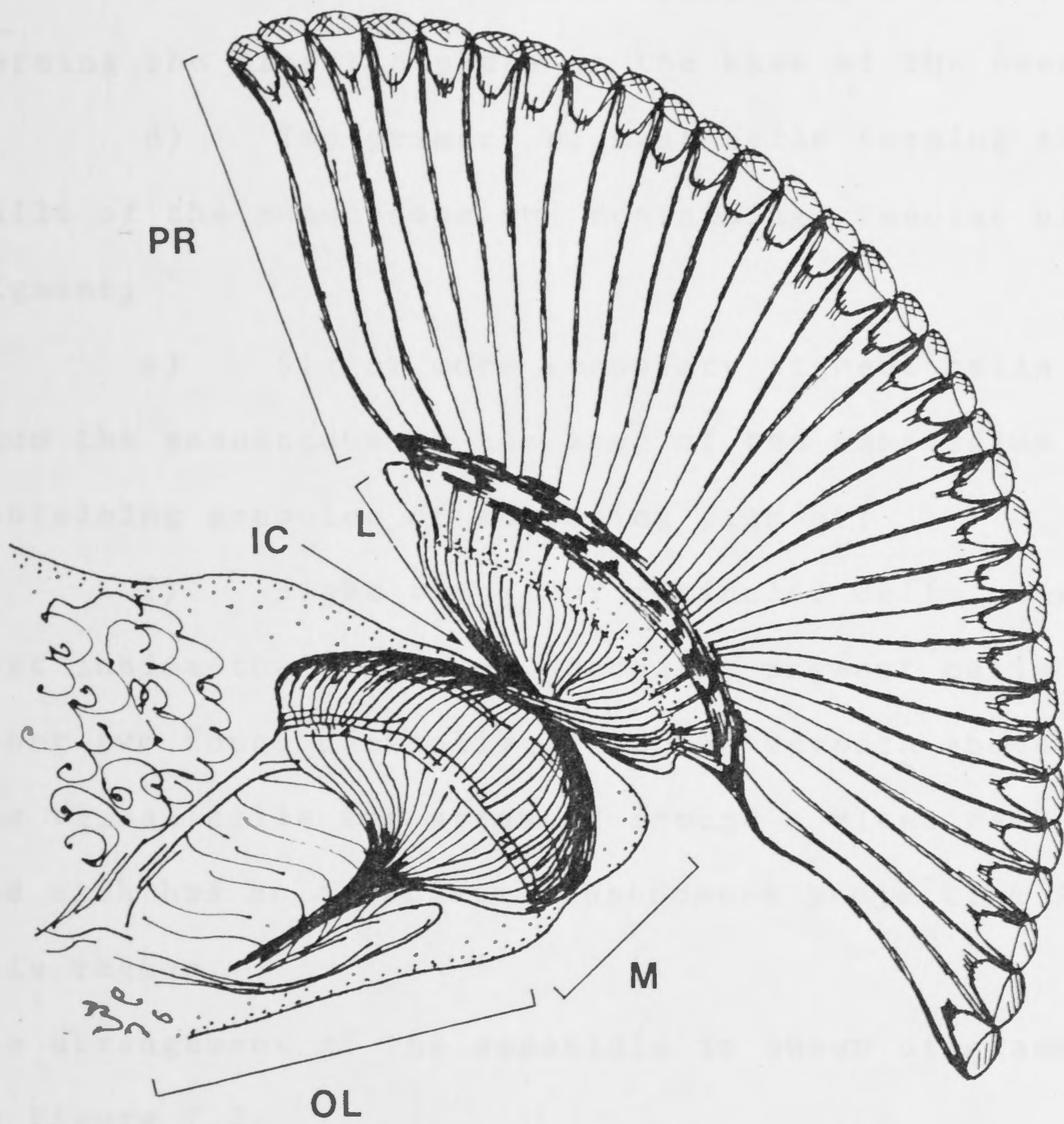


FIGURE 7.1

DIAGRAM SHOWING THE COMPOUND EYE OF DIPTERA

PR - peripheral retina; L - lamina; IC - intermediate chiasm; M - medulla; OL - optic lobe.

Redrawn from Trujillo-Cenóz (1972) and Meyerowitz & Kankel (1978).

c) Four cone cells (also called Semper cells) forming the dioptric plate at the base of the pseudocone;

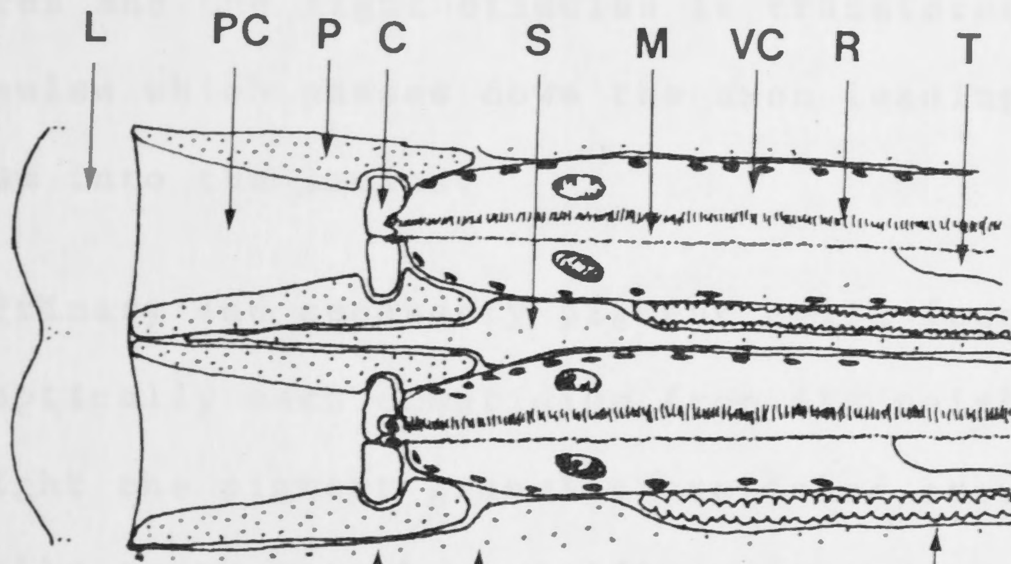
d) Two primary pigment cells forming the lateral walls of the pseudocone and containing granules of screening pigment;

e) Six or more secondary pigment cells extending from the pseudocone to the base of the ommatidium and also containing granules of screening pigment;

f) Eight visual or retinular cells, six arranged just inside the circle of secondary pigment cells and the other two found centrally, with the seventh above the eighth. The visual cells are arranged around a clear central matrix and each has an associated rhabdomere projecting into this region.

The arrangement of the ommatidia is shown diagrammatically in Figure 7.2.

The photosensitive pigment, rhodopsin, is found in the rhabdomeres of the visual cells. Rhabdomeres are made up of tubuli (microvilli) in precise array (reviewed by Carlson & Chi, 1979). The rhabdomeres from each visual cell are arranged asymmetrically within the central cavity of the ommatidium (Melamed & Trujillo-Cenóz, 1968; Boschek, 1971; Braitenberg & Strausfeld, 1973; Stavenga, 1975), and groups of rhabdomeres may be sensitive to different wavelengths of light (White, 1978). At the distal end of the visual cells the apices of the rhabdomeres (termed retinular caps) pass into the cone cells which form the dioptric apparatus. Light passes through the lens and



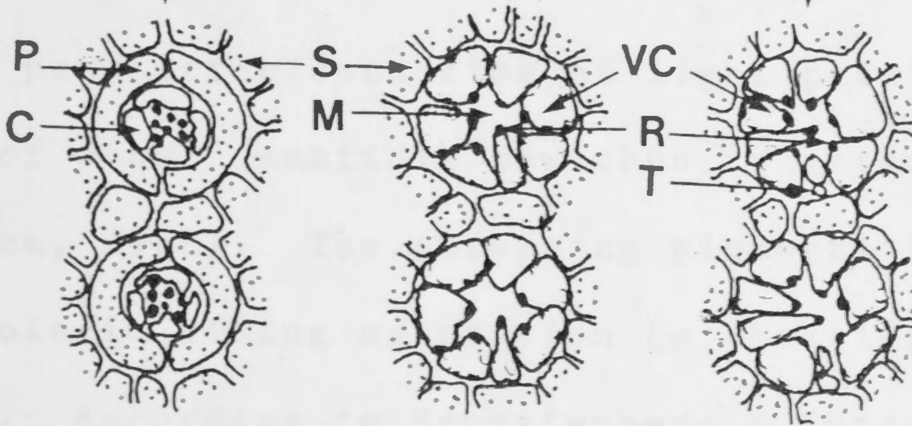


FIGURE 7.2

DIAGRAM OF TWO OMMATIDIA FROM A FLY RETINA

Longitudinal section (a) and associated cross sections (b) through adjacent ommatidia.
L - crystalline lens; PC - pseudocone; P - primary pigment cell;
C - cone cell; S - secondary pigment cell; M - central matrix;
VC - visual cell; R - rhabdomere; T - trachea.

Redrawn from Stavenga (1975).

pseudocone to the cone cells where the image is formed. This is detected by the photosensitive pigment in the rhabdomeres and the light stimulus is transformed into a nerve impulse which passes down the axon leading from the ommatidium into the lamina.

Primary and secondary pigment cells function to isolate optically each ommatidium from its neighbours. In bright light the pigment granules are found around the visual cells so that each ommatidium responds only to light coming almost axially through the lens. Under less bright illumination, the granules move to the region of the pseudocone, permitting detection of light passing through the lenses of other ommatidia and thus brightening the image (Wolken, 1968). The screening pigments thus play an important role in aiding adaptation to changing light intensities. According to Hengstenberg & Götz (1967) this is mainly through enhancing pattern contrast sensitivity, rather than visual acuity.

In the eyes of wild type *D. melanogaster*, Shoup (1966) identified two types of pigment granule which she termed Type I and type II. She concluded, on the basis of a developmental study of wild type and mutants, that ommochrome is bound to Type I granules found in the primary and secondary pigment cells. Type II granules in the secondary pigment cells were thought to carry the red pteridine pigments (the drosopterins). Although Shoup (1966) reports two distinct granule types for ommochrome and

pteridine pigments, Nolte (1961) suggested that xanthommatin and drosopterin are bound to the same granules in parts of the secondary pigment cells. Nolte (1961) also reported somewhat larger granules in the eyes of the drosopterin-deficient mutant sepia of *D. melanogaster*, to which he felt sepiapterin was bound.

In the eyes of the blowfly *Calliphora erythrocephala*, Langer (1967, 1975) found red and yellow/brown pigment granules in the primary and secondary pigment cells. The granules in the eyes of the white-eyed mutant chalky were colourless and more variable in size, shape and electron density following osmium tetroxide fixation, than those in wild type. Langer (1967) attributed the differences in granule colour to the oxidised (yellow) and reduced (red) forms of xanthommatin. He claims that there are no pteridine binding granules in the pigment cells of the eyes of this fly. This contention is based largely on micro-spectrophotometric measurements of pigment granules which do not show the characteristic spectrum of sepiapterin. However, as noted by Ziegler & Harmsen (1969), sepiapterin undergoes a bathochromic shift when bound to granules and the spectrum of the bound pigment is therefore different from that of sepiapterin in solution. Nonetheless, Langer (1975) claims that pteridines usually occur unbound or loosely bound in the eyes of Diptera, exceptions being the Type II drosopterin-binding granules of *D. melanogaster* reported by Shoup (1966) and small sepiapterin-binding granules apparently present in the visual cells of *C. erythrocephala*

and *M. domestica* (Langer, 1975).

Varying degrees of granule and pigment cell abnormality are associated with disruption of pigment synthesis among the eye colour mutants of dipteran species. Shoup (1966) identified abnormal pigment granule types, termed Type III and Type IV, in the eyes of xanthommatin-deficient mutants of *D. melanogaster*. Type III granules were thought to reflect the failure of ommochrome synthesis in the red-eyed mutants vermilion and cinnabar. Type IV granules were found in the eyes of those mutants which lacked pigment of both classes, such as white and the double mutant vermilion;brown of *D. melanogaster* as well as the white-eyed mutant of the moth *Ephestia kühniella*. Shoup (1966) suggests that Type IV granules may represent autophagic vacuoles in which materials resulting from the complete failure of pigment synthesis are hydrolysed. Sun *et al.* (1972) found granules of abnormal morphology in the eyes of the mutants clot and sepia of *D. melanogaster*. Granules in the eyes of the eye colour mutants of *C. erythrocephala* were of different morphology and frequency from those in wild type (Langer, 1967, 1975). It might therefore be expected that the eyes of *L. cuprina* eye colour mutants would also show abnormal pigment granule morphology. In addition to abnormal granule morphology, Nolte (1961) reports gross distortions of pigment cell shape and location in the eyes of the pteridine-deficient *D. melanogaster* mutants raspberry and prune and it is possible that such defects could be found among the pigment mutants of other species.

In this chapter, the results of a preliminary ultrastructural examination of the peripheral retina of wild type and eye colour mutants of *L. cuprina* is presented. Particular attention is paid to the pigment granules. Pigment granule morphology is considered through two approaches. The first involves a comparison of pigment granules in wild type and the seven different eye colour mutant strains, so that the possible functions of the granules can be deduced from information concerning the biochemistry of these mutants reported in the previous chapters. In a second approach, the non-autonomous mutant yellow and the autonomous mutant topaz[1] (Chapter 4) were fed a diet supplemented with 3-hydroxykynurenine so that the nature of the granules formed could be compared with that in flies not fed 3-hydroxykynurenine. Previously, Schwabl & Linzen (1972) had shown that pigment granule morphology in cinnabar of *D. melanogaster* is normalised when this non-autonomous mutant (probably equivalent to yellow; Chapters 4 and 5) has been provided with 3-hydroxykynurenine throughout larval life (so that brown pigment is made). These two approaches allow an interpretation to be made of the role of the different granule forms seen in the eyes of *L. cuprina* strains.

7.2 *MATERIALS AND METHODS*

7.21 EXPERIMENTAL ANIMALS

Adult flies 12 - 24 hours after emergence were used in all experiments described in this Chapter. All eyes were light-adapted. Feeding of 3-hydroxykynurenine was performed exactly as described in Section 4.23

7.22 PREPARATION OF MATERIAL FOR MICROSCOPY

Compound eyes were dissected as described in Section 5.25 and immediately plunged into a modified Karnovsky fixative (see Glauert, 1975), consisting of 2.5% glutaraldehyde, 2.0% formaldehyde, 0.12M sodium phosphate buffer (pH 7.4) with 0.06M D-glucose and 4mM calcium chloride. Eyes were left in this pre-fixative overnight at 0 - 4°C. The following day, eyes were washed several times in the same buffer (including glucose and calcium chloride) at 0°C for 2 - 2½ hours, post-fixed in 2% osmium tetroxide in the same buffer (including glucose and calcium chloride) at 0°C for 2 hours, washed several times in distilled water at room temperature, dehydrated through a graded series of ethyl alcohol in water and finally polymerised in epoxy resin (Spurr, 1969).

