THE ISOLATION AND STUDY OF TWO MUTANTS AFFECTING MOTOR ACTIVITY IN *DROSOPHILA MELANOGASTER*

by

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ABSTRACT

Mutants of Drosophila melanogaster which are paralysed by exposure to one temperature, but recover mobility at another temperature may aid in the investigation of the neural and muscular components which govern motor activity. With the help of a mechanical screening method, a recessive sex-linked temperature-sensitive paralytic mutant (para^{ts} -53.9) was discovered among the progeny of ethyl methane-sulphonate-treated males and attached-X females. para^{ts} flies which had been raised at 22°C were paralysed within 5 seconds after transfer to 29°C, but quickly regained mobility when returned to 22°C. When left at 29°C for prolonged periods, the flies gradually regained mobility. Further studies indicated that the time required for recovery following an increase in temperature was directly related to the magnitude and rate of the temperature rise. Temperature-sensitive paralysis was seen only in adult flies.

The abnormal movements which are characteristic of the behavioural mutants Hk^{1P}, Hk^{2T}, Sh^{5}, when linked to para^{ts} in males, were quickly stopped and started by temperature shifts from 22°C to 29°C and 29°C to 22°C, respectively. The possible significance of these observations is discussed.

para^{ts}/M(1)0 females exhibited temperature-sensitive paralysis. The possibility that the chromosome bearing the M(1)0 mutation might also carry a deletion or mutant allele of para^{ts} has not yet been investigated.

A sex-linked dominant mutation which caused abnormal movements of the head and appendages under ether anaesthesia as well as shuddering

movements in unetherised flies was also discovered. The mutation was called Shuddering (Shu - 55.1). The shuddering movements could be temporarily suppressed by feeding the flies media containing LiCl but not NaCl, NH_4Cl or KCl.

The evidence presented in this and other studies suggests that the effects of $para^{ts}$ and Shu mutations upon motor activity are mediated through their effects upon the nervous system.

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INTRODUCTION

Comparative neuroanatomy was founded in the Alexandrian schools of medicine in 300 B.C. Since that time, knowledge of the nervous systems of numerous animals has been enlarged through many disciplines. The current interest in neurobiology represents a renewed awareness of the diversity of nervous systems which can now be analysed with a large assortment of technical and biological tools. In view of the dramatic success of genetic analyses of microbial systems, many investigators now wonder whether the mysteries of the nervous system may also be amenable to a similar strategy.

Although the effects of single mutations upon behaviour have been studied for some time (McEwen, 1918), an organised pursuit of single mutations which affect a specific nervous function was first launched in Drosophila melanogaster by Benzer (1967). His search has yielded many nonphototactic mutants. Those which are characterisable by abnormalities in their electroretinograms have been assigned to five cistrons of the X chromosome by genetic mapping and complementation tests (Hotta and Benzer, 1970).

Brenner (1970) pointed out that the morphogenesis of a nervous system probably requires extensive genetic regulation. Mutations affecting regulation are therefore likely to be found among behavioural mutants. Accordingly, Brenner has conducted a search for nonspecified behavioural deviants using a Nematode which possesses 50 axons and a ganglion comprised of 20 neurons. 350 mutants have been characterised and 20 functional groups have been defined. Electron microscopic studies of some of the mutants have revealed alterations of the nerve

circuitry. Unfortunately, the species has not been accessible to electrophysiological probes.

Whiting (1932) was able to determine that the expression of sex specific behaviour in the wasp Habrobracon juglandis depended upon the genetic composition of the head, by studying gynandromorphs. Genetic mosaics have also been analysed by Ikeda and Kaplan (1970b) and Hotta and Benzer (1970) to determine the regional specificity and autonomy of behavioural mutants of Drosophila. In the latter studies, the frequency of genetic mosaics was increased by using the somatically unstable ring chromosome In(1)w^{vC} (Hinton, 1955) or the mutation claret-nondisjunctional (Lewis and Gencarella, 1952).

