ETUDES DU DEVELOPPEMENT DE LA DROSOPHILE: VARIATIONS ON A THEME

by

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ABSTRACT

As a holometabolous organism which has been highly exploited genetically, the fruitfly, Drosophila, is an excellent model for the study of development in higher organisms. Conditional mutations that are sensitive to temperature differences were used to investigate problems of gene action in different tissues, regulation of specific tissue determination and differentiation, and the genetic regulation of development and functional integration of the nervous system.

The first problem, a classical question in developmental biology, attempts to determine whether control of the activity of a structural gene is directly imparted by the tissue in which the gene product is active or whether, in fact, a freely flowing evocator of gene action exists.

A temperature-sensitive ras allele has an effective lethal phase at 29°C about 12 hours after pupation and a temperature-sensitive period (TSP) for lethality beginning midway through the third larval instar and ending around pupation. The mutation also alters the quantity of pteridines present in the eyes, testes and malpighian tubules at 29°C. The TSP for pigment production in the malpighian tubules occurs in the egg and first instar larvae, and in eyes and testes after pupation. The demonstrated autonomy of the mutant in the eyes implies the tissue-specific functioning of the gene. It is suggested that the different TSPs for a single mutation indicate tissue-specific activation of a gene at different times during development, although the possibility of activation of preformed polypeptides has not been eliminated.

There exists in Drosophila a class of mutations called "homeotic" which cause changes in determination of the imaginal discs. The second problem investigated concerns the possibility of isolating a temperature-sensive homeotic mutant for the purpose of studying genes which regulate specific pathways of differentiation. The homeotic mutant, ss^{a40a}, was found to have a temperature-sensitive transformation of the arista segment of the antennal complex to a tarsus of the leg. In a selected stock, penetrance was complete so that at 29°C, normal aristae were produced, whereas at 17°C, complete tarsi developed in all flies. "Shift" studies revealed a temperature-sensitive period in the third larval instar. The temperature-initiated action of ss^{a40a} does not appear to act on a ts receptor site within the disc cells. In combination with another homeotic mutant, Antennapedia, the entire antennal complex is transformed to a complete leg at 22°C.

Mutants affecting the nervous system were sought for the purpose of investigating the genetic regulation of the development and function of the nervous system. A temperature-sensitive mutation, $para^{ts}$ of *Drosophila melanogaster* causes an immediate but reversible paralysis only of adults when shifted from 22°C to 29°C. The mutation is a sex-linked recessive mapping 2.8 units to the left of *f*. Wild-type flies observed for two hour periods exhibited normal mobility at all temperatures between 22° and 35°C. From 22° to 25°C, $para^{ts}$ flies were wild-type in walking, climbing and flying ability. At one degree intervals above 25°C, $para^{ts}$ flies became increasingly debilitated and at 29°C, complete paralysis occurred. After prolonged maintenance at 29°C, recovery of some activity could occur at that

temperature. Extensive studies of behavior of mosaics at 29°C revealed a requirement of the + allele in the head for mobility and a thoracic component for proper leg movement. Normal electroretinograms were obtained at both 22° and 30°C. The results suggest a temperature-sensitive defect in the central nervous system.

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This becomes the most difficult part of the thesis. How does one thank Dave Suzuki? As my major professor, he has been more than an excellent scientist and teacher. He has been patient, considerate, enthusiastic, jovial, and more than helpful in every way. In short, he has been and is a friend. I can say no more.*

The work presented in Chapters 5 and 6 was done in collaboration with Rodney Williamson. I wish to thank him for this and also for his helpful discussions on the rest of the work presented in this thesis.

To the rest of the members of the lab, past and present, too numerous to mention, for their help and discussions, but more important for their camaraderie and for providing the unique atmosphere in which this work was done, my special thanks. It has been fun.

Chapters 3, 4 and 5 of this thesis have been published in the Proceedings of the National Academy of Sciences, U.S.A. Chapter 6 is in press in Developmental Biology.

* except maybe to add that he's a good quarterback.

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REVIEW

In the past 20 years, bacteria and bacteriophages have been intensively analyzed by molecular biologists. Their spectacular success in elucidating the molecular nature of gene structure and function has overshadowed phenomena in multicellular organisms. However, with an understanding of the basics of replication, coding, transcription and translation, interest has been renewed in problems inherent to eukaryotic multicellular organisms.

As one of the earliest and most extensively exploited organisms genetically, the fruit fly, *Drosophila melanogaster*, is unique for the study of development. As a holometabolite, it experiences a complete metamorphosis from a larval crawling form to an adult which walks, flies and sees. A brief review of the life cycle is shown in Figure 1. The anatomical structures of the adult and larvae are completely different, and the differences between the two stages with respect to obtaining food, locomotion, and behavior in response to external stimuli are obvious. Nevertheless, the larval stage anticipates the requirements for formation of the adult organism. During early embryonic development, packets of cells (imaginal cells or discs), which will give rise to most of the external structure of the fly during pupation, are set aside.

The discs are formed from ectodermal cells during blastoderm formation (about 3 hours postfertilization). Cell proliferation in the discs continues until the late third instar when the final size and shape are attained (Figure 2). Each disc consists of a large

FIGURE 1

General life cycle of *Drosophila melanogaster*.

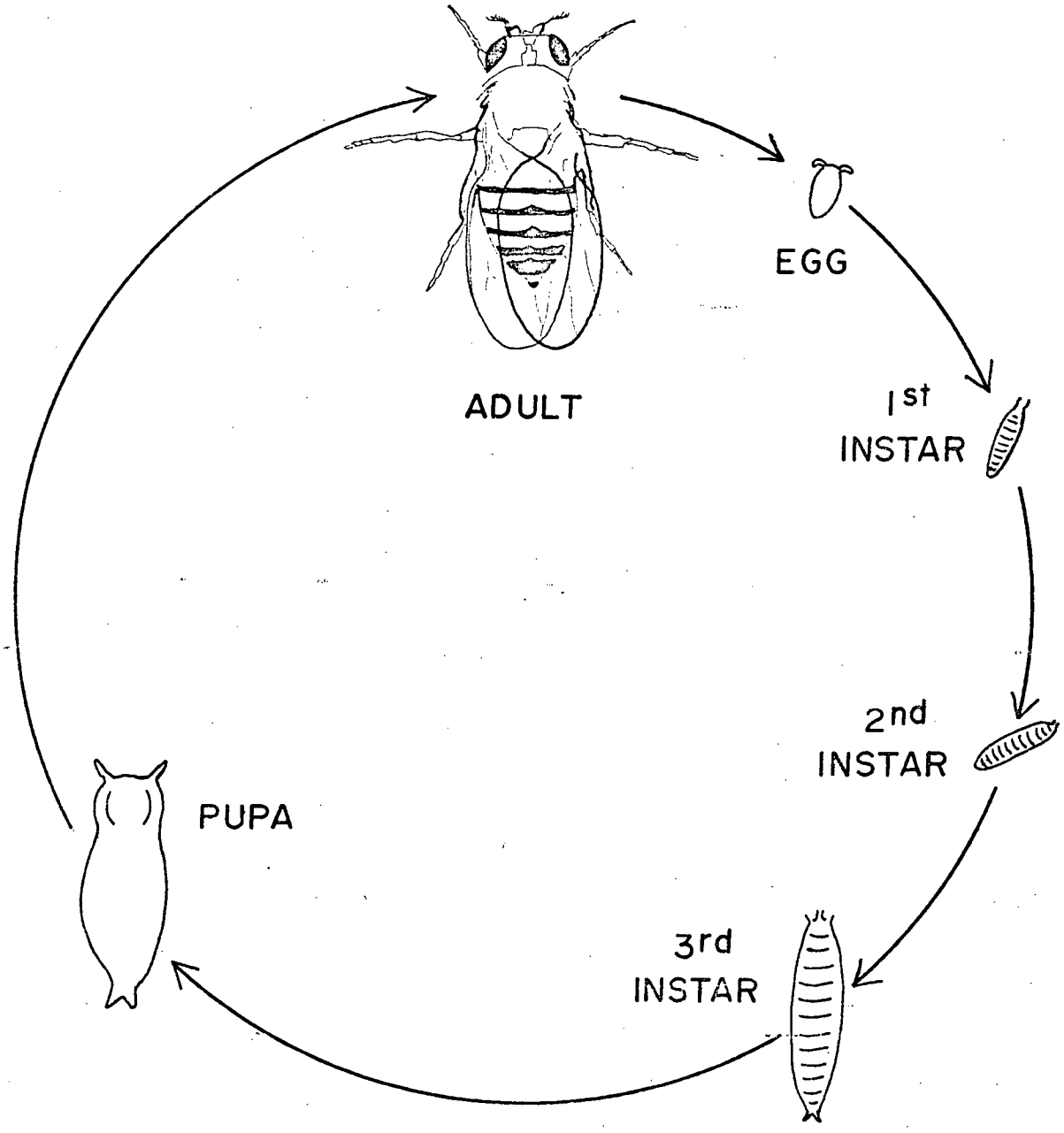
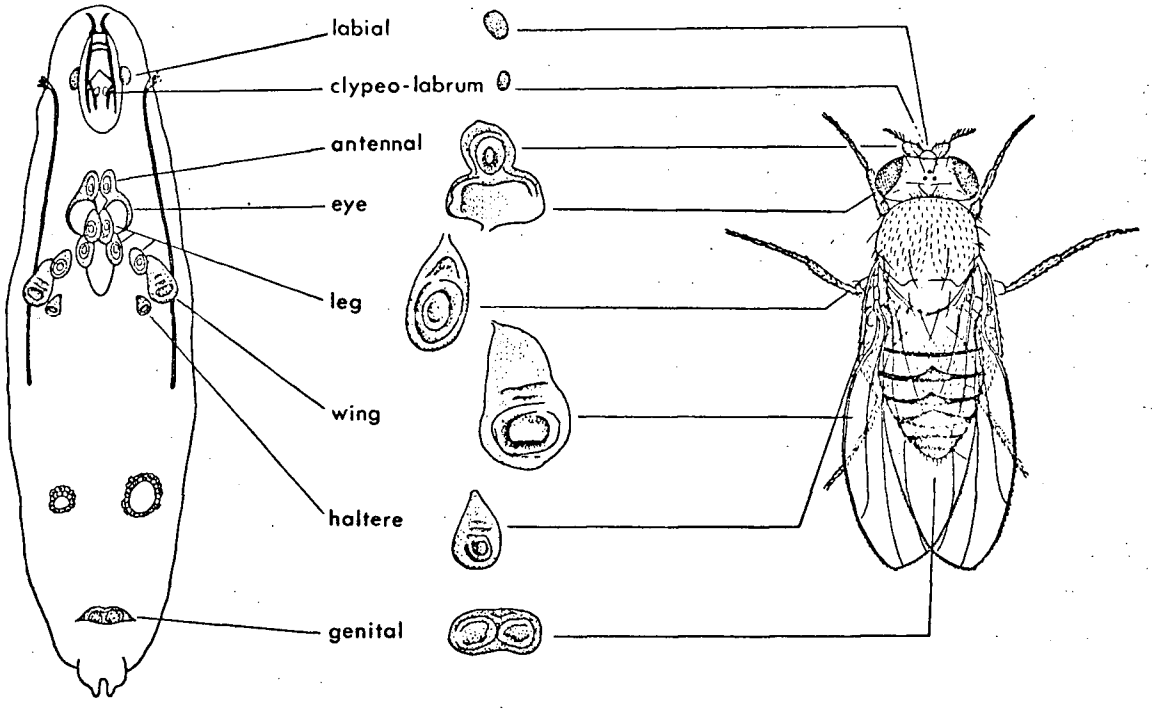

ADULT
EGG
1st
INSTAR
2nd
INSTAR
3rd
INSTAR
PUPA

FIGURE 2

Imaginal organ rudiments: their position in mature third instar larva and the corresponding adult structures which they form. Adopted from Fristrom *et al*. (1969).


labial
clypeo-labrum
antennal
eye
leg
wing
haltere
genital

number of small, histologically similar cells. Three pairs of discs give rise to the adult head: the labial discs which give rise to the proboscis (Wildermuth and Hadorn, 1965), the clypeo-labrum (Gehring and Seippel, 1967) and the eye antennal discs. The antennal portion gives rise to the antennae, prefrons, rostrum and palpi (Gehring, 1966); the eye disc generates the rest of the head capsule, dorsal and posterior to the frontal suture including the eyes and vibrisse (Ouweneel, 1970a) (see Figure 3 for the anatomy of the adult fly).

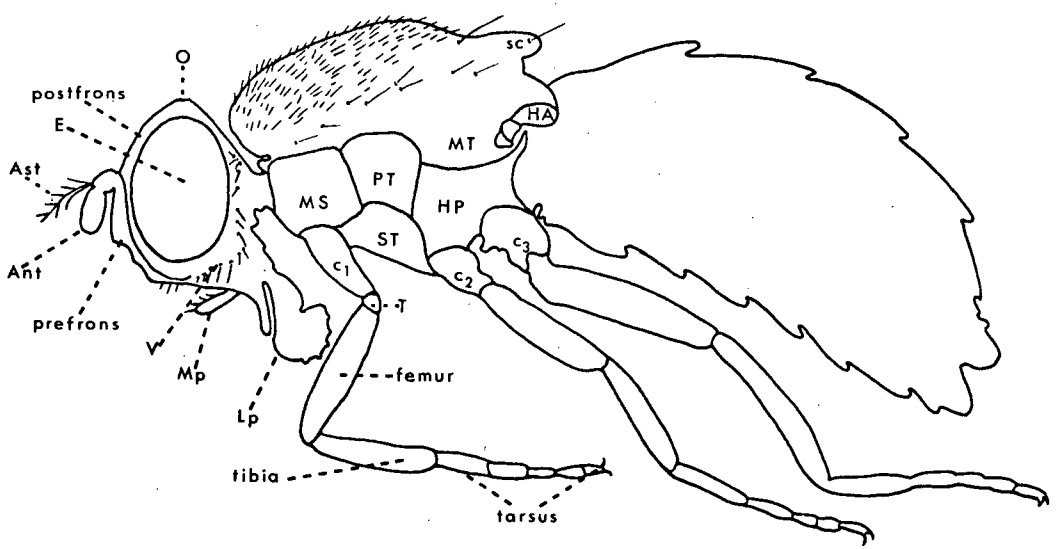
The thorax arises from six pairs of discs. Three pairs of discs produce the six legs, the ventral thorax and the sternopleura (Stern et al., 1963). The humeral discs become the humeral regions of the prothorax (Lamprecht and Remensberger, 1966). Wing (Hadorn and Buck, 1962) and haltere discs (Loosli, 1959) produce the wings and halteres respectively, as well as the dorsal parts of the thorax.

The unpaired male or female genital disc forms both the genital apparatus (excluding the gonad), and the last abdomenal segment (Hadorn et al., 1949). The remaining adult abdomenal epiderm arises through proliferation of paired dorsal and ventral histoblast cells located within each abdomenal segment (Bodenstein, 1950).

The imaginal discs can be readily identified by their position, size and shape. The final differentiation of the discs into adult structures is triggered during pupation by the release of the hormone, ecdysone (Mandaron, 1970). Discs provide a magnified illustration of the phenomena of a "determined" stage which commits undifferentiated cells to a specific fate and "differentiation" which is the actual realization of that fate. The discs can be readily manipulated in a number of ways. Thus, whole or fragmented discs may be

FIGURE 3

General anatomy of adult fly: lateral view.
Ant - antenna; Ast - arista; C_{1-3} - $coxa_{1-3}$; E - eye; HA - haltere; HP - hypopleura; Lp - labial palpus; MP - maxillary palpus; MS - mesopleura; MT - metanotum; O - ocellus; PT - pteropleura; SC - scutellum; ST - sternopleura; T - trochanter; V - vibrissae.


O
postfrons
E
Ast
Ant
prefrons
V
Mp
Lp
MS
PT
ST
c1
T
femur
tibia
tarsus
c2
c3
HP
MT
HA
sc

implanted into adult hosts where proliferation without differentiation occurs or into larval hosts where both processes ensue. Recently, methods for culturing of discs *in vitro* have been established. In addition, it is possible to isolate large amounts of disc tissue at a level that permits quantitative biochemical studies (Fristrom and Mitchell, 1965).

In most organisms, the processes of determination and differentiation follow one another in rapid succession, whereas in holometabolous organisms, these events may be widely separated temporally. Hadorn (1965) has defined determination as the "process which initiates a specific pathway of development by singling it out from among the various possibilities for which a cellular system is competent". It is, therefore, the regulative, rather than the structural aspect of differentiation.

A. Determination of Imaginal Discs

The processes of determination and transdetermination have been reviewed extensively (Wildermuth, 1970; Gehring, 1968; Ouweneel, 1970b). Hence, only a succinct review will be presented in the next few pages.

Among the basic problems of development is the question of whether determination occurs under the influence of an external stimulus provided by separate cells or group of cells, or whether it occurs within a cell without the influence of inducing factors. It would appear that the latter is the more likely possibility since embryonic or young 1st instar larval imaginal discs, cultured either *in vitro* (Gottschewski, 1960) or in an adult abdomen (Garcia-Bellido,

1965; Hadorn et al., 1968; Schubiger et al., 1969), are determined in the same fashion as in situ. That is to say, once the cell or small group of cells are recognizable as presumptive disc cells, they undergo further determination autonomously.

Several lines of evidence indicate that the actual time of determination is early in embryogenesis. Removal of imaginal cells of young embryos by surgical extirpation or irradiation leads to the absence of specific adult structures (Geigy, 1931; Howland and Child, 1935). In addition, various chemical treatments administered during embryogenesis can induce phenocopies of known disc mutants. Elegant studies have illustrated that 3 hour old embryos have been subjected to the determinative process. In these studies, cells from the anterior half of a three hour old embryo genetically marked with yellow (a mutant causing yellow body color, see Lindsley and Grell, 1968) were mixed with cells from whole embryos marked with the dark body color, ebony. These mixtures were then implanted into larval hosts, and it was shown that cells marked with yellow gave rise only to structures found in the anterior half of the fly, whereas ebony cells gave rise to both anterior and posterior structure (Chan and Gehring, 1971). It would appear, then, that determination had occurred by 3 hours postfertilization.

Somatic recombination can be used to obtain spots of homozygous mutant tissue in an otherwise heterozygous (wild-type) background (see Stern, 1968, for a review). Cells exhibiting a mutant phenotype in one area presumably result from a single somatic crossover event which was subsequently amplified mitotically. Bryant and Schneiderman (1969) found that the induction of somatic crossing

over in three hour old embryos yielded marked clones of cells which were confined to a single appendage. Although clones were found which extended over several segments of the leg, for example, they were never found to extend uninterrupted from the leg to adjacent parts of the body. It seems, therefore, that certain cells of the blastula are already determined to form a specific part of an adult structure, such as a leg. However, since the patches often extended through several segments of the leg, it is believed that the cells within a given disc Anlage have, at these early stages, the potential of producing many cell types within that appendage.

Several lines of evidence indicate that the developmental potential of each disc cell becomes increasingly restricted as larval life ensues. For instance, if somatic crossing over is induced at various times during larva growth, the extent of mosaic patches which arise are restricted to increasingly smaller areas of a given structure as the larva matures (Bryant and Schneiderman, 1969). In earlier studies, Becker (1957) found that certain cells of the eye disc from late second instar larva still have the option to differentiate into either ommatidia or into vibrissae. By the third instar stage, these same cells had lost this potential, and were committed to form either ommatidia or vibrissae. By the late third larval instar stage, regional specificity is superimposed upon the prior determined state of a given disc. Thus, cells are committed to form specific regions and structures of the adult appendage. A disc may be removed from a third instar larva and cut into fragments which can then be implanted into other host third instar larvae. Such fragments are found to differentiate into specific segments of the adult structure (Schubiger, 1968).

Similarly, when a small area of cells is removed from explanted discs by microbeam treatment, and the discs implanted into third instar larvae, adult appendages are formed which lack a specific structure (Ursprung, 1957, 1959). Using these techniques, it has been possible to construct anlagen plans or fate maps of cells in the imaginal discs for: eye antenna (Vogt, 1946a; Gehring, 1966), wing (Hadorn and Buck, 1962), haltere (Loosli, 1959), leg (Bodenstein, 1941), male foreleg (Nöthiger and Schubiger, 1966; Schubiger, 1968), female genital (Hadorn and Chen, 1956; Ursprung, 1957), male genital (Hadorn, et al., 1949; Ursprung, 1959; Lüönd, 1961). Thus, a disc from a late third instar larva, though made up of histologically similar cells, is, in fact, comprised of cells already rigidly programmed to form different parts of a specific adult appendage and the cells committed to form one specific area are no longer able to generate parts of the organ supplied by other areas. Thus, by the end of the third instar, cells from different areas within a given disc bear the same relationship to one another as cells in whole discs did to each other in the late embryo.

Nothing is known about the nature of determination within an individual cell because single cells haven't been amenable to experimentation. However, some information on state of determination of individual cells has been deduced by mixing cells from different discs. In such experiments, cells from two or more discs are mechanically or chemically dissociated, then mixed and reaggregated. The mosaic pellet is then transplanted into an adult host for several days to allow time for possible cell sorting, and then injected into a larval host where it is allowed to differentiate during metamorphosis. Alternatively,

the pellet can be injected directly into the larval host. The cells from different discs are marked genetically with mutations affecting color mutants and often bristle and hair morphology.

If cells from two genetically different discs of the same type (i.e., leg) are mixed, allowed to aggregate and then tested, they form patterns similar to the in situ patterns except that they are phenotypic mosaics (Nöthiger, 1964; Tobler, 1966; Garcia-Bellido, 1966a). This indicates that the cells from the same region of a given disc type are able to co-operate to form a specific adult structure. On the other hand, when genetically marked cells from two different disc types, for example, wing and genital, are mixed and implanted, upon metamorphosis no integrated mosaics are formed (Nöthiger, 1964). Thus, individual cells must be determined to the extent that like cells will aggregate, whereas unlike cells will sort out, perhaps by cell migration. Moreover, if proximal and distal cells from the leg disc (Tobler, 1966) or the wing disc (Garcia-Bellido, 1966a) are mixed, segregation is also observed. Therefore, even cells from different areas of a given disc are able to segregate from one another. Finally, during these mixing experiments, in a few cases (Nöthiger, 1964) a single cell or small number of cells from one disc type (genital) became trapped in a large area of cells of another disc type (wing). These trapped cells differentiated autonomously.

Is the ability of unlike cells to segregate from each other a property inherent to larval disc cells, or is it acquired only in the metamorphosing cells? The ability of larval disc cells to segregate was rather convincingly demonstrated by Garcia-Bellido (1967). He mixed dissociated wing and leg disc cells from third instar larvae,

and placed the pellets in adult hosts which were starved so that no proliferation occurred. After two to ten days, they were examined histologically and the wing discs were found to have reaggregated into folded sheets like the pattern found in in situ third instar discs. Furthermore, Kuroda (1969) showed that in re-associated eye-antennal disc cells cultured in vitro, the presumptive omatidial cells were able to sort out and cluster separate from the presumptive antennal cells.

Although the cells of a specific disc from late third instar larva are determined to form only a small region of the adult structure, it has not been proved that the developmental potential of these cells cannot be further restricted. That is, they may not have reached their final stage of determination. In mixing experiments involving genetically marked leg discs, Garcia-Bellido (1966a) found small mosaics between bristle and bract cells (specialized hair cell). Since isolated brachts have not been observed in mixing experiments, it was argued that they are induced by the bristle cells. Furthermore, Lees and Waddington (1942) have shown that a bristle which is composed of two cells (the shaft and the socket cells) is formed during the early pupal stages by unequal division of the bristle mother cell. It seems, therefore, that in some cases further restrictions of developmental potential occurs during early pupa.

In summary, the adult body structures are derived from a series of discrete packets of cells. These discs are ectodermal in origin and are initially determined to produce a given adult structure at blastoderm formation. Their initial determined state is further restricted during larval growth, such that specific cells within a

disc become programmed to produce particular sections of the adult appendage. Once the initial determination is established, further development is autonomous.

B. Transdetermination

The experiments described so far have shown that once a state of determination is imposed, that state is stable and hereditary to all daughter cells. The length of time that this state of determination would be retained in disc cells which were dividing but not differentiating soon became of interest. Hadorn and members of his group have maintained some lines of disc blastemas by culturing *in vivo* in adult fly abdomens for up to seven years. When these disc blastemas are tested by implanting fragments into larvae, the metamorphosed structures formed are the same as that of the original disc. Sometimes, when these *in vivo*-cultured blastemas are tested, structures arise which are different from the program dictated by the original determination event. For example, cultures of presumptive genital disc cells occasionally produce leg or antennal structures (Hadorn, 1963). The new structures were called allotypic in contrast to the normally produced autotypic structures (Hadorn, 1965). The process whereby one state of determination is exchanged for another state of determination has been called *transdetermination*.

If and when transdetermination occurs, not all the cells of the test implant undergo transdetermination. Moreover, there is a sharply defined boundary between the transdetermined (allotypic) and normal (autotypic) cells, although they may lie adjacent to one another. An intermediate or mosaic structure has never been described on the

border between the allotypic and autotypic structures. Like autotypic cells, allotypic cells pass on their program for altered differentiation to their daughter cells over several generations (Gehring, Mindek and Hadorn, 1968). From the size and shape of allotypic regions within a disc, it is generally believed that transdetermination occurs in several cells concomitently. However, it has been impossible to successfully transplant single cells. In addition, it is difficult to identify a single allotypic cell in a wild-type background. If transdetermination occurred in a single cell, cell multiplication during late third instar or early pupa would lead to at least a small cluster of clonally-related cells. By genetically marking the implanted cells, it was possible to show that the allotypic structures always arose from the implanted disc cells and not from host cells (Gehring, 1968).

The question then arose, do allotypic structures arise from already determined autotypic cells or from undetermined cells? From the state of determination fixed in late third instar discs, one would assume that the allotypic structures arise from determined autotypic cells. Gehring (1967) induced somatic crossing over in larvae heterozygous for several genetic markers. He then implanted the eye-antennal disc into adult hosts for several days and then transplanted the cells back into larval hosts. After metamorphosis, several clones of marked cells contained both autotypic and allotypic cells. In several cases, the palpus which is an autotypic structure formed by the antennal disc had both wild-type and marked cells and the clone of marked cells was continuous with a large area of allotypic wing cells. From this it was concluded that somatic crossing over occurred in an autotypic cell which proliferated and then underwent a transdetermination event to

give rise to the allotypic cells of the clone.