Semi-thin (1.0 - 1.5 μm) and thin (silver - gold) sections were cut on a Reichert OmU2 ultramicrotome. Semi-thin sections were examined without post-staining or

following post-staining in the basic fuchsin - toluidine blue O stain of Alsop (1974) using a Leitz Ortholux II light microscope. The sections were mounted on coated copper grids, post-stained in uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1973) and examined in a Hitachi HU12 electron microscope.

7.3 *RESULTS*

7.31 LIGHT MICROSCOPY OF THE EYES OF WILD TYPE *L. CUPRINA*

The peripheral retina of the wild type strain of *L. cuprina* has the characteristics of that in other dipteran species. Figure 7.3a shows a low magnification light micrograph of a longitudinal section through a wild type retina. In the higher magnification micrograph (Figure 7.3b) it can be seen that both primary and secondary pigment cells contain round, osmiophilic granules. The secondary pigment cells surround eight visual cells and the unfused rhabdom of *L. cuprina* has the same asymmetric array of rhabdomeres around a central matrix as seen in other flies. This can be seen in the cross section shown in Figure 7.3c. As discussed by Stavenga (1975), tracheae were found associated with each ommatidium (Figure 7.3a). Other structures characteristic of the dipteran ommatidium can be seen in Figure 7.3b and in the electron micrograph in Figure 7.3d. These include the corneal lens,

pseudocone, cone cells, rhabdomeres and their associated retinular caps.

7.32 ELECTRON MICROSCOPY OF THE EYES OF WILD TYPE AND MUTANTS

All micrographs presented in this chapter show longitudinal sections of parts of ommatidia at the level of the cone cells.

In the primary pigment cells of wild type *L. cuprina* eyes all pigment granules appear similar to the Type I granules of *D. melanogaster* (Shoup, 1966) (Figure 7.4a). These granules are round, very electron dense and membrane-bound (Figure 7.4b). The granules in the secondary pigment cells are also very electron dense and membrane-bound but appear less regularly shaped and often have a lighter staining crescent at the periphery (Figure 7.4c).

The primary pigment cells of the ommochrome-deficient mutants with yellow eyes (yellowish - Figures 7.4 d-f; yellow - Figures 7.5a-c; topaz^1 - Figures 7.5d-f) contain almost empty membrane-bound structures roughly the size of the granules found in wild type (Figures 7.4d,e; 7.5a,b; 7.5d,e). These structures contain a small amount of electron dense material and are similar to the Type III granules of Shoup (1966). In addition to similar empty granules, the secondary pigment cells of these mutants usually contain membrane-bound granules with a fibrous substructure (Figures 7.4d,f; 7.5a,c; 7.5d,f)

similar to an immature form of the Type II granules described by Shoup (1966) in *D. melanogaster*. Similar structures were not seen in wild type eyes of *L. cuprina*.

In the two partially pigmented (orange-eyed) mutants tangerine (Figures 7.6a-c) and topaz^2 (Figures 7.6d-f), similar empty Type III granules are seen in both cell types but more frequently in secondary pigment cells (Figures 7.6a and 7.6d). As well, both cell types of these mutants contain a scattering of round, electron dense granules similar to the Type I granules of wild type (Figures 7.6b,c,e). However, the size and shape of the Type I granules in tangerine and topaz^2 are less regular than in wild type and the granules are more frequent in secondary pigment cells of tangerine and primary pigment cells of topaz^2 than in their primary and secondary (respectively) pigment cells. The fibrous "Type II" granules are also occasionally seen in the secondary pigment cells of these mutants, though in very low frequency (Figures 7.6a,d). In addition, the primary pigment cells of tangerine (Figure 7.6a) were filled with variably staining irregularly shaped membrane-bound vesicles which were not seen in wild type, topaz^2 or the yellow-eyed mutants.

The eyes of the mutant white contain no screening pigment of either class (Chapters 4 and 6) and the pigment cells are devoid of structures similar to any of the granules described above (Figures 7.7l-c). Instead, there are (in low frequency) membrane-bound bodies similar in size to the granules seen in wild type. These bodies

have a granular substructure (Figures 7.7b,c). They are very similar to the Type IV granules described by Shoup (1966) for *D. melanogaster* and *Ephestia kühniella* white-eyed mutants.

Both the primary and secondary pigment cells of the mutant grape have a very different structure from that of wild type (Figures 7.7d-f). Electron transparent, membrane-bound vesicles almost completely fill both cell types (Figure 7.7d). Scattered among these vesicles are a few Type I granules (Figures 7.7e,f) mainly in the primary pigment cells. In the case of the primary pigment cells, the only strain which has an ultrastructure approaching that in grape is tangerine (Figure 7.7a) but the membrane-bound vesicles filling the cells of the two mutants are of different electron densities, frequency and shape. The ultrastructure of the secondary pigment cells in grape is unique among the strains of *L. cuprina* examined.

7.33 RESULTS OF FEEDING 3-HYDROXYKYNURENINE TO YELLOW AND TOPAZ[1]

It was seen in Section 4.33 that the mutant yellow is non-autonomous when fed a diet supplemented with 3-hydroxykynurenine, while the mutant topaz[1] is autonomous. Since these two strains have similar visible and ultra-structural phenotypes, they were chosen for a study of the effects of feeding 3-hydroxykynurenine and consequent pigment formation by yellow on the pigment granules.

The results of this study (Figure 7.8) show that almost normal pigment granules are made by yellow, which has already been shown to produce about 25% of the wild type level of xanthommatin under these conditions (Chapter 4). After feeding on 3-hydroxykynurenine, the empty Type III granules have almost disappeared from the pigment cells of yellow (Figures 7.8a-c) suggesting that xanthommatin has been deposited on both of these types of granule. In contrast, the pigment cells of topaz[1] are the same whether the dietary supplement was made (Figure 7.8d) or not (Figure 7.5d).

7.4
DISCUSSION

The morphology of the eye of *L. cuprina* is similar to that described in other flies (as seen in Section 7.31). The unfused rhabdom has the asymmetrical arrangement of rhabdomeres seen in most dipterans. Boschek (1971) noted that this asymmetry is not seen in a primitive dipteran, *Wilhelmia equina* and recently Zeil (1979) has shown that another species, *Bibio marci*, which is thought to be less advanced than the drosophilids and calliphorids, also has the symmetrical arrangement of rhabdomeres. Shoup (1966) has drawn the rhabdomeres of *D. melanogaster* in relatively symmetrical array (her Figure 1), but this seems unlikely in view of other studies of *D. melanogaster* which show the same asymmetry as seen in *L. cuprina* (Wolken,

1968; Sun *et al.*, 1972; Stavenga, 1975; Franceschini, 1975).

The eye colour mutants of *L. cuprina* exhibit some pigment cell and pigment granule abnormalities in common with those of their homologues in *D. melanogaster*, as studied by Shoup (1966) (Section 7.32). Granules similar in morphology to all four types described by Shoup (1966) were found; however the relationship between these granule types seems to be different from that described in *D. melanogaster*.

In *L. cuprina*, ommochrome is apparently deposited on two sorts of granules. In the presence of xanthommatin these granules have very similar morphologies, with one sort being marginally less round in shape and sometimes having a lighter staining crescent of material at the edge (Figure 7.4c). In the absence of xanthommatin (as in the yellow-eyed mutants) these granules appear to be represented by the fibrous granules similar to the Type II granules of Shoup (1966) (which were found in the secondary pigment cells of the yellow-eyed mutants of *L. cuprina*) as well as by the empty Type III granules (found in both pigment cell types of these mutants). Evidence for the conclusion that the fibrous granules bind xanthommatin is found in the study of the non-autonomous mutant yellow which had been raised on a diet supplemented with 3-hydroxykynurenine. These flies had produced xanthommatin during development (Chapter 4). In the eyes of these flies, the Type III granules had almost disappeared, to be replaced by many Type I granules. In addition, the fibrous "Type II" granules

were rare. A number of these were seen, generally more heavily staining than those in the untreated controls (compare Figures 7.8a-c with Figures 7.5a-c), suggesting that xanthommatin deposition was incomplete on these granules.

There are apparently no sepiapterin-binding granules in the eyes of *L. cuprina*. The fibrous granules which resemble the drosopterin-binding Type II granules of Shoup (1966) are now thought to carry xanthommatin in *L. cuprina* (from the studies described above with the non-autonomous mutant yellow). Sepiapterin may be in solution in the pigment cells of *L. cuprina*. This is consistent with the claim by Langer (1967, 1975) that sepiapterin is not bound to granules in the eyes of *C. erythrocephala*. This possibility was also discussed in Chapter 6 where a difference was noted in the colour of extracts from sepia of *D. melanogaster* (where sepiapterin is known to be granule bound; see Ziegler & Harmsen, 1969) and extracts from *L. cuprina*. The results presented in the present chapter do not rule out the possibility that sepiapterin is bound to some of the granules which also bind xanthommatin, as suggested by Nolte (1961) for drosopterins.

It should be noted that the age of the flies used in this study is not ideal for consideration of sepiapterin deposition. For reasons discussed in Section 2.4, eyes from flies 12 - 24 hours after emergence were examined. Although xanthommatin synthesis is probably nearing completion

at this time (Chapter 3), it is the time of rapid sepiapterin deposition (Chapter 6). A small difference in age could therefore account for quite a large difference in amount of sepiapterin during this time. It might be better to compare older flies in such a study, at a stage where both xanthommatin and sepiapterin levels are stable.

The grossly abnormal cell structure of grape and to a lesser extent tangerine cannot be explained in terms of the known biochemical abnormalities of these two mutants. Nolte (1961) has noted that the pteridine-deficient mutants raspberry and prune of *D. melanogaster* have gross abnormalities of secondary pigment cells, which involve both the shape and the relative dispositions of the cells within the ommatidia. In grape, the position of the pigment cells in the eye is apparently not altered, but the cells are altered in shape, since they more completely fill the peripheral retina than those of wild type. Grape is unique in being affected in both pigment cell types. It is not possible to say whether the membrane-bound vesicles which fill the pigment cells of grape and are found in the primary pigment cells of tangerine are the main effect of the genetic lesion or a secondary result of the failure of pigment synthesis. Some other *D. melanogaster* mutants have abnormal eye colour and eye morphology, but these have not been studied ultrastructurally. They include glass and Moiré, in which the eye has a smooth appearance, and lozenge, where the eye is of altered shape (Lindsley & Grell, 1968).

The dark eye of the grape strain has been attributed to the absence of the brightening effect of the yellow pteridine sepiapterin (Chapter 6). However, it is possible that the colour of the eyes of grape flies is due in part to an alteration in the reflection and refraction characteristics of the eye caused by abnormal pigment cell structure. A comparison with the ultrastructure of the *D. melanogaster* mutants Moiré and glass might be instructive in this respect. Since tangerine flies have an eye phenotype frequently indistinguishable from that of topaz[2] and yellow reared on 3-hydroxykynurenine-supplemented medium (in both of which pigment cell morphology is normal), the abnormal subcellular morphology of the primary pigment cells of tangerine apparently does not contribute to the colour in this strain.

In the eyes of white flies, membrane-bound vesicles with granular substructure were found. These are very similar to the Type IV granules found by Shoup (1966) in the eyes of white-eyed strains (white and vermilion;brown of *D. melanogaster* and *wa* of *Ephestia kühniella*). Shoup (1966) proposes that these Type IV granules are the sites of destruction of materials not used in pigment synthesis. The study reported in this chapter does not present any new evidence for or against this proposition.

The work reported in this chapter allows a preliminary correlation of granule types with the nature of the pigment they carry to be made. It seems that the two abnormal pigment granules seen in yellow-eyed mutants

result from the failure to bind xanthommatin. These may therefore be either immature "pregranule" forms, to which xanthommatin is bound during development if formed, or aborted pigment granules which result from the destruction of "pregranules" which have failed to bind xanthommatin. A developmental study considering the time of appearance of the different granule forms during eye development would help to clarify the relationships between granules seen in wild type and mutants. A second approach which would provide useful information concerning the nature of the granules would be to isolate pigment granules using subcellular fractionation techniques and to examine their morphology and composition after isolation. Another approach which might help resolve the question of the location of the pigments within the pigment cells would be an immunocytochemical one, involving the production of antibodies to xanthommatin and sepiapterin and subsequent treatment of sections.

FIGURE 7.3

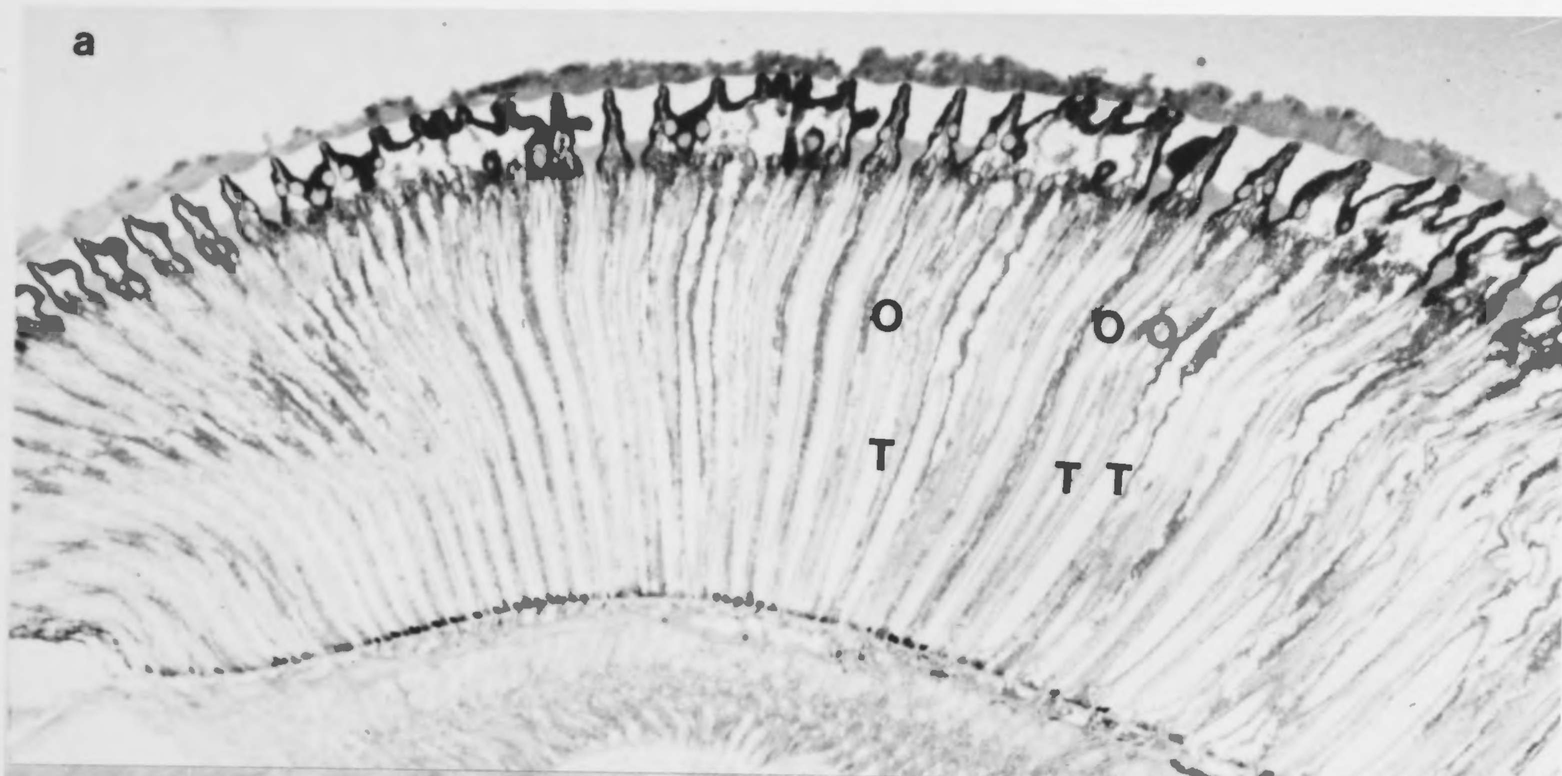
STRUCTURES IN THE EYES OF WILD TYPE *LUCILIA CUPRINA*

(a) Light micrograph of a longitudinal section through the peripheral retina, showing the columnar arrangement of ommatidia (O) and associated tracheae (T) when sectioned in the appropriate plane. Mag. = 95x