The genetic manipulations made possible by the variety of chromosome aberrations and mutations in Drosophila melanogaster, together with the accumulated knowledge of the organism, sustain its usefulness as a tool for genetic study. Furthermore, the neurophysiology of the organism is now accessible to intracellular electrical recording techniques (Ikeda and Kaplan, 1970a). The use of mutations to probe the adult motor nervous system was initiated by Kaplan and Trout (1969) and Ikeda and Kaplan (1970a,b). The existing knowldge of this system and its relationship to muscular physiology make it a promising area for molecular investigation.

The first step towards a genetic study of neural and muscular function is the isolation of mutants. Paralysis is both a logical and a feasible mutant phenotype to look for. The extensive documentation of conditional mutations whose phenotypes are temperature-dependent (Suzuki, 1970) suggested a search for temperature-dependent paralysis. A mutation which allows mobility at one temperature, but is reversibly

paralysed by another provides a basis for large scale screening methods for its detection. If the fly recovers mobility after it is returned to the "permissive" temperature, the mutation may be perpetuated as a stock.

By screening for temperature-sensitive paralysis, individuals exhibiting various kinds of debilitated motor activity might also be detected. With the recovery of each behavioural mutant, the probability that one of them will be accessible to an existing means of investigation increases. The same rationale applies to the paralytic phenotype itself. From a variety of defects which cause paralysis, a new access to the neuromuscular system may be found.

METHODS AND MATERIALS

General Procedures.

Complete descriptions of the mutations affecting behaviour will be given; other stocks used routinely have been described by Lindsley and Grell (1968). Unless specified, the medium was the standard mixture used at the California Institute of Technology.

In experiments requiring media containing various molarities of LiCl, NH_4Cl, KCl and NaCl the appropriate salt solution was diluted ten fold with standard media and blended with a Sorvall Omnimixer.

Crosses were made in shell vials or quarter and half pint milk bottles. The standard laboratory temperature was $21.5 \pm 1^{\circ}C$.

Screening devices for recovery of paralysed flies.

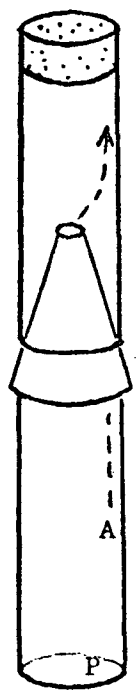
Theoretically a paralysed individual should be easy to find within a group of active flies. Unfortunately, a caged population of normal flies usually includes many which stay perfectly still at any one moment. Because each fly goes through its own cycles of activity and inactivity, the membership of the motionless segment of the population is constantly changing. For this reason, direct attempts to determine which motionless fly was actually paralysed proved to be impractical. Two types of apparatus were designed to overcome this problem.

Considerable success in separating mobile from immobile flies was achieved by placing the flies in an empty shell vial which was capped with a paper funnel leading upward into another shell vial containing medium (Figure 1). The flies, driven by their tendency to move upward and the attraction of the medium, climbed into the upper

FIGURE 1

Type I-a screening device for isolating paralysed flies. The device consists of an upper and lower vial facing mouth to mouth separated by an upwardly directed paper funnel. The upper vial contains media.

An active fly (A) climbs into the upper vial. After many flies have left the lower vial, the paralysed fly (P) is more easily noticed among the diminished population.



vial. The cone was a fairly efficient valve which prevented migrants from returning. Immobile individuals were selected from the diminished population of the lower vial. This assembly will be referred to as the type I-a screener. A modification of this design is shown in Figure 2. Vials positioned above and below funnels cut out of $\frac{1}{2}$" plexiglass were held in place by foam rubber cushions. This device (type I-b) permitted easy handling of the vials and provided an unobstructed view of the flies.

When a large population of flies is confined to a small space, the flies tend to cluster, their activity seems aimless and their antigeotactic behaviour diminishes. Consequently, the type I designs imposed a limit of a few hundred flies per vial for optimal screening efficiency.