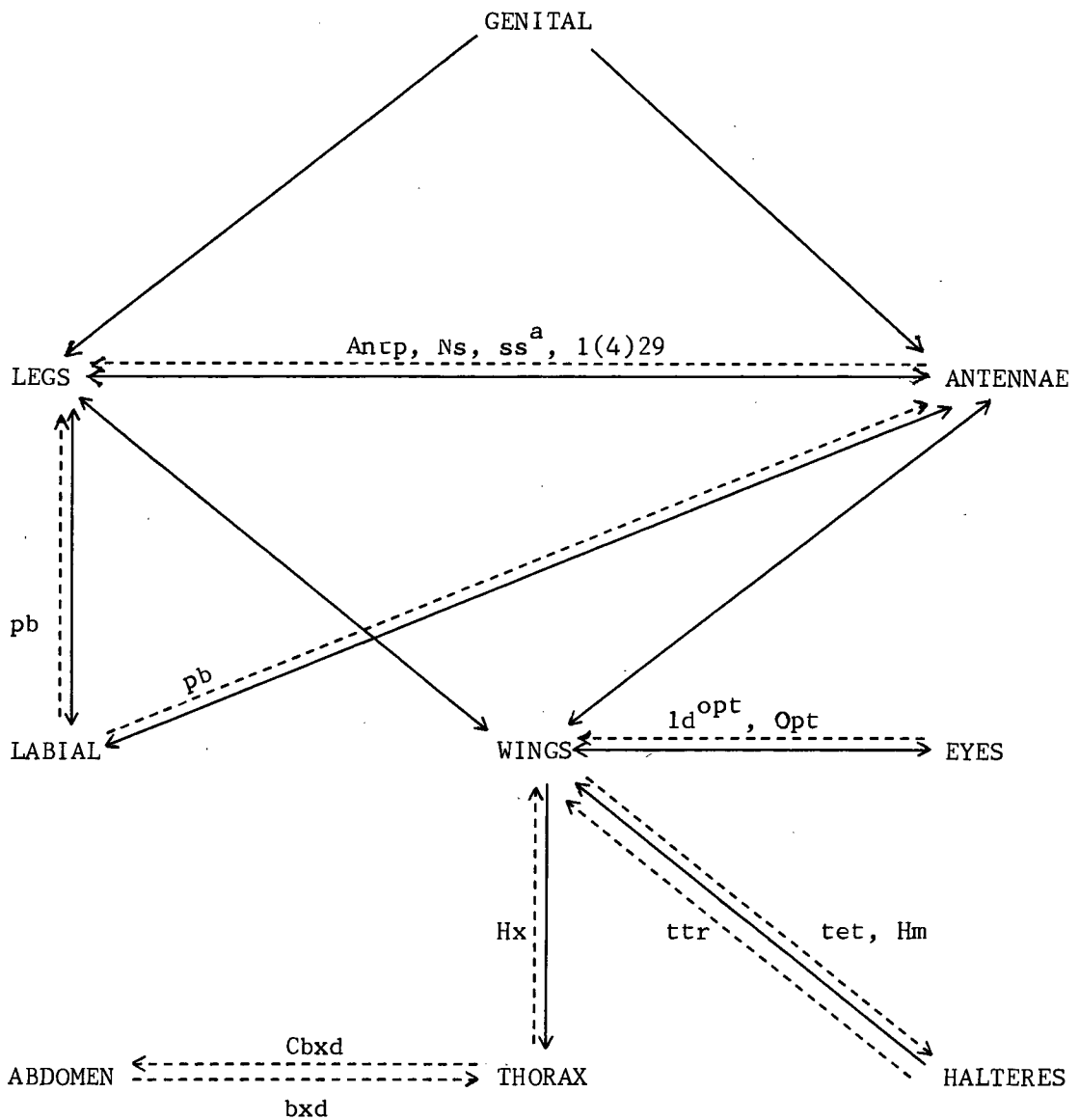
Transdetermination has been observed in all disc types which have been cultured *in vivo* (Dubendorfer, 1969). Disc cells from any larval stage and even embryonic tissue (Hadorn *et al.*, 1968) will undergo transdetermination once they have completed their imaginal development in an adult host.

It seems that the rate of transdetermination varies from one type of disc to another. For instance, the genital disc undergoes transdetermination events only after it has been through several generations of adult hosts (Hadorn, 1966), whereas a labial disc will undergo transdetermination after only one generation in an adult host (Wildermuth, 1968). Wildermuth (1968) also offered evidence for differences in the rate of transdetermination induced in discs from various species of Drosophila.

The most interesting fact associated with transdetermination is that it is a directed process. That is, once programmed by determinative events, a group of cells can undergo transdetermination to form only one or two specific allotypic structures. The change from a given autotypic structure to an allotypic structure is predictable for both direction and frequency in all cases. For example, genital disc cells undergo transdetermination to form either leg or antennal structures. Allotypic cells may themselves undergo transdetermination again to give rise to a second order of allotypic structures. For example, allotypic leg cells may form wing structures. The direction of these further transdeterminative events is also predictable. It is possible to construct a diagram showing the directions of transdetermination starting with any given autotypic or allotypic structure (Figure 4).

FIGURE 4

A diagram indicating the directions in which transdetermination occurs with the corresponding transformations caused by homeotic mutants. ——— transformations observed in transdetermination; ----- transformations caused by homeotic mutants.
For the full name of the homeotic mutants, see Table 1.


GENITAL
Antp, Ns, ss^{a}, 1(4)29
LEGS
ANTENNAE
pb
pb
LABIAL
WINGS
ld^{opt}, Opt
EYES
Hx
ttr
tet, Hm
Cbxd
ABDOMEN
THORAX
HALTERES
bxd

It is interesting to note that many, but not all, of the possible steps in transdetermination are reversible.

Transdetermination is known to occur within a disc, that is, cells from a specific region of a disc which are determined to give rise to a specific part of the adult appendage, sometimes produce structures that generally arise from other cells within that disc (Gehring, 1966).

One of the possible explanations of transdetermination, namely, somatic mutation, is generally ruled out as a possibility because the rate at which cells undergo transdetermination is much higher than the somatic mutation rate. From analyses of clones (Gehring, 1967), it has been shown that allotypic structures can be made up of cells of two different phenotypes indicating that the allotypic structures arise from two or more cells. The possibility that mosaic allotypic structures arise from two independent transdeterminative events followed by aggregation is discredited because no salt and pepper mosaics are found. Instead, cells seem to group in mosaic clones within an allotypic structure. Thus, transdetermination is not due to somatic mutation.

The possibility that the culture medium affects transdetermination cannot be ruled out, although it seems unlikely. Disc cells that are cultured in larvae undergo transdetermination with the same frequency and the same direction as cells cultured in adult flies, even though the composition of the hemolymph varies (Hadorn, 1963). In addition, it has been demonstrated that the frequency of transdetermination is directly related to the extent of all proliferation and not to the length of time spent in the hemolymph (Tobler, 1966). Apparently

the ability of the programmed disc cells to undergo transdetermination is a property inherent to the discs themselves. However, Wildermuth (1968) has shown that the culture medium does have an effect on transdetermination in certain cases where discs from one species of Drosophila were cultured in another species.

As mentioned, transdetermination seems to be associated with cell proliferation. By two different but unrefined techniques, it has been demonstrated that there is a linear correlation between growth rate and the frequency of transdetermination (Tobler, 1966; Wildermuth, 1968; Mindek, 1968). Wildermuth (1968) gave good evidence that transdetermination is restricted to dividing cells. He observed that a labial disc which was cultured in an adult host for several days then implanted into a metamorphosing larva gave rise to a complete proboscis (generally this structure is formed from a pair of labial discs). The new half of the proboscis (a mirror image of the old half) arose by cell proliferation from the original labial disc. The new half of the proboscis could be distinguished from the old half by observing the orientation of the implant early in differentiation. Allotypic structures frequently arose in these transplants and they always arose on the <u>newly-formed half</u> of the proboscis. Because of this correlation of transdetermination with cell proliferation, Hadorn has hypothesized that in cells undergoing transdetermination, specific carriers of determination which are normally replicated and passed on to their daughter cells to form autotypic structures, become diluted out when proliferation is rapid, thereby allowing for a shift in gene expression.

One can speculate whether transdetermination occurs as a change from one determined state directly to another determined state

or if the cells go from a state of determination to an undetermined stage (de-determination) and then choose a new state of determination. If the latter possibility is true, the mechanism of transdetermination might be more closely related to the mechanism of regeneration, except that in transdetermination one course of differentiation is exchanged for another. True regeneration (as distinguished from organ duplication) has been known to exist in insects (see review, Bulliere, 1971) and has recently been discovered in Drosophila (Schubiger, in press). It is most interesting that some cases of transdetermination have been reported in vertebrate tissues which normally undergo regeneration (see Hay, 1968).

C. Homeotic Mutants

There exists a class of mutations whose phenotypic effects resemble the changes resulting from transdetermination. These mutations have been termed "homeotic" (Goldschmidt, 1945). Homeosis is defined as the replacement of one organ structure by another structure from an homologous organ (Bateson, 1894). One example of this is the replacement of part of the antenna by a leg tarsal segment in the mutant aristopedia (Balkaschina, 1929).

Several different homeotic mutants have been described. These mutants, along with the structural modifications they effect, are listed in Table 1. Some of the homeotic mutants cited may not truly fit the definition of homeosis owing to the difficulty in demonstrating homology between eye and wing tissue for example.

In these homeotic mutants, as in transdetermination, the transformations of one structure into another is always complete. That

TABLE 1

Known Homeotic Mutants

Mutant	Locus	Reference	Tissue Affected	New Phenotype
proboscopedia (pb)	3-47	Bridges & Dobzhansky (1932)	proboscis	antenna or tarsus
Antennapedia (Antp)	3-48	Lewis, 1956	antenna	leg
Nasobemia (Ns)	3-48	Gehring, 1966	antenna	leg
Polycomb (Pc)	3-48	Hannah-Alana, 1958	legs	extra sex combs
Multiple sex comb (Msc)	3-48	Tokunaga, 1966	2nd & 3rd legs	1st leg
Extra sex comb (scx)	3-48	Hannah & Stromnaes, 1955	2nd & 3rd legs	extra sex comb
tetraltera (tet)	3-48.5	Goldschmidt, 1940	wing	haltere
tetraptera (ttr)	3-51.3	Astauroff, 1929	haltere	wing
aristapedia (ss^a)	3-58.5	Balkaschina, 1929	arista	tarsus
bithorax (bx)	3-58.8	Bridges & Morgan, 1923	metathorax	mesothorax
Contrabithorax (Cbx)	3-58.8	Lewis, 1964	mesothorax	metathorax

TABLE 1 (continued)

Mutant	Locus	Reference	Tissue Affected	New Phenotype
Contrabithoraxoid (Cbxd)	3-58.8	Lewis, 1968	metathorax	abdomen (1st)
Ultrabithorax (Ubx)	3-58.8	Lewis, 1964	metathorax	mesothorax
bithoraxoid (bxd)	3-58.8	Lewis, 1964	abdomen (1st)	metathorax
postbithorax (pbx)	3-58.8	Lewis, 1964	metathorax	mesothorax
loboid-ophthalmoptera (ld^{oph})	3-102	Kobel, 1969	eye	wing
Haltere mimic (Hm)	T (2,3)	Lindsley & Grell, 1968	wing	haltere
lethal (4)29 (l(4)29)	4	Gehring, 1969	antenna 2nd & 3rd legs	coxa, trochanter 1st legs, late pupal lethal
Hexaptera (Hx)	2	Herskowitz, 1949	prothorax	wing (or leg)
extra sex comb (esc)	2-54.9	Slifer, 1942	2nd & 3rd legs ♂	1st legs
Ophthalmoptera (Oph)	2-68	Goldschmidt & Lederman-Klein, 1958	eye	wing
reduplicated sex combs (rsc)	1	Lindsley & Grell, 1968	2nd & 3rd legs ♂	extra sex comb

is, no structures are formed which are intermediate between the autotypic and allotypic structures. This would seem to indicate that in both transdetermination and in homeotic mutants, selection of one pathway of determination and differentiation excludes the expression of other possible pathways.

As was the case for allotypic structures that resulted from transdetermination, the allotypic structures produced by homeotic mutants are identical to their autotypic counterparts in all respects. Similarly, the cellular affinity of the allotypic cells is the same as their autotypic counterparts. Thus, if genetically marked cells from third instar antenna discs of the homeotic mutant aristapedia are mixed with cells from a normal leg disc and implanted into a larva, the marked allotypic tarsal cells aggregate and differentiate with the wild-type leg cell to form a salt and pepper mosaic leg tissue (Garcia-Bellido, 1968).

Finally, ordinary morphological mutations which affect normal legs or wings have the same effect on the allotypic structures produce by the homeotic mutants aristapedia (Braun, 1940), bithorax and proboscopedia (Villee, 1946) and loboid ophthalmoptera (Ouweneel, 1970). It is concluded, therefore, that the new structure generated by a homeotic mutation is biologically complete and indistinguishable from its autotypic counterpart.

All homeotic mutants that have been tested, either by generation of mosaics or by transplantation, have been shown to be autonomous (Braun, 1940; Vogt, 1946b; Lewis, 1964; Roberts, 1964; Garcia-Bellido, 1968). It is most interesting to note that most of the known homeotic mutants map genetically within a rather restricted segment of

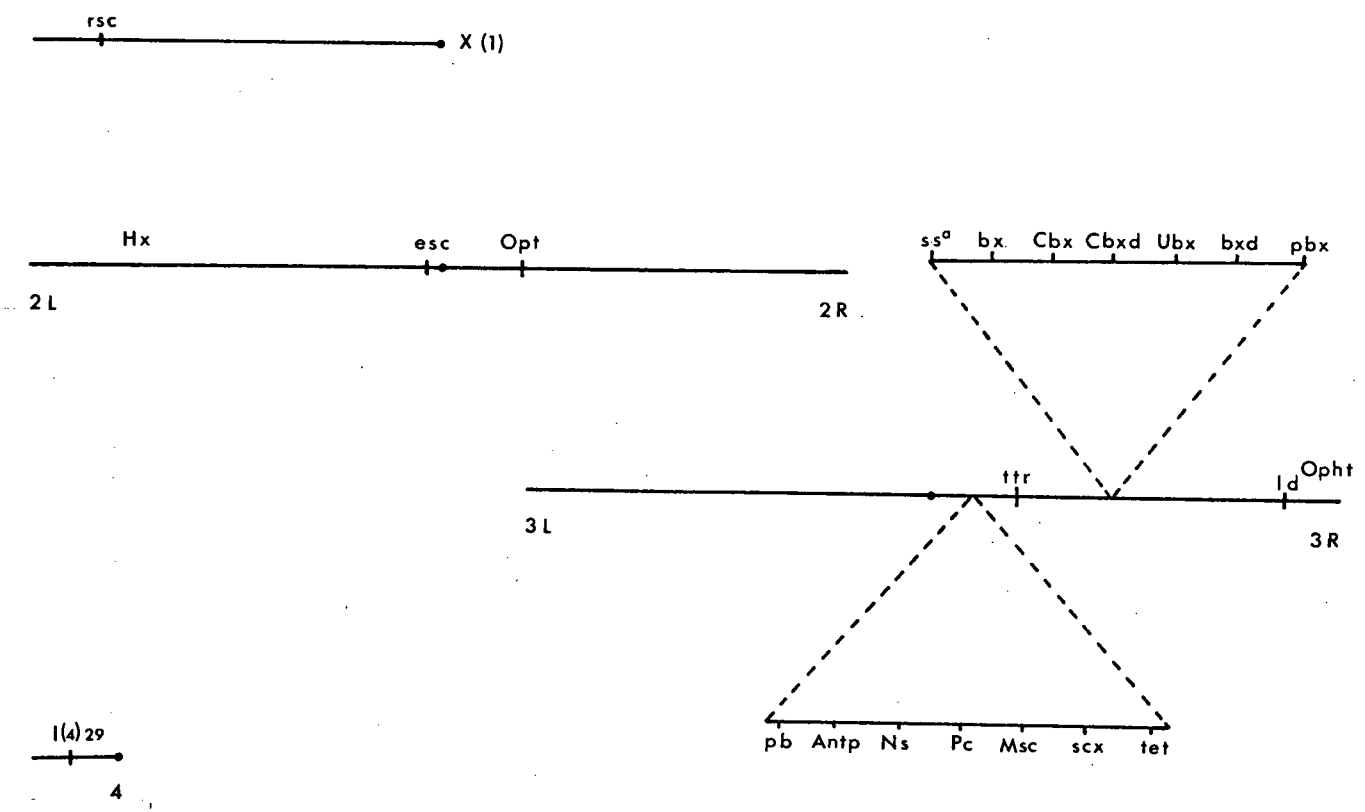
the Drosophila chromosomes. Of the 22 mutants known, 17 reside on the 3rd chromosome between 47 and 58 map units, a genetic segment which comprises less than 5% of the total Drosophila genome (Figure 5). Moreover, within this short region, there are two clusters of mutations; one between 47 and 48.5 contains 7 mutants and the other between 58.5 and 58.8 contains 7 mutants. Of the eight remaining mutants, one maps on the third chromosome at 102, another at 51.3 and a third, haltere mimic, is associated with the translocation between the second and third chromosome and thus may also reside on the third chromosome. This leaves five homeotic mutants known which do not reside on the third chromosome. Three of these are located on the second chromosome, one is on the X-chromosome and one maps on the fourth chromosome. These observations suggest that this small region on the right arm of the third chromosome is an important area involved with regulation of disc determination.

The transformations caused by the homeotic mutations parallel the directed changes encountered in transdetermination (see Figure 3). However, some cellular transformations found in transdetermination experiments have no counterpart among the homeotic mutants. This could be due to the fact that some homeotic mutants are lethal and therefore not readily recovered. For example, the mutant, 1(4)29, which converts part of the antenna to the coxa of a leg and the second and third leg to the first leg structure, is generally a pupal lethal (Gehring, 1970). Similarly, spineless aristopedia (which convert the arista to a tarsus) is lethal when raised at 29°C (Grigliatti, unpublished data).

The converse is also true. Not all of the structural transformations caused by homeotic mutations have been observed in trans-

FIGURE 5

General position of homeotic mutants on the Drosophila genome. X(1) - sex chromosome; 2L, 2R - left and right arms of the second chromosome; 3L, 3R - left and right arms of the third chromosome; 4 - the fourth chromosome.
For the full name of the homeotic mutants, see Table 1.


rsc
X (1)
Hx
esc
Opt
2 L
2 R
ss^a
bx
Cbx
Cbxd
Ubx
bxd
pbx
ttr
ld
Opht
3 L
3 R
pb
Antp
Ns
Pc
Msc
scx
tet
l(4) 29
4

determination experiments. For example, the transformation of wing tissue to halteres (contrabithorax, tetraltera, and haltere mimic) has never been observed in transdetermination experiments. Nevertheless, the striking resemblance in characteristics at the phenotypic level produced by transdetermination or homeotic mutations, strongly suggests that the same molecular processes are being affected in the two processes.

The best phenotypic expression of the homeotic mutation, ophthalmoptera, is generated in the presence of other mutations which reduce the number of eye facets. The degenerated eye facets are often replaced by duplicated head structures which must arise by cell proliferation. Kobel (1968) suggested that these additional cell divisions might lead to the formation of allotypic structures by the same mechanism as transdetermination. Various theories as to the cause of homeotic mutations have been advanced. However, the causes of homeotic mutations and their relationship to transdetermination are still unresolved.

D. *In Vitro* Cultures of Discs

In addition to *in vivo* culture of imaginal discs, various attempts have been made at maintaining the discs *in vitro*. However, it is difficult and has been relatively unsuccessful until recently. Schneider (1964) was able to culture eye antennal discs in a medium which was defined except for the addition of serum albumin. The discs were cultured along with both cerebral hemospheres of the cephalic ganglion, the ventral ganglia, ring gland, aorta and lymph gland. She was able to obtain some differentiation of the antennal structure and a few cultures (about 10%) developed ommatidia and cornea and showed

some pigment deposition. A similar result was obtained by Hanly et al. (1957). Hanly and Hemmert (1967) were able to show that the extent of bristle development in cultured eye discs was temporally related to the attachment of the eye disc to the cephalic ganglia. Kuroda (1969b), using a synthetic medium (K-6) (Kuroda and Tamura, 1956), was able to demonstrate the differentiation of ommatidia from eye discs in cultures to which ecdysone analogs were added. Fristrom et al. (1970) grew leg and wing discs in Schneider's medium supplemented with ecdysone and reported that evagination of the leg discs took about 10 hours, approximately 4 hours longer than in vivo.

Growing eye-antennal, wing and leg discs plus the cerebral complex of a defined medium (S), Mandaron (1970) reported no differentiation or evagination of the discs in the absence of ecdysone. However, in the presence of ecdysone, most of the discs evaginated and differentiated the normal bristles, hairs or claws characteristic of the epidermis and, in addition, there was some deposition of chitin by the leg structures. Interestingly, no musculature was found in the leg structures that were formed and these legs did not grow in length.

Leg and wing discs cultured without the brain or ring glands in a different medium (M) again showed no evagination (Mandaron, 1971). However, with ecdysone, the discs evaginated and thus he suggested that evagination is "an active process related to an intrinsic property of the disc". This is substantiated by several studies in vivo which showed that evagination coincides with the change of disc cell shape from columnar through cuboidal to flattened cells (Fristrom et al., 1970; Poodry and Schneiderman, 1970). Fristrom et al. (1970) also reported that actinomycin D and puromycin inhibit evagination initiated

by ecdysone and therefore suggest that molecular synthesis is needed for normal evagination.

Instead of using the criteria of differentiation, Robb (1969) developed a defined media based on the efficiency and continuity of labelled uridine uptake. Discs cultured in his medium show linear (though decreasing) incorporation of ^{3}H-uridine into RNA for 48 hours.

Recently, Schneider has developed a media in which cells derived from embryonic tissue will proliferate. To date, disc cells have not been able to grow in this media (W. Gehring and C. Laird, personal communication).

These recent advances promise to make extensive studies of discs *in vitro* quite possible in the near future. In fact, these recent advances in culturing of discs has already stimulated some initial biochemical investigations in RNA and protein synthesis in discs.

E. Biochemistry Review

Study of the biochemistry of disc development became feasible with the development of culture mediums for *in vitro* studies and through the development of a system for mass isolation of discs (Fristrom and Mitchell, 1965). The general patterns and dynamics of protein and RNA synthesis in third instar leg discs have been investigated and found to resemble those in other eukaryotic cells (Fristrom and Knowles, 1967; Fristrom, Brothers, Mancebo and Stewart, 1968).

The effects of ecdysone on RNA synthesis *in vitro* have been investigated (Fristrom *et al.*, 1970; Raikow and Fristrom, 1971) and have shown that ecdysone stimulates an increase in both the rate of

RNA precursor uptake and the rate of RNA synthesis. It causes an increase in heterogenous RNA synthesis, a preferential increase in ribosomal RNA synthesis and accelerates the processing of 38S ribosomal RNA precursors to 28S and 18S subunits. It is suggested that there may be a causal connection between increased RNA synthesis and evagination of the discs and also that the increase in ribosomal RNA synthesis may be in connection with the transport of messenger RNA to the cytoplasm.

No difference in the species of protein or RNA synthesized in different discs or discs in various stages of development have been reported. It is clear that a great deal of work is needed before we begin to understand the molecular basis of determination and differentiation.

CHAPTER 2

GENERAL INTRODUCTION

Determination and differentiation of imaginal discs poses questions of fundamental importance to an understanding of development (see Chapter 1). We may pose several questions worthy of investigation: (1) what is the role of cell communication in the regulation of the determinative process? (2) how does the development of neural tissue proceed? (3) what are the mechanisms involved in neural integration? and (4) does the development of the nervous system affect the differentiation of other, non-neural tissues?

In a living organism, determination appears to be a step-wise process by which the possible pathways of differentiation open to a given cell become increasingly limited. However, cultured discs from late third instar or prepupae seem capable of switching from an initial program to an alternate pathway of differentiation. Whether the determination of an imaginal disc as a complete entity is firmly barred from alternative fates only at the onset of differentiation, or whether there is a lock put on the initial decision, remains to be seen. If the latter is correct, then transdetermination would involve an initial de-determination with subsequent redifferentiation. In this respect, transdetermination would be a process akin to regeneration.

The method whereby cells recognize each other has been studied by mixing experiments. Upon mixing cells of different third instar discs, only cells of the same type of disc aggregated. Indeed, the specificity was so great that cells from different regions of the same disc apparently segregate from one another. The question then

arises as to how cells are able to define and recognize "self" versus "non-self". It may be possible that cells of different disc types share some of the early events associated with anlagen determination so that, for example, particular cells from the young leg disc recognize as "self" some cells of the immature genital disc. If some common cell types exist among developing discs early in larval life, perhaps the ontogenic relatedness of discs could be correlated with the specific stepwise pattern observed in transdetermination.

There is some evidence that cell migration may act as a mechanism of metamorphosis (Tokunaga, 1962). Thus, the sorting out and aggregation phenomena observed in implantation studies may be of significance to disc development *in vivo*.

Once a cell has been initially determined, subsequent determination and differentiation can occur autonomously. How do external factors, such as hormones, affect a cell or group of cells? Do hormones act as temporal control of the onset of subsequent steps of determination, or do they control the direction of the determinative process? Intrinsic factors, such as the rate of cell division, have been shown to be correlated with the frequency of transdetermination. Whether the increase in the rate of cellular proliferation *results* from the transdetermination process or whether it is the principal *cause* or initiator of the transdetermination process, has not been established. If cell proliferation does trigger transdetermination, then one might ask if the rate of cell division can affect the choice of the new pathway of differentiation.

What role does intercellular communication within a disc play in the determination and differentiation of that disc? An

efficient biological system demands that each cell must recognize the position it will occupy in the adult structure in order to complete the standardized pattern of the adult framework. It seems reasonable, therefore, that the cells must not only know that they are determined for a particular state, i.e., "legness", they must also know what their positional relationship is to their neighboring cells and to the disc as a whole in order to produce a specific structure of the adult appendage. The problems of pattern formation and prepatterns have been considered in an excellent review (Stern, 1968) and theoretical models on how positional information and pattern formation might occur have been discussed (Wolpert, 1969). It would seem that communication between cells within a disc may be instrumental in the determination of the final phenotype. Indeed, there is anatomical evidence for gap junctions that allow cytoplasmic connection between disc cells (Poodry and Schneiderman, 1970). Transdetermination appears to occur in a number of cells simultaneously and it therefore seems reasonable to assume the prevalence of a system of intercellular communication within discs. Additional support for such a scheme has been presented (Grigliatti and Suzuki, 1971).

The origin of disc cells is ectodermal. The adult appendages which they produce are primarily epithelial yet they have well developed musculature and neural connections. The question arises as to whether the disc cells are entirely epithelial, or whether they contain presumptive muscle and/or presumptive nerve cells. Early workers observed "mesenchymal cells" juxtaposed to the disc epithelium and thought they may have "invaded" the disc tissue (Newby, 1942; Auerbach, 1936). In transplantation experiments, only the genital disc differentiates

muscular tissue, i.e., the contracting ejaculatory duct (Hadorn et al., 1949). Muscles have never been observed in metamorphosed transplants of leg or wing cells, although both are supplied with muscles in situ (Hadorn and Buck, 1962; Schubiger, 1968). Poodry and Schneiderman (1970), using the electron microscope, detected disoriented aggregates of perhaps partially histolized muscle cells in metamorphosed transplants of leg discs. They further identified adepithelial cells in third instar discs in situ which they suggest may give rise to muscle tissue. However, careful examination of these cells throughout development must be made before any definitive conclusions may be drawn. Even if these adepithelial cells represent presumptive muscle cells, how they come to be found in developing discs is still unknown. Mutants affecting muscle development and function might be extremely valuable in analyzing the relationships of the muscle and ectodermal cells.

Some third instar discs posses a neural connection to the brain, for example, the first and second leg discs, while others, for example, the third leg disc, lack this association (Auerbach, 1936; Bodenstein, 1950). Recent experiments in the housefly, Musca domestica, have shown that the attachment of nerves to muscles is associated with muscle development in the legs (Bhaskaran and Sivasubramanian, 1969). The significance of neural connection for muscle differentiation and tissue regeneration in insects has been reviewed by Nüesch (1968). It would be of interest to establish what role, if any, innervation plays in the determination and/or differentiation of the discs. On the other hand, it may be that neural connection to the discs is important in the determination or differentiation of the adult nervous system. Our information on the nervous system of Drosophila, in general, its

development and its functional integration, is lacking. The study of mutants affecting the development of the nervous system, the innervation of disc and muscle tissue, interneural and neuro-myal transmission and neural degeneration and regeneration may provide a valuable foundation for the understanding of the nervous system of higher organisms in general.

Of the several problems discussed above, three have been chosen as the material for this thesis. The first, a classical problem in developmental biology, attempts to determine whether control of the activity of a structural gene is directly imparted by the tissue in which the gene product is active, or whether, in fact, a freely flowing evocator of gene action exists. That is, given a gene whose activity is required in several different organs during development, is this gene activated concomitantly in all organs in response to a general humoral factor within the body, or is the gene activated at different times during development in different tissues in response to tissue-specific triggers?

The second problem investigated concerns the possibility of isolating a temperature-sensitive homeotic mutant that expresses a wild-type phenotype at the permissive temperature and a fully penetrant mutant phenotype under restrictive conditions. Such a mutant might provide the firm foundation on which various biochemical techniques can be investigated or refined for the purpose of approaching a molecular understanding of determination and the regulation of differentiation in higher organisms. Studies related to the temperature-sensitive aspects of such a mutant may provide information as to the time of action of regulatory genes and may also provide information

on cell communication and pattern formation.

The third problem is the possibility of isolating mutants affecting the nervous and/or muscle systems of Drosophila. Such mutants may be used to begin to investigate some of the problems just mentioned as to the development of the nervous system, its functional integration, and the effect of innervation on the development of various tissues.

A more complete introduction to each of these problems is presented at the beginning of its respective chapter.