(b) Higher magnification of part of (a) showing the distal end of two ommatidia. This figure illustrates the osmiophilic granules associated with the primary pigment cells (granules labelled P) and secondary pigment cells (granules labelled S) and also shows the corneal lens (L), pseudocone (PC), cone cell (C), rhabdomere (R) and associated retinular cap (RC). Mag. = 1,600x

(c) Light micrograph of a cross section through the peripheral retina showing the asymmetric arrangement of the rhabdomeres (R) around the central matrix (M) of each ommatidium. Mag. = 1,300x

(d) Electron micrograph of the area shown in (b), at the level of the cone cell. This picture illustrates, at the resolution of the electron microscope, most of the structures indicated in (b), in addition to supposed lipid droplets (LD), mitochondria (MT) and a cone cell nucleus (N). Mag. = 13,000x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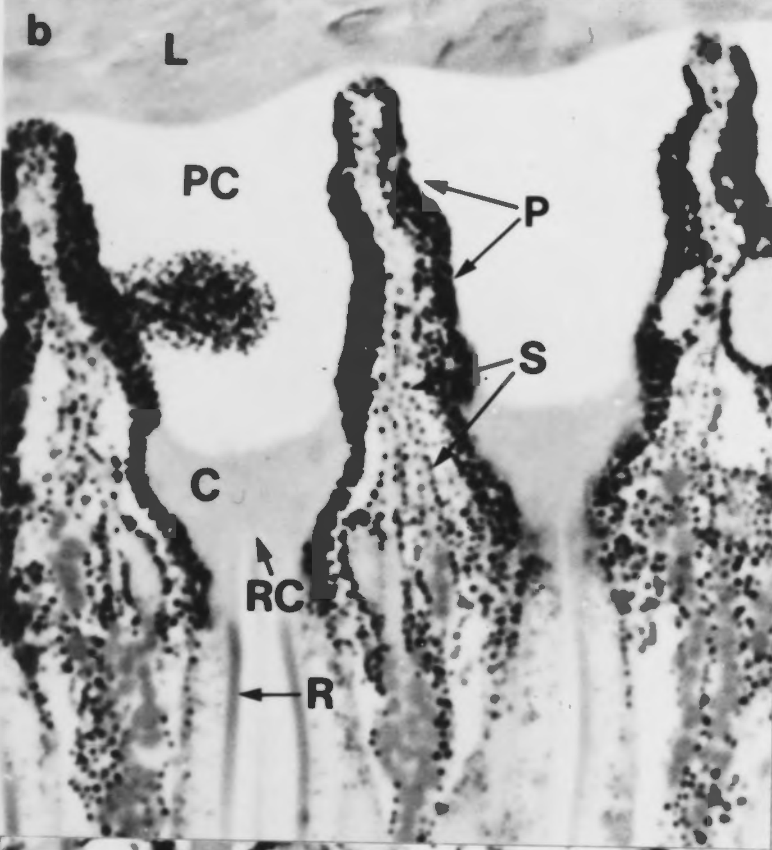
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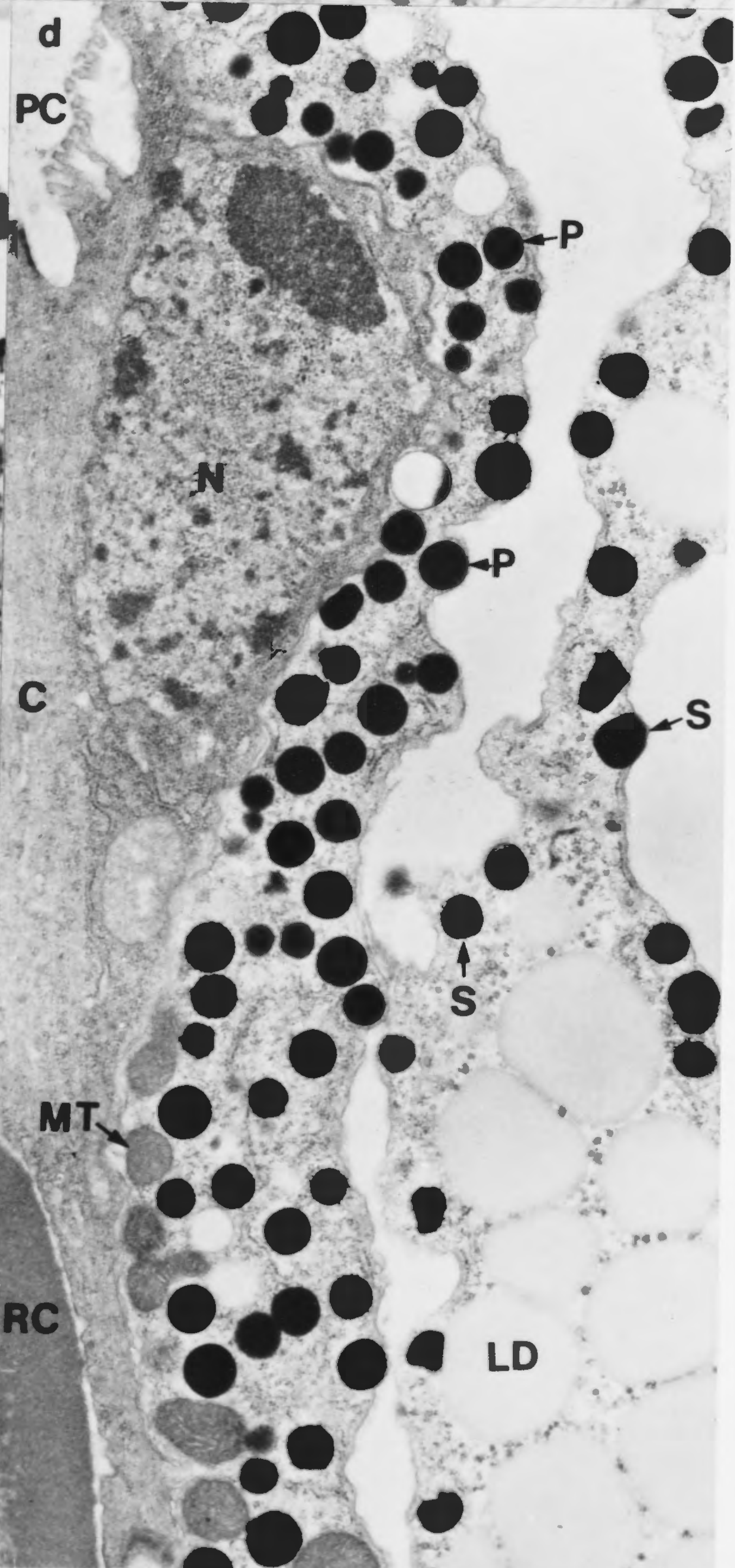
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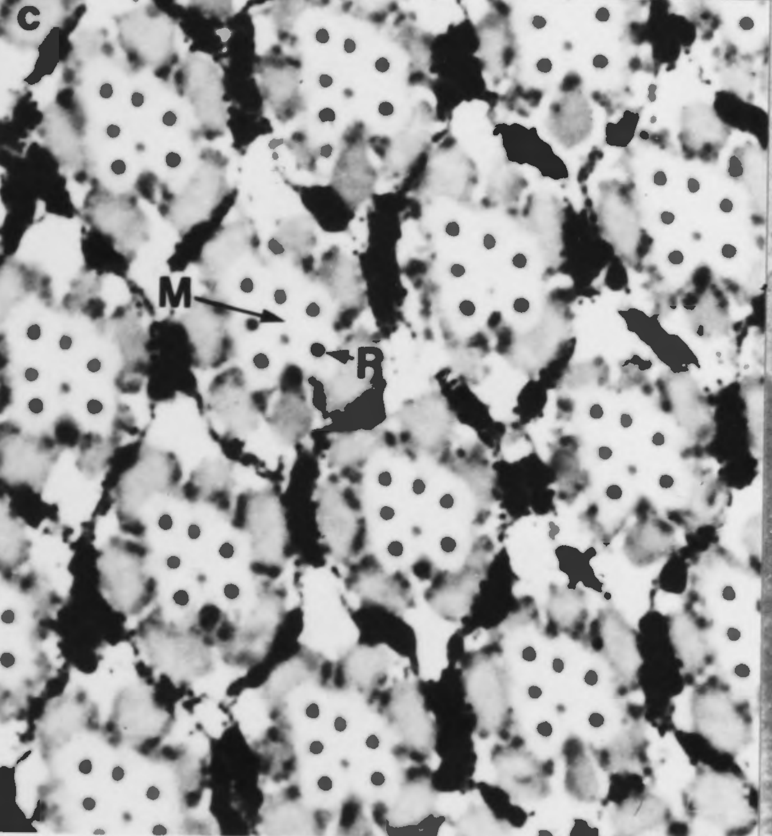
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FIGURE 7.4

ELECTRON MICROGRAPHS OF PIGMENT CELLS OF WILD TYPE AND YELLOWISH STRAINS

(a) - (c) Electron micrographs of wild type illustrating the ultrastructure of Type I pigment granules in primary and secondary pigment cells. The electron dense, membrane-bound pigment granules in primary pigment cells are designated P in (a) and shown at higher magnification in (b). Less regularly shaped, membrane-bound pigment granules with a lighter staining crescent at their periphery found in secondary pigment cells are designated S in (a) and shown in higher magnification in (c).

Mag. of (a) = 30,000x Mag. of (b) & (c) = 77,000x

(d) - (f) The yellow-eyed mutant yellowish, showing the almost electron transparent nature of membrane-bound type III pigment granules in the primary pigment cells. These granules have a small amount of electron dense material at one end and are designated P in (d) and shown at higher magnification in (e). Also shown in (d) is the fibrous substructure of pigment granules (immature forms of Type II granules) in secondary pigment cells (S in (d) and at higher magnification in (f)).

Mag. of (d) = 30,000x Mag. of (e) & (f) = 77,000x

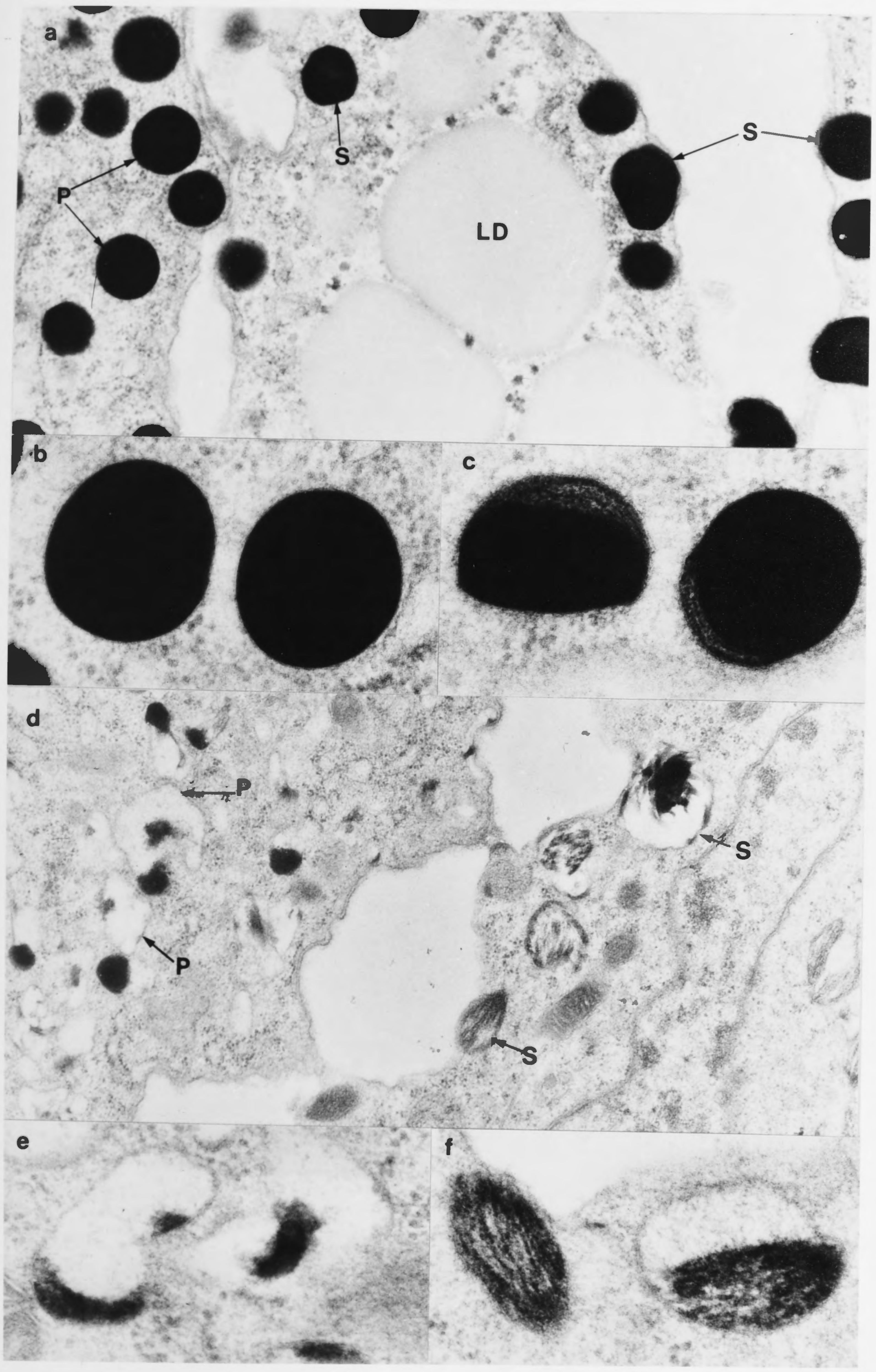
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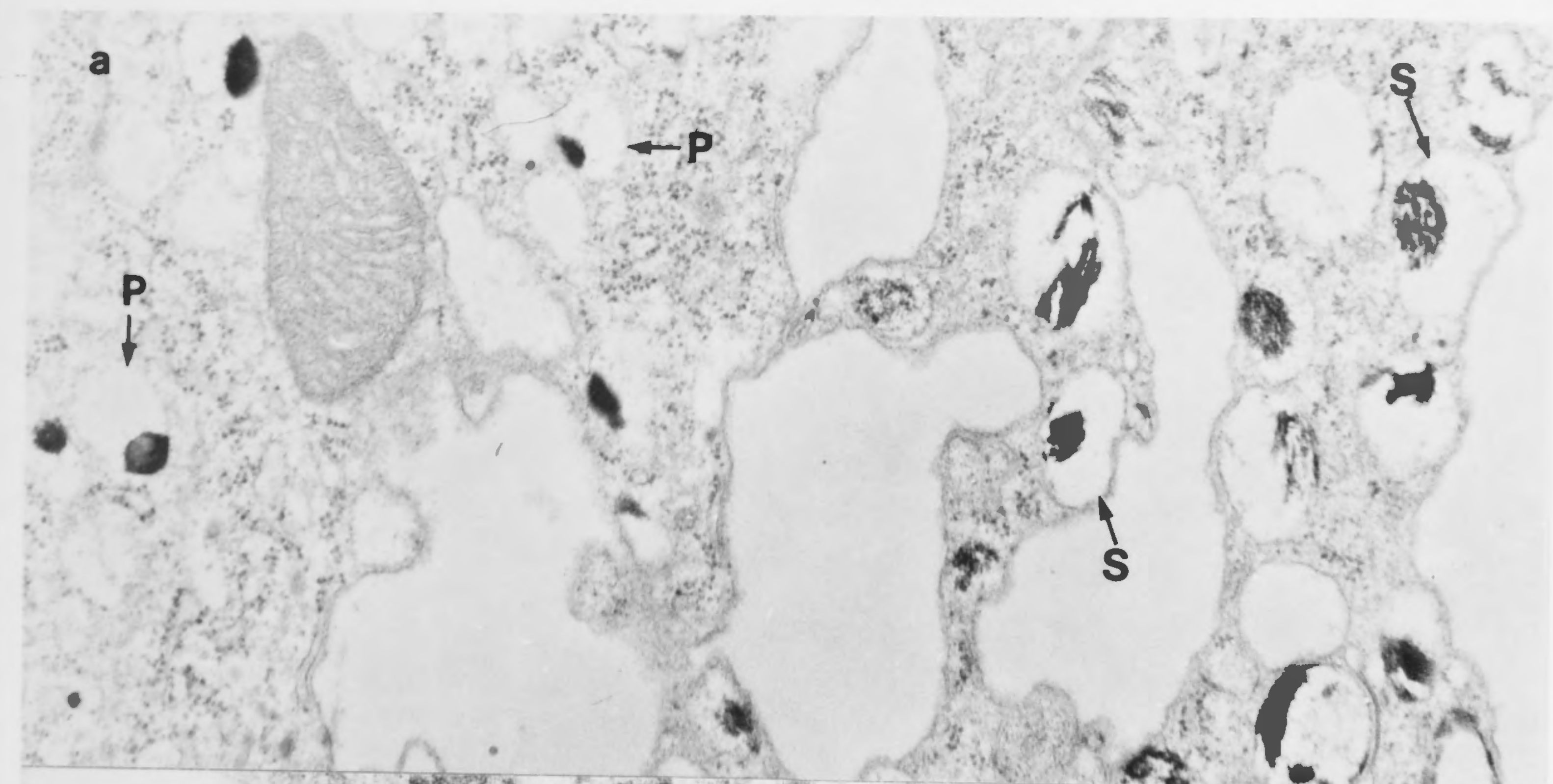
FIGURE 7.5