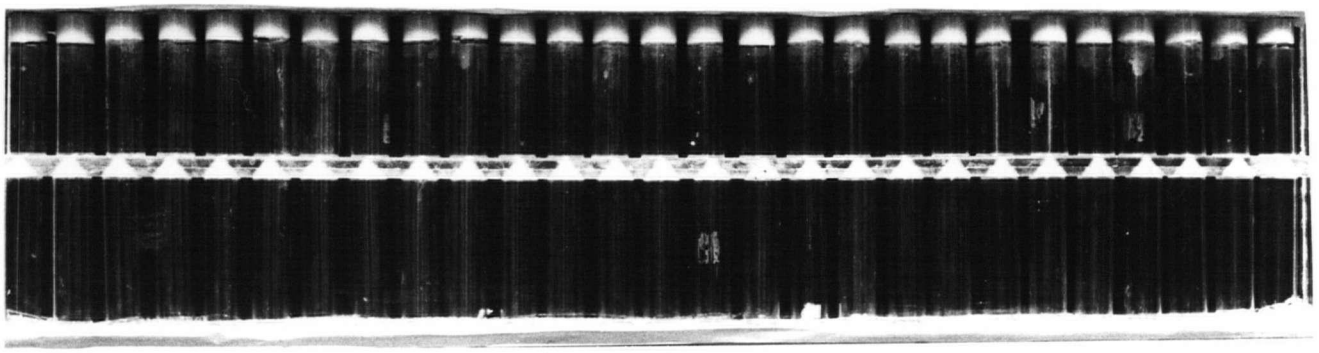
Benzer (1967) found that nonphototactic mutants could be selected by counter current distribution from a population of several hundred flies placed in an 18 x 150 mm test tube. However, each group of flies to be tested must be put through several operations and tubes.

A second limitation of the type I device was the dependence on a constant behaviour pattern, namely migration through the funnel into the upper vial. Many flies were capable of climbing, but simply remained in the lower vial. Some showed no antigeotactic tendency while other flies stopped inside the funnel at its lower edge.

The efficiency of the screener was somewhat improved by selecting for a strain of flies characterised by its ability to reach the upper vial quickly. Mutants were induced and selected from this stock which will be referred to as F.C. for fast climbers. However, heterogeneous behaviour was never eliminated. Consequently, a more efficient

FIGURE 2

Type I-b screening device for isolating paralysed flies. The device works on the same principle as the type I-a. In this case, the upper and lower vials are pushed against funnels of plexiglass with foam rubber cushions.



separating device was needed.

The type II screener (Figures 3 and 4) is basically an elongated box with a diagonal barrier (b) extending the length of the box and a drawer (d) fitting into the acute angle between the barrier and the bottom of the box. The diagrams of the end view of the box, shown in Figure 4, illustrate the sequence of operations used to isolate an immobilised individual (P) from a large population. Rotation of the box clockwise 90° and then counterclockwise 90° (i.e., from position 1 to 2 to 3) causes P to fall into the drawer. The activity of the population is greatly increased by introducing a small quantity of vinegar and detergent solution onto the left side of the barrier (3). All flies which are not inside the drawer can be eliminated by rotating the box 90° and shaking the active flies into the solution (4). Finally, P can be selected from among the small number of individuals which remain in the drawer (5).

The design minimises the deleterious effects of crowding, as well as the number of physical obstacles which tend to inhibit free movement. Six thousand flies at a time could be readily screened in the type II box shown in Figure 3.

Screening procedure for dominant temperature-sensitive mutants.

The efficiency of the type I screener depended upon the speed with which a large proportion of the flies would enter the upper vial. In this respect, Oregon-R was better suited to the apparatus than Samarkand. The efficiency of separation was further improved by occasionally regenerating the stock with flies which had reached the upper vial most quickly.

FIGURE 3

Type II screening device for isolating paralysed flies. Outside dimensions 6" x 6" x 18" ($\frac{1}{4}$" plexiglass).