The utility of conditional mutations whose expression is temperature-dependent is amply illustrated in these problems. They allow one to overcome the problem that certain homeotic, neural and muscle mutations might be lethal. In addition, the conditional aspect provides a means for determining the interval during which the gene product is used. This, coupled with experiments using somatic crossing over, might allow one to determine the duration or interim between the transcription of a given gene and the time at which the product is utilized or converted to a functional form. Not only would such assays permit the investigation into possible roles of masked messenger RNA, et cetera, in development, but they also allow the investigator to control the onset of the change and the selection of the developmental pathway. Thus, the isolation of temperature-sensitive mutations in *Drosophila melanogaster* adds new depth to the investigations of classical topics and promises to open up new areas of interest in Drosophila.

CHAPTER 3

A TEMPERATURE-SENSITIVE MUTATION AFFECTING LEVELS OF PTERIDINES IN *DROSOPHILA MELANOGASTER*

I. Introduction

A major question in developmental genetics is whether a single gene whose function is known to be required in different tissues, is activated concomitantly in all of these tissues in response to a single trigger or responds to tissue-specific stimuli at different developmental stages. Since many of the fluorescent pigments found in adult eyes and testes and in larval and adult malpighian tubules of *Drosophila melanogaster* are regulated by the same set of genetic loci (Ziegler, 1961), a single mutation affecting pigmentation in all three tissues might shed light on the problem. Flies carrying a recessive sex-linked mutation, $1(1)E6^{ts}$ (Suzuki, 1970), cause lethality at 29°C but result in a mutant eye color of viable adults at 22°C. Subsequent tests have indicated that, in addition to temperature-sensitive (ts) lethality, the presence and quantity of pteridines in the eyes, testes and malpighian tubules are also affected by temperature. The level of pigment in these organs was measured in flies grown at 29°C during different developmental intervals in order to delineate the temperature-sensitive period for the presence of pigment.

II. Materials and Methods

A. Genetic Analysis

The mutation 1(1)E6^{ts} (abbreviated as E6) was induced by ethyl methanesulfonate and found to be viable at 17°C and 22°C, but was lethal at 29°C. At 22°, the eye color of E6 males was obviously mutant, whereas homozygous females were visually indistinguishable from wild-type. The lethal was initially localized genetically (Suzuki, 1970) close to v (33.0) (the symbol of a mutation is followed by its genetic position in parenthesis; for a complete description of the mutants used, consult Lindsley and Grell, 1968) between cv (13.7) and v and subsequently positioned to the left of v within 0.7 map units by the markers lz (27.7) and v. A test of allelism of E6 with ras (32.8) revealed that E6/ras females were viable at 29° but had a mutant eye color. In order to determine whether ts lethality and the ts pigment phenotypes are caused by a single mutation, females heterozygous for E6 and m (36.1) were mated at 29° and the male offspring scored for the presence of surviving crossover progeny with the mutant eye phenotype. This experiment showed that E6 was 3.67 units to the left of m and that no separation of lethality from the pigment variant was found among 3,421 males scored. If, in fact, the lethal and eye phenotypes result from separate mutations, an upper limit of 0.09 map units (Stevens, 1942) separates the two sites; this makes it probable that the two phenotypes result from a single mutation at the ras locus.

B. Developmental Analysis

The "effective lethal phase" (Hadorn, 1961), that is, the time at which E6 flies die when raised at 29°, was established by maintaining

eggs from the E6 stocks collected within 2 hours of deposition at 29°. The developmental stages reached were determined by inspecting the culture at 12 hour intervals. It was found that obvious autolysis and death occurred prior to the formation of any distinguishable adult structures, approximately 12 hours after puparium formation.

The actual developmental interval of sensitivity to the high temperature (TSP) during which growth at 29° irrevocably commits the flies to death was established by reciprocal "Shifts" at successive 12 hour intervals in which 29° cultures were shifted to 22° (Shift-down) and vice versa (Shift-up). The first Shift-down which gave a reduction in survival was taken to indicate the beginning of the TSP and the first culture in a Shift-up which yielded viable adults delimited the end of the TSP. By these criteria, the TSP was shown to begin midway through the third larval instar and terminate just prior to pupation (Figure 6).

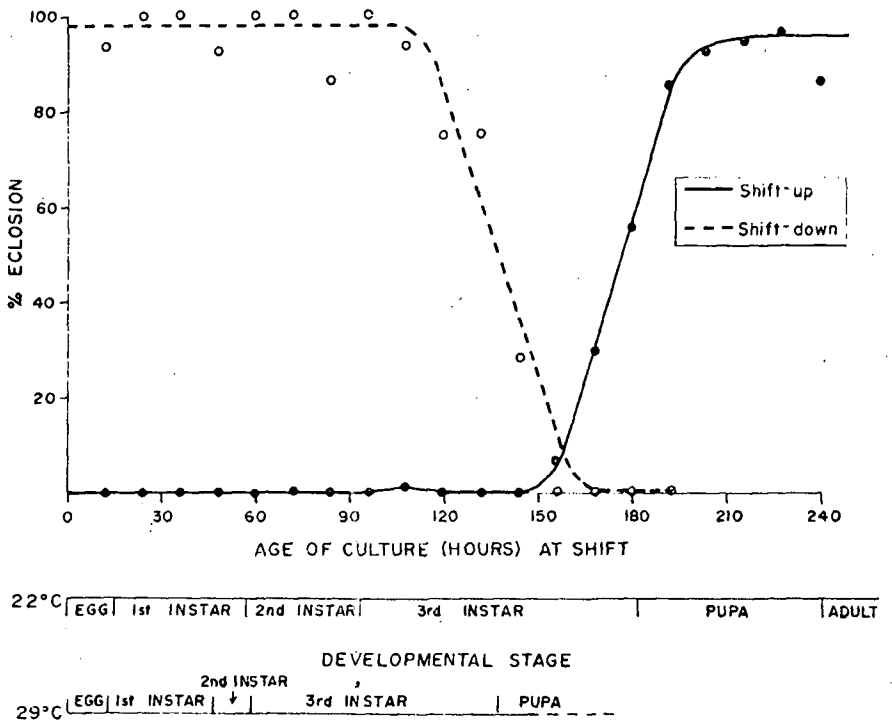
It was immediately noted that the eyes of flies hatching from a culture shifted up just after the TSP for lethality were dark brown in color. This suggested that, 1) defective pigment synthesis in testes and malpighian tubules might also be affected by high temperatures, and that, 2) TSPs for mutant pigment syntheses might be established. The Shift studies carried out to determine the TSPs for pigment production will be described with the Results.

C. Spectrofluorometric Analysis of Pigment Production

The fluorescent pigments (FP) of the various tissues were separated by thin layer chromatography, using MN 300 cellulose with $CaSO_4$ binder (Machery and Nagel) spread to a thickness of approximately 250u on glass plates. The plates were developed by ascending chromato-

FIGURE 6

Percent of E6 pupae which eclose after Shift-ups and Shift-downs at different times during development. The developmental states present at each culture age at 22°C and 29°C are shown at the bottom.


100
80
60
40
20
% ECLOSION
0
30
60
90
120
150
180
210
240
AGE OF CULTURE (HOURS) AT SHIFT
Shift-up
Shift-down
22°C
EGG
1st INSTAR
2nd INSTAR
3rd INSTAR
PUPA
ADULT
DEVELOPMENTAL STAGE
29°C
EGG
1st INSTAR
2nd INSTAR
3rd INSTAR
PUPA

graphy in the dark at 22° for three to four hours in solvents of either propanol: 1% NH_3 water (2:1), or on occasion, butanol: acetate: H_2O (20:3:7). The latter solvent had the advantage of resolving FP-3 to FP-6 better than the propanol: NH_3 water solvent; however, it did not resolve (or only poorly resolved) FP-7 to FP-9. Consequently, the propanol: NH_3 water solvent was used for the bulk of the experiments. The plates were allowed to dry for one hour in a fume hood and were then examined with a U.V. light source and scored visually. The quantity of each fluorescent pigment was determined spectrofluorometrically using an Aminco Bowman scanning fluoromicrophotometer with a Varicord recorder.

Eye pigments were obtained from intact heads of adult flies decapitated by a microscalpel. The heads were placed directly onto the thin layer cellulose plates and crushed with the end of a glass rod in order to release pigment directly onto the cellulose thin layer plates (Hadorn and Mitchell, 1961).

Pigments from the testes and malpighian tubules were more difficult to obtain. Testes were removed from adult flies by microdissection in Drosophila Ringer's solution (Ephrussi and Beadle, 1936) and transferred to the thin layer plates with a minimum of accompanying D. Ringer's solution. One pair of testes from a single fly was used per run. Malpighian tubules of either 3rd instar larvae or adult flies were dissected and chromatographed in the same manner. While the fluorescent pigment of malpighian tubules from single individuals (4 strands) was sufficient for quantitation, generally malpighian tubules from two identically-treated individuals were pooled to increase the amount of pigment per run and the accuracy of our measurements. Crushing of the testes and malpighian tubules was not necessary for good recovery of

fluorescent pigment.

III. Results

A. Temperature effects on eye and testis pigmentation.

The TSP for eye and testis pigmentation was determined in the following manner: 15-20 prepupae from a 22° culture were collected within a 15 minute interval and shifted up to 29°. Starting twelve hours after this Shift-up, different vials of pupae were shifted down again at successive one hour intervals until 70 hours after prepupal formation. Adult flies from each vial were counted and their eyes and testes analyzed for pigment production as described above. The percent eclosion (number of adults/number of pupae) for each vial was calculated in order to show that the TSPs for lethality and eye-testes pigmentation are definitely separable.

The heads of a total of 362 E6 and 173 Oregon-R flies raised at different temperatures (17°C, 22°C, 29°C) were chromatographed and compared. It was found that anywhere from 7 to 11 fluorescent pigments could be separated from both the mutant and + strain, depending on the preparation and the quality of the thin layer plate. Generally, 8 pigments were resolved easily with the propanol: ammonia solvent. These pigments were designated as FP-1 for that fluorescent pigment running closest to the origin (smallest Rf) to FP-8 for the fluorescent pigment running nearest the front. No attempt was made to characterize the pigments by their absorption or fluorescent spectra. However, by comparing our plates with the data and descriptions of other workers (Hadorn, 1958, 1962; Narayanan and Weir, 1964), some pteridine pigments could be tentatively identified. The two eye pigments consistently affected in E6 by growth at high temperature were FP-1 and FP-8; FP-1 corresponds to drosopterin. The drosopterin level in the mutant raised at 29° was

decreased by about 30% as compared to Oregon-R (Figure 7A).

At 22°, E6 female eyes were visually wild-type whereas males were distinguishably mutant; at 17°, the eyes of both females and males were indistinguishable from wild-type. Chromatographically, drosopterin levels in flies raised at 17° were 80% in E6 males and 90% in E6 females of those in comparable Oregon-R flies. It is interesting to note that the drosopterin level in females raised at 29° was approximately equal to that in males raised at 22° and the level in females raised at 22° approximated that of males raised at 17°. From Shift studies, the TSP for eye pigment formation was determined to begin 52-56 hours after pupation at 29°. The end of the TSP was not measured but it was assumed to continue until eclosion (Counce, 1957).

The pigment content of the testis differed somewhat from that of the eye and malpigian tubules. Only five fluorescent pigments were consistently separable from the testes of both the E6 mutant and Oregon-R males. The drosopterin pigment was either missing, or masked by the bright blue FP-1 (isoxanthopterin) of the testes in both the E6 mutant and Oregon-R males. While FP-2 was present in E6 males grown at 22° or 17°, it was absent in the E6 males shifted to 29° at the onset of pupation (Figure 7B). The level of FP-1 found in males raised at 22° or 17° never reached the level of the wild-type controls.

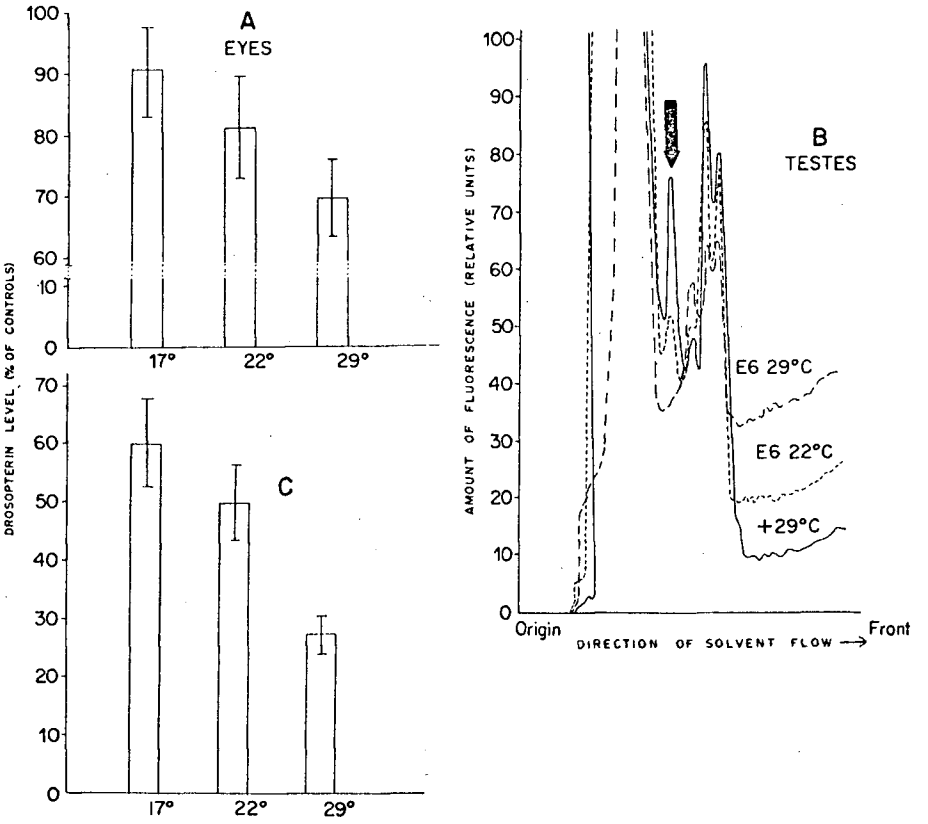
The Shift studies showed that the onset of the TSP for pigment synthesis in testes at 29° occurred 56-60 hours after pupation which was very close to the TSP for eye pigment. The end of the TSP for pigment formation in testes was not determined. The percent eclosion in the various vials varied from 70% to 90% of the rate of survival determined for E6 at 22°. Therefore, the TSP for lethality was definitely prior to

FIGURE 7

(A) The drosopterin (FP-1) levels (with confidence limits) in the eyes of E6 females relative to the level of Oregon-R females raised at 29°C.

(B) The distribution of fluorescent pigments in thin layer chromatograms of testes of E6 and Oregon-R males raised at different temperatures. The arrow indicates the pigment (FP-2) measured to determine the TSP.

(C) The drosopterin (FP-1) levles (with confidence limits) in the malpighian tubules of E6 flies relative to the level in Oregon-R flies raised at 29°C.


A
EYES
C
B
TESTES
DROSOPTERIN LEVEL (% OF CONTROLS)
AMOUNT OF FLUORESCENCE (RELATIVE UNITS)
17°
22°
29°
E6 29°C
E6 22°C
+29°C
Origin
Front
DIRECTION OF SOLVENT FLOW

the TSPs for eye and testis pigmentation.

B. Temperature effects on malpighian tubule pigmentation

The effects of temperature on pigment production in the malpighian tubules was determined by growing cultures of E6 at the restrictive temperature until 3rd instar and then shifting them down to 17°. Pigments in malpighian tubules from either 3rd instar larvae or adult E6 flies were chromatographed following the Shift-down and compared with those of E6 larvae or adults raised entirely at 17° and Oregon-R flies from high or low temperatures. The beginning of the TSP for pigment synthesis in the malpighian tubules was determined by collecting eggs laid within one hour at 29° and shifting individual cultures down to 17° at successive four hour intervals until the 36th hour. The malpighian tubules of 3rd instar larvae were scored for pigment by quantitative TLC. The end of the TSP for malpighian tubule pigment synthesis was delineated by sequential Shift-ups at successive 12 hour intervals of cultures established from two hour egg lays. Shift-ups were carried out from the time of egg deposition to late third larval instar stages and malpighian tubules removed from late third instar larvae were assayed for pigmentation.

The fluorescent pigments found in the malpighian tubules were the same as those in the eyes. FP-1 (drosopterin) was nearly absent in malpighian tubules in third instar E6 larvae raised at 29°, while it was detectable in E6 larvae raised at 22° and increased to approximately 50% of that found in Oregon-R larvae at 17° (Figure 7C). FP-8 was not present in sufficient quantity in either Oregon-R or E6 malpighian tubules to permit quantitative comparisons.

Shift studies indicated that the TSP for pigment synthesis was from approximately 12 hours after egg deposition at 29° to about 120 hours at 17°. The end of the TSP for pigment synthesis (delineated at 17°) corresponded to the end of the 2nd larval instar.

To summarize, for flies growing at 22°, the TSP for malpighian tubule pigmentation was 12 hours to 90 hours, for lethality 153 hours to 187 hours, for eye pigmentation 224 hours to eclosion, and for testis pigmentation 228 hours to eclosion (Figure 8).

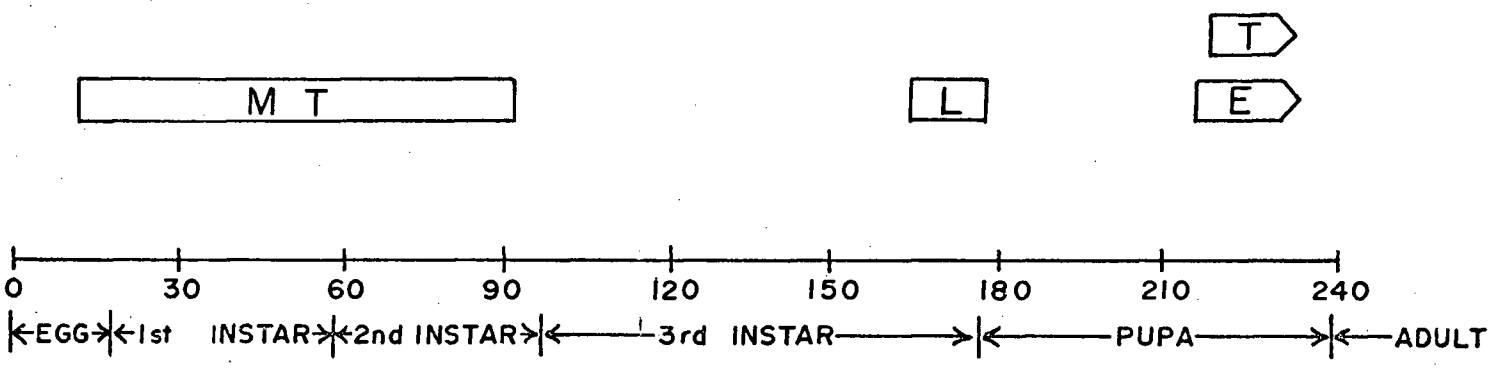
C. Autonomy of pigment formation in different organs and pigment turnover

Since the TSPs for pigment synthesis varied from organ to organ, it is tempting to conclude that the time of gene activity varies from organ to organ. Indeed, Stern and Tokunaga (1968) demonstrated the autonomous pleiotropy of the spl mutation in eyes and mesonota. However, in our experiments, the presence of pigment in a tissue does not preclude transport of the molecules from other tissues and therefore, the presence of pigment in a tissue cannot be assumed to reflect its synthesis in that tissue. A demonstration of tissue autonomy for pigment production would strengthen the suggestion that pigments are synthesized at the site of deposition.

The first experiment consisted of a series of Shift studies. Cultures collected within a two hour period at 29° were shifted down to 22° at 80 hours (mid-3rd instar) just prior to the TSP for lethality. Half of these flies were left at 22° until eclosion (Group A) while the other half was shifted back up 12 hours after pupation (Group B). Thus, flies in Group B were kept at 29° except during the TSP for lethality.

FIGURE 8

The temperature-sensitive periods in E6 for lethality and pigment levels in the eyes, testes and malpighian tubules (on a 22°C time scale).



AGE (HOURS AT RT) AND DEVELOPMENTAL STAGE FOR EACH TSP

Concomitantly, eggs were collected at 22° and half were shifted up 12 hours after pupation (Group C) and the other half left at 22° until eclosion (Group D). The adult flies that emerged from these Shift studies were scored for eye, testis and malpighian tubule pigmentation.

The results of the Shift studies are shown in Table 2. It can be seen that the mutant malpighian tubule phenotype was determined irreversibly by early exposure to 29° whereas growth at low temperatures up to pupation did not affect eye or testis pigmentation after a Shift-up. These experiments ruled out significant transport of pigment from the malpighian tubules to the eyes and testes and vice versa, a result supporting the autonomy of each tissue.

Tissue autonomy of pigment synthesis was also tested in mosaic females using the unstable ring X chromosome, $\underline{In(1)w^{vc}}$ (Hinton, 1955). $\underline{In(1)w^{vc}}/\underline{1(1)E6^{ts}}$ females were raised continuously at 29° and the adults scored for the presence of mutant eye tissue. Since the somatic loss of $\underline{In(1)w^{vc}}$ produced cells hemizygous for E6, the observation that mutant patches of eye tissue were readily detected in a wild-type background constituted the unequivocal genetic proof of autonomy of E6. Several bilateral mosaics for external genitalia were also observed. Both approaches suggest that the E6 gene is acting autonomously in all three organs.

TABLE 2

Phenotypes of malpighian tubules and eyes after each kind of Shift (see Figure 3).

Temperature Shifts	Type of Shift	Phenotype		
		Malpighian Tubules*	Eyes**	Testes*
A	Shift-down before TSP	mutant	wild-type	wild-type
B	Shift-down before and after TSP	mutant	mutant	mutant
C	Shift-up after TSP	wild-type-like	mutant	mutant
D	22°C control	wild-type-like	wild-type	wild-type

* based on chromatographic profile
** determined visually

IV. Discussion

It has been shown that a mutation which results in a mutant phenotype in different tissues acts autonomously in each tissue (Stern and Tokunaga, 1968). Our experiments have corroborated this and have demonstrated that the amounts of those fluorescent pigments affected by E6 are sensitive to temperature in different organs at different times during development (Figure 8). Since the genetic mapping studies indicated that a single mutation was responsible for both lethality and pteridine synthesis or deposition, at least three separable TSPs have been delineated during development. We assume that the lethal effect of E6 occurred in tissues other than the organs scored chromatographically since mutant patches of eye and external gonadal tissue were observed in mosaic flies raised at 29°C.

The significance of these results is dependent upon the interpretation of the TSP in molecular terms. In micro-organisms, ts lethality has been shown to be a consequence of missense mutation which results in the thermolability of the polypeptide gene product (Jockusch, 1966). The genetic properties of ts lethal mutations in Drosophila strongly suggest a basis for temperature-sensitivity similar to that in micro-organisms (Suzuki, 1970).

The genetic proof that expression of the E6 phenotype in each organ was autonomous suggests that the E6 locus does indeed function in the organs in which pigment was measured. However, it should be stressed that the concentrations of the pigments measured were quite likely an indirect result of the activity of the E6 locus since many different pteridine and ommachrome eye mutants affect the amount of drosopterin produced (Hadorn and Mitchell, 1951). Furthermore, the complete via-

bility of white eyed mutants which are totally lacking in pigments shows that the fluorescent compounds *per se* are not necessary for viability. Hence, it could be suggested that a single period of genetic activation (transcription) occurs early in development in all tissues; the resultant gene product could be a long-lived masked messenger RNA (Tyler, 1967) or an inactive polypeptide (Neurath, 1964; Steiner and Oyer, 1967; Steiner *et al.*, 1967) with a temperature-sensitive translational or activation mechanism, respectively. Although these possibilities cannot be eliminated, their contrivance and special assumptions required, render them unattractive. Waddington's experiments (Waddington and Robertson, 1969) showed that pigment synthesis in optic discs was suppressible by actinomycin D treatment in early pupation, a result strongly supporting the hypothesis that the synthesis of RNA and proteins involved in pigment formation occur close to the time of pigment production in the eye itself. It is therefore suggested that the *E6* locus is activated in different organs in response to tissue specific stimuli at different developmental stages.

The occurrence of the TSP for a mutant phenotype following pupation suggests a number of experiments. The effects of ecdysone on macromolecular synthesis in imaginal discs may be tested on the induction of pigmentation in explanted and transplanted imaginal discs. The discs in which *E6* manifests its lethality may also be indirectly detected using somatic elimination of the unstable ring X chromosome. For example, the recovery at 29° of viable mosaics in which one eye was wild-type while the other was mutant in color or which carried male genitalia indicates that lethality due to *E6* does not occur in tissues derived from eye and genital imaginal discs. Nolte (1959, 1961) has shown by

light and electron microscopy that $\underline{ras}^2$, a non-ts allele of E6 which also manifests depressed levels of drosopterin, has a disorientation of the secondary pigment cells of the eye and some irregularly shaped pigment granules. Of immediate interest is whether temperature affects the normal formation of secondary pigment cells and pigment granules or transport and deposition of pigment in the eyes of the E6 mutant.

CHAPTER 4

THE TEMPERATURE-SENSITIVE HOMEOTIC MUTANT, ss^{a40a}

I. Introduction

Hadorn's (1965, 1967) demonstration of "transdetermination" in Drosophila, that is, a change in determination of larval imaginal discs which results after repeated transplants through adult hosts, has renewed interest in the problem of disc determination. His experiments demonstrated that the rigid program in the discs could, nevertheless, be altered at the phenotypic level. Furthermore, the sequence in changes was highly specific, that is, genital discs could not be transdetermined directly to wings, rather they first had to be altered to legs and then to wings. Biochemical studies of transdetermination are difficult owing to the mechanical limitations of transplantation techniques. However, a class of mutations called "homeotic" mimics the kinds of phenotypic changes in determination resulting from serial transplantation (Goldschmidt, 1945).

By correlating the time of X-ray-induced somatic crossing over during antennal disc development, the switch in disc determination from antennal to leg triggered by the homeotic mutant, Antennapedia, was inferred to occur after the beginning of the 3rd larval instar stage (Postlethwait and Schneiderman, 1969). The expression of the homeotic mutant, proboscopedia (pb), was found to be dependent upon temperature (Villee, 1944). By shifting cultures from one temperature to another, the temperature-sensitive period (TSP) for the expression of this mutation was also found to occur in the third instar stage

(Vogt, 1946a). Extensive genetic, developmental and biochemical studies of the mechanism of homeotic effects have been hampered by low penetrance and variable expressivity of such mutants. This chapter concerns the developmental effects of a selected strain of the temperature-sensitive (ts) homeotic mutation, $\underline{ss}^{a40a}$, which was completely penetrant.

II. Materials and Methods

In a search for a fully penetrant ts homeotic mutant, all homeotic mutations currently available from stock centers were tested for their penetrance and the degree of expression of the mutant phenotype at 29°, 22° and 17°C. Flies homozygous for an allele of spineless-aristapedia, $\underline{ss^{a40a}}$ (see Lindsley and Grell, 1968), a mutant which transforms the arista of the antennal complex to a tarsus-like structure characteristic of the distal part of legs, were totally wild-type when raised at 29°C and showed a high penetrance of the mutant phenotype at 17°C. Individuals expressing a total transformation of aristae to tarsi at 17°C were selected and inbred for 8 generations to produce the stock reported here. The fourth and fifth antennal segments and the arista (the sixth antennal segment) were tarsus-like at 17°C. The normal legs generally did not show swelling and fusion of the four tarsal joints usually characteristic of $\underline{ss^{a}}$ flies and in males, the sex combs were neither enlarged nor present on the second pair of legs. The bristles were essentially wild-type.