ELECTRON MICROGRAPHS OF OTHER YELLOW-EYED MUTANTS

(a) - (c) The mutant yellow showing primary (P in (a) and at higher magnification in (b)) and secondary (S in (a) and at higher magnification in (c)) pigment granules similar to the granules in corresponding cells of the mutant yellow (Figure 7.4 d - f). Mag. of (a) = 30,000x Mag. of (b) & (c) = 77,000x

(d) - (f) The mutant topaz[1] illustrating primary (P in (d) and at higher magnification in (e)) and secondary (S in (d) and at higher magnification in (f)) pigment granules characteristic of yellow-eyed mutants. Mag. of (d) = 30,000x Mag. of (e) & (f) = 77,000x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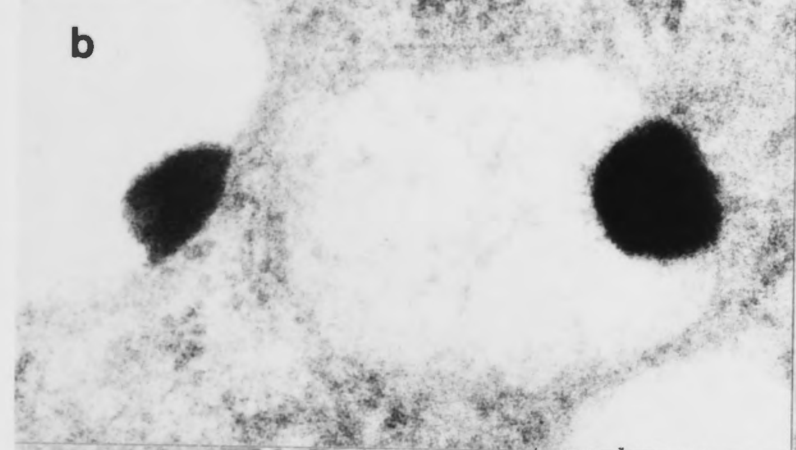
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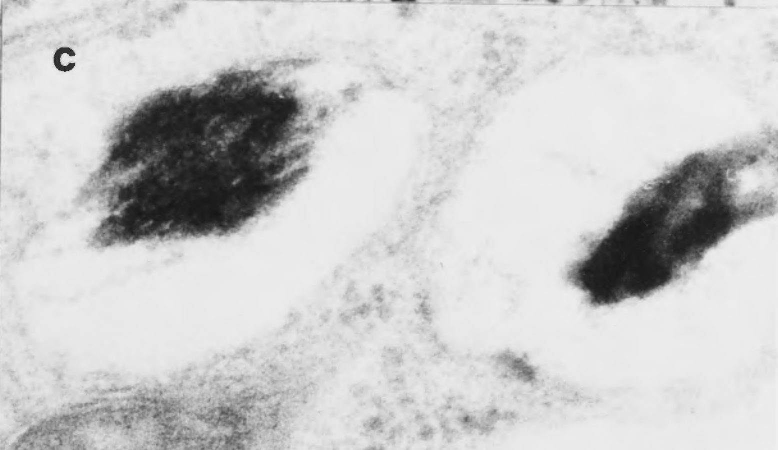
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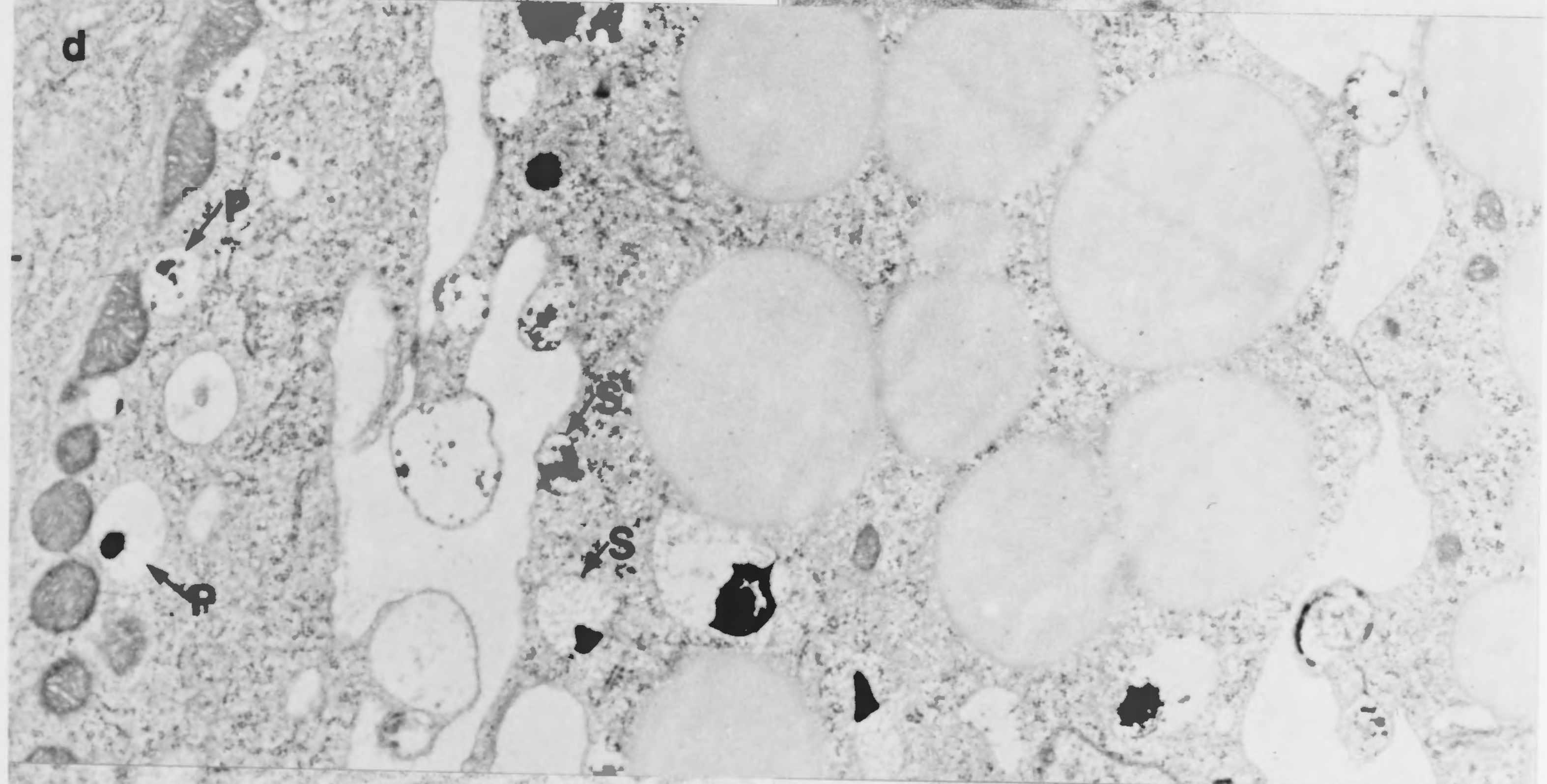
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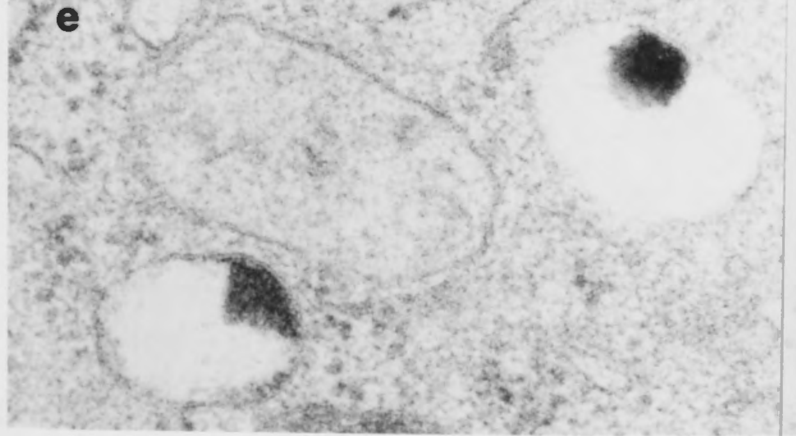
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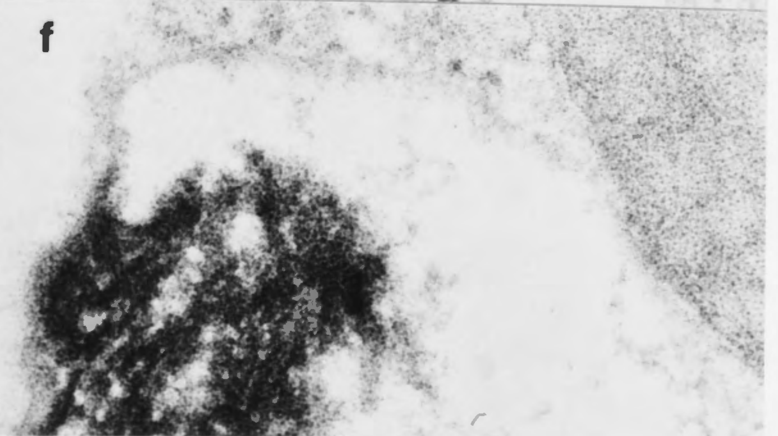
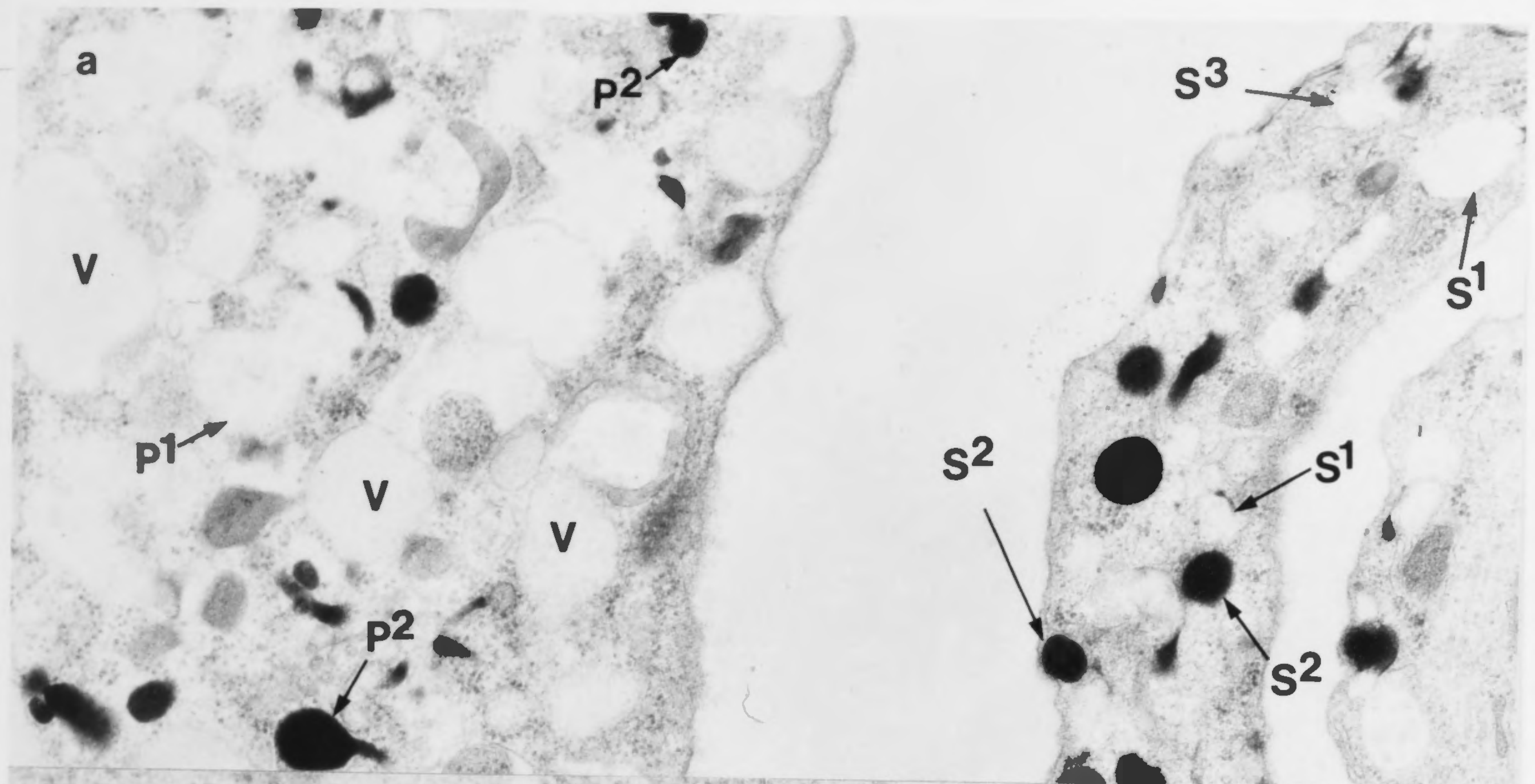

f

FIGURE 7.6

ELECTRON MICROGRAPHS OF THE ORANGE-EYED MUTANTS

(a) - (c) The mutant tangerine, illustrating various types of pigment granules in this mutant: supposed type III granules in primary (P^1) and secondary (S^1) pigment cells (a); irregularly shaped supposed Type I granules in primary (P^2 in (a) and at higher magnification in (b)) and secondary (S^2 in (a) and at higher magnification in (c)) pigment cells; fibrous "Type II" (S^3 in (a)) granules in secondary pigment cells. Also shown are irregularly shaped, variably stained, membrane-bound vesicles in primary pigment cells (V in (a) and at higher magnification in (b)). Mag. of (a) = 30,000x Mag. of (b) & (c) = 77,000x