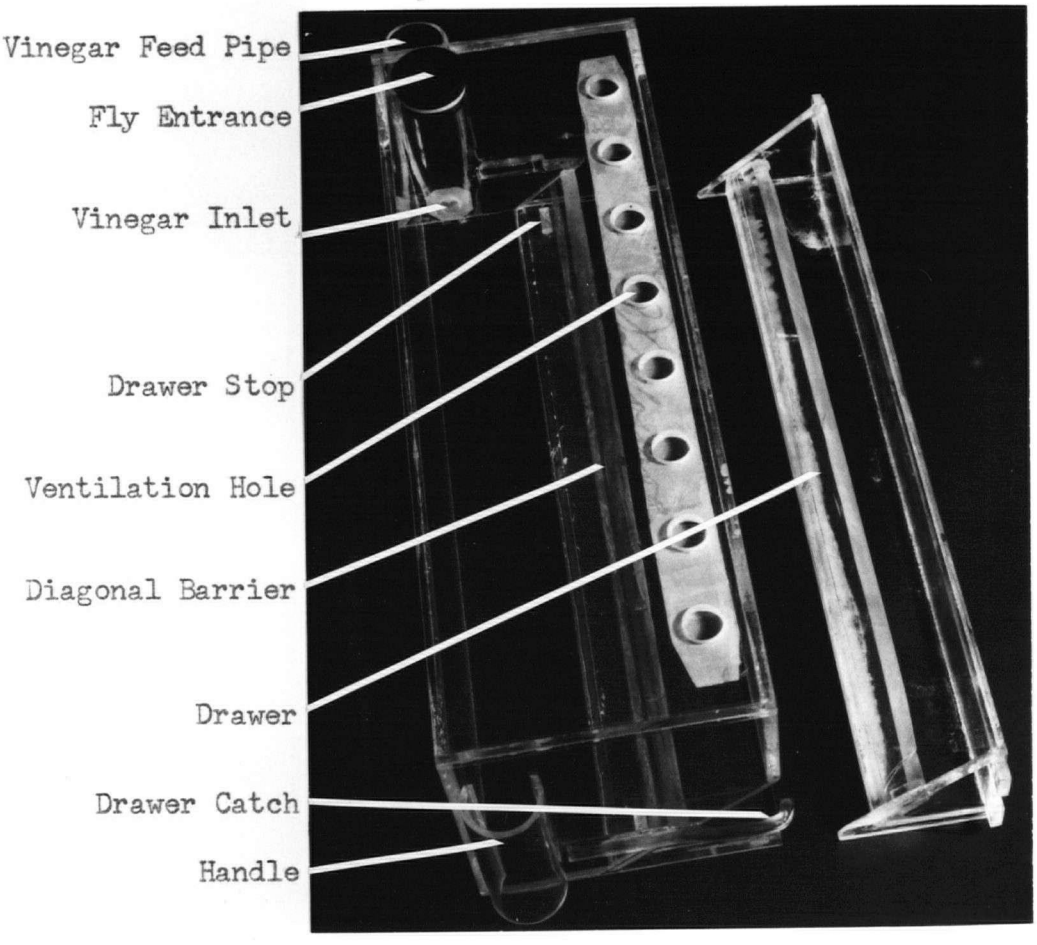
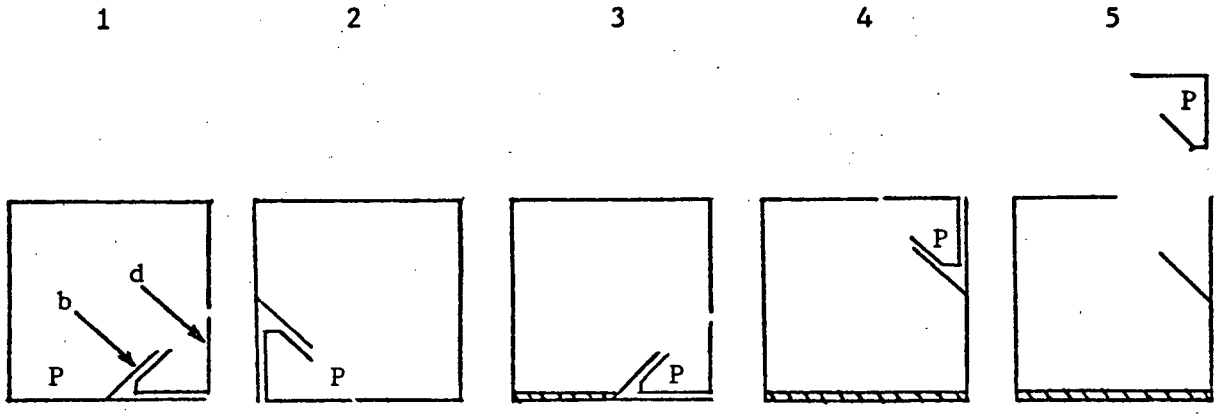