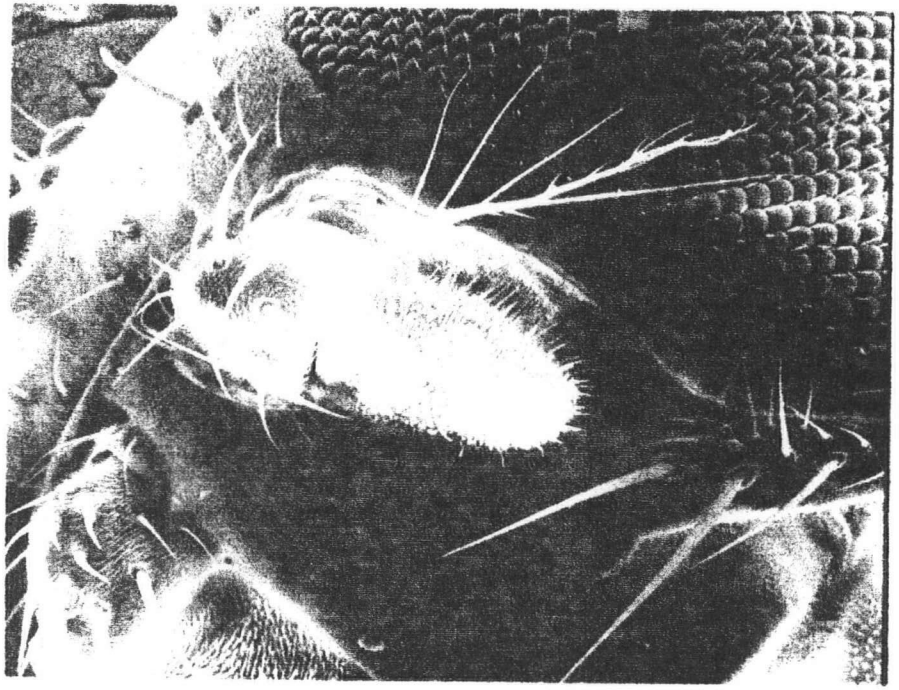
Virtually all of the flies had normal aristae when raised at 28°C (Figure 9a). At 22°C, about 85% of the flies had a protrusion of a leg-like structure from the third antennal segment with a normal though somewhat shorter arista extending from the tarsal elements (Figure 9b). Expressivity of the mutant phenotype at 22°C varied from a slight swelling at the proximal end of the arista to a leg-like structure which was approximately half the total length of the arista. About 15% of the flies grown at 22°C had normal aristae with no leg-like structures. At 17°C, the aristae of over 98% of the flies were completely transformed to tarsal segments of the leg (Figure 9c). Although

FIGURE 9

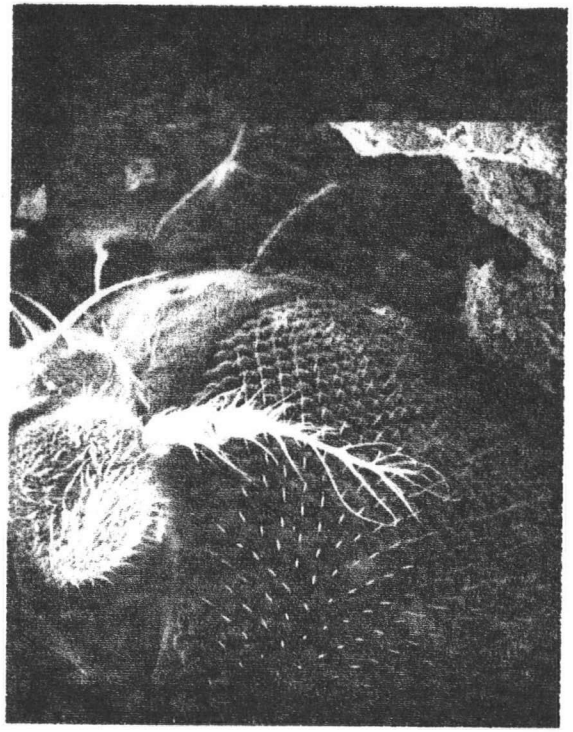
A scanning electron micrograph of $\underline{ss^{a40a}}$ raised at:

a) 28°C
b) 22°C
c) 17°C
d) a wild-type Oregon-R raised at 22°C, magnification approximately 280X.

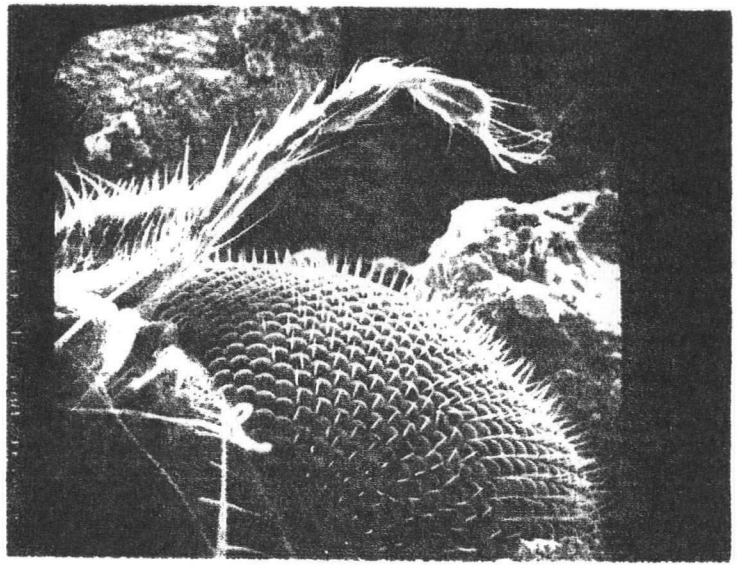
a



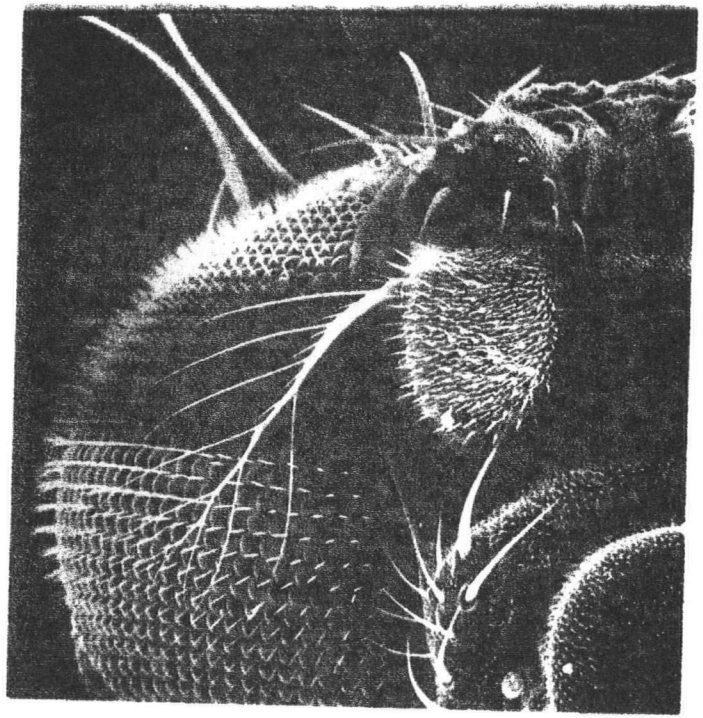
b



c



d



transformation of aristae to leg-like structures was not complete in a few flies, the mutation was 100% penetrant at 17°C.

The temperature-sensitivity of $\underline{ss}^{a40a}$ permitted a delineation of the actual developmental interval during which the expression of mutation could be altered. This was accomplished by collecting eggs on petri plates for a one hour period, transferring groups of 50 to 75 eggs to individual vials and shifting such cultures established at 17° and 28°C to 28° and 17°C, respectively, at successive times (Suzuki, 1970). This provided a crude delineation of the TSP, which was then determined more accurately by a second series of experiments which were carried out in the following manner. Asynchronously developing cultures were established at 28° and 17°C. As soon as the first pupa was detected, the cultures were shifted to 17° and 28°C, respectively. Newly formed prepupae were collected at 1 hour intervals, placed into vials and left to develop with no further shifting. In this manner, the interval between the shift and pupation could be accurately defined. The phenotypes of flies hatching after the shift were then determined.

The developmental interval during which temperature affected the expression of $\underline{ss}^{a40a}$ was determined from the shift experiments by the following rationale. Flies hatching from cultures shifted down prior to the TSP will pass through that interval at the low temperature and therefore will express the mutant phenotype, whereas cultures shifted down after the TSP will yield wild-type adults. Reciprocal results are expected from the shift-up experiment. The TSP could be readily timed in this way. The phenotypes of flies shifted within the TSP were of special interest. The discs producing jointed appendages represent concentric rings of cells which evaginate to form each segment. We may

ask what cells within the disc are affected by temperature. The observation of a change at the aristal base at 22°C rather than a salt and pepper mosaic of leg and aristal structures showed a polarity of effect on the adult organ. If the events leading to the transformation of aristae to leg structures resulted from a wave or gradient of determinative substance passing through the antennal disc, then one might expect that aristae of flies shifted up during the TSP would show a leg-like structure in the place of the proximal portion of the arista with a normal aristal segment protruding distally from a leg-like base (Pattern I, Figure 10). Similarly, flies hatching from cultures shifted down during the middle of the TSP might be expected to show a normal arista from which a leg-like structure protruded distally (Pattern II, Figure 10). Therefore, flies hatching from shift cultures were scored for the relative amount and location of leg and arista tissue and each arista was placed into one of ten categories shown in Figure 10.

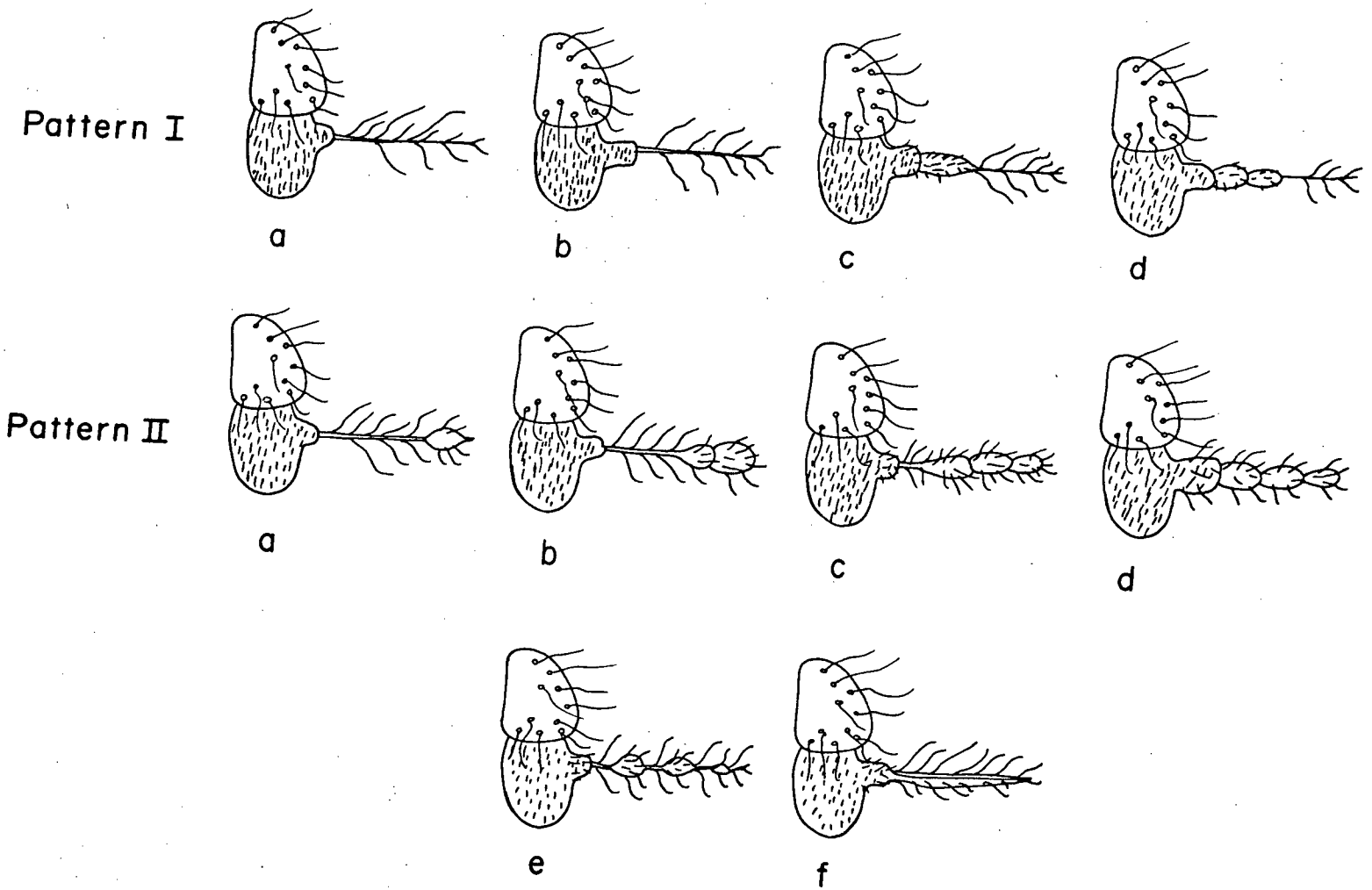
Finally, in order to enhance the change of the antennal complex to leg structures, $\underline{ss^{a40a}}$ was combined with the homeotic mutant, $\underline{Antp^{B}}$ (Lewis, 1956) which converts the antenna into leg structures. The structure of $\underline{ss^{a40a}}$ +/$\underline{ss^{a40a}}$ $\underline{Antp^{B}}$ was compared with $\underline{ss^{a40a}}$ +/ + $\underline{Antp^{B}}$ and $\underline{ss^{a40a}}$ +/$\underline{ss^{a40a}}$ + at 22°C.

FIGURE 10

Diagram of the entire antennal complex. Shows the possible phenotypes of flies hatched from various shift experiments.

Pattern I (a) normal arista; (b) proximal 1/4 of arista transformed to tarsus, distal 3/4 normal arista; (c) proximal 1/2 transformed to tarsus, distal 1/2 arista; (d) 3/4 tarsus, 1/4 arista.

Pattern II (a) proximal 3/4 of arista is normal while distal 1/4 is transformed to tarsus; (b) proximal 1/2 of arista normal, distal 1/2 transformed to tarsus; (c) proximal 1/4 arista, distal 3/4 tarsus; (d) complete transformation to tarsus; (e) salt and pepper pattern of mixed arista and tarsus tissues; (f) stripes of arista and tarsus tissues.


Pattern I
a
b
c
d
Pattern II
a
b
c
d
e
f

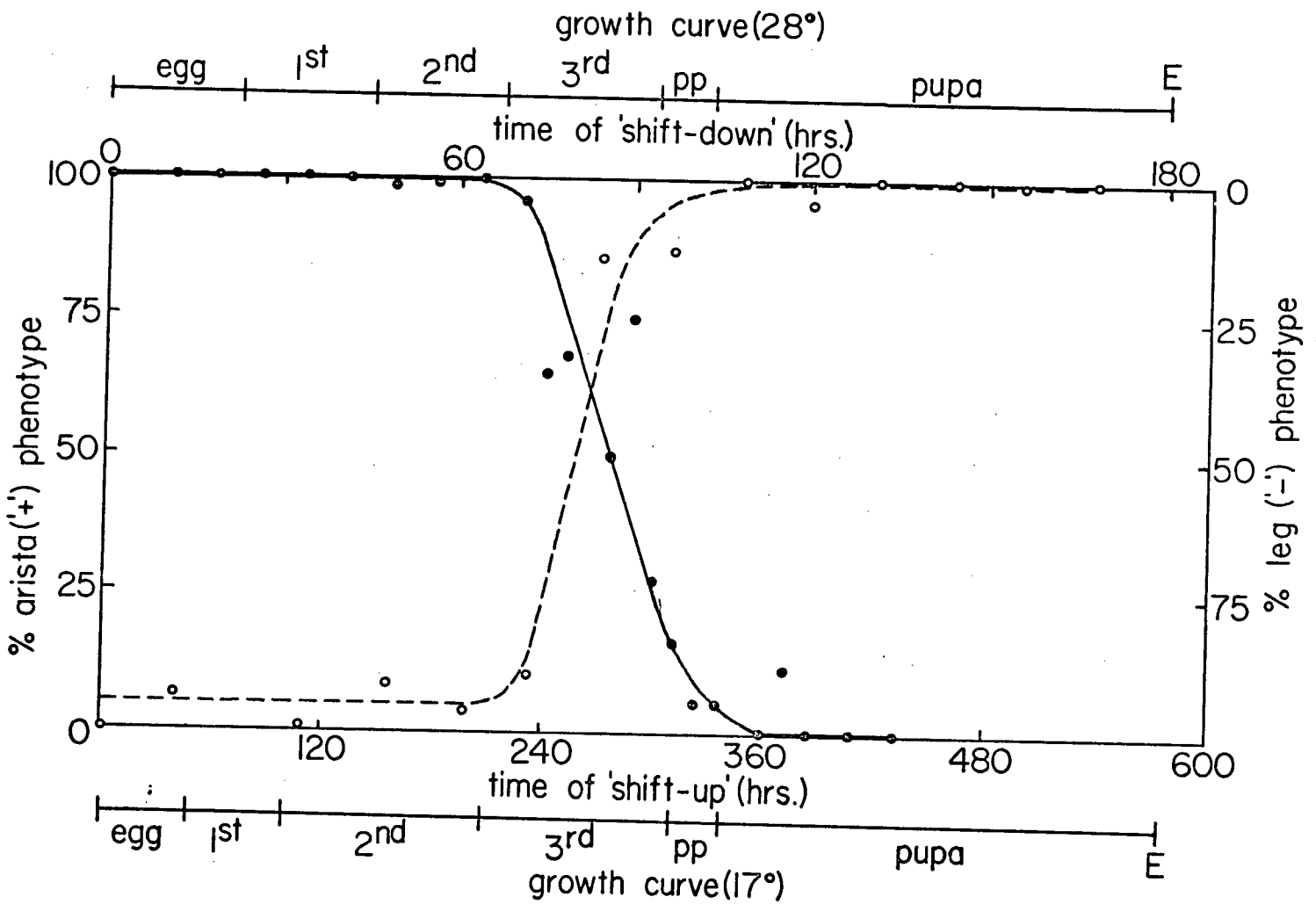
III. Results and Discussion

These studies demonstrate that highly penetrant ts homeotic mutations can be recovered. This result is of considerable importance since bulk preparations of discs in a homogeneous state are required for biochemical studies of the mechanisms of determination. The mutant expression of $\underline{ss}^{a40a}$ occurs at 17°C; therefore, the first culture in shift-up experiments which produces adults with a leg-like transformation of the arista indicates the beginning of the TSP. Similarly, the first shift-down culture which yields wild-type flies signals the end of the TSP. By these criteria, the TSP was approximately 6 hours in duration at 28°C and 18 hours in duration at 17°C, and occurred during the second quarter of the third larval instar stage (Figure 11). This is a very short TSP and may be suggestive of a critical event during disc development when the final "lock" on the fate of those cells occurs.

The TSPs of the other two alleles of spineless-aristapedia and of proboscopedia also have been shown to occur sometime during the third larval instar stage (Vogt, 1946a; Villee, 1943). Mosaic spots generated by somatic crossovers have shown that $\underline{Antp}^{R}$ also affects the antennal discs during the third larval instar stage (Postlethwait and Schneiderman, 1969). Clearly, the change in disc fates caused by homeotic mutants occurs during the third larval instar long after the initial determinative events have occurred (Gehring, 1969). These mutants suggest that critical determinative events occur early in the third larval instar at a time when extensive cell division and proliferation take place in the imaginal discs. We are currently attempting to determine whether ts homeotic mutations which affect earlier determina-

FIGURE 11

Results of shift studies - closed circles are from shift-up experiment; open circles from shift-down experiment.


growth curve(28°)
egg
1st
2nd
3rd
pp
pupa
E
time of 'shift-down' (hrs.)
0
60
120
180
100
75
50
25
0
% arista('+') phenotype
0
25
50
75
% leg ('-') phenotype
120
240
360
480
600
time of 'shift-up' (hrs.)
egg
1st
2nd
3rd
pp
pupa
E
growth curve(17°)

tive events can be recovered. What is still uncertain is whether the homeotic mutations represent a class of genes which are active from the post embryonic stages (after the time of initial determination of the discs) through the 3rd instar stage; or whether, in fact, these genes are active only during the third larval stage of development. Experiments are under way which may distinguish between these two alternatives.

It is interesting to note that of the homeotic mutants which are affected by temperatures, all are sensitive to cold temperature and only one, proboscopedia, is also affected by exposure to high temperature. In micro-organisms, cold sensitivity of mutations appears to be a property more common among those concerned with regulation or molecules which can self-assemble (Guthrie, *et al.*, 1969). It is of interest whether this suggests the regulatory mechanism of homeotic loci. Selection for cold-sensitive homeotic mutations may increase the yield of such mutants.

Transplantation experiments have shown that the mutant ss^{a} (an allele of ss^{a40a}) acts autonomously (Braun, 1940). This indicates that the temperature-modifiable changes in determination of the ss^{a40a} does not result from a freely circulating "humoral" factor.

Shift studies during the TSP of N^{60g11}, a temperature-sensitive mutation which disrupts eye facet arrangement, revealed a wave of facet orientation that proceeded from the posterior rim of the eye anteriorly (Foster and Suzuki, 1970). It was asked whether such a wave or gradient of determination also occurred in the aristae in response to ss^{a40a}. Shifts up and down were carried out at different times during the TSP and the aristae of the adults examined. It was found that individuals shifted up during the early part of the TSP had leg-

like structures only in the proximal part of the transformed arista, whereas after shifts up at successively later times within the TSP, the leg structures increased distally as the size of the arista retreated. However, unlike the facet pattern, shifts down during the TSP did not yield a reciprocal pattern. Shifts down early in the TSP yielded aristae that were transformed to legs proximally with aristal tissue distally. Successively later shifts down gave more arista proceeding proximally. These results are summarized in Table 3 and indicate that the change in determination is not simply the reflection of a wave or gradient of determination passing through the disc or to a ts receptor site for such a gradient. The results may indicate that there is significant cell movement within late third instar discs. Alternatively, there could be a site within the antennal disc which, in response to cold temperature, produces a substance which alters the final determination of the existing pre-determined aristal cells.

Flies which were genotypically $\underline{Antp}^{B}$ $\underline{ss}^{a40a}$/+ $\underline{ss}^{a40a}$ had the entire antennal complex transformed into a complete leg-like structure at 22°C (Figure 12). The mutant phenotype was completely penetrant. In a few cases, the transformation was so complete that the males had sex combs on their antennal legs. These two mutations supposedly affect different segments of the antenna and when studied separately in the original stocks, expression of each mutant at 22°C was quite low. The fact that the two mutants interacted for 100% expressivity and a total transformation of antenna to leg, leads us to ask whether cell communication within discs may be important in determining the final phenotype. This question is currently under investigation.

TABLE 3

Expected and actual results from shifts during the TSP.*

	Shift down	Shift up
A	Pattern I d ⟶ a	Pattern I a ⟶ d
B	Same as above	Pattern II a ⟶ d
C	Pattern II d ⟶ a	Pattern I a ⟶ d

A. These are the actual results from the shift experiments. These do not agree with either of the expected results if the change in determination is due solely to a wave or gradient of determination passing through the discs.

B. These are the results expected from shift experiments if a gradient of determination passes from anterior to posterior through the disc.

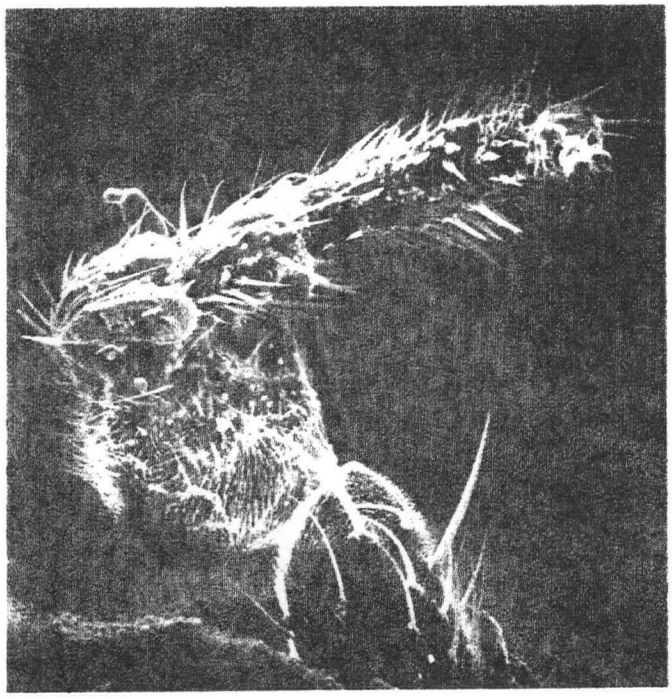
C. These are the results expected from shift experiments if a gradient of determination passes from posterior to anterior through the disc.

* The term "Pattern" refers to diagrams in Figure 10.

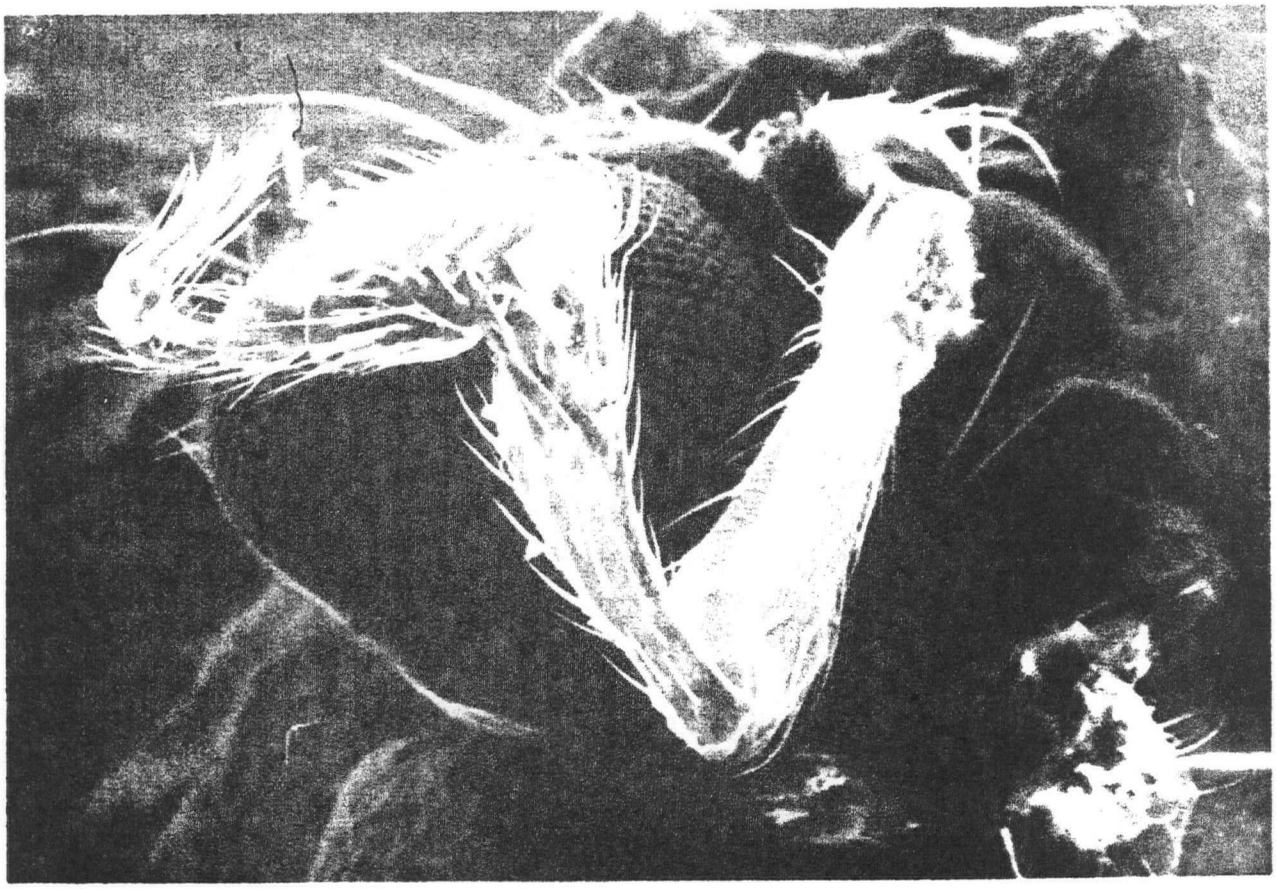
FIGURE 12

A scanning electron micrograph of $\underline{Antp}^{B}$, $\underline{ss}^{a40a}$/+ $\underline{ss}^{a40a}$ raised 22°C, magnification approximately 210X.

a



b



CHAPTER 5

A TEMPERATURE-SENSITIVE MUTATION (*para*^{ts}) CAUSING ADULT PARALYSIS IN *DROSOPHILA MELANOGASTER*

I. Introduction

The genetic regulation of neural structure and function and its relationship to behavior are of fundamental biological interest. The ready induction and recovery of mutations affecting behavior and their genetic manipulation in Drosophila are now being exploited in a number of laboratories (Hotta and Benzer, 1969, 1970; Pak *et al.*, 1969; Ikeda and Kaplan, 1970a). By selecting flies manifesting the phenotype of paralysis, we felt that mutations affecting nerves and/or muscles could be efficiently obtained. However, flies exhibiting such a mutant phenotype would not be expected to be viable; therefore, we searched for mutations that showed conditional paralysis that was temperature-dependent (Williamson *et al.*, 1970; Grigliatti *et al.*, 1970). This chapter concerns the discovery and properties of such a mutation, paralytic-temperature-sensitive (*para*^{ts}), in *Drosophila melanogaster*.