(d) - (f) The mutant topaz^2 showing the various types of pigment granules characteristic of the orange-eyed mutants: symbols in (d) are the same as those in (a). Higher magnification micrographs of supposed Type I granules in primary and secondary pigment cells are shown in (e) and (f) respectively. Mag. of (d) = 30,000x Mag. of (e) & (f) = 77,000x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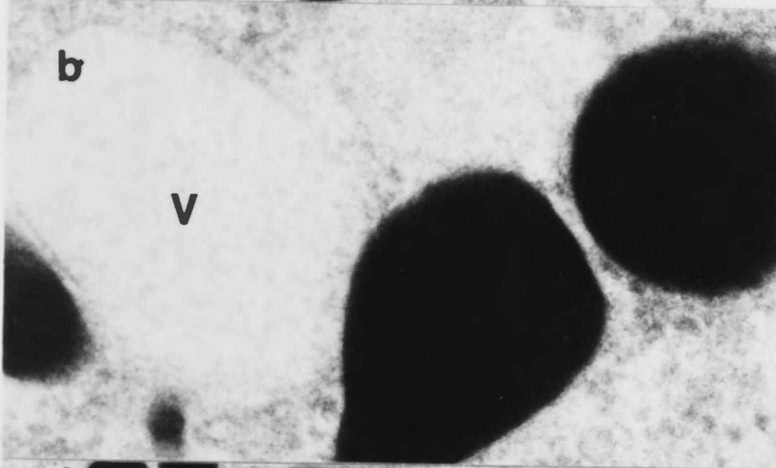
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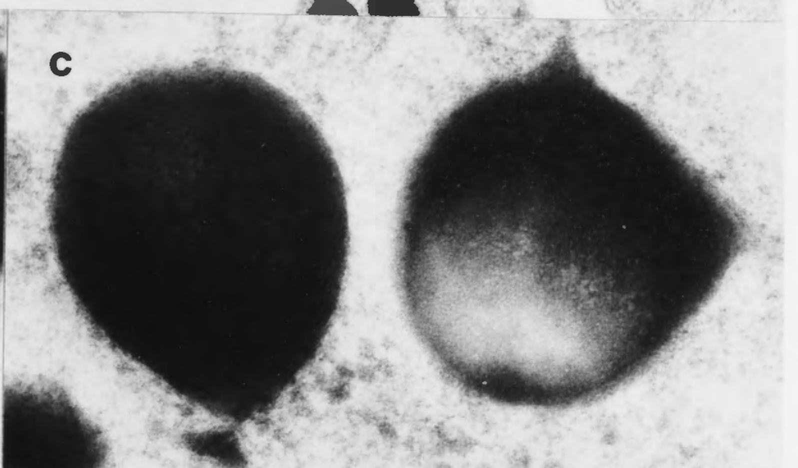
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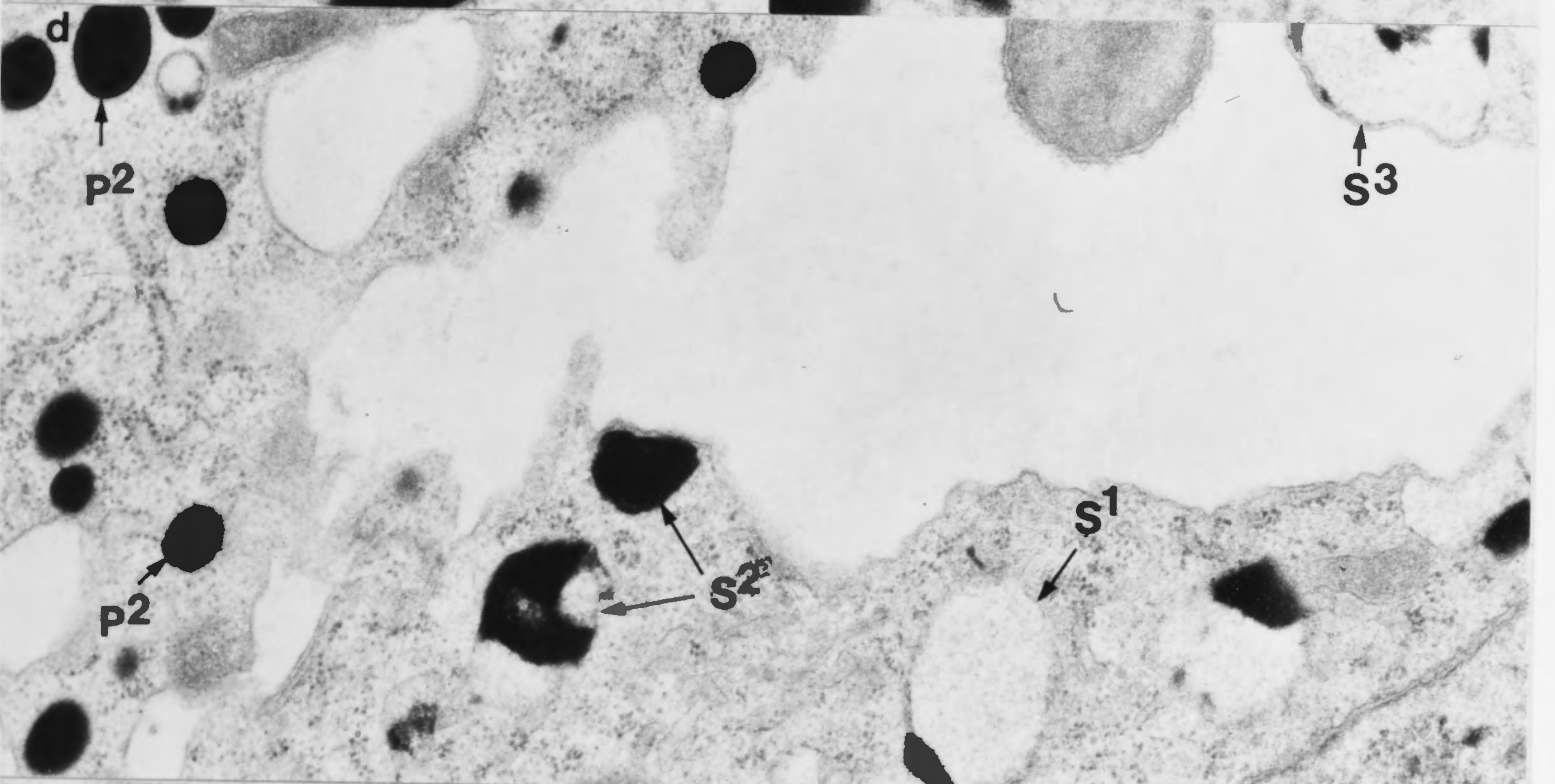
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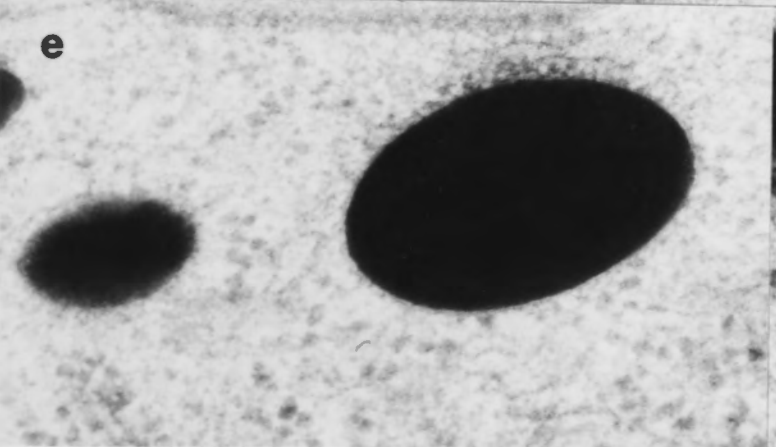
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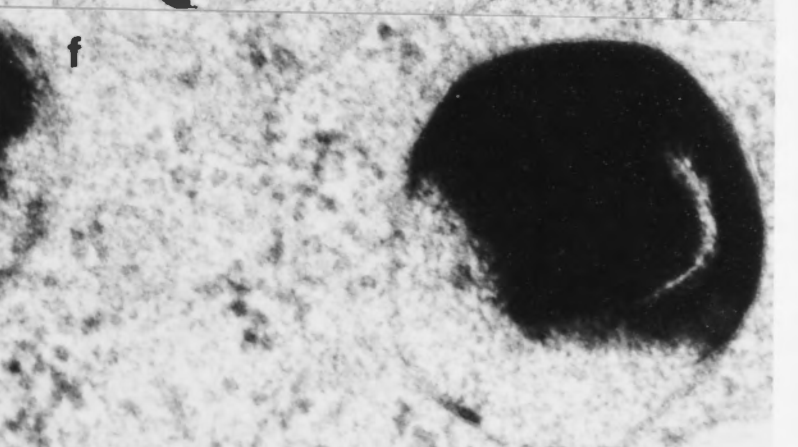
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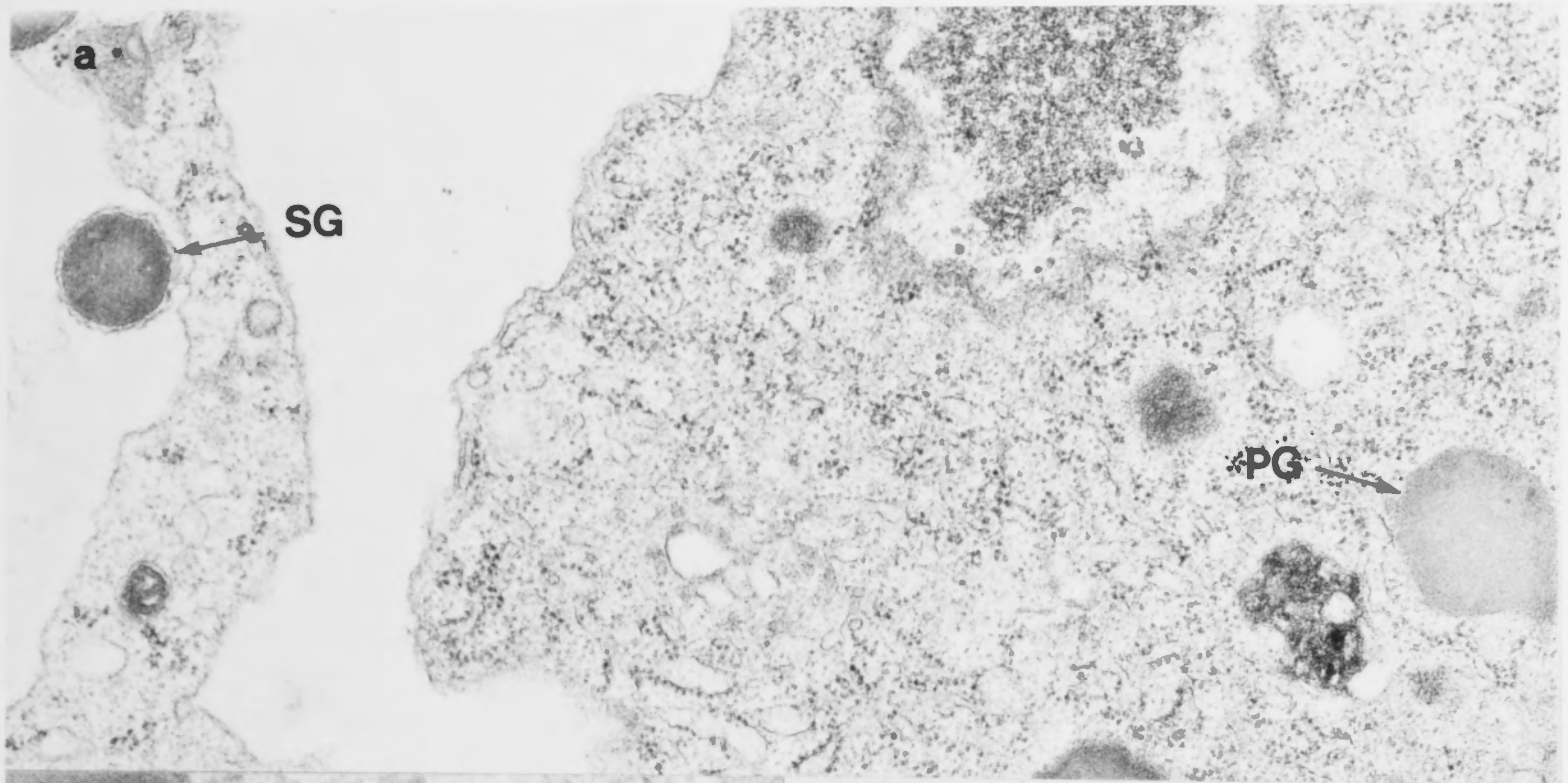
FIGURE 7.7

ELECTRON MICROGRAPHS OF THE MUTANTS WHITE AND GRAPE

(a) - (c) The mutant white, which lacks screening pigments. Supposed Type IV granules with a granular substructure are present in primary (PG in (a) and at higher magnification in (b)) and secondary (SG in (a) and at higher magnification in (c)) pigment cells, in low frequency. Mag. of (a) = 30,000x Mag. of (b) & (c) = 77,000x