FIGURE 4

End views of the type II screening device during the isolation of paralysed flies. Steps 1 to 5 represent successive stages in the procedure. The label b refers to a diagonal barrier; d is a drawer; P is an immobilised fly. The hatched area represents a vinegar and detergent solution. The operation of this device is explained on page 8 .



F.C. Oregon-R males were left for 24 hours in half pint milk bottles containing two discs of Whatman No. 1 filter paper dampened with 1 ml of 0.025M ethyl methanesulphonate (EMS) in a 1% sucrose solution (Lewis and Bacher, 1968). The mutagen treated males were then mated with F.C. Oregon-R females in quarter and half pint milk bottles (10 to 15 pairs per bottle). These flies were transferred to fresh bottles every 3 to 8 days. Eggs were never collected beyond 16 days after treatment.

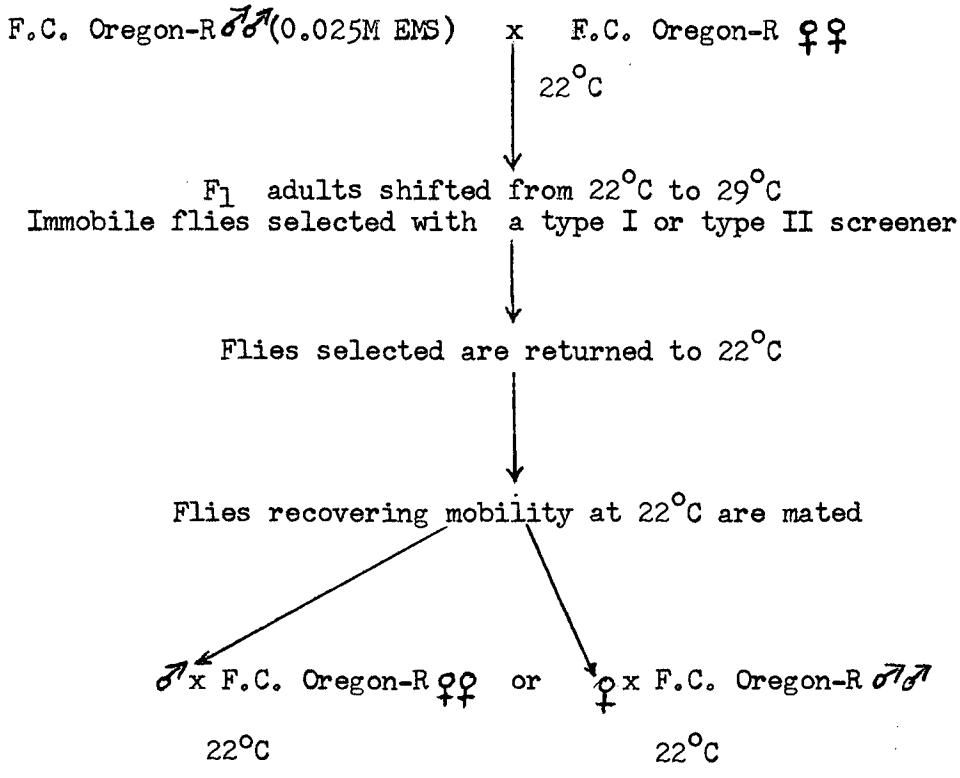
The progeny were raised at 22°C. After eclosion, they were placed into a type I or type II screener which had been prewarmed to 29°C. After 10 minutes to 2 hours at 29°C, the population was screened for immobile flies. Each immobile fly which recovered mobility at 22° was then crossed to 3 F.C. Oregon-R flies in a shell vial. The progeny of the fertile individuals were again raised at 22°C and then tested for the inheritance of paralysis by direct transfer to a 29°C vial. This procedure is outlined in Figure 5.