II. Materials and Methods

Newly eclosed adult Oregon-R males were fed the mutagen, ethyl methanesulfonate (0.025M) dissolved in a 1% sucrose solution (Lewis and Backer, 1968). Twenty-four hours later, they were mated at 22°C to attached-X-bearing females in quarter pint bottles. Adult progeny were then placed in a preheated plexiglass screening apparatus which allowed the ready separation of immobilized flies from those retaining normal movement (Williamson, 1971). One to eight thousand adults at a time were placed in the box and left for 1/4 to 2 hours before selection. All motionless flies were then returned to 22°C; any which regained mobility were mated (males to XX females, females to Oregon-R males) and all offspring of fertile individuals were tested for paralysis at 29°C. The specific properties and details of analysis of the ts paralytic mutant recovered will be discussed in the next section.

III. Results

Table 4 shows the results of the screening. The bulk of the immobilized flies recovered were dead or sterile at 22°C. However, out of an estimated quarter of a million flies screened, one was detected which carried a mutation causing a temperature-sensitive paralysis.

A. Genetic Properties

The ts paralyzed fly was a male and all of its male progeny were paralyzed upon shifting to 29°C whereas the females were not, thereby showing this mutation to be sex-linked. Females heterozygous for the mutation were not paralyzed whereas homozygous females were. Consequently, the mutation was named paralytic-temperature-sensitive, $\underline{para}^{ts}$. The mutation was mapped genetically by crossing $\underline{para}^{ts}$ males to females carrying the markers (see Lindsley and Grell, 1968) (followed by their genetic positions): y (0.0), cv (13.7), v (33.0), f (56.7) and car (62.5) and testcrossing the F_1 females at 22°C. The $\underline{para}^{ts}$ and + male progeny of the testcross were separated at 29°C and then scored for the visible markers. The mutation was readily located 2.8 units to the left of f (2,993 males scored).

B. General Biological Properties

The effects of temperature on $\underline{para}^{ts}$ flies were then studied in detail. At 22°C, the mutants exhibited normal walking and flying ability. When adult $\underline{para}^{ts}$ flies were shifted from 22°C to 29°C, complete paralysis was induced in less than 5 seconds; upon shifting the paralyzed flies back to 22°C, mobility was recovered in less than 2 seconds. Paralysis and recovery could be induced repeatedly in the

TABLE 4

Results of screening adult offspring of mutagenized flies for paralysis at 29°C.

Type of flies	Number
Total flies screened (estimated)	250,000
Immobilized at 29°C	293
Dead	200
Recovered mobility at 22°C	93
Fertile at 22°C	34
Temperature-sensitive paralytic mutations	1

same individuals with no apparent harm. Flies maintained at 29°C for several hours recovered normal mobility immediately upon shifting down to 22°C. While paralysis was initially complete at 29°C, a recovery of some movement at this temperature could be seen. After 30 minutes at 29°C, the flies were able to right themselves and regained limited walking ability. After an hour or more the flies were capable of climbing up the sides of the vials. However, it must be emphasized that these flies were visibly weak and never regained the strength and coordination which $\underline{para}^{ts}$ flies showed at 22°C. Flies which had regained mobility after 2 hours at 29°C immediately recovered wild-type behavior upon shifts to 22°C and were paralyzed again upon shifting back to 29°C. Preliminary tests suggest that $\underline{para}^{ts}$ flies left at 29°C for 12 hours, then shifted down to 22°C for 5 minutes and back up to 29°C are not paralyzed. Thus, initial 29°C recovery remains temperature labile, whereas long-term recovery at 29°C suggests a different basis for movement.

In order to determine whether $\underline{para}^{ts}$ also affected larval mobility, two tests were carried out. A direct test was made by placing 3rd instar larvae reared at 22°C into Drosophila Ringer's (Ephrussi and Beadle, 1936) solution at 29°C. The larvae continued to move normally for several hours and were totally unaffected by the increased temperature. A second method compared the relative fitness of $\underline{para}^{ts}$ individuals with wild-type flies at 29°C. Attached-X-bearing females (which were wild-type with respect to $\underline{para}^{ts}$) were crossed to $\underline{para}^{ts}$/Y males for 24 hours at 22°C. The inseminated females were allowed to lay their eggs for 3 days in fresh bottles at 29°C. The F_1 progeny were left to develop at 29°C until adults began to emerge, whereupon 219 late pupae

were collected, placed in fresh vials and half were shifted to 22°C. Twelve hours later, the flies had eclosed and the ratio of $\underline{para}^{ts}$/Y males to wild-type females was scored. At 22°C, the sex ratio was 97♂:122♀, showing that $\underline{para}^{ts}$ males had competed successfully with their wild-type sisters up to pupation at 29°C (females tend to hatch sooner than males). In the sample left at 29°C, a ratio of 83♂:215♀ was obtained. However, when the number and sex of adults found dead in the food and unhatched in their pupal cases was scored and included in the sex ratio, the ratio returned to 230♂:240♀. It was concluded that the $\underline{para}^{ts}$ mutation does not interfere with the development of the larval or early pupal stages and therefore is expressed only in differentiated adults. The fact that some males did eclose at 29°C could be accounted for by the earlier observation that $\underline{para}^{ts}$ adults recover some mobility after prolonged exposure to 29°C. The hatched males had obviously recovered sufficiently to crawl out of their pupal cases.

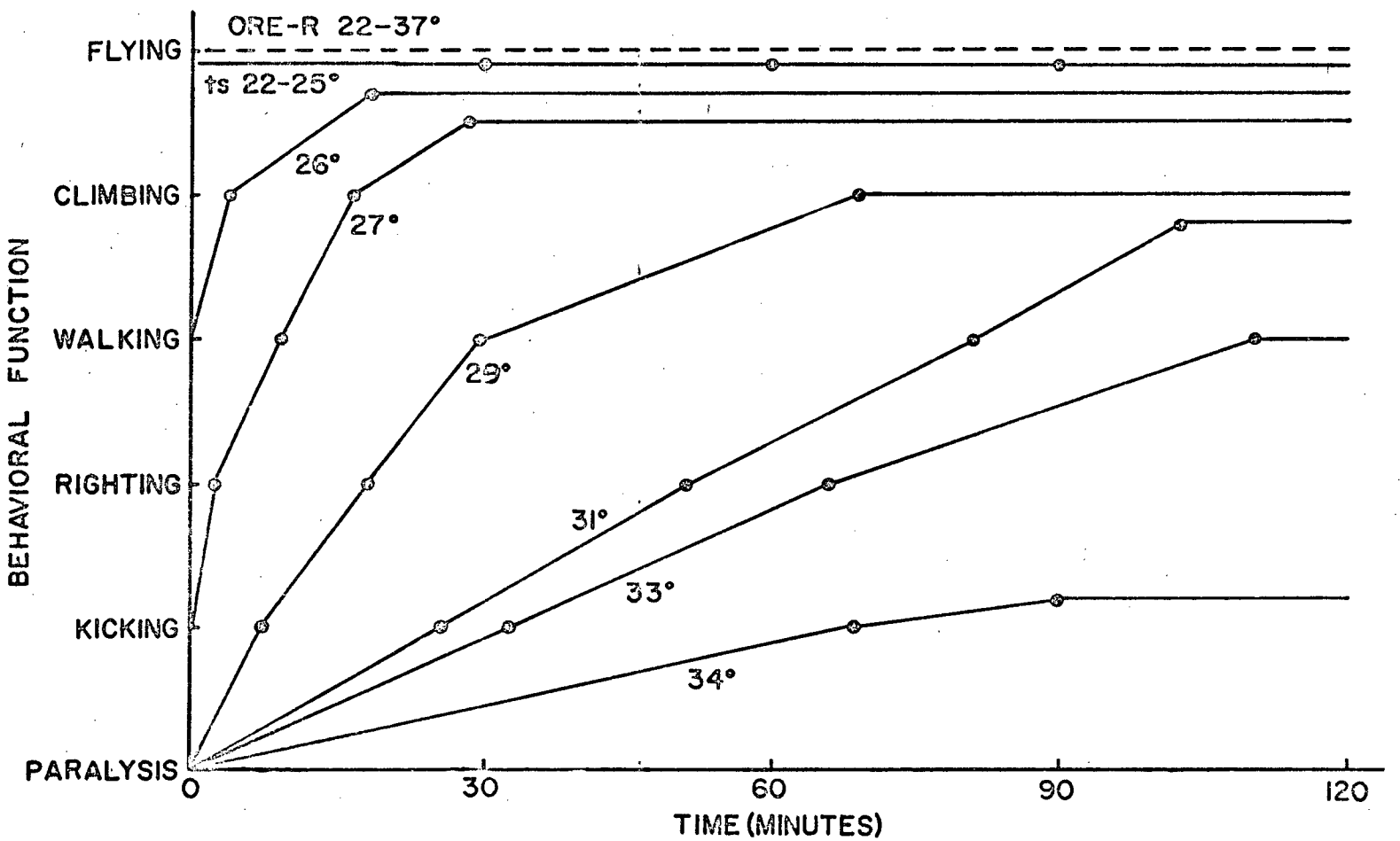
The effect of prolonged exposure of $\underline{para}^{ts}$ adults to different temperatures was then examined in order to determine the degree to which movement, co-ordination and recovery of activity were affected over a wide temperature range. A dissecting microscope was mounted over two identical water-tight chambers immersed in a temperature-controlled circulating water bath. Adult $\underline{para}^{ts}$ and + flies (at least 40 each) which had been raised at 22°C were placed into separate chambers and observed continuously for two hours at each temperature. Observations were made on different flies at one degree intervals between 22°C and 35°C. All movements from flying to tarsal twitching of individual legs were recorded; however, for analytical purposes, the behavior during

recovery has been classified into six categories: complete paralysis, kicking, ability of flies to right themselves, walking, climbing and flying. The results are presented in Figure 13. The units separating the categories on the ordinate are arbitrary so that the shapes of the curves are not especially significant. Oregon-R wild-type flies were not visibly affected over the entire range of temperatures from 22°C to 37°C. On the other hand, $\underline{para}^{ts}$ flies behaved normally only up to 25°C. They were visibly debilitated between 26° and 28°C and completely paralyzed at higher temperatures. With increasing temperatures, the phenotypic effects of the mutation became more severe and the length of time required for recovery was prolonged. For example, at 29°C, flies began to climb after 70 minutes, whereas those at 31°C took 105 minutes to regain the same ability. At 33°C, the flies showed only a weak capacity for walking toward the end of the observation period and at 34°C, only 5% of the flies were able to even right themselves. It should be added that after two hours at any temperature up to 33°C, all $\underline{para}^{ts}$ flies shifted down to 22°C recovered. However, after 2 hours at 34°C, only 10% of the flies remained alive after shifting down to 22°C.

Since $\underline{para}^{ts}$ flies can be maintained at 33°C for up to 2 hours, it was assumed that all vital organs still function in the paralyzed fly. Indeed, the heart, which can be easily seen through the dorsal wall of the abdomen, was observed to beat quite normally in paralyzed individuals. Similarly, slight movements of the abdomen which might result from gas exchange through the spiracles could be observed in $\underline{para}^{ts}$ flies at 29°C.

FIGURE 13

Recovery of behavioral activity of $\underline{para^{ts}}$ flies at different temperatures during a 2 hour interval. Each point represents the time at which at least half of the flies observed exhibited the behavioral trait.


ORE-R 22-37°
FLYING
ts 22-25°
26°
CLIMBING
27°
WALKING
29°
RIGHTING
31°
33°
KICKING
34°
PARALYSIS
BEHAVIORAL FUNCTION
0
30
60
90
120
TIME (MINUTES)

C. Tissue Specificity

It was then asked whether the $\underline{para}^{ts}$ mutation functions autonomously and whether any tissue specificity of the mutation could be determined. These questions could be answered by generating somatic mosaics of $\underline{para}^{ts}$ and + tissue. The ring chromosome, $\underline{In(1)w}^{vC}$ (see Lindsley and Grell, 1968), is somatically unstable and is lost with a high frequency in mitotically dividing nuclei (Hinton, 1955). By marking a normal rod X chromosome bearing $\underline{para}^{ts}$ with $\underline{y}$ (a recessive mutation which produces yellow cuticle and bristles), the loss of the ring chromosome (which carried the $\underline{y}^{+}$ and $\underline{para}^{ts^{+}}$ alleles) could be detected on the exterior chitin as yellow patches in a wild-type background (Bryant and Schneiderman, 1969). $\underline{In(1)w}^{vC}$,+ +/$\underline{In(1)dl\text{-}49}$,$\underline{y}$ $\underline{w}$ $\underline{spl}$ females were crossed to $\underline{y}$ $\underline{para}^{ts}$/$\underline{sc}^{8}$·Y,$\underline{y}^{+}$ males at 22°C. Mosaics of $\underline{In(1)w}^{vC}$,+ +/$\underline{y}$ $\underline{para}^{ts}$ zygotes were then recovered and the exact area of yellow tissue of each mosaic recorded. Each mosaic was then placed in a coded vial, and another person allowed to note the behavior of each fly under a dissecting microscope for 20 minutes at 29°C. Non-mosaic wild-type females were placed at random in coded vials as controls. After 150 mosaics had been scored, the data on the location of the mutant patches and the behavior at 29°C were compared.

The relation between specific behavioral patterns and the location of the patches was quite clearcut. From the mosaic data one could accurately predict the behavior of each fly at 29°C. A total of 300 mosaics was scored with the sizes of yellow tissue ranging from tiny patches involving one or two bristles to almost complete $\underline{y}$ $\underline{para}^{ts}$/O males. A detailed analysis of the mosaic data will be presented in the next chapter. The pertinent data has been summarized by placing the

mosaics into the following classes based on the location of yellow tissue: I - abdomen, II - head, III - thorax and IV - head-thorax. Each class could be further subdivided into specific subgroups which differed in behavioral patterns (Figure 14).

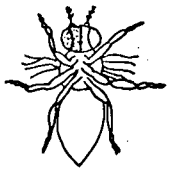
A description of the behavior of each class of flies indicated in Figure 14 can be seen in Table 5. All Class I mosaics were wild-type in behavior at 29°C. Flies of the reciprocal class in which only the abdomen was wild-type were completely paralyzed. Complete head mosaics (Class IIb) assumed a normal stance at 29°C but could not move, thus showing that $\underline{para^{ts^{+}}}$ head tissue is necessary for normal leg movement. Flies with mutant tissue in the legs only (Class IIIa) could move, but the legs were stiff during movement. This class is actually a composite of 38 different mosaics involving from 1 to 5 mutant legs. Of these, 19 had one mutant leg, 11 had mutant regions on 2 legs, 5 on 3 legs, 1 on 4 and 2 on 5 legs. Bilateral thoracic mosaics (Class IIIb) moved their legs on the wild-type side, but the mutant legs were paralyzed in an extended position. Mosaics having completely mutant thoraces (Class IIIc) were paralyzed, as were the complete head and bilateral thoracic mosaics (Class IVc). In addition, 18 flies having mutant tissue on the dorsal surface of the thorax only, were completely normal in mobility at 29°C. In another, the entire dorsal portion of the thorax and two legs were mutant yet the four + legs moved normally. These data clearly demonstrate dual components for normal leg movement - wild-type head for motion and wild-type thorax and legs for the posture and normal movement of each leg.

The use of mosaics to locate the regions governing normal mobility depends upon the detection of mutant tissue on the external

FIGURE 14

Classification of mosaic females generated by somatic loss of In(1)w^{vC} according to the ventral location of mutant tissue. The stippled area represents y $para^{ts}$ tissue in which the ring is lost.

I
abdomen only



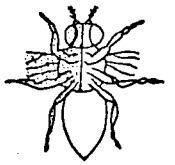
II a
bilateral head

II b
complete head

III a
leg only

III b
bilateral thorax

III c
complete thorax



IV a
bilateral head-thorax

IV b
bilateral head
complete thorax

IV c
complete head
bilateral thorax

IV d
complete head-thorax

TABLE 5

A correlation of the position of mutant patches and the pattern of paralysis of mosaic females at 29°C.

Class	Number of Mosaics	Location of Mutant Patch	Behavioral Characteristics at 29°C
I	6	Abdomen	wild-type
IIa	9	Bilateral head	walks, climbs in helical pattern
IIb	5	Entire head	stands normally, cannot move
IIIa	38	Legs only	walks in stilted manner, legs stiff
IIIb	30	Bilateral thorax	mutant legs paralyzed, wild-type legs continue to move
IIIc	7	Entire thorax	legs paralyzed
IVa	17	Bilateral head and thorax	mutant legs paralyzed, wild-type legs continue to move
IVb	8	Bilateral head, entire thorax	completely paralyzed
IVc	26	Entire head, bilateral thorax	completely paralyzed
IVd	9	Entire head and thorax	completely paralyzed

surface of the fly. It has been assumed that the external mutant patch is an indication that the underlying internal tissue is also mutant. Indeed, a good correspondence has been found in certain non-neural and some neural tissues (Ikeda and Kaplan, 1970a). Supporting evidence for this contention derives from our screening of 216 phenotypically non-mosaic $\underline{In(1)w^{vC}}$,+ +/$\underline{y}$ $\underline{para^{ts}}$ females and over 200 phenotypic $\underline{y}$ $\underline{para^{ts}}$/O males at 29°C. If, in fact, considerable internal mosaicism existed with was not indicated externally, we would have expected many of the externally non-mosaic females to exhibit aberrant behavior and some of the presumed X/O males to move at 29°C. This was not observed. All of the X/O males were completely paralyzed at 29°C. Twelve females initially screened as non-mosaic were observed to walk abnormally at 29°C; upon re-examination, 10 were found to be missing a leg, 1 had a mutant patch which had been overlooked and only 1 appeared to be completely wild-type.

When different classes of mosaics (Classes IIb, IIIc, IVc and IVd) were compared with $\underline{para^{ts}}$ flies for 2 hours at 29°C, there were no detectable differences in the rates of recovery of co-ordinated movement in the mutant areas. This finding, in conjunction with the observation that Class IIb, IIIc and IVb mosaics (Figure 14) cannot move at 29°C, shows that paralysis is not induced by the loss of a freely circulating factor necessary for movement. Moreover, the ability of Class I and IIa mosaics as well as the wild-type side of Classes IIB and IVa mosaics to move, rules out the existence of a freely circulating inhibitor of movement produced by the $\underline{para^{ts}}$ mutation.

D. Visual and Flight Response

Flies having mutant tissue around one eye (Class IIa, Figure 3) could walk at 29°C, but upon climbing vertically, invariably followed a helical path, always keeping the mutant eye up. We asked whether this behavior indicated blindness in the mutant eye. This could be tested easily by examining its optomotor response at 29°C. Wild-type flies invariably turn in the direction of moving stripes (Kalmus, 1948). When both eyes and ocelli of the wild-type flies were painted, these flies no longer showed a positive optomotor response. The wild-type eye and ocelli of bilateral head mosaics (Class IIa, Figure 14) were then painted. At 22°C, the flies showed a positive optomotor response, whereas the results at 29°C were ambiguous. On occasion, the flies did respond positively but much less strongly and more slowly.

We therefore measured the electrical response of the eyes of $\underline{para}^{ts}$ flies to light by recording the electroretinogram (ERG) (Hotta and Benzer, 1969, 1970). The ERG was obtained by Drs. Yoshiki Hotta and Seymour Benzer. A positive ERG was obtained at both 22° and 30°C. This result indicated that the eyes of $\underline{para}^{ts}$ flies do transduce light into electrical responses at 30°C.

IV. Discussion

The genetic and biological properties of the mutation, $\underline{para}^{ts}$, point to a specific defect in the nervous system of adults which regulates both flight and walking. The rapidity with which paralysis and recovery can be induced by temperature shifts argues against a direct involvement of de novo macromolecular synthesis. Co-ordinated activity at 29°C gained after a 2 hour exposure to 29°C is eliminated by a 5 minute exposure to 22°C (shift-down-and-up). This suggests that early recovery from restrictive temperatures does not involve processes different from those affected by an initial shift-up.

The lack of effect of $\underline{para}^{ts}$ abdomens on paralysis of flies having a wild-type thorax and head (Class I) shows the absence of some freely circulating inhibitor of mobility which might be made in any part of the fly. Furthermore, the complete autonomy of each half of bilateral thoracic mosaics (Class IIIb) in both paralysis and recovery time rules out a temperature-induced humoral inhibitor or temperature-resistant promoter of movement. On the assumption that the external phenotype demarcates the boundaries of internal nervous tissue of the same genotype, the mosaics demonstrate two components that govern normal movement; the head and the ventral thorax including wings. These regions correspond to the location of the cephalic and thoracic ganglia.

The normal ERG obtained at 30°C indicates that light can be transduced by the eye into electrical responses and rules out any generalized physiological effect of temperatures on CO_2 levels. The mutant, Hyperkinetic-1, $\underline{Hk}^1$, has a defect which causes rapid rhythmic impulse generation in the motor areas of the thoracic ganglion (Ikeda and Kaplan, 1970a). These impulses induce a characteristic leg twitching

in lightly etherized flies (Ikeda and Kaplan, 1970a). The double mutant $\underline{Hk}^1$ $\underline{para}^{ts}$, exhibits leg shaking at 22°C which immediately stops upon shifting to 29°C. This suggests that $\underline{para}^{ts}$ causes a disorder which affects neural tissue. Recent studies on the time of appearance of activity of acetyl choline esterase and choline acetyl transferase during development (Dewhurst *et al.*, 1970), prompted a study of these enzymes in $\underline{para}^{ts}$ flies. Activity of both enzymes was not reduced at 30°C (Williamson *et al.*). Of crucial interest is a determination of whether transmission within a neuron, between neurons or across a neuromyal junction is affected by high temperatures. Such studies are currently under investigation by Dr. K. Ikeda.

The phenotype of temperature-dependent paralysis permitted the recovery of a hereditary behavioral defect. Selection schemes for paralytic larvae can also be readily constructed. Methods for large-scale mutagenesis and screening should make the search for such mutations feasible and promise to yield a variety of mutations of considerable biological interest.

CHAPTER 6

DEVELOPMENTAL ANALYSIS OF THE PARALYTIC MUTATION, *para*^{ts}

I. Introduction

Numerous physiological and cytological investigations into the structure and function of nerves and muscles have been carried out, yet the genetic control of these tissues has not been well characterized. It occurred to us that such information might be obtained by the recovery of mutations in Drosophila which exhibit a phenotype of paralysis, which could result from a lesion in either the nerves or muscles. However, since paralysis would be a lethal phenotype, selection for a reversible temperature-dependent paralysis was carried out. A recessive, sex-linked mutation, paralytic-temperature-sensitive, *para*^{ts}, was recovered from a quarter of a million zygotes (Williamson *et al.*, 1970; Grigliatti *et al.*, 1970).

The genetic properties of *para*^{ts} was described in the previous chapter. This chapter presents a detailed analysis of the relationship between the position of mutant tissue and the pattern of aberrant behavior at 29°C, with a view to an anatomical localization of the lesion caused by *para*^{ts}.

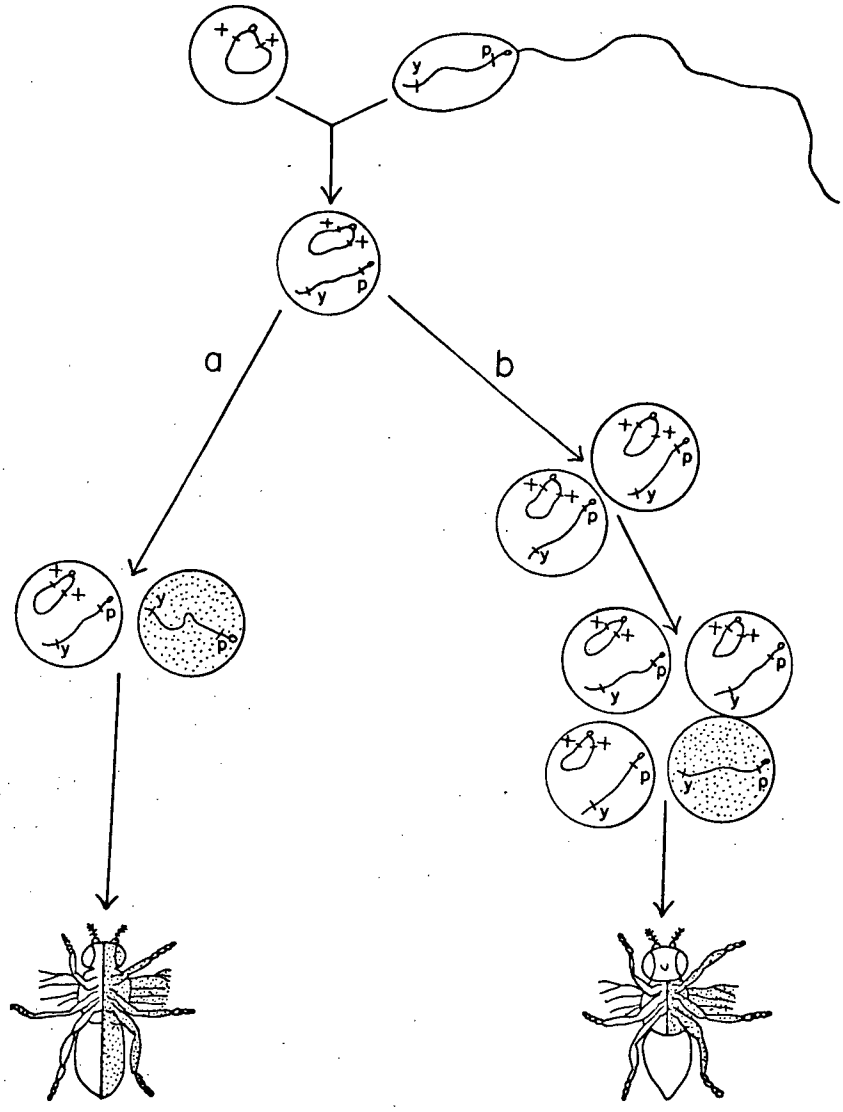
II. Materials and Methods

The X chromosome aberration, $\underline{In(1)w^{vC}}$, is a ring which is somatically unstable and lost with a high frequency in mitotically dividing nuclei (Hinton, 1955). This permits the generation of individual mosaics of X/O (male) and X/$\underline{In(1)w^{vC}}$ (female) cells in a zygote which has an initial genotype of X/$\underline{In(1)w^{vC}}$. X/O cells can be recognized externally in such mosaics when the normal rod X chromosome carries $\underline{para^{ts}}$ and the mutation, $\underline{y}$ (a recessive which produces yellow cuticle), since $\underline{In(1)w^{vC}}$ carries the dominant alleles, $\underline{y}^{+}$and $\underline{para^{ts}}^{+}$. It can be seen that the absence of the ring chromosome can be detected by the yellow phenotype in a wild-type background (Figure 15). Loss of the ring chromosome during the first division of the zygote generates a bilateral gynandromorph in which the half of the fly which is hemizygous (X/O), male, and yellow, and can express the $\underline{para^{ts}}$ phenotype (Figure 15a). The other half of the fly is female and wild-type since it is still heterozygous for the yellow and $\underline{para^{ts}}$ genes. Loss of the ring chromosome later in development results in a smaller patch of yellow, X/O tissue (Figure 15b). The assumption that tissue underlying the cuticular surface is genotypically similar to the exterior does have some experimental support (Ikeda and Kaplan, 1970b;Hotta and Benzer, 1970; Merriam, personal communication).