(d) - (f) The mutant grape, illustrating the high concentration of membrane-bound vesicles filling primary (PV in (d) and at higher magnification in (e)) and secondary (SV in (d) and at higher magnification in (f)) pigment cells. Also shown are occasional Type I pigment granules seen in primary (P in (d) and at higher magnification in (e)) and secondary (higher magnification in (f)) pigment cells. Mag. of (d) = 30,000x Mag. of (e) & (f) = 77,000x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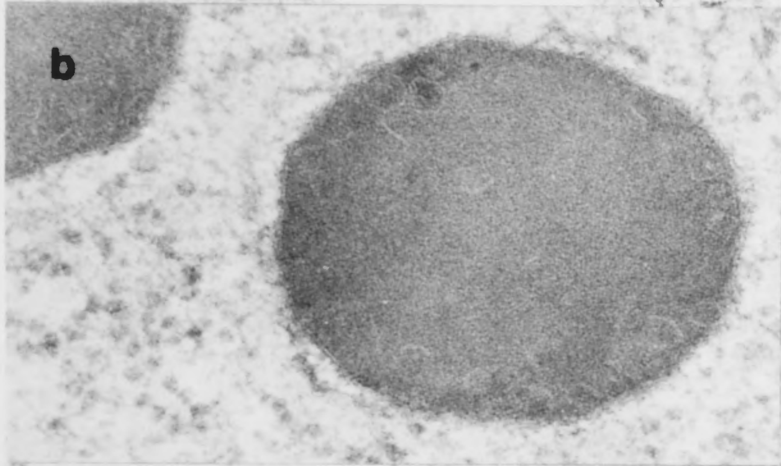
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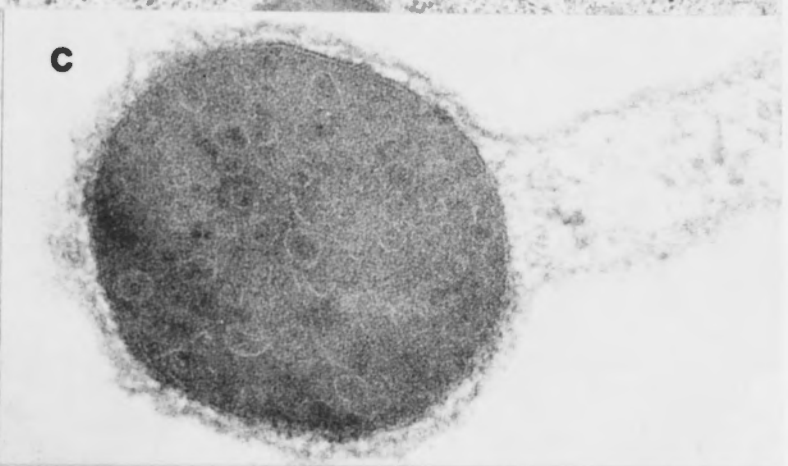
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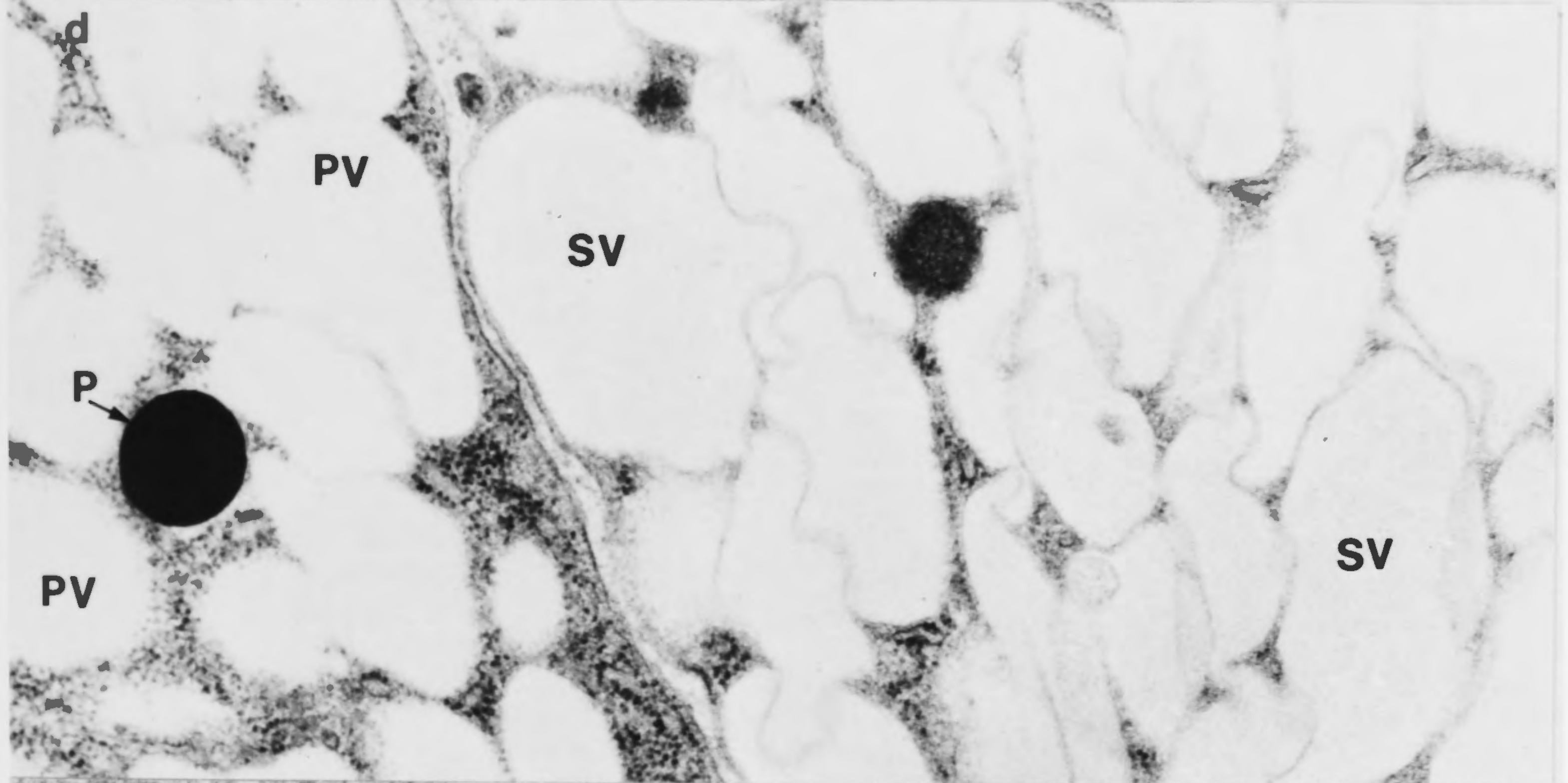
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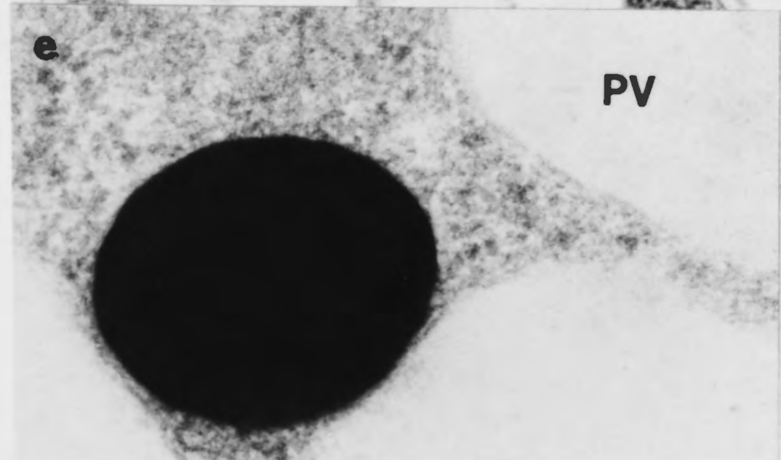
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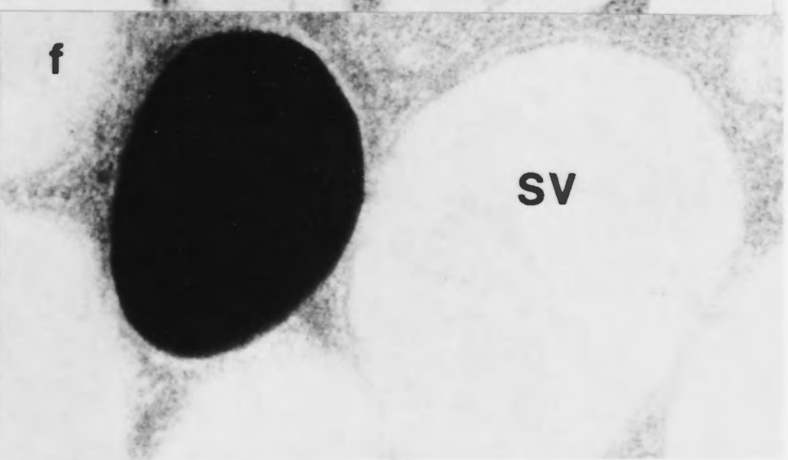
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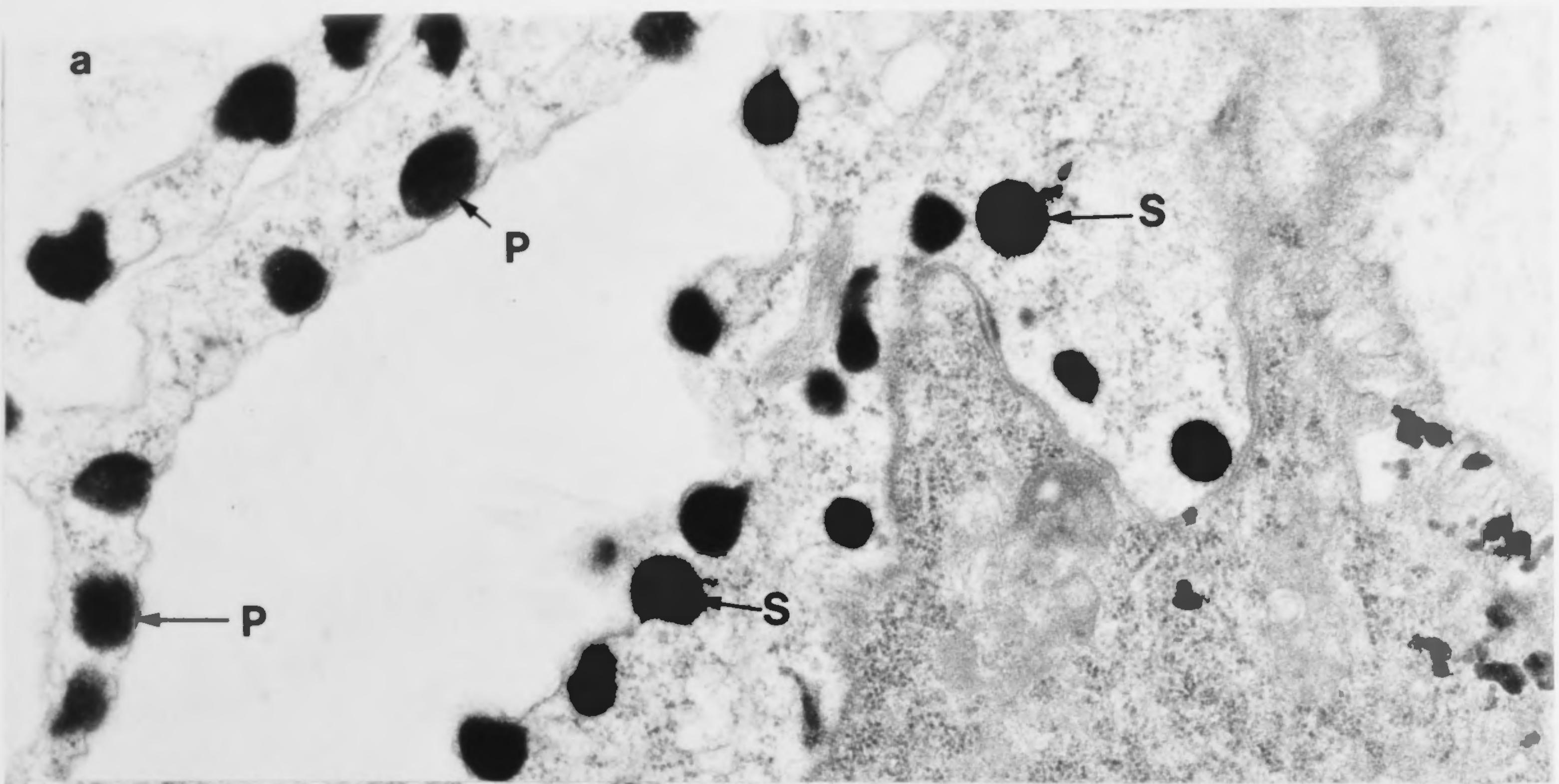
FIGURE 7.8

ELECTRON MICROGRAPHS OF MUTANTS FED A DIET SUPPLEMENTED WITH 3-HYDROXYKYNURENINE

(a) - (c) The mutant yellow (non-autonomous) illustrating irregularly shaped Type I granules present in primary (P in (a) and at higher magnification in (b)) and secondary (S in (a) and at higher magnification in (c)) pigment cells. These indicate a normalisation of pigment granule morphology (c.f. Figure 7.5a).
Mag. of (a) = 30,000x Mag. of (b) & (c) = 77,000x