Screening procedure for sex-linked recessive and autosomal dominant mutations.

A search for sex-linked mutants was also conducted (Figure 6). EMS-treated Oregon-R males were crossed to F.C. females carrying an attached-X chromosome ($\hat{X}\hat{X}$). This permitted the recovery of male progeny carrying mutagenised paternal X chromosomes. All screening was carried out in a type II apparatus. The selected males and females were individually crossed to F.C. $\hat{X}\hat{X}$/Y females and F.C. Oregon-R males, respectively. Their progeny were tested for inherited temperature-sensitive paralysis as described above.

FIGURE 5

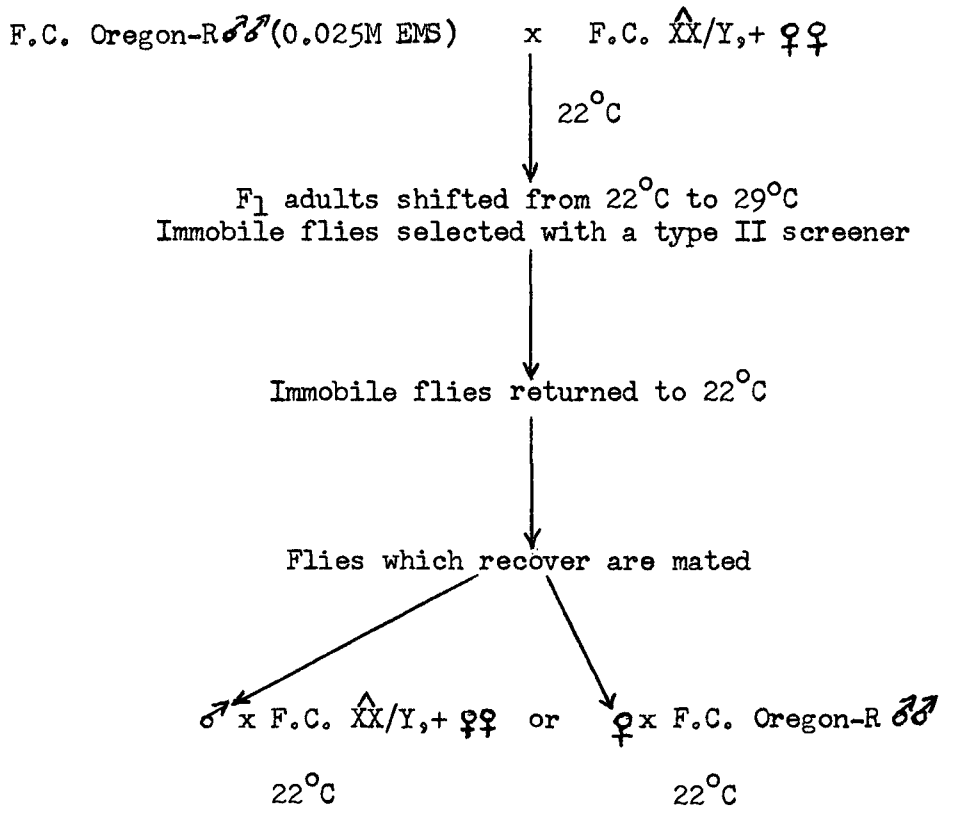
Procedure for detection of a dominant temperature-sensitive paralytic mutant.



Tested for paralysis induced by a 22°C to 29°C shift
Tested for recovery by returning flies to 22°C

FIGURE 6

Procedure for detection of a sex-linked recessive or autosomal dominant temperature-sensitive paralytic mutant.



Tested for paralysis induced by a 22°C to 29°C shift
Tested for recovery by returning flies to 22°C

Recovery and genetic mapping of a shuddering mutant