In the actual cross, $\underline{In(1)w^{vC}}$,$\underline{y}^{+}$,$\underline{para^{ts}}^{+}$/$\underline{In(1)dl\text{-}49}$,$\underline{y}$ $\underline{w}$ $\underline{spl}$, females were mated to $\underline{y}$ $\underline{para^{ts}}$/$\underline{sc^{8}}$·Y,$\underline{y}^{+}$males (for a complete description of the mutations used, consult Lindsley and Grell, 1968). Mosaics of $\underline{In(1)w^{vC}}$,+ +/$\underline{y}$ $\underline{para^{ts}}$ zygotes were recovered at 22°C. Females in which there was no loss of the ring in cuticular tissues were phenotypically wild-type and were also tested for possible paralysis or

FIGURE 15

The method for generation of somatic mosaicism by loss of <u>In(1)w^{vC}</u>: (a) early loss
(b) late loss during cleavage divisions.
The stippled area is mutant X/O tissue.


a
b

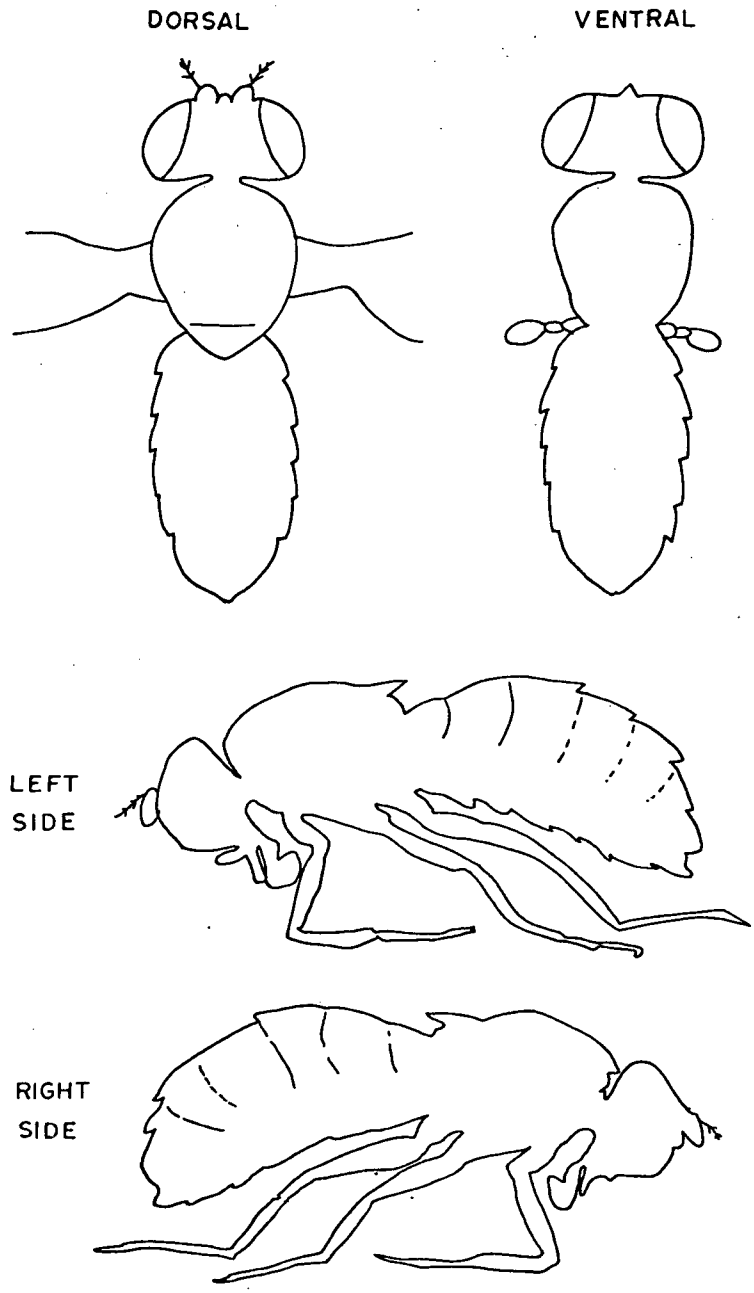
debilitation at 29°C which could be an indication of undetected internal mosaicism. In addition, completely yellow, patroclinous, X/O males which could result from loss of the ring X chromosome during maternal meiosis or loss in all cells which generate external cuticle were also recovered. All patroclinous males were also tested for activity at 29°C to determine possible internal mosaicism.

The area of yellow tissue in each mosaic was recorded on diagrams as shown in Figure 16. Each mosaic was then placed into a coded vial. The behavior at 29°C was observed for up to 20 minutes. Non-mosaic, Oregon-R wild-type females were also placed at random in coded vials as controls.

Methods for the optomotor response, electroretinogram, and flight response will be detailed as needed in the Results.

FIGURE 16

Diagrams used for delineating the area of mosaicism during the scoring procedure.


DORSAL
VENTRAL
LEFT SIDE
RIGHT SIDE

III. Results

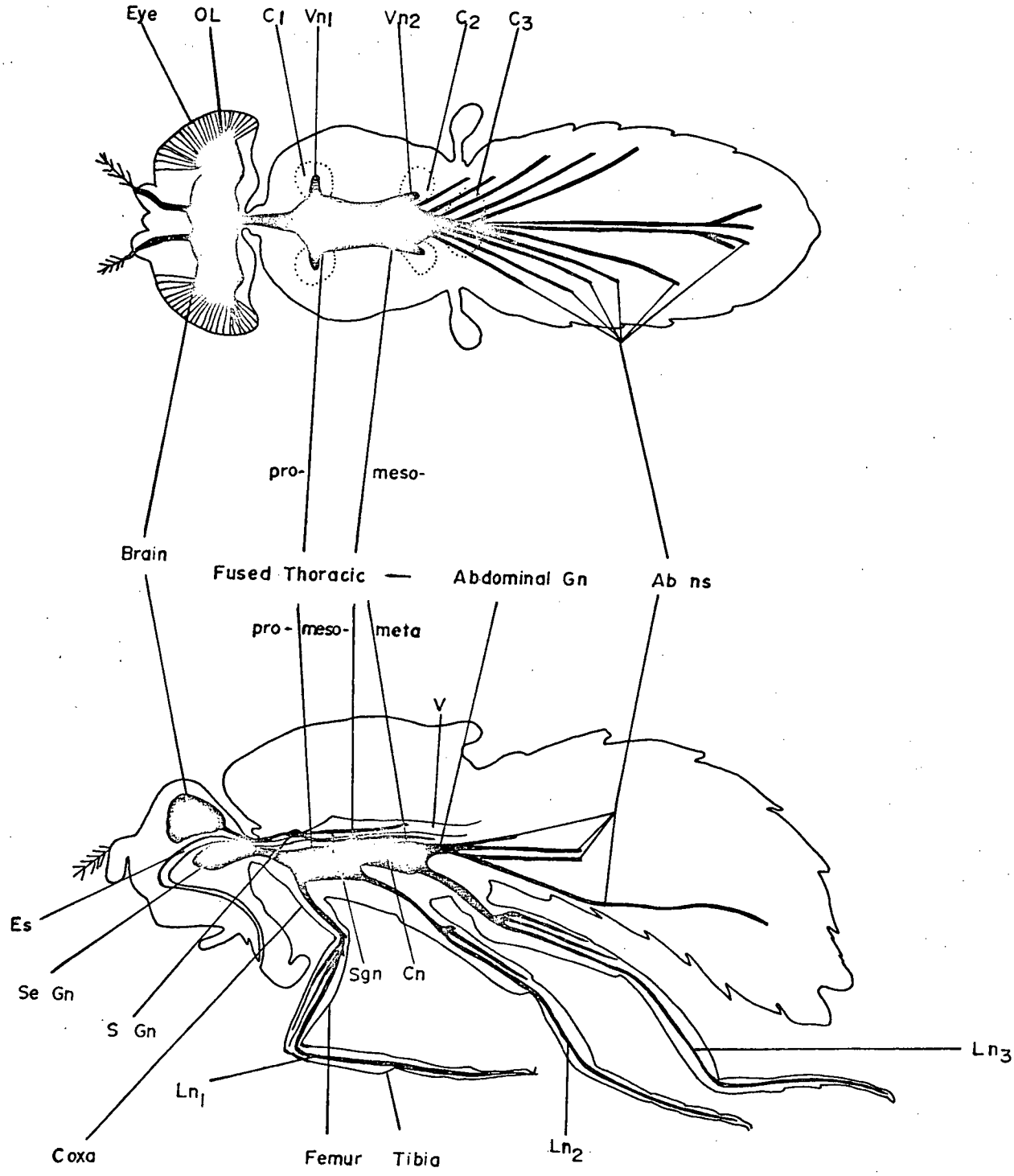
A total of 371 mosaics was detected. The amount of yellow tissue ranged from tiny patches involving one or two bristles to almost complete y $para^{ts}$/O males. Of these, 310 were selected for observation at 29°C.

Many of these mosaics could be placed into one of the following classes based on the location of the mutant tissue: I - abdomen-only mosaics, II - head-only mosaics, III - thorax-only mosaics, IV - head-thorax mosaics. These four classes were further subdivided into ten specific subgroups (Figure 14).

Since the data point to a neural lesion, it is instructive at this point to describe the basic elements of the nervous system with respect to their position in the fly. An anatomical description of the nervous system in Drosophila has been reviewed by Poulson (1950), Bodenstein (1950), and Miller (1950) and is shown in Figure 17. The central nervous system (CNS) consists of the brain and subesophageal ganglion which is fused to the posterior end of the thoracic ganglia. The brain, with its optic lobes, lies dorsal to the esophagus but is connected to the subesophageal ganglion which lies ventral to the esophagus. The subesophageal ganglion is connected to the thoracic ganglia or ventral nerve cord, via a pair of fused nerves running through the cervical connection ventral to the esophagus. Each of the three fused thoracic ganglia gives off, among others, a pair of ventral nerves which innervate each pair of legs. The abdominal ganglion is fused to the caudal portion of the metathoracic ganglion and innervates the abdomen, genitalia and internal reproductive organs. The stomodeal ganglion of the sympathetic or visceral nervous system, rests between

FIGURE 17

(a) Dorsal view of a lateral section, and
(b) sagittal section of Drosophila adult.
Darkened areas are nervous tissue.
Symbols: Ab ns - abdominal nerves; C_{1-3} - coxal cavity; Cn - crop nerve; Es - esophagus; Gn - ganglion; Ln_{1-3} - leg nerves, first, second and third; meso - mesothoracic ganglion area; meta - metathoracic ganglion area; OL - optic lobe; pro - prothoracic ganglion area; S Gn - stomodeal ganglion; Se Gn - subesophageal ganglion; Sgn - salivary gland nerve; V - ventriculus; Vn_{1-2} - ventral nerves one and two innervating the first and second leg respectively. Adapted from Miller (1950).


Eye
OL
C1
Vn1
Vn2
C2
C3
pro-
meso-
Brain
Fused Thoracic
Abdominal Gn
Ab ns
pro-
meso-
meta
V
Es
Se Gn
S Gn
Sgn
Cn
Ln3
Ln1
Coxa
Femur
Tibia
Ln2

the esophagus and aorta dorsal to the thoracic ganglia. It sends two nerves anterior to innervate the protocerebrum of the brain and the esophagus, as well as posterior nerves to innervate the cardia, the stalk of the crop, and the salivary gland.

The fly can be divided into the three body parts: head, thorax, and abdomen. Since no ganglion of the central nervous system is located in the abdomen, it was of interest initially to determine whether mutant tissue in the abdomen affected the behavior of otherwise wild-type flies. Conversely, would the presence of wild-type tissue in the abdomen in any way rescue an otherwise $\underline{para}^{ts}$ fly? The results are shown in Table 6. Seventeen flies had totally wild-type head and thoracic tissue and abdomens ranging from completely mutant to mosaic for small mutant patches (Class I, Figure 14). Of these, one fly had totally mutant abdominal tissue. Its behavior at 29°C was indistinguishable from wild-type, that is, it could walk and climb the vertical sides of the vial normally, and was even observed to fly. Sixteen flies had wild-type head and thoracic tissue but mosaic abdominal tissue that ranged from yellow male genitalia through patches or bands of yellow tergites and sternites to almost totally yellow abdomens. All 16 of these mosaics displayed normal behavior at 29°C. Therefore, mutant tissue in the abdominal region does not visibly alter the behavior of otherwise + flies.

Eighteen flies had totally mutant tissue in the head and thorax, and abdominal mosaicism ranging again from almost totally wild-type to almost totally mutant abdomens. Upon exposure to high temperature, all of these mosaics were paralyzed with a characteristic contraction of all legs.

TABLE 6

Behavior at 29°C of mosaics in which the phenotype of abdominal tissue differed from the head and thorax.

No. Flies	Phenotype of Body Regions			Behavior at 29°C
	Head	Thorax	Abdomen	
1	+	+	mutant	+ (flew)
16	+	+	mosaic	all +
18	mutant	mutant	mosaic	all paralyzed - legs generally contracted
16	mosaic	mutant	+ or mosaic	all paralyzed - legs generally contracted

Sixteen individuals in which the thorax was totally mutant while the head tissue was mosaic, had abdominal tissue which ranged from either totally + to mosaic with only small patches of + tissue. At 29°C, these mosaics were all paralyzed, and again the legs were contracted. All of these data suggest that wild-type tissue in the abdomen in no way rescues otherwise $\underline{para^{ts}}$ flies from paralysis. Since the presence or absence of $\underline{para^{ts}}$ tissue in the abdomen does not counteract the genotype of the head and thorax with respect to walking or flight behavior in any way, we can dispense with any further consideration of mosaicism in the abdomen from this point on.

Attention was then focussed on the behavior of 17 flies with mosaic heads and wild-type thoraces (Class IIb, Figure 14) at 29°C. Of these, two flies had completely mutant heads and wild-type thoraces and abdomens (Table 7). Both were paralyzed and did not walk at 29°C although they maintained a normal stance. If the vial was tapped with sufficient force to topple them, they would often right themselves immediately but could not walk or move; furthermore, even gentle prodding with a blunt probe failed to induce walking. Thus, these two mosaics had a wild-type posture but simply did not move. A third fly having a small patch of + tissue above the left eye, was very similar to the preceding in both its stance and behavior phenotype.

Five flies were bilateral head mosaics; that is, either the left or the right side was completely mutant both dorsally and ventrally (Class IIa, Figure 14). They all walked normally at 29°C; however, when they climbed the vertical surface of the vial, they invariably followed a helical pathway always keeping the mutant side of the head up. The possibility that these mosaics were blind in the eye on the

TABLE 7

Behavior of head mosaics at 29°C.

No. Flies	Location of Mosaicism			Behavior at 29°C
	Head	Thorax	Abdomen	
2	completely mutant	+	+	paralyzed - legs normal posture
1	small + spot above left eye	+	+	paralyzed
5	left or right bilateral	+	+	all walk - climb - helix
7	single quadrant mosaic	+	+	all +
2	mutant antennae	+	+	all +

mutant side of the head was tested using both an optomotor response and an electroretinogram which will be discussed later.

Seven mosaics had mutant tissue in one of four quadrants of the head: dorsal right, dorsal left, ventral right or ventral left. All of these mosaics exhibited normal behavior at 29°C, including climbing in a relatively straight line or diverging randomly from a straight line. Finally, two mosaics were found that had mutant antennal tissue only. Both of these mosaics exhibited normal walking and climbing behavior.

The final gross segmental mosaic class involved flies with completely mutant thoraces (Class IIIc, Figure 14). Nine flies with completely or almost totally mutant thoraces and wild-type head tissue were recovered (Table 8). Seven flies had completely mutant thoraces. Of these seven, five were totally paralyzed at 29°C, another, though paralyzed, occasionally pawed with the first left leg, and the seventh was paralyzed on the left side, but was able to drag itself forward slowly in a right diagonal direction. In contrast to the head-thorax mosaics of Table 6, the legs of these mosaics were not always contracted but varied from normal posture to contraction of the coxa, femur, and tibia. The movement of two of the mosaics may suggest the presence of internal + tissue not represented in the chitinous surface. This will be discussed later. Finally, two other thoracic mosaics which each had only a small patch of + tissue in the dorsolateral area of the thorax near the humerals, were paralyzed at 29°C. Thus, $\underline{para}^{ts}$ tissue located only in the thorax was sufficient to paralyze the flies at 29°C.

In summary of the results from mosaics considered so far, flies with completely mutant head tissue are unable or unwilling to

TABLE 8

Behavior at 29°C of thoracic mosaics.

No. Flies	Location of Mosaicism		Behavior at 29°C
	Head	Thorax	
7	+	completely mutant	5 paralyzed - legs extended 1 paralyzed - legs extended, 1L paws 1 left side paralyzed - walks in rt. diagonal
2	+	mutant with small dorsolateral + patches	2 paralyzed

walk but the fly retains a normal stance. Incomplete head mosaics show varying degrees of activity. Mosaics with mutant thoraces only are paralyzed, generally unable to stand, and vary in the amount of contraction in the legs, from normal posture to full coxa-femur-tibia contraction. Complete head-thorax mutants are paralyzed, and their legs are generally contracted. Finally, mutant abdominal tissue does not affect the walking, climbing or flying ability of otherwise wild-type flies.

From the preceding analysis, we would expect that flies with completely mutant thoraces and varying degrees of mosaicism in the head would be paralyzed at 29°C. Indeed, sixteen individuals with mosaic heads, completely yellow thoraces, and either + or mosaic abdomens were all paralyzed at 29°C. Two of these 16 individuals did move their first legs sporadically, but neither of them was able to right itself.

Wild-type thoracic abdominal tissue cannot rescue a fly with a mutant head (Class IIb, Figure 14) from paralysis at 29°C. Similarly, wild-type head abdominal tissue cannot rescue a thoracic mutant (Class IIIc, Figure 14) from paralysis at 29°C. These facts suggest that paralysis at 29°C cannot be due to the absence of a freely circulating factor necessary for walking. The behavior of bilateral mosaics within a body segment can perhaps give us additional information on the tissue autonomy of $\underline{para}^{ts}$. Autonomy in Drosophila is defined by the phenotypic reflection of a tissue's genotype in spite of its juxtaposition to cells of a different genotype. If the mutation is indeed autonomous, then thoracic mosaics can provide information about the regional specificity of $\underline{para}^{ts}$ and perhaps indicate affected

areas within these regions.

Twenty-eight complete bilateral thoracic mosaics (Class IIIb, Figure 14) were recovered. Fourteen were totally mutant in the left half and 14 in the right half of the thorax (Table 9). Legs on the wild-type side invariably were active whereas the mutant legs were paralyzed. In order to facilitate the analysis of their behavior at 29°C, and to compare their behavior with other mosaic classes, each mosaic was placed into one of the following six categories based on the amount of activity at 29°C; paralyzed, very weak, weak, moderate, strong, or very strong (Table 9). These are somewhat subjective divisions and are defined as follows: paralyzed - flies did not move; very weak - attempted to move using the wild-type legs, but could not right themselves or walk; weak - slowly pulled themselves with their wild-type legs; moderately-active - able to walk continuously using their + legs, generally walked in a diagonal direction pulling themselves forward and dragging the mutant side, or in a circular fashion with the + legs on the outside arc of the circle; strong - walked faster and were generally more active then the preceding class, and sometimes, unsuccessfully attempted to pull themselves up the vertical surface of the vial; very strong - pulled themselves a short distance up the vertical surface of the vial. In most cases, individuals could be readily placed into one of these categories.

None of the bilateral thoracic mosaics was either completely paralyzed or wild-type in behavior. Clearly then, the wild-type half cannot restore total activity to the mutant side, nor does the mutant side suppress movement on the wild-type side. However, the variable expression of movement in what appeared to be similar mosaics could

TABLE 9

Categorization of 29°C activity of bilateral head-thorax and thorax mosaics.

	Class	Characteristics	Number of Mosaics	
			bilateral thorax only	bilateral head-thorax
a	paralyzed	no movement	0	2
b	very weak	attempted to move using + legs	1	9
c	weak	slowly pulled themselves with + legs	6	7
d	moderate	moved continuously using primarily + legs; moved diagonally or in circle	13	5
e	strong	more active than preceding, some use of mutant legs, unsuccessful climbing attempts	2	4
f	very strong	very active, pulled themselves short distances up a vertical surface	6	2
		Total	28	29

reflect either some non-autonomy or variable internal mosaicism. In order to quantify the extent of behavioral activity, each category was assigned a digital value from 0 (paralyzed) to 5 (very strong). By multiplying the number of flies in each category by its value and dividing the sum of these products by the number of mosaics, an average of 3.2 was obtained. This value indicates that, on the average, bilateral thorax mosaics exhibit a moderate amount of activity at 29°C.

It may then be asked whether all three legs on each side of bilateral thoracic mosaics behaved similarly. The front, middle and hind legs on each side of bilateral thoracic mosaics were each placed into one of the five categories of behavioral activity seen in Rows A and C (Table 10). The legs on the wild-type side of the mosaic generally exhibited normal walking ability or, in a few cases, a slight debilitation. On the other hand, the legs of the mutant side were strikingly enfeebled. It is of considerable interest to note that the first leg on the mutant side, though debilitated, invariably showed much more activity than the second and third legs, which were both inactive (Figure 18). This may suggest a difference in innervation between the first leg and the second and third.

In comparing activity of bilateral head-thorax mosaics (Class IVa, Figure 14) with bilateral thoracic mosaics (Class IIIb, Figure 14), a further comparison of the behavior of the first with the second and third legs could be made. Twenty-nine bilateral head-thorax mosaics were found and they were classified by the same criteria applied to bilateral thorax-only mosaics (Table 9). Addition of head mosaicism ipsilaterally clearly reduced activity of the thoracic mosaics. Thus, activity of a quarter of the thoracic mosaics was weak or less whereas

FIGURE 18

Activity of each leg in bilateral thoracic mosaics at 29°C.
——— + legs, ----- mutant leg 1, -•-•- mutant leg 2,
····· mutant leg 3.

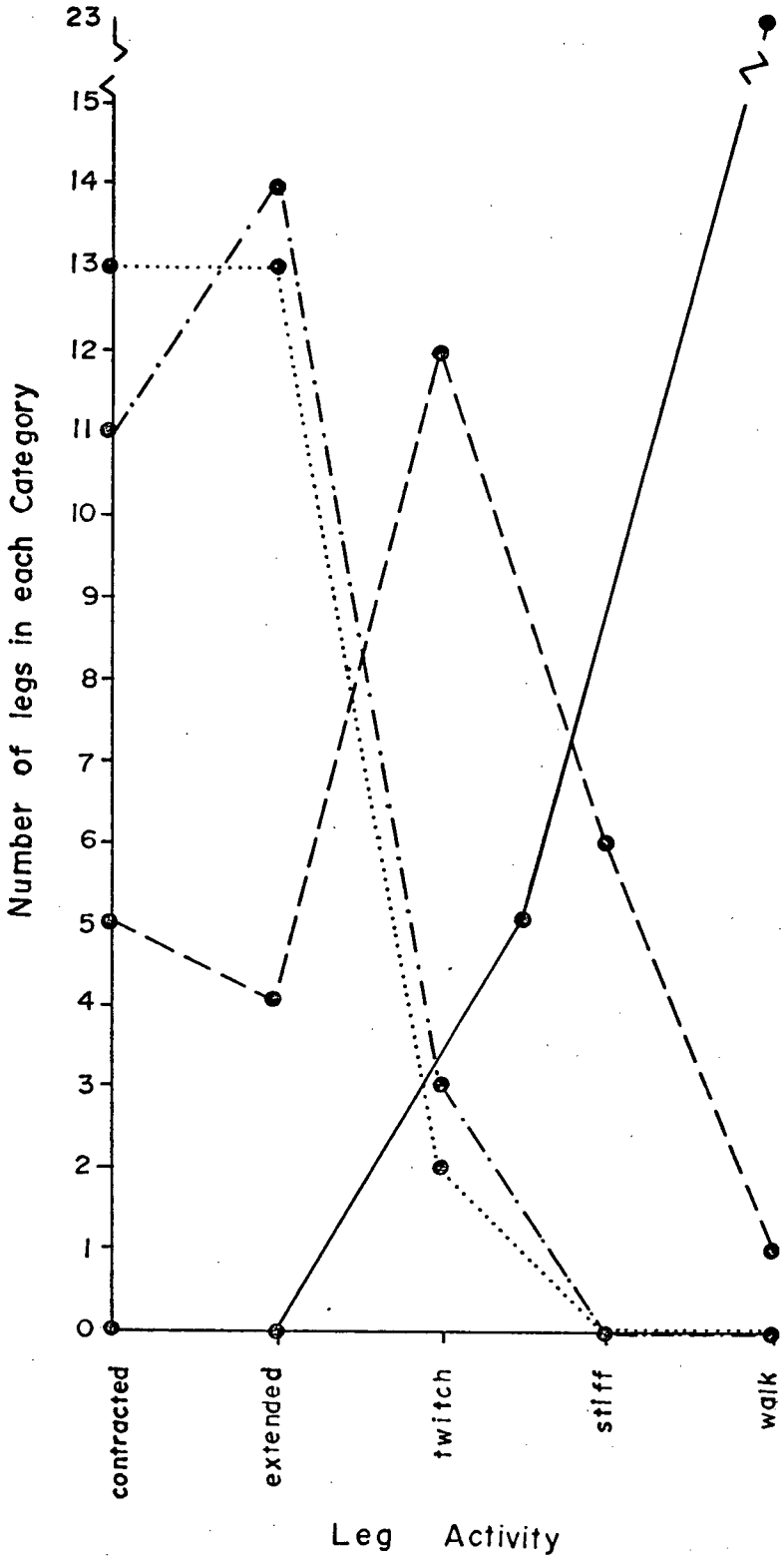

Number of legs in each Category
23
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
contracted
extended
twitch
stiff
walk
Leg Activity

FIGURE 19

Behavior of bilateral thoracic and head-thoracic mosaics at 29°C. ——— thoracic, ----- head-thoracic; letters correspond to classes of activity shown in Table 17.