(d) The mutant topaz^{1} (autonomous) showing that there has not been any significant normalisation of pigment granule morphology (c.f. Figure 7.5d). Mag. = 30,000x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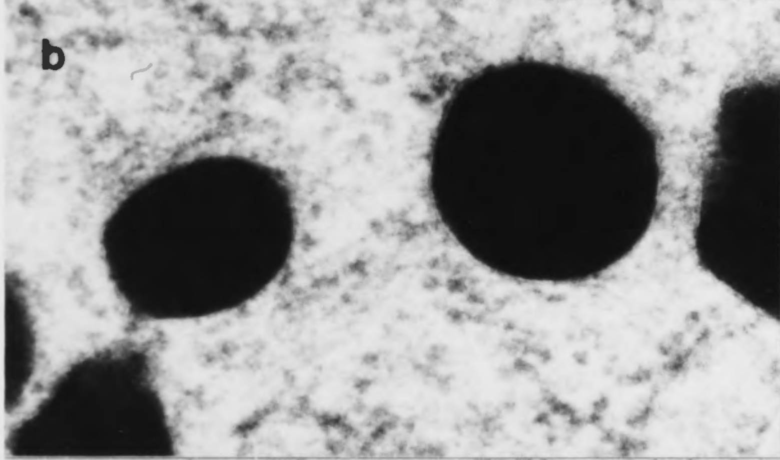
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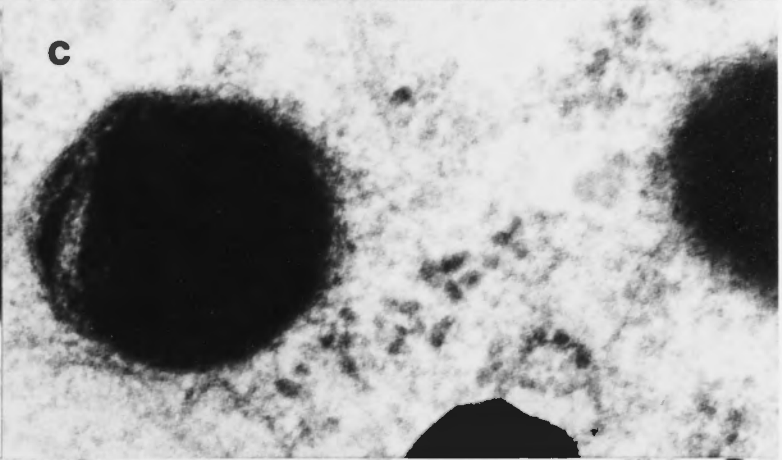
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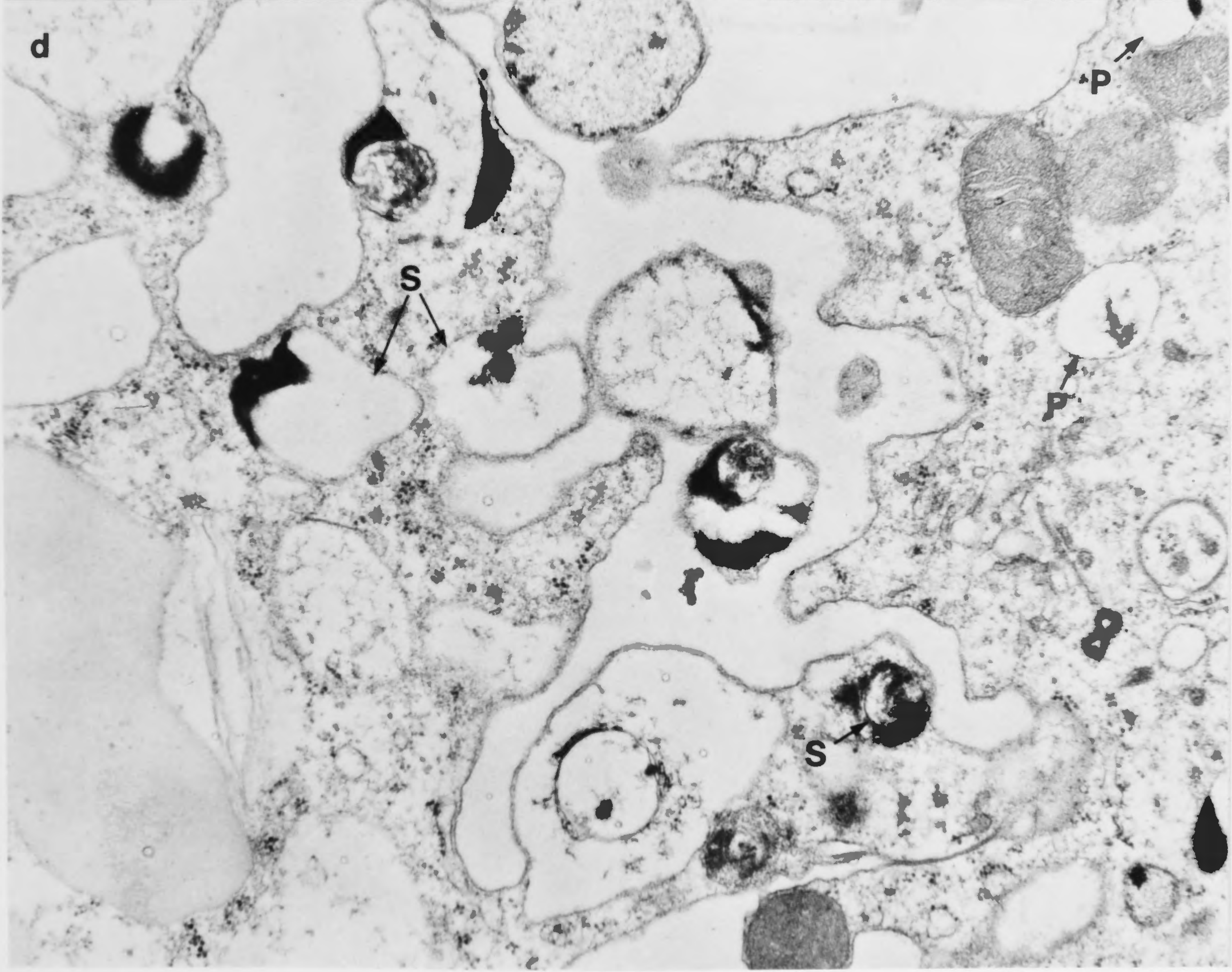
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CHAPTER 8
GENERAL DISCUSSION

8.1 *THE EYE PIGMENT MUTANTS OF* LUCILIA CUPRINA

It is now possible to draw some conclusions about the functions of some of the eye pigment loci in *L. cuprina* and to compare them with the loci of *D. melanogaster* which have been studied in detail. Of the six loci examined, four (yellowish, yellow, tangerine and topaz) are exclusively concerned with the production of ommochrome, one (grape) appears to be primarily concerned with the production of pteridines, and one (white) is concerned equally with both biosynthetic pathways.

The evidence suggesting that the yellowish locus contains the structural gene for tryptophan oxygenase, or is at least intimately concerned with its regulation, is summarised in Chapter 4. Yellowish was shown to resemble vermilion of *D. melanogaster*, the structural gene for tryptophan oxygenase in this species (see Tobler *et al.*, 1971; O'Brien & MacIntyre, 1978). To show that the yellowish locus does in fact contain the sequences coding for tryptophan oxygenase, several approaches may be taken. Firstly, the activity of tryptophan oxygenase should be directly proportional to the number of yw^+ genes in the genome. Heterozygous yw/yw^+ insects would be expected to have half the wild type activity and segmental aneuploid stocks, carrying three yw^+ genes, to have 1.5 times the wild type activity. As Finnerty (1976) points out, such a test is not conclusive, because of the possibility of

dosage compensation and other regulatory mechanisms to suppress gene expression, especially in the case of aneuploid stocks. Cases of loci, known not to be structural genes, with a similar dose response, have been documented (Finnerty, 1976). Secondly, attempts could be made to detect strains of *L. cuprina* with electrophoretic variants of tryptophan oxygenase. These must be shown to map at the yellowish locus. Thirdly, there may be the absence of material cross-reacting with antibody to purified tryptophan oxygenase in yellowish mutants. A combination of these approaches would provide reasonably conclusive evidence whether or not the structural gene for tryptophan oxygenase was located at the yellowish locus.

Equally clear evidence suggests that the yellow locus either contains the structural gene for kynurenine hydroxylase, or is intimately concerned with its regulation. This evidence in presented in Chapters 4 and 5. In all the factors examined, yellow mutants resemble cinnabar mutants of *D. melanogaster*. Cinnabar is probably the structural gene for kynurenine hydroxylase in that species (Sullivan *et al.*, 1973). Similar tests to those described for yellowish could be performed to determine the role in the production of kynurenine hydroxylase activity of the yellow locus.

The tangerine locus may be involved in the production of phenoxazinone synthase. The structural gene for phenoxazinone synthase in *D. melanogaster* may be cardinal

or karmoisin or both (Sections 1.32 and 4.1). Extensive studies of this enzyme have not been made in this species because of the difficulties experienced in assaying its activity. Similar problems were found with the *L. cuprina* enzyme and so some uncertainty is felt about ascribing a role to the tangerine locus in its production. Although tangerine pupae accumulate 3-hydroxykynurenine, as do those of cardinal of *D. melanogaster* (Howells *et al.*, 1977), several factors suggest that the role of tangerine in determining phenoxazinone synthase activity may be a more complex one than that of the structural gene. Firstly, tangerine larvae have 3-hydroxykynurenine levels slightly lower than wild type (as judged by the value in young pupae) suggesting a role for the tangerine locus in larval life. Since xanthommatin synthesis is not present during larval life, effects of the lack of phenoxazinone synthase activity would not be expected to be felt until midway through pupal life. Secondly, the accumulation of 3-hydroxykynurenine by tangerine pupae during pupal life is not as substantial as the accumulation seen in cardinal, which has three times the wild type level at emergence (compared with 1.5 times the wild type level at emergence in tangerine). This build up of excess 3-hydroxykynurenine in tangerine in no way makes up for the low level of xanthommatin and there is no compensatory build-up of tryptophan or kynurenine to make up the difference in tangerine insects, as seen for cardinal (Summers, 1974). These differences in the biochemical phenotype of tangerine and cardinal suggest that these mutants may not be homologous. The

tangerine phenotype cannot be explained in terms of an uptake/storage defect (Chapter 5). It is interesting that the primary pigment cells of tangerine eyes contain similar membrane-bound vesicles to those in the eyes of grape flies and this may indicate that tangerine is not simply a structural gene locus. This gene may be involved in the regulation of the final step in xanthommatin biosynthesis. This possibility makes it an interesting mutant for future studies of this step. Studies of phenoxazinone synthase may be easier in *L. cuprina* than in *D. melanogaster* because of the higher activity of the enzyme (about twice that of the *D. melanogaster* enzyme; Chapter 3).

The role of the topaz locus in ommochrome production remains unclear. The two features in which the topaz mutants were found to be abnormal are firstly their failure to accumulate 3-hydroxykynurenine into the larval malpighian tubules *in vivo* and secondly the low level of *in vivo* conversion of [^{3}H]-kynurenine to [^{3}H]-3-hydroxykynurenine in larvae and adult eyes (although not in adult bodies). It is not yet possible to say whether either defect is primarily responsible for the mutant phenotypes of the two topaz alleles. It seems unlikely that it is the low kynurenine hydroxylase activity *in vivo* which is solely responsible. Topaz^{1} was shown to be autonomous when supplied with 3-hydroxykynurenine in the diet throughout larval life, unlike yellow, which could make pigment after this treatment. This shows that the defect in topaz^{1} cannot be overcome merely by the provision of the product of

kynurenine hydroxylase activity. In addition, kynurenine levels in the topaz mutants are not raised, suggesting that there is no build-up of kynurenine due to the enzyme block, unlike the situation in yellow. Note also that the topaz mutants do accumulate substantial levels of 3-hydroxykynurenine during both larval and pupal development. If the primary defect causes the failure of the malpighian tubules to accumulate pigment precursors, these tubules must play a more active role in bringing about pigmentation than was previously attributed to them. What this role might be will be discussed in more detail in the next section of this chapter. It is also possible that none of the biochemical aspects of the topaz phenotypes found to be different from wild type is the primary result of the genetic defect. There is a marked difference in the xanthommatin level of $topaz^1$ and $topaz^2$ (3% and 25% of the wild type level respectively; Table 4.1) which suggests that in the critical aspect $topaz^2$ should have a phenotype midway between that of $topaz^1$ and wild type. In the features examined so far, $topaz^1$ and $topaz^2$ are virtually indistinguishable.

The white locus clearly affects both ommochrome and pteridine pigment production in a way similar to that of white of *D. melanogaster*. Both larval and adult accumulation of ommochrome precursors (kynurenine and 3-hydroxykynurenine) into specific tissues is affected, although these compounds can be synthesised by white insects since both are detectable in pupae. Similarly,

pterin and isoxanthopterin could be detected in white pupae, showing that at least part of the pteridine biosynthetic pathway functions in these mutant insects. Again, it is not possible to determine the significance of the low *in vivo* kynurenine hydroxylase activity in determining the white phenotype. The results might be interpreted as support for the suggestion of Ghosh & Forrest (1967) that kynurenine hydroxylase requires a pteridine cofactor, but the observation that pteridines are present in white pupae five days after pupariation (when kynurenine hydroxylase is almost certainly active; Chapter 3) does not support this theory. Like white of *D. melanogaster*, white of *L. cuprina* is unable to accumulate 3-hydroxykynurenine normally in the cells of either the malpighian tubules or the adult eyes. From the kinetics of uptake of [^{3}H]-3-hydroxykynurenine into adult eyes, it may be that in this mutant, storage of 3-hydroxykynurenine within the cells, rather than the uptake process, is affected. Since the topaz mutants are defective in 3-hydroxykynurenine accumulation in larval malpighian tubules but not in adult eyes, the processes may be somewhat different in the two tissues. This question could be resolved by performing, with larvae, short-term uptake experiments similar to those performed with adults. Further exploration of the white phenotype is also needed to reveal the nature of the defect which causes the failure in sepiapterin synthesis. The most likely possibility seems to be a similar uptake or storage defect which affects a pteridine precursor. The precise precursor which must be transported and stored in the cells

is not yet known. In *D. melanogaster* it is most likely to be a compound early in the pathway, before the action of GTP cyclohydrolase, since this enzyme appears to be located in the pigment cells in this species (Evans & Howells, 1978). Greater knowledge of the details of pteridine biosynthesis is needed before this problem can be clarified.

The other mutant strain examined in this thesis is the dark-eyed mutant grape. This is clearly deficient in the production of sepiapterin, although pterin and isoxanthopterin have been found in extracts of grape pupae and isoxanthopterin seems to operate at the wild type level (Chapter 6). The pteridine biosynthetic pathway in this strain may be blocked beyond the branch point from dihydroneopterin triphosphate (see Figure 1.3). Mutation at the grape locus also has the effect of reducing ommochrome production, perhaps through the effect on the uptake of ommochrome precursors into the eye (Chapter 5). Perhaps the most striking effect of the grape mutation is on the ultrastructure of the eye (Chapter 7). Whether the distorted morphology of the pigment cells is the primary result of the genetic lesion or whether it follows from the disruption to pigment synthesis remains to be shown. It is noted that the eyes of white, which has no pigment synthesis in adults, do not show this altered morphology, suggesting that it is not an inevitable result of deficiency in pigment synthesis. The primary pigment cells of tangerine, which has normal sepiapterin production, resembled those of grape. This ultrastructural aspect of pigment synthesis needs to be investigated further. The function of the grape

locus could be clarified by a number of approaches. A developmental electron microscopic study would indicate the time at which the abnormal morphology becomes apparent, perhaps parallelling the development of the pigment cells or after the deposition of pigment begins. Studies of the enzymes of pteridine biosynthesis in grape could indicate the level at which the pathway is blocked. Similarly, the uptake of pteridine precursors into the pigment cells of grape eyes should be considered, to examine the possibility that this is the primary defect. Until such studies have been performed, the function of the grape locus remains undefined, like those of most of the *D. melanogaster* eye colour mutants affected in pteridine pigment synthesis.

When comparing eye pigment synthesis in *L. cuprina* with that in *D. melanogaster* a number of differences have been found. The most obvious and fundamental is the absence of drosopterins in *L. cuprina* but there are a number of other less dramatic differences. Differences, for example, in the biochemical phenotype between the topaz mutants of *L. cuprina* and scarlet of *D. melanogaster* and also between tangerine of *L. cuprina* and cardinal of *D. melanogaster* have been discussed previously (Chapters 4 and 5 and Section 8.1). It also seems that the tissue localisation of some of the xanthommatin biosynthetic enzymes may be different in *L. cuprina* from that in *D. melanogaster*. Both tryptophan oxygenase and kynurenine hydroxylase have been found in the eyes of *D. melanogaster* (see Table 1.2),

while the results for *L. cuprina* (Chapters 3 and 5) suggest that these two enzymes have only low activity in the eyes. Consequently, for *D. melanogaster*, synthesis of xanthommatin starting from tryptophan is possible in the eyes (although uptake of kynurenine and 3-hydroxy-kynurenine is obviously important), but for *L. cuprina* synthesis of xanthommatin starting from tryptophan within the eyes may be restricted, placing more emphasis on the uptake of 3-hydroxykynurenine from the haemolymph. Thus in the pigment cells of *D. melanogaster* efficient uptake of tryptophan and kynurenine may be of critical importance in xanthommatin formation while in *L. cuprina* the uptake of 3-hydroxykynurenine may be much more significant.