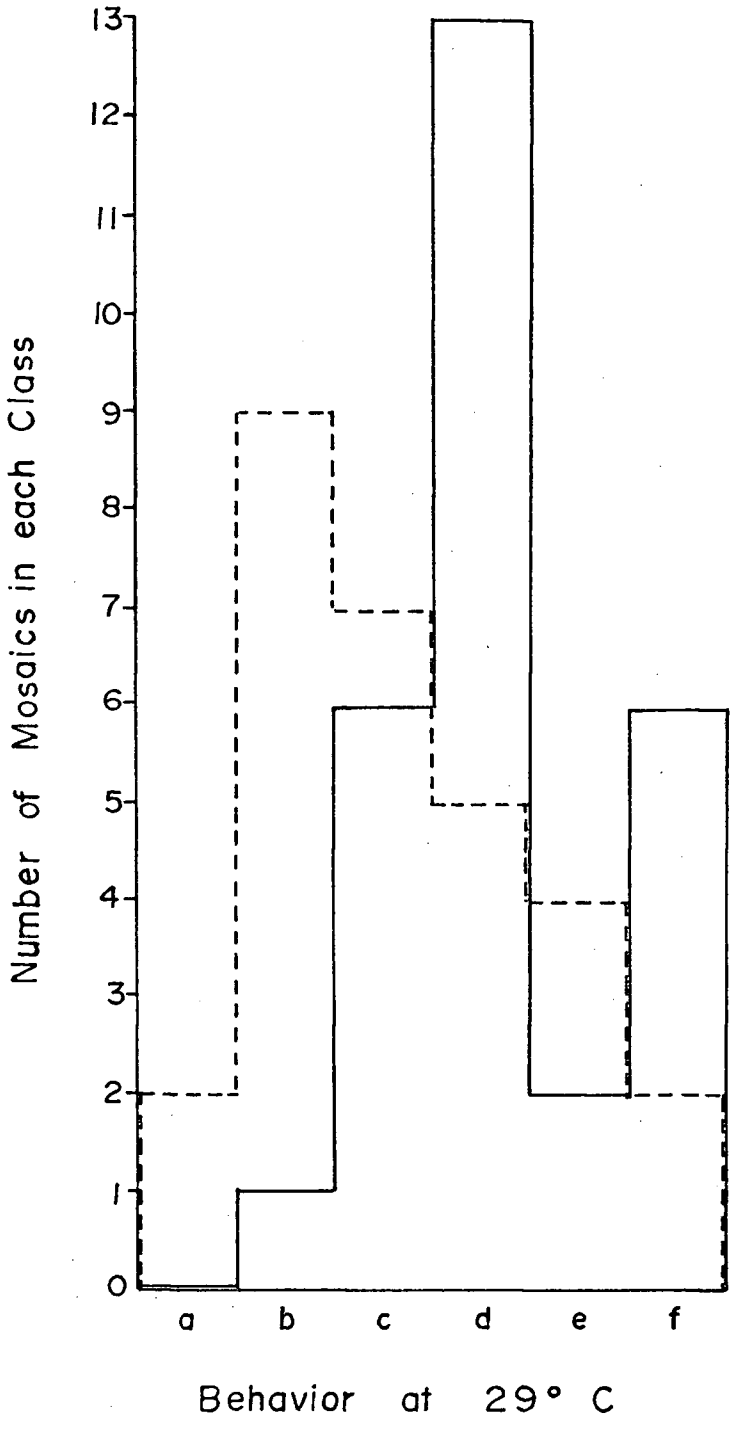

Number of Mosaics in each Class
0
1
2
3
4
5
6
7
8
9
10
11
12
13
a
b
c
d
e
f
Behavior at 29° C

TABLE 10

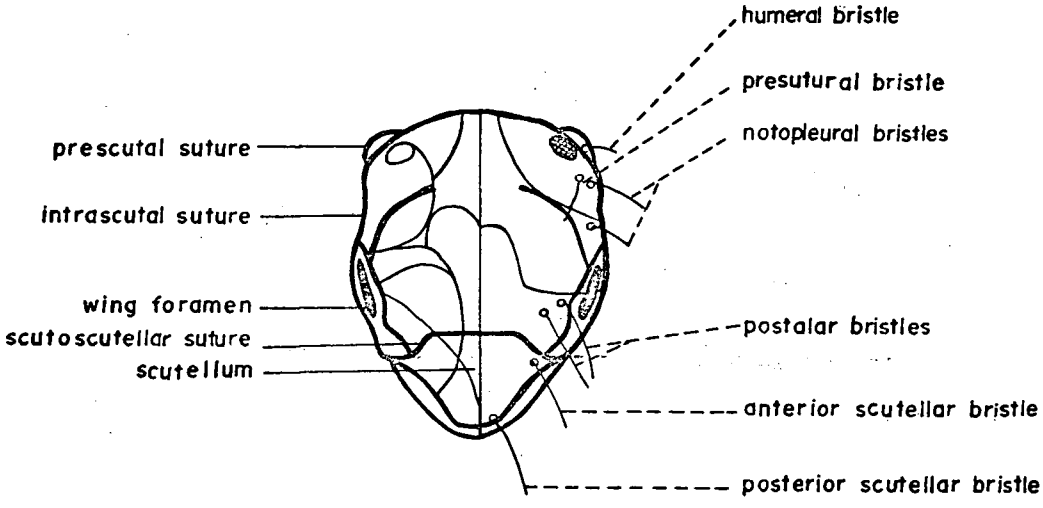
Leg activity at 29°C in bilateral head-thorax and thorax mosaics*.

Tissue Type	Row	Type of Mosaic	Leg	Number of mosaics in each category of activity: Contracted	Extended	twitch	stiff	walk	total
mutant	A	thorax	1st	5	4	12	6	1	38
			2nd	11	14	3	0	0	28
			3rd	13	13	2	0	0	28
	B	head-thorax	1st	11	3	7	7	1	29
			2nd	13	12	1	3	0	29
			3rd	13	14	1	1	0	29
wild-type	C	thorax	1st	0	0	← 5 →		23	28
			2nd	0	0	← 5 →		23	28
			3rd	0	0	← 5 →		23	28
	D	head-thorax	1st	2	5	3	4	15	29
			2nd	2	5	3	4	15	29
			3rd	2	6	2	4	15	29

* Classes are delineated as follows: contracted - coxa, femur and tibia, or at least femur and tibia were contracted; extended - preceding joints not contracted but the leg did not move; twitch - occasional sporadic flexure of the joints but the leg was not used in walking; stiff - the leg though used for walking did not show normal articulation of the joints; and walking - normal use of the leg.

FIGURE 20

Location of different mutant patches on the dorsal surface of the thorax. The cross hatched patch is the only region not encompassed by mutant tissue.


humeral bristle
presutural bristle
notopleural bristles
prescutal suture
intrascutal suture
wing foramen
scutoscutellar suture
scutellum
postalar bristles
anterior scutellar bristle
posterior scutellar bristle

almost two-thirds (18/29) of the head-thorax mosaics were weak or less. Also, six out of 28 of the thoracic mosaics were very strong whereas only two out of 29 of the head-thorax mosaics were. This can be seen in Figure 19. The digital index of behavior of the head-thoracic mosaic was 2.2, a further indication of the decreased activity relative to the thorax-only mosaic (3.2).

A comparison of movement of legs in head-thorax mosaic reveals two interesting points: (1) while there is somewhat more debilitation of the mutant first leg when the head is also mosaic, it still retains considerably more activity than either the second or third legs (Rows B and D, Table 10); (2) addition of mutant tissue to the head of thoracic mosaics results in a reduction of movement of the legs on the wild-type side. This suggests that there is some contralateral connection from the subesophageal ganglion to the thoracic ganglia. Furthermore, it seems that there is an ipsilateral connection from the head to the thorax which may indirectly affect the activity of the first pair of legs.

The extent to which the thorax is involved in mobility might be delineated by the behavior of mosaics carrying mutant patches on the dorsal surface of the thorax. Fifteen of such mosaics, all of which behaved normally at 29°C, were observed. No mosaic which was mutant over the entire dorsal surface of the thorax only was found. However, when the mutant patches on the dorsal surface of the thorax were superimposed (Figure 20), they overlapped to extend over an area laterally down to the wings and over the entire dorsal surface of the thorax with the exception of a small patch of tissue on the right anterior dorsal surface of the thorax above the humeral bristles. However,

several mosaics were found that had mutant tissue in that same area on the left side of the thorax. From the behavior of these mosaics, it can be inferred that mutant tissue on the dorsal surface of the thorax in no way debilitates the fly. This offers further support for an effect of $\underline{para}^{ts}$ on the CNS since the thoracic and abdominal ganglia lie in the ventral portion of the thorax.

The different pattern of behavior of the first pair of legs from that of the second and third legs, made the early development of each leg of interest. Thirty-seven mosaics with mutant tissue in the legs only (Class IIIa, Figure 14) were examined and the frequency with which each leg had mutant tissue was determined (Table 11). There was a curious absence of mosaics with mutant tissue in the second leg only. Also, there were no mosaics with mutant tissue simultaneously in the first and second legs or in the first and third legs even though eight flies had mutant tissue in both the second and third legs. Since mosaics with mutant tissue in the third leg alone but none in the second leg alone were recovered, we conclude that whenever mutant tissue is generated in the second leg, the third leg also contains mutant tissue. Therefore, it is possible that the third leg cells are determined later in development than the second leg cells, and that, in fact, the presumptive second leg cells give rise to the presumptive third leg cells. Since we recovered nine mosaics with all three legs on one side mutant, it is quite probable that the first and second leg cells are derived from a small number of the same progenitor cells. We did not delineate the area of mutant tissue in the leg mosaics to permit an estimate of the number of cells from which the three leg discs were derived. In addition to the developmental data, it is

TABLE 11

Frequency of mutant or mosaic tissue in individual legs of leg-only mutants.

	mosaic in one leg only			mosaic in two legs			mosaic in three legs	total of mosaics in each leg		
	1	2	3	1,2	2,3	1,3	1,2,3	1	2	3
Number of mutant or mosaic	12	0	8	0	8	0	9	21	17	25

interesting to note that though all of the leg-only mosaics could walk at 29°C, some were slightly enfeebled (Table 12).

Thirty mosaics of both the head and thorax with different degrees of mosaicism ranging from small patches of mutant tissue in both the head and thorax to large patches covering most of the head and thorax were studied. The behavior of each of these mosaics was completely predictable on the basis of the results from mosaics in single body segments already discussed. In addition, 21 mosaics were isolated with completely mutant heads and only small patches of wild-type thoracic tissue. All were paralyzed as expected.

Eighteen mosaics were found that had completely mutant heads and bilaterally mosaic thoraces (Class IVc, Figure 14). All were paralyzed at 29°C. In 17, the legs on the mutant side were contracted whereas those on the wild-type side assumed normal posture. Thus, combined mutant tissue in the head and thorax ensures the contraction of the mutant legs.

Four flies had completely mutant heads, wild-type thoraces, and mosaic legs. In one of these flies, only the first right leg was mutant. It did not walk at 29°C and the first right leg was contracted. The first left leg showed some slight femur-tibia contraction and both the second and third pair of legs had normal posture. The second fly had mosaicism in both the second and third pair of legs. It took a few very small and slow steps, listing to the left and moving slightly counterclockwise. Fly 3, like the first, had a mutant first right leg. The first right leg was completely contracted, and the fly took two very small and slow steps using the other legs then did not move. Fly 4 also had a mutant first right leg which was contracted. The fly

TABLE 12

The behavior at 29°C of individual mutant legs in leg-only mosaics.

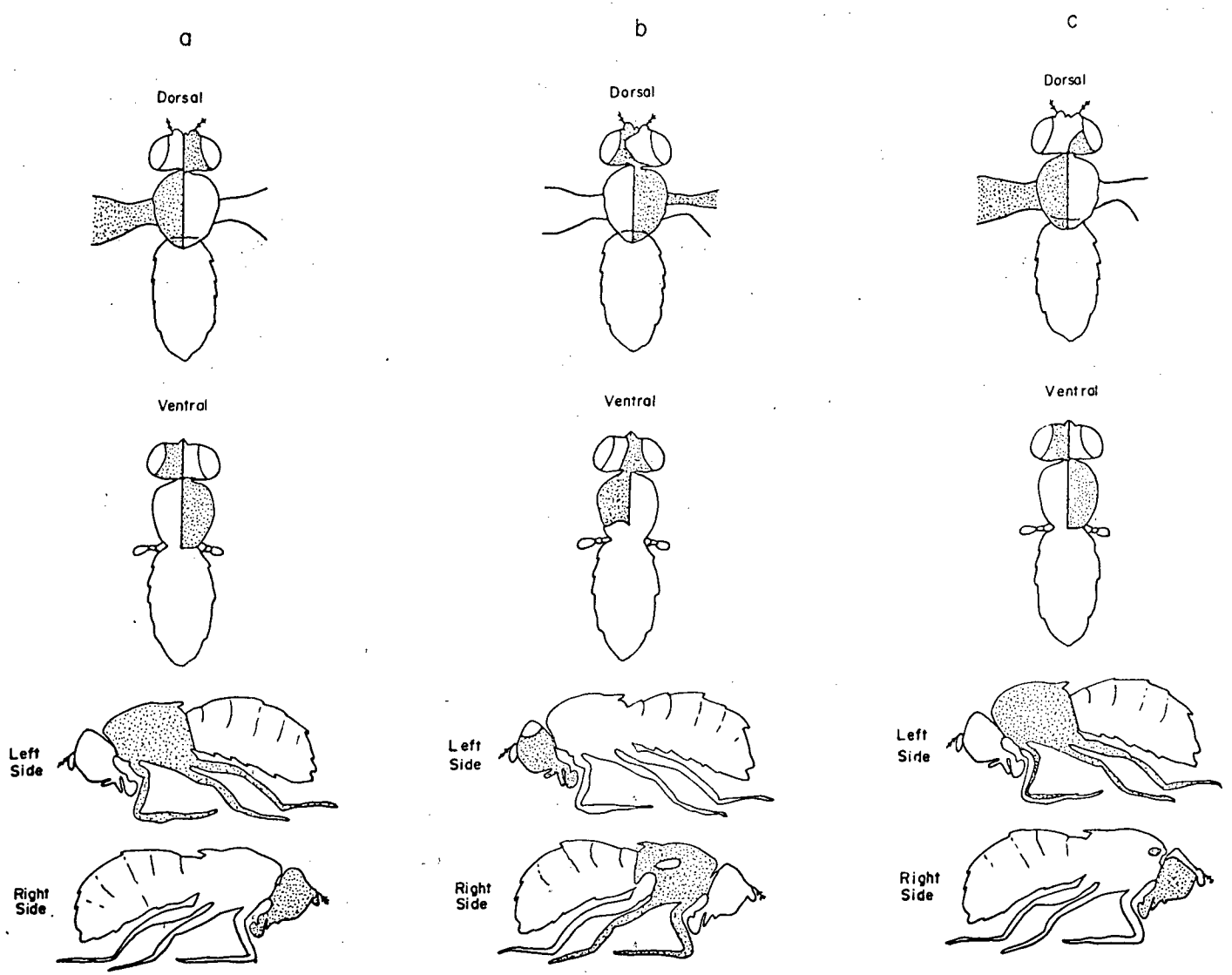
	Number of mosaics in each category of activity					
leg	contracted	extended	twitch	stiff	walk	total
1	0	0	0	4	2	6
2	0	2	0	6	2	10
3	0	7	0	5	5	17

did not move at all, but just stood in one spot and listed to the left. These four flies reconfirm the inability of head mutants to move at 29°C and demonstrate the specificity of the head-leg mutant combination for the leg contraction.

Three individuals that were bilateral head mosaics and bilateral thorax mosaics were found in which the mutant patches in the body segments were in opposite halves of the body (Figure 21). The behavior of these flies is particularly interesting. If the nerves joining the cephalic ganglion with the thoracic ganglia are crossed, then one would predict that the mutant legs would be paralyzed and contracted while the wild-type legs should pull the fly as in a bilateral thorax mosaic. On the other hand, if the innervation of the thorax from the head is ipsilateral - not forming a chiasma - then one would predict that the fly would not walk at 29°C and that both the wild-type and the normal legs would assume the normal posture. The first individual had the right half of the head and the left half of the thorax mutant (Figure 21a). The left legs were completely contracted, i.e., coxa, femur, and tibia; the first right leg was missing, and the second and third right legs showed a more normal posture with some femur-tibia contraction. The fly never moved at 29°C. The second individual had the left half of the head and the right half of the thorax mutant (Figure 21b). It was paralyzed on the right side with some use of the first right leg, less use of the second right leg, and least use of the third right leg. It moved by dragging itself with the left legs and slightly using the first right leg. The last individual in the group, like the first, had mutant tissue in the right half of the head and left half of the thorax and in addition the first left

FIGURE 21

Location of mutant tissue in head-thorax catercorner mosaics. The stippled area indicates mutant X/O tissue.


a
b
c
Dorsal
Dorsal
Dorsal
Ventral
Ventral
Ventral
Left Side
Left Side
Left Side
Right Side
Right Side
Right Side

was mosaic and the first right was very slightly mosaic (Figure 21c). It moved using the right legs, and it had some use of the first left leg and the second left leg was stiff. The results from these three individuals do not strictly conform to either prediction. It is likely that there is a cross innervation of head to thorax but perhaps linear innervation exists as well.

Interpretation of data using mosaics depends on the assumption that external phenotypes delineate internal tissue of the genotype. Indeed, a good correspondence between external and internal mosaicism has been found in some neural and non-neural tissues (Hotta and Benzer, 1970; Ikeda and Kaplan, 1970b; Merriam, personal communication). We can test this contention further in two ways: (1) by determining how many individual mosaics exhibit behavior that digresses markedly from the patterns established by the majority of the mosaics, and (2) by testing the behavior of non-mosaic females and yellow (XO) patroclinous males that were generated along with the mosaics. The non-yellow females have not lost the ring X chromosome in any external chitinous cells whereas the yellow males have lost the ring X chromosome in all the external chitinous cells. If internal mosaicism in neural tissue exists, then some females might be enfeebled at 29°C, while some males should not be completely paralyzed at 29°C. Over 200 yellow patroclinous males were observed at 29°C and all were paralyzed. Over 300 wild-type females were tested at 29°C and of these 12 walked abnormally. Upon re-examination, 10 were found to be missing a leg, one had a mutant patch that had been overlooked, and only one completely wild-type, and therefore true exception, was found.

At first this fly walked and then, after about 10 seconds, it

slowed down and walked occasionally. The femur and tibia of the third pair of legs often contracted, the second pair of legs was stiff, and the first pair of legs would sometimes show femur-tibia contraction. We conclude that this fly must indeed have had some internal mosaicism.

Of the more than 300 mosaics observed, only seven deviated to some extent from the behavior patterns predicted on the basis of the general observations. Fly 1 (Figure 22a) was expected to be immobile. However, it did move very slowly using the first pair and second left legs. This very weak activity might be expected if the ventral patch of + tissue in the head extended dorsally through the cephalic ganglia. In another exception (Figure 22b), the right side was predictably paralyzed. However, the fly dragged itself using the left legs and, what is surprising, could even climb the vertical side of a vial. Again, this suggests more wild-type tissue internally than was indicated by the external chitinous structure. A third mosaic (Figure 22c) was expected to be virtually immobilized, and the left side was, in fact, paralyzed. However, it dragged itself with the right legs. We conclude, therefore, that it had some wild-type tissue in the head not delineated by the external mosaicism or perhaps that the patch of + tissue on the right surface of the head continued down through the brain and subesophageal ganglion but not to the external ventral surface.

On the basis of the visible mosaicism, another fly (Figure 23a) was expected to move very slowly with the second and third right legs extended; however, it walked well, climbed, and even flew once. While it is true that from the external mosaicism we would predict that the cephalic as well as a good part of the thoracic ganglia were

FIGURE 22

Location of mutant tissue in three head-thorax mosaics which behaved unexpectedly at 29°C. The stippled area indicates mutant X/O tissue.

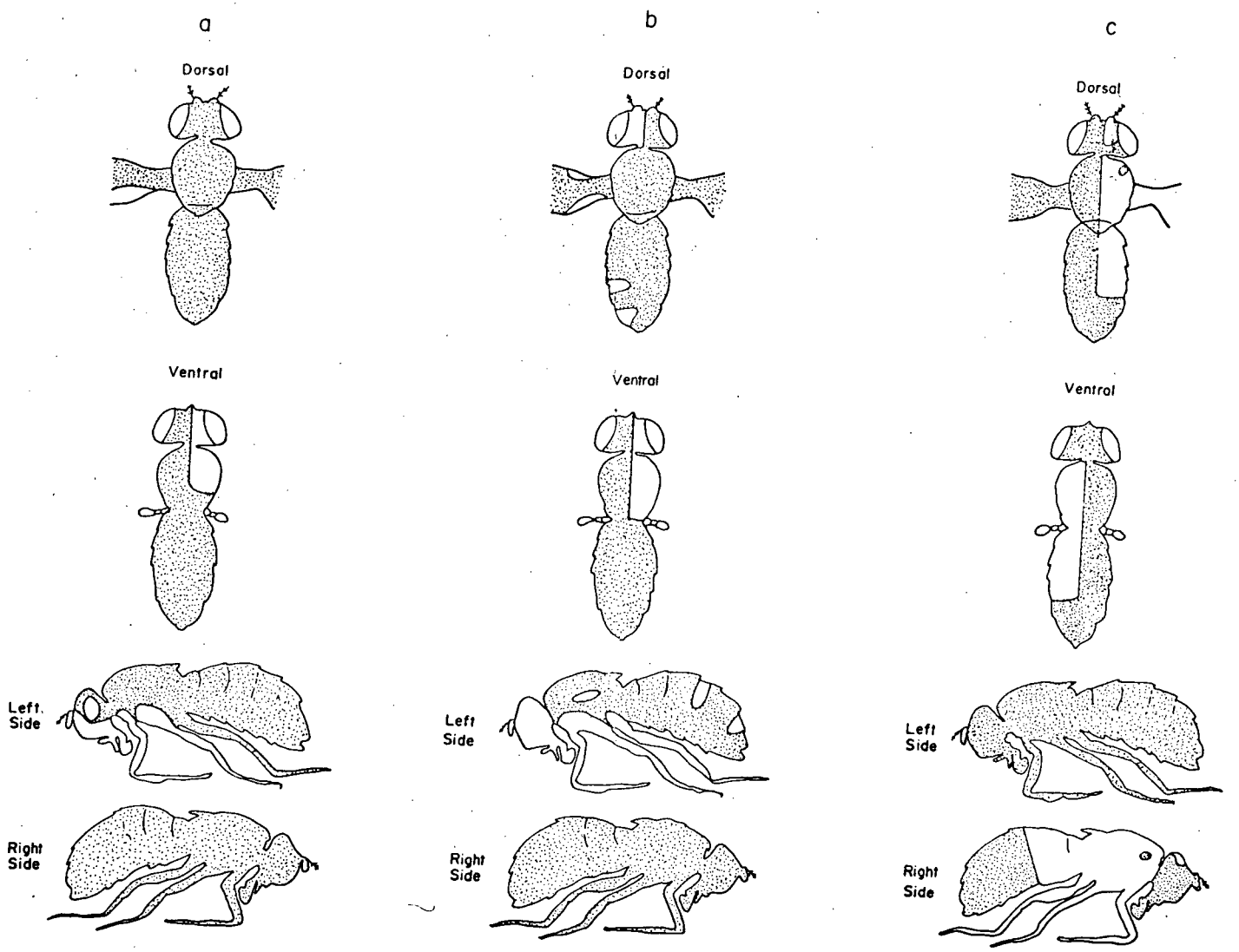
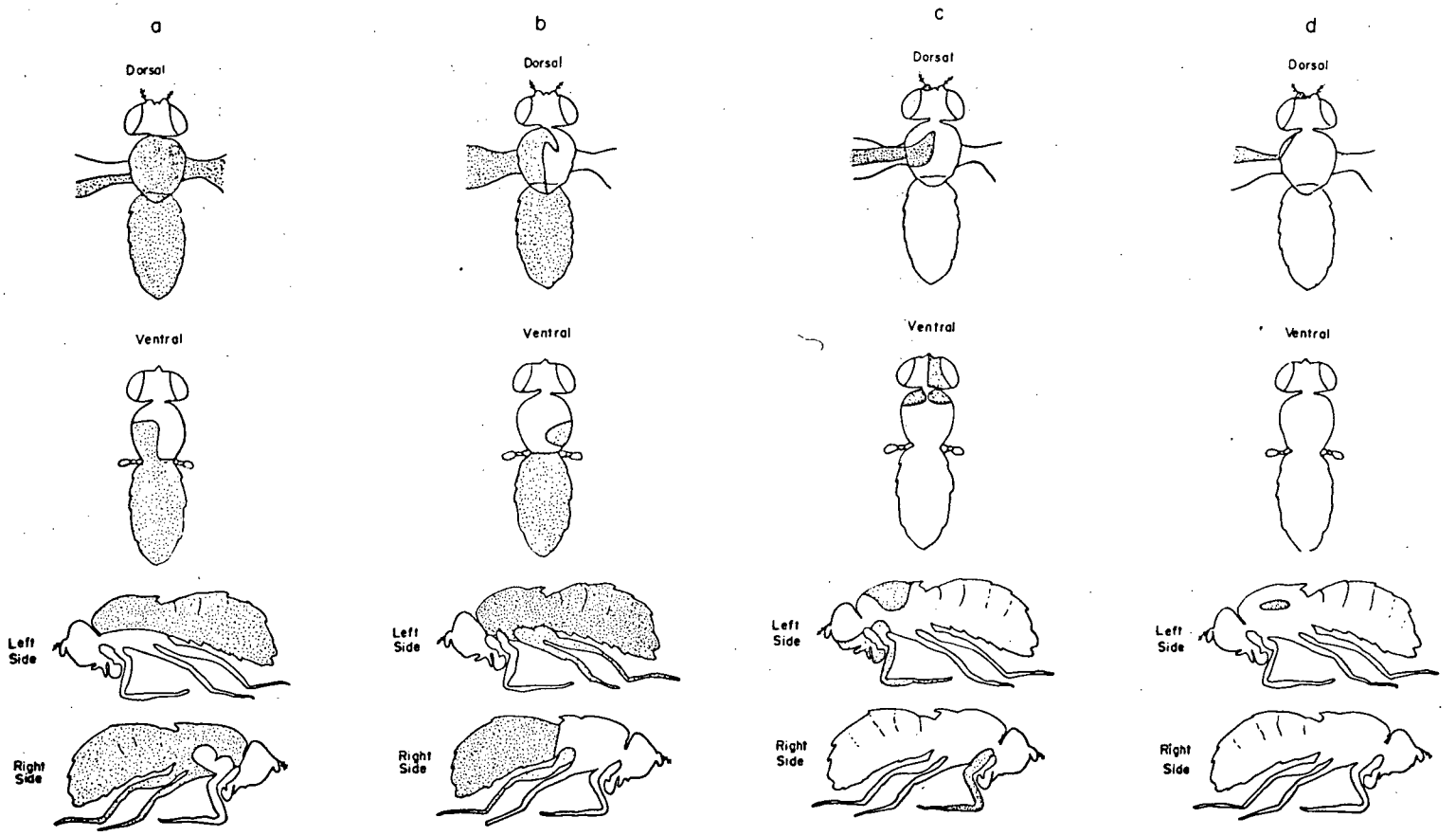

a
Dorsal
Ventral
Left Side
Right Side
b
Dorsal
Ventral
Left Side
Right Side
c
Dorsal
Ventral
Left Side
Right Side

FIGURE 23

Location of mutant tissue in four head-thorax mosaics which behaved unexpectedly at 29°C. Lightly stippled area indicates mosaic tissue.


a
b
c
d
Dorsal
Dorsal
Dorsal
Dorsal
Ventral
Ventral
Ventral
Ventral
Left Side
Left Side
Left Side
Left Side
Right Side
Right Side
Right Side
Right Side

probably wild-type, we did not expect that the fly would be quite as active as it was. Another fly (Figure 23b) walked fairly rapidly. What was unexpected was that it used its first and second left legs well and listed to the right when walking. A sixth fly (Figure 23c) was expected to be fairly active. However, it crawled forward very slowly at first and was then motionless except for cleaning behavior with the third pair of legs. Finally, the seventh fly (Figure 23d) was expected to exhibit normal behavior. It did walk fast. However, it slowed down occasionally, the second pair of legs seemed slightly stiff and it was reluctant to climb the vertical surface of the vial.

In summary, seven of the more than 300 mosaics observed at 29°C exhibited behavior that diverged somewhat from the behavior we would have predicted, based on their markings and the behavior of the other mosaics. One of approximately 500 non-mosaic females and yellow patroclinous males exhibited non-predictable behavior. This evidence indicates that there is a very good correlation between the amount of external and internal mosaicism in this system.

When $\underline{para}^{ts}$ flies are shifted from 22°C to 29°C, they are initially paralyzed. However, they will recover some co-ordinated activity over a two hour period at that temperature. A possible measure of non-autonomy could be the rate of recovery of mosaic flies at 29°C. Mosaics with $\underline{para}^{ts}$ tissue in the head-only did seem to recover co-ordinated activity at 29°C faster than complete $\underline{para}^{ts}$ flies (Figure 24). Mosaics with totally mutant head and thorax, but wild-type abdomen (Class IVd, Figure 14), wild-type head and mutant thorax (Class IIIc, Figure 14), and mutant head and bilateral thorax (Class IVc, Figure 14) did not recover co-ordinated activity any faster than totally $\underline{para}^{ts}$

flies (Figure 24).

This finding, in conjunction with the observation that thorax-only (Class IIIc), bilateral head-mutant thorax (Class IVb) and mutant head-bilateral thorax (Class IVc) mosaics could not move at 29°C, shows that paralysis is not induced by the loss of some freely circulating factor necessary for movement. Moreover, the fact that abdominal mosaics (Class I), bilateral head mosaics (Class IIa), as well as the wild-type sides of bilateral thorax and head-thorax mosaics (Classes IIIb and IVa) can move, rules out the existence of some freely circulating inhibitor of movement produced by the $\underline{para}^{ts}$ mutation. Thus, the mutation seems to be autonomous.