8.2 *ROLE OF THE LARVAL MALPIGHIAN TUBULES IN THE PRODUCTION OF SCREENING PIGMENT - A PROPOSAL*

A phenotypic connection between larval malpighian tubules and adult eyes in Diptera is well documented (see Section 1.34). On the basis of evidence provided by the topaz mutants, in which xanthommatin synthesis is deficient and accumulation of 3-hydroxykynurenine in the larval malpighian tubules (but not the adult eyes) fails, it is now proposed that the role of the malpighian tubules in pigment formation is an active one.

This proposed role of the malpighian tubules in actively promoting xanthommatin formation is apparently mediated by 3-hydroxykynurenine and probably involves an effect on phenoxazinone synthase. 3-hydroxykynurenine has previously been suggested as an activator of phenoxazinone synthase in moths and flies (Muth, 1969; Linzen, 1974; Yamamoto *et al.*, 1976). Evidence supporting the importance of 3-hydroxykynurenine in mediating a role for the malpighian tubules can be found by examining the phenotypes of several of the mutant strains. Two mutants with low larval 3-hydroxykynurenine, yellowish and yellow, also have low adult phenoxazinone synthase activity and this was also found for their *D. melanogaster* homologues, vermilion and cinnabar (Yamamoto *et al.*, 1976). Kynurenine is apparently not directly involved in bringing about this correlation since yellow has high levels of this metabolite.

The properties of the topaz and white mutants of *L. cuprina* suggest that the correlation of low adult phenoxazinone synthase activity is not with larval 3-hydroxykynurenine level *per se*, but probably with the level of 3-hydroxykynurenine within the malpighian tubules. 3-hydroxykynurenine could be accumulated within the tubules in two ways: having been synthesised in other tissues, it could be taken up from the haemolymph, or it could be synthesised from kynurenine within the tubules, in which case kynurenine or tryptophan would need to be taken up from the haemolymph. In the white and topaz mutants, which have low activity of phenoxazinone synthase, the

first mechanism is apparently not operative, since dietary [^{3}H]-3-hydroxykynurenine could not be accumulated in the tubules (Chapter 5). It seems likely that *in situ* production of 3-hydroxykynurenine would also be low in these strains, since larvae of these mutants had a very reduced level of conversion of [^{3}H]-kynurenine to [^{3}H]-3-hydroxykynurenine *in vivo* (Chapter 5). A likely interpretation of this result is that the defect in 3-hydroxykynurenine uptake extends to kynurenine uptake as well, so that any activity of the kynurenine hydroxylase located in the tubules would be inaccessible to the [^{3}H]-kynurenine. Thus it seems probable that the level of 3-hydroxykynurenine within the malpighian tubules of the *L. cuprina* mutants white, topaz^1 and topaz^2 is negligible, which is supported by the observation that the colour of the tubules is abnormal in these mutants (Section 2.3), and that this low level of tubule 3-hydroxykynurenine is correlated with reduced adult phenoxazinone synthase activity.

Grape mutants also have a reduced level of 3-hydroxykynurenine in larvae (Chapter 4), but there is no apparent barrier to its accumulation in the tubules, since the accumulation of dietary [^{3}H]-3-hydroxykynurenine in the tubules of these insects was similar to wild type (Chapter 5). In agreement with this normal level of accumulation within the tubules is the observation that phenoxazinone synthase activity in these insects was also similar to wild type (Chapter 4). The only mutant in which the level of phenoxazinone synthase activity is not

correlated with tubule accumulation of 3-hydroxykynurenine is tangerine. Tangerine insects have substantial larval levels of 3-hydroxykynurenine and can accumulate it in the tubules, and yet have reduced levels of phenoxazinone synthase activity. As discussed in Section 8.1, this deficiency in phenoxazinone synthase can probably be attributed to a lesion in the structural gene itself or in a regulator gene for this enzyme.

Thus there is strong circumstantial evidence that 3-hydroxykynurenine accumulated in the larval malpighian tubules has some effect on adult phenoxazinone synthase activity. There are a number of ways in which this effect might be mediated. 3-hydroxykynurenine might be modified to an active effector form within the tubules. Such modification might be chemical alteration of the 3-hydroxykynurenine molecule itself or binding of 3-hydroxykynurenine to a carrier protein. This activated form of 3-hydroxykynurenine might then be released from the tubules (during the reorganisation which occurs with metamorphosis) and carried in the haemolymph to the developing eye. There it would have an effect on phenoxazinone synthase activity, perhaps by turning on the phenoxazinone synthase gene or by activating a presynthesised, inactive precursor of phenoxazinone synthase. Alternatively, 3-hydroxykynurenine might have its effect on gene expression in the malpighian tubule cells themselves, causing them to produce an effector which would later be released from the tubule cells and act as described above for the modified 3-hydroxykynurenine.

In this case it would seem likely that 3-hydroxykynurenine would have to enter the nucleus in the tubule cells to alter gene expression.

This proposal implies that in *L. cuprina* (and probably other dipteran species) the regulatory processes involving larval 3-hydroxykynurenine would occur before pigmentation begins and would prevent the formation of the pigment synthesising machinery. A low level of tubule 3-hydroxykynurenine at pupariation would act as an indicator that a larva had experienced a tryptophan deficiency. At a low larval level of tryptophan, little kynurenine would be formed (due to the allosteric nature of tryptophan oxygenase activity) and hence little 3-hydroxykynurenine would be stored. In this case, unnecessary synthesis of phenoxazinone synthase and perhaps pigment granules, would be prevented. If 3-hydroxykynurenine in larval tubules is high, due to an excess of free tryptophan from the breakdown of dietary proteins, the onset of xanthommatin biosynthesis would be assured because of this regulation.

Why might a regulatory strategy of this type have evolved? The answer might lie more in protecting the insect from a dietary excess of tryptophan than from a dietary deficiency. Because tryptophan is an essential amino acid in Diptera (Wigglesworth, 1972), adequate levels must be stored during larval life for the later synthesis of adult proteins. To ensure that adult development will not be

jeopardised, dipterans may have evolved mechanisms to store excess tryptophan in larval proteins. The 3-hydroxykynurenine regulatory system would provide an early warning that this excessive storage of tryptophan has occurred and that some tryptophan will have to be disposed of during metamorphosis.

This idea is compatible with the theory presented by several authors (Bückmann *et al.*, 1966; Linzen, 1967, 1974; Leibenguth, 1970; Fuseau-Braesch, 1972; Sullivan & Kitos, 1976) that ommochromes represent inert, oxidised, insoluble end-products of tryptophan (Linzen, 1967); that is, that ommochromes are detoxification products of tryptophan. The indole structure of tryptophan is highly reactive and in high concentrations tryptophan may well be detrimental to insect development. Excess tryptophan arising from protein breakdown during metamorphosis cannot be excreted. During pupal life, therefore, the insect risks a detrimental accumulation of free tryptophan. To prevent this happening, the ommochrome deposition system is activated in advance. It is proposed, therefore, that 3-hydroxykynurenine in late larvae/early pupae serves as a signal, via the malpighian tubules, that the tryptophan level will rise during pupal life and so ommochrome production will be necessary. In the evolutionary context, ommochrome production perhaps arose first as a detoxification mechanism and its adaptation as a protective pigment (that is, its accumulation in specific tissues such as the eyes) may have come later.

As well as this proposed feed-forward control provided by the levels of 3-hydroxykynurenine on phenoxazinone synthase activity, there appears to be a form of feed-back control on the breakdown of tryptophan-containing proteins, when the levels of tryptophan metabolites (kynurenine, 3-hydroxykynurenine and tryptophan itself) rise too high during metamorphosis, as in some mutant strains. The efficiency of this mechanism is demonstrated by the calculations in Table 8.1 which show that the accumulation of metabolites of the tryptophan → ommochrome pathway in wild type during pupal life is much higher than the amounts of these metabolites which accumulate in yellowish, yellow and tangerine. It is possible that this control could work at the level of protein synthesis in the larva, so that the tryptophan content of the proteins synthesised in these mutant strains is lower than in wild type. However, in *D. melanogaster*, the larval proteins of the vermilion mutant were found to have the same tryptophan content as those of wild type (Green, 1949) and this mutant also accumulated less of the pathway metabolites than wild type (Ryall & Howells, 1974). This suggests that a mechanism which prevents the breakdown of proteins rich in tryptophan during metamorphosis is the more likely control. It is interesting, when comparing the mutants of *D. melanogaster* and *L. cuprina*, that this regulated release of tryptophan was not found for cardinal of *D. melanogaster* (from data in Summers, 1974), whereas, as Table 8.1 shows, it was found for tangerine of *L. cuprina*. This is another reason for

TABLE 8.1

ACCUMULATION OF METABOLITES OF THE TRYPTOPHAN → OMMOCHROME PATHWAY DURING PUPAL LIFE IN *LUCILIA CUPRINA* STRAINS

METABOLITE[a]	STRAIN			
	Wild Type	Yellowish	Yellow	Tangerine
Tryptophan	-4.3	26.8	-5.8	-7.4
Kynurenine	-2.4	0.4	21.1	1.5
3-Hydroxykynurenine	-7.0	2.3	3.1	36.3
Xanthommatin[b]	93.0	3.0	4.2	11.2
TOTAL	79.3	32.8	22.6	41.6

a Expressed as nmoles/insect; value at emergence minus value at pupariation (from results presented in Chapter 4).

b Value for xanthommatin multiplied by two, since 2 molecules tryptophan → 1 molecule xanthommatin.

regarding tangerine as perhaps not strictly homologous with cardinal (as discussed in Section 8.1).

The possible functions of the white and topaz proteins in relation to this hypothetical scheme of feed-forward regulation by 3-hydroxykynurenine would be in taking up 3-hydroxykynurenine into the larval malpighian tubules and maintaining the level within this tissue. Thus the white protein might provide an uptake mechanism, or a storage matrix to which 3-hydroxykynurenine is bound after its entry into the cell. This is compatible with the data for accumulation of 3-hydroxykynurenine in the eyes and tubules of the white mutant, and is discussed in more detail in Chapter 5. The topaz protein might be involved in the uptake or storage processes in the tubules but not the eyes. Alternatively, the topaz protein might be an enzyme, carrier protein or receptor molecule which produces the active form of 3-hydroxykynurenine which is held within the tubule cells.

Data relevant to a possible role for the malpighian tubules in eye pteridine production was not obtained in the course of this work. Such a role could conceivably exist, acting to "detect" excessive accumulation of guanine nucleotides in RNA during larval life. Guanine has been shown to be detrimental to insect development (el Kouni & Nash, 1977) and its breakdown to uric acid requires a deamination step (see Figure 1.4). Since ammonia is not produced during metamorphosis (see Section 1.2), insects may avoid this step by converting excess guanine to pteridines during

metamorphosis, in the same way that tryptophan is converted to ommochromes. Thus guanine, guanosine, other pteridine precursors or perhaps isoxanthopterin, might act in the same way as 3-hydroxykynurenine in ensuring that the pteridine synthesising machinery is fully active at the appropriate time in pupal life. The role of the white gene in this process could be very similar to its role in ommochrome production; as an uptake or storage protein for the relevant effector compound in both the tubules and the eyes. There might be proteins with functions similar to that of the topaz protein, which act primarily in uptake or storage of the relevant compound in the tubules only.

Since both pteridine and ommochrome production in the eyes might be controlled by similar feed-forward mechanisms mediated by the malpighian tubules, it is possible to see how many of the loci involved in eye pigmentation might have a role in both pathways, through these regulatory mechanisms. The same or similar proteins might be responsible for the release of the active form of the regulator substances from the tubules or for the response of the eyes to this form. Many loci could be necessary, containing regulatory and structural genes for the proteins involved.

8.3 *FURTHER STUDIES WITH* LUCILIA CUPRINA

Further experimental work arising directly out of the studies reported in this thesis has been outlined in each chapter and in Section 8.1 and requires no further discussion. This work includes the definitive experiments to establish whether or not structural genes involved in pigmentation are located at the yellowish and yellow loci, a detailed examination of the pteridine biosynthetic enzymes in *L. cuprina* and further ultrastructural studies. However, the proposal linking the functions of the larval malpighian tubules with eye pigmentation, outlined in the previous section, opens a broader experimental area in which *L. cuprina* provides a useful experimental system for detailed study. The approaches which might be used will be discussed in this final section.

Further information on the potential role of 3-hydroxykynurenine in the activation of phenoxazinone synthase could be gained by a study of the non-autonomous mutants yellowish and yellow. If 3-hydroxykynurenine levels in the tubules early in pupal life are critical in determining ommochrome production, there may be a stage during development in the puparium after which provision of 3-hydroxykynurenine can no longer cause xanthommatin to be produced. One way to test this would be to inject enough 3-hydroxykynurenine to cause pigmentation into larvae, pupae of various ages and adults, to see whether the capacity for pigment production is lost as development proceeds.

Especially interesting would be the stage of development between the time of rearrangement of the malpighian tubules and the time of onset of xanthommatin production.

To clarify further the role of 3-hydroxykynurenine, it would be interesting to use autoradiographic techniques to follow the fate of injected 3-hydroxykynurenine in the insect. This could indicate, for example, whether 3-hydroxykynurenine enters the nucleus in either tubule cells or eyes; what other tissues it enters; and its fate after entry into the tubules. Radioactively-labelled 3-hydroxykynurenine could also be used to attempt to identify 3-hydroxykynurenine-binding granules within the tubules or the eyes. Isolation of these tissues from *L. cuprina* in sufficient quantities for biochemical analysis is certainly easier than their isolation from *D. melanogaster*.

Ultimately, if the hypothesis proposed in Section 8.2 is correct, an effect of 3-hydroxykynurenine at the level of gene expression must be observed in either the malpighian tubules or the adult eyes. Given the recent advances in techniques for mRNA isolation, for the synthesis of copy DNA to mRNA using reverse transcriptase, for restriction endonuclease analysis of DNA, for DNA sequencing and for cloning specific fragments of DNA in bacteria, it is feasible to search for evidence of altered gene expression. It would be interesting, for example, to compare the mRNAs produced in the tubule cells of yellowish or yellow larvae raised with and without a dietary supplement of

3-hydroxykynurenine.

It is necessary to examine the importance and nature of uptake/storage processes in pteridine pigment production, with a view to establishing whether any pteridine precursor or product might have a role similar to that proposed for 3-hydroxykynurenine in regulating the pteridine biosynthetic pathway. A full understanding of the white mutation and the ways in which the two pathways interact can only be achieved when this information is available for pteridine biosynthesis.

This discussion has revealed again the usefulness of eye pigmentation, particularly ommochrome formation, in the study of gene expression during development (as discussed initially in Chapter 1). As outlined above, the potential role of 3-hydroxykynurenine in influencing gene expression opens up a whole range of experiments and techniques which might be used in the future. The biosynthesis of the pteridine pigments and the nature of the interactions between pteridine and ommochrome production are still largely unexplored areas, giving additional scope for useful experiments.

The basic information now available for *L. cuprina*, as a result of the studies described in this thesis, will allow these studies to be performed with this species, which is often more suitable for the kind of work proposed than *D. melanogaster*. Successful completion of such

studies should go far towards providing an understanding of gene regulation during pigment synthesis and therefore a conceptual framework to apply to more complex systems of differentiation and development in higher organisms.

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