The observation that five bilateral head mosaics all walked normally at 29°C, but invariably followed a helical pathway always keeping the mutant eye up when climbing the vertical side of a vial, prompted us to ask whether this behavior was an indication of blindness in the mutant eye. If the flies were indeed blind, their attempts to balance the amount of light striking the two eyes might result in a helical climbing pattern. This suggestion could be tested quite easily by examining the optomotor response (Kalmus, 1948) of these mosaics at 29°C. When presented with a moving pattern of stripes, wild-type flies will invariably turn and walk in the direction in which the stripes are moving. This was tested by placing a fly whose wings had been amputated into a cylinder whose walls were striped vertically (Figure 25). The cylinder was then rotated to the right or the left. Wild-type flies gave a strong positive response at both 22°C and 29°C. When the eyes and ocelli of wild-type flies were covered with white enamel paint, they ceased to show a positive optomotor response at both 22°C and 29°C.

FIGURE 24

Activity recovered with time at 29°C by different mosaics.
——— *para*^{ts} control, ----- mosaics,
(a) head only mosaics
(b) thorax only
(c) complete head and bilateral thorax, and
(d) abdomen only

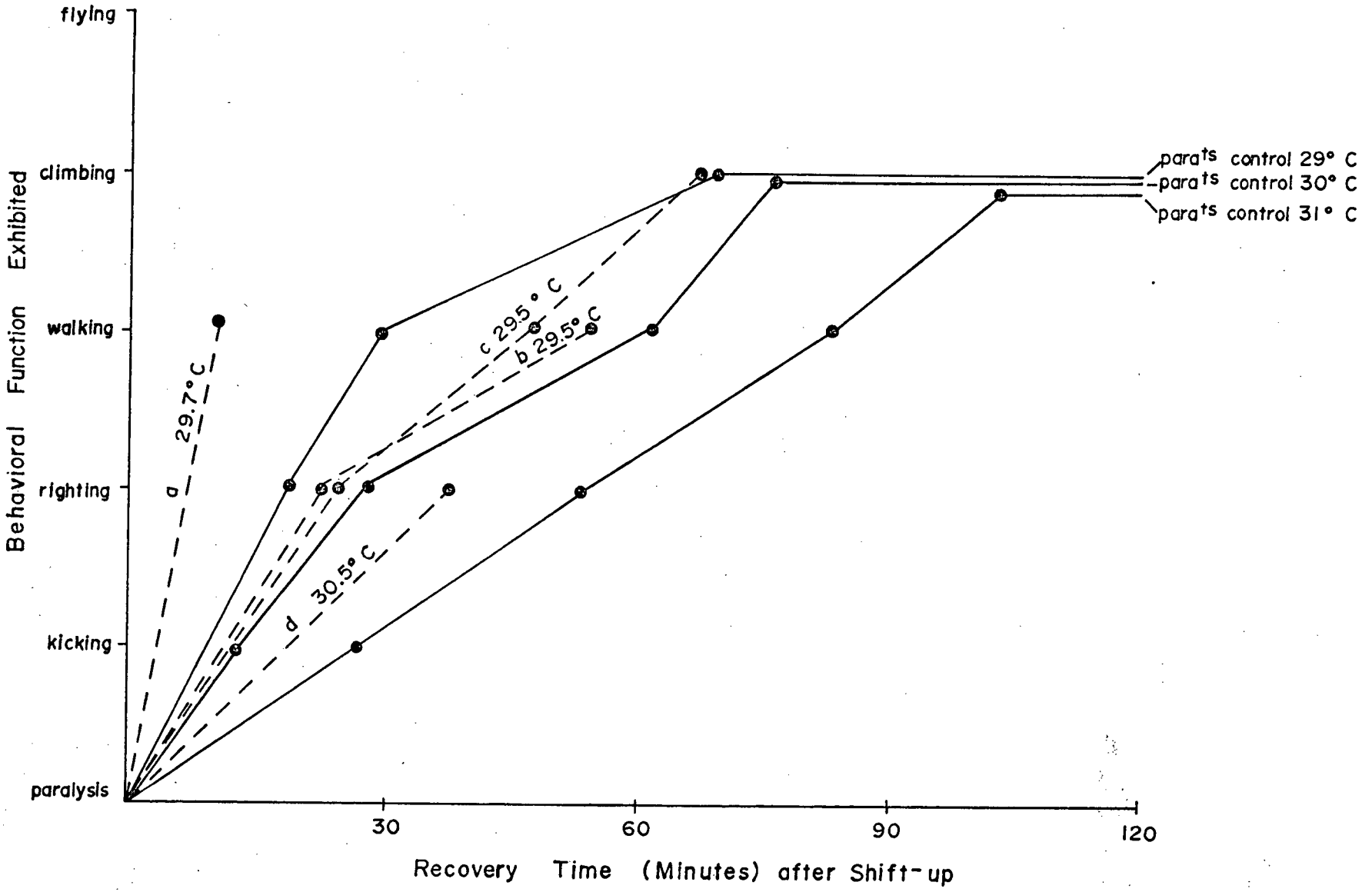
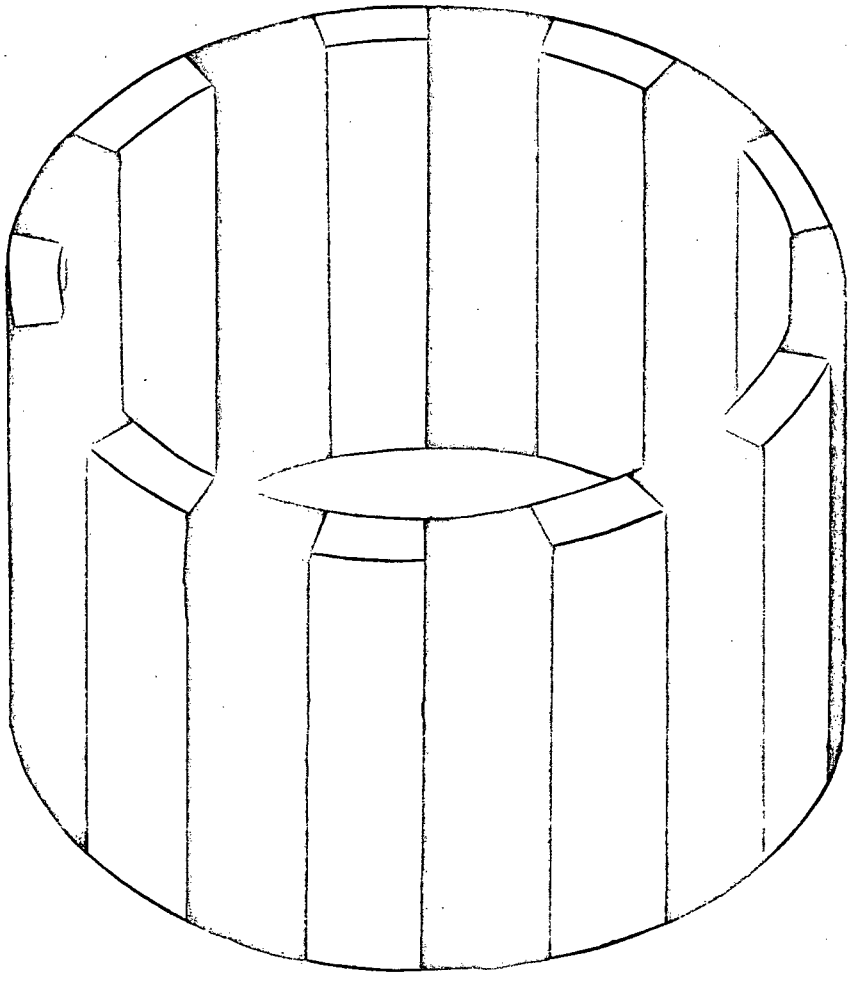
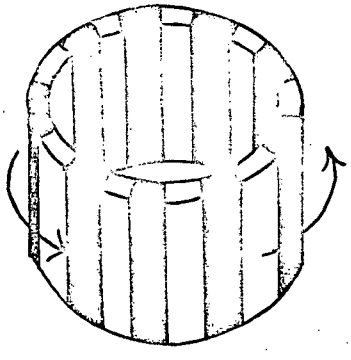
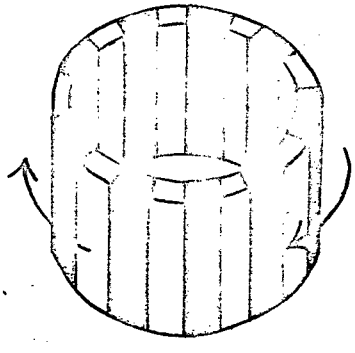

flying
climbing
walking
righting
kicking
paralysis
Behavioral Function Exhibited
para^ts control 29° C
para^ts control 30° C
para^ts control 31° C
a 29.7° C
c 29.5° C
b 29.5° C
d 30.5° C
30
60
90
120
Recovery Time (Minutes) after Shift-up

FIGURE 25

A diagram of the device used to test the optomotor response. The hollow cylinder with alternating black and white striped is rotated either clockwise or counterclockwise. A wild-type fly placed in center of the cylinder will walk in the same direction in which the cylinder is rotated.





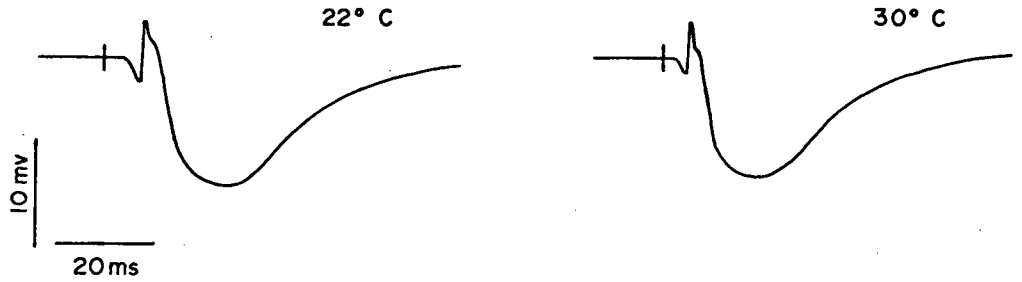


We then painted only the eye on the wild-type side and the ocelli of bilateral head mosaics. At 22°C, these painted mosaics still exhibited a strong positive optomotor response, whereas the results at 29°C were ambiguous. Most of the time, the mosaics did not respond to changes in the direction of rotation of the stripes. On a few occasions the flies did stop moving when the direction of the rotation of the stripes changed, and occasionally did orient themselves in the general direction of the new rotation of the stripes. Thus, on a few occasions these flies did respond positively but much less strongly and more slowly. They were clearly debilitated but our evaluation of the extent of their response was too subjective to make a definitive assessment of their vision.

A more definitive test of sight is the response of the eyes of $\underline{para}^{ts}$ flies to short flashes of light as shown by an electroretinogram (ERG) (Hotta and Benzer, 1969). The ERG was obtained by the generosity of Dr. Yoshiki Hotta through the co-operation of Dr. Seymour Benzer. The ERG was positive and remained unchanged for 20 minutes at both 22°C and 30°C (Figure 26). This indicated that the eyes of $\underline{para}^{ts}$ flies do transduce light into impulses at both 22°C and 30°C and that these impulses can at least travel down the axons of the retinula cells. Since the optomotor response was nil or at best very weak, these signals may not reach or be recognized by the brain with any regularity. It must now be asked whether the optic nerves are transmitting the impulses to the brain and/or whether the cephalic ganglia can interpret such messages and send the appropriate messages of response to the thoracic ganglia and legs. At this time attempts are being made to determine if interneural and neuro-myal transmission occurs in $\underline{para}^{ts}$

FIGURE 26

Electroretinogram of $\underline{para}^{ts}$ at 22°C and 30°C showing the response to a short (20 μs) flash of light.


22° C
30° C
10 mv
20 ms

flies at 29°C.

The effect of $\underline{para}^{ts}$ on the activity of afferent neurons was studied by examining flying ability. The tips of toothpicks were trimmed and glued perpendicular to the center of the dorsal surface of the thorax. When these tethered flies were lifted, they began to "fly" as soon as their feet left a solid surface. At 22°C, $\underline{para}^{ts}$ flies initiated flight immediately upon lifting, whereas at 29°C, flight was not initiated. However, once flight was initiated at 22°C, upon shifting to 29°C, flight would continue for about 5-10 seconds. Bilateral head and full thorax mosaics, as well as bilateral head-thorax mosaics, terminated flight upon shifting from 22°C to 29°C and were unable to initiate flight at 29°C. However, bilateral head-only mosaics can fly at 29°C. This implies that a wild-type thorax and at least partially wild-type head are needed to initiate flight at 29°C.

A more interesting question is whether a fly which had initiated flight at 22°C and was shifted to 29°C, would terminate flight when only its mutant legs touched a solid object. Although bilateral head-thorax mosaics cannot initiate flight at 29°C, if flight is initiated at 22°C, upon shifting to 29°C they will continue to fly for periods up to 15 seconds. Three bilateral head-thorax mosaics, and two bilateral head-full thorax mosaics were tested. Flight was initiated at 22°C, then the flies were shifted to 29°C and less than 7 seconds later, the mutant legs were allowed to touch a plate. In all cases, the flies terminated flight immediately. This may indicate that the afferent neurons and the inhibitory neurons to the flight muscles still function at 29°C. An alternate explanation exists; since the flies are able to see, perhaps the proximity of the approach-

ing platform caused the cessation of flight in a learned response to distance and landing. A similar experiment was attempted with complete $\underline{para^{ts}}$ flies. On occasion, flight initiated at 22°C was not terminated at 29°C when the flies' feet touched the platform. In retrospect, since some flights, once initiated at 22°C, continue for only several seconds when the fly is shifted to 29°C, and paralysis takes a few seconds (less than 5 seconds) to be complete, these experiments seem of dubious value in the information they may yield.

If the afferent neurons of $\underline{para^{ts}}$ tissue do indeed function, bilateral thorax and bilateral head-thorax mosaics (which can move at 29°C) might be expected to attempt to crawl away if touched on the mutant side. Using a small hair, the legs and body of the mutant side and alternatively the wild-type side, were prodded. When the fly was touched on the mutant side there was generally no reaction. Occasionally the fly made a weak attempt to crawl away. However, the intrusion of the hair on the + side of the body <u>always</u> caused a directed movement away from the probe. Likewise, if a hair was placed in the direct path of the mutant side of the body, the fly continued in the direction in which it was travelling, allowing the mutant legs to brush by the hair. If the same hair was placed in the path of the + side of the body, the fly invariably veered in a direction away from the object. These tests seem to indicate that the afferent neuron on the mutant side of the body is not working, or if it is functioning, the thoracic ganglia cannot sort out the message. These possibilities cannot be definitively resolved without the use of electrophysiology.

Finally, one experiment was performed to test the possibility that inhibitory neurons were malfunctioning at 29°C and thus induced

the paralysis. Wild-type flies can walk after decapitation. If the lack of movement in complete head mosaics is caused by the malfunctioning of inhibitory neurons, then decapitation might remove the inhibition and permit walking. Only two complete head mosaics were found and at the time were not used for this experiment. However, four complete head and bilateral thorax mosaics were available for the experiment. This class of mosaics is inactive at 29°C, while bilateral thorax mosaics are fairly active. Upon decapitation of the bilateral head-thorax mosaics at 29°C, leg movement on the wild-type side was not initiated. However, this does not critically rule out the possibility that inhibitory neurons are affected by the $\underline{para}^{ts}$ mutations.

IV. Discussion

The inability of abdominal genotype to affect behavior of a head and thorax of a different genotype (Table 6) and the complete autonomy of each half of bilateral thoracic mosaics shows the absence of a freely circulating inhibitor of movement which might be made in all parts of the fly. Similarly, the fact that Classes IIIc, IVc and IVd mosaics (Figure 14) were paralyzed at 29°C and did not recover coordinated activity any faster than complete $\underline{para}^{ts}$ flies suggests that paralysis is not due to a temperature-sensitive humeral factor necessary for movement. The more rapid recovery of complete head-only mosaics than complete $\underline{para}^{ts}$ flies does not negate the above conclusions. Only two of such mosaics were found and though the recovery times were repeatedly obtained for each, there was considerable variation from one individual to another and we consider the sample size to have been too small to be definitive at this point. The recovery data taken in conjunction with the behavior of several classes of mosaics all point to the autonomy of the $\underline{para}^{ts}$ mutation.

The genetic and biological properties of the $\underline{para}^{ts}$ mutation point to a specific defect in the nervous system of the adult which regulates both flight and walking. The autonomous activity of the mutation permitted an analysis correlating behavior with the position of somatic mosaicism. This localized the components involved in the paralytic effect of $\underline{para}^{ts}$ to cephalic and ventral thoracic areas which correspond to the sites of the cephalic (brain and subesophageal ganglion) and thoracic ganglia (Figure 17). The fact that complete head mosaics as well as mosaics with completely mutant head tissue and mosaic legs could not walk at 29°C suggests that the mutation

certainly affects the CNS. The mobility of leg mosaics minimizes the possibility that the mutant has defective muscles. The positive ERG of *para*^{ts} flies at 29°C shows that mutant eyes can transduce light into impulses, yet the poor optomotor response of the mutant eye in bilateral head mosaics suggests that the brain does not receive or cannot interpret these messages. The epistasis of *para*^{ts} over *Hk-1*, a mutation whose phenotype results from an abnormal pattern of nerve firing (Ikeda and Kaplan, 1970a) supports the contention that *para*^{ts} causes a lesion in the central nervous system (Suzuki *et al*., 1971). Electrophysiological tests are being attempted to determine whether there is transmission across neuro-myal and interneural junctions at 29°C.

The ability of the eyes to transduce light into impulses led us to ask whether other sense receptors, or afferent neurons in general, functioned at 29°C. The observation that tethered bilateral mosaics as well as *para*^{ts} flies, with few exceptions, terminated flight at 29°C, would indicate that sense receptors and afferent neurons function at 29°C in *para*^{ts}. However, the observations from the prodding experiments on bilateral mosaics suggest that if, in fact, the afferent neurons function at 29°C, then their messages are not received and/or interpreted by the ganglia of the CNS. The question of whether or not afferent neurons function at 29°C remains unresolved.

On the assumption that *para*^{ts} does indeed affect nerves, inferences on CNS circuitry can be made. The existence of an ipsilateral and/or contralateral connection of the subesophageal ganglia with the prothoracic ganglion and its relationship to the behavior of the first pair of legs is of interest. While the fused nerves of the

subesophageal ganglia send off a pair of cervical nerves which innervate the muscle about the neck, it is unlikely that they also innervate the first pair of legs. The addition of mutant head tissue ipsilateral to the mutant tissue of bilateral thorax mosaics reduced the activity of the mutant first leg but still did not render it as debilitated as the second and third legs. This suggests that while the head tissue does affect the behavior of the first leg, its control is not direct. The results might be rationalized as a simple additive effect of mutant tissue. Perhaps the $\underline{para}^{ts}$ mutation, while inducing paralysis, may allow occasional neuronal activity (i.e., it may be leaky). In a bilateral thorax mosaic (Class IIIb), the incoming signals from the wild-type head may affect some firing of the motor neurons to the first leg. The amount of signal reaching the mesothoracic ganglia from the head could be reduced or garbled by the partially mutant prothoracic ganglia and thus may not influence firing of the motor neurons associated with the mesothoracic and metathoracic ganglia. The addition of mutant tissue to the head might lessen the number and strength of the signals reaching the prothoracic ganglion and thus have a reduced effect on the first leg.

The proposal that the $\underline{para}^{ts}$ mutation allows some, though greatly reduced, neural activity may be supported by the results from the optomotor response. In the bilateral head mosaics in which the + eye was painted, there was a positive (though much less strong) optomotor response occasionally. The opticon (optic lobe of the brain) on one side of the head, in addition to being connected to the protocerebrum, is connected to the opticon of the opposite side of the head by fibres in the optic tract. The occasional weakly positive - or allow-

ing for anthropomorphism, perhaps confused - response may be due to an occasional signal reaching the wild-type side of the brain from the eye on the mutant side of the head.

The fact that the wild-type legs of bilateral head-thorax mutants are more debilitated than the + legs of bilateral thorax mosaics suggest that there is contralateral connection of the head and thorax. This contralateral connection is clearly not a direct innervation and control of the right side of the thorax by the left half of the head. It may be that there is simply an efficient exchange of information contralaterally as well as ipsilaterally and the lowered activity of bilateral head-thorax mosaics may simply be an additive effect resulting from an increasing loss of CNS activity.

As stated, the mobility of leg-only mosaics minimizes the likelihood that the mutant has defective muscles. However, while the leg-only mosaics were not paralyzed, they were often visibly affected. If it is true that the ventral nerves originating in the thoracic ganglia continue to the distal segments of the legs without synapsis with neurons extending from the leg discs, then the observed abnormal behavior of mutant legs on a wild type thorax is puzzling. There is always the possibility that internal mosaicism was not detected, since the invagination of the leg buds during embryogenesis occurs in the proximity of the ventral nerve formation. Alternatively, the effect of $\underline{para}^{ts}$ on interneural transmission may be postsynaptic. Interneural and neuro-myal transmission being similar, $\underline{para}^{ts}$ may also have some postsynaptic effect on neuro-myal transmission, though its effect may be reduced in muscles. Finally, the afferent neurons from the sensory receptors may be affected in leg-only mosaics with the result

that the fly tends to favor the mutant leg.

The restriction of the effect of $\underline{para}^{ts}$ to the adult fly is of considerable developmental significance. Power (1952) noted that during pupation there is no manifest regression of CNS material which can be correlated with histolysis of specifically larval tissue. After the onset of pupation, there is an increase in the amount of tissue associated with the neuropile of the CNS and a concomitant decrease in cortex material. Thus, there is a decrease in the size or amount of neuron bodies of the cortex with a concomitant increase in their aggregated fibrular processes which make up the neuropile. The adult specificity of $\underline{para}^{ts}$ suggests the possibility of separate genetic regulation of movement in larvae and adults.

Since $\underline{para}^{ts}$ flies survived prolonged exposure to high temperature, it was assumed that the vital organs still functioned in the paralyzed fly. Indeed, the heart, which could be easily seen through the dorsal wall of the abdomen, was observed to beat quite normally in paralyzed individuals. According to Miller (1950), there appears to be a dorsal median nerve present in the first chamber of the heart. No nerve connection has been traced, but the heart may be innervated from the stomodeal ganglion of the sympathetic nervous system. This suggests that the $\underline{para}^{ts}$ mutation has little or no effect on the autonomic nervous system.

The use of somatic mosaicism to delineate anatomical sites affected by a mutation depends upon the validity of the assumption that external genotype reflects the genotype of cells underlying the chitin. The resolution of questions such as: where in the CNS does the $\underline{para}^{ts}$ lesion occur; is the autonomic system affected; are

inhibitory neurons affected; and, is the effect of $\underline{para}^{ts}$ indeed post-synaptic, will require a method for distinguishing internal mosaicism. One promising method is the use of quinacrines to stain heterochromatic bodies (Lewis, personal communication). If such a heterochromatic body was inserted into the unstable ring X chromosome, its loss could be detected. Alternatively, a distally located heterochromatic element on a wild-type rod X chromosome could be eliminated by mitotic crossing over which would also homozygose $\underline{para}^{ts}$. Other methods of cell markers using enzyme variants may also be possible (Hotta and Benzer, 1970).

The mosaic analysis has led us to conclude that $\underline{para}^{ts}$ affects the CNS. However, behavioral mutants exhibiting this phenotype could quite easily affect muscles as well as sensory neurons. Several mutants affecting one organ of sensory input, the eye, have been isolated and described (Hotta and Benzer, 1969). Ikeda and Kaplan (1970a)have described a mutant, Hk-1, which results in a lesion in specific motor neurons of the thoracic ganglia associated with the legs. Thus, mutations affecting behavior may provide a useful probe into the properties of elements of the nervous system.

The property of temperature-sensitivity made it possible to detect and recover the behavioral defect of paralysis. The availability of methods for mutagenesis and screening on a large scale make the search for mutations affecting nerves and muscles based on a paralytic phenotype quite feasible. Selection schemes for paralytic larvae can also be readily constructed. Thus, the technique of isolating temperature-dependent behavioral mutants promises to yield various mutations of considerable biological interest.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Three problems of gene action during development have been investigated: (1) the temporal activity of a single gene known to function in several different tissues, (2) the effect of a gene known to affect determination or regulation of differentiation of a specific imaginal disc and (3) the genetic regulation of the nervous system. The availability of temperature-sensitive mutations permitted the analysis of each of these problems.

With regard to the first problem, it was demonstrated that the product of a specific gene which is required in at least three different tissues during development is produced at different times during development in each tissue. These results, in conjunction with those of other workers, led to the conclusion that the gene is probably transcribed at different times during development in the different tissues in response to tissue specific stimuli. It may be worth noting that in the life of any given cell, the gene is only turned on and off once, whereas in the animal as a whole, there appears to be a succession of activation and inactivation. Some evidence was presented that suggested that this gene may affect membrane and/or pigment granules in differentiating eye tissue.

The temperature-sensitive mutation, $\underline{ss^{a40a}}$, was shown to affect the choice of the pathway of differentiation of the antenna. When raised at 28°C, $\underline{ss^{a40a}}$ flies grew normal aristae. However, when raised at 17°C during the third instar, there was a change in determination of the cells which produce the arista so that a tarsus was

produced. Shift studies showed that this effect was not due simply to a gradient of a freely flowing determinative substance. This seems to offer evidence against the simple models for positional information and subsequent determination suggested by Wolpert (1969). However, one may invoke cell migration as an effector of final phenotype or a more complex model of positional information may be posed. The shift studies also showed that the gene product was active during third instar. This information, in conjunction with induced somatic crossing over experiments, may tell us when the gene was transcribed.

The combination of $\underline{ss}^{a40a}$ and $\underline{Antp}^{B}$ produced a complete leg at 22°C. Since each of these two genes alone exhibit poor expression at 22°C, and are supposed to affect different segments of the antennal structure, it was suggested that their effect, when combined, may be evidence for cell communication in imaginal discs. It is possible to test whether this extreme phenotype is due to cell communication or is an epigenetic effect. Antennal disc cells from differently marked $\underline{ss}^{a40a}$ and $\underline{Antp}^{B}$ stocks, can be dissociated, mixed and implanted into a larval host. The metamorphosed structure is then compared to the structure formed from + $\underline{ss}^{a40a}$/+ $\underline{ss}^{a40a}$; $\underline{Antp}^{B}$ +/+ +; + +/+ + and $\underline{Antp}^{B}$ $\underline{ss}^{a40a}$/+ $\underline{ss}^{a40a}$ disc cells treated in a similar manner but not mixed. If cell communication occurs, the mixed implant should resemble $\underline{Antp}^{B}$ $\underline{ss}^{a40a}$/+ $\underline{ss}^{a40a}$ control. If the mixed cells look like the + $\underline{ss}^{a40a}$/+ $\underline{ss}^{a40a}$ and $\underline{Antp}^{B}$ +/+ + controls, then epigenesis may be the cause of the extreme phenotype observed in the combinant at 22°C.

The high penetrance and expressivity of $\underline{ss}^{a40a}$ at the two temperatures, make it ideal for initiating biochemical studies with an

aim to understanding the molecular basis for determination and regulation of differentiation in higher organisms.

The last study was an attempt to determine whether mutants affecting the nervous system and/or muscles could be obtained. Such a mutant was successfully obtained. From the studies so far, it seems that this mutant affects the central nervous system and affects only adults. The fact that its effects are confined to the adult suggests either that in adults and larvae the mode of neural transmission or that the functional anatomy is quite different. The existence of a neural mutant (*para*^{ts}) which acts only in adults, suggests that other mutants of the nervous system might be found to affect only the larval stages. Likewise, there might be a class of neural mutants that affect both adults and larvae. Some of the nerve or muscle mutants that affect either adults or larvae may acquire the additional value of functioning as animal models for some human diseases. For instance, one could select for mutants which cause degeneration of only the adult nervous system as a model for Huntington's Chorea.

The *para*^{ts} mutations quickly disrupts neural activity, and thus it probably does not depend on any *de novo* macromolecular synthesis for its effect. It may be argued that mutants which require a long exposure to high temperature to cause paralysis may be lesions in macromolecules whose continual synthesis is required for normal neural activity.

We have seen that Drosophila provides an excellent model for the study of development in higher organisms. The advent of the widespread use of temperature-sensitive mutants in Drosophila has brought a new dimension to the analytical power of genetics in Drosophila and at the same time has opened up new areas for investigation.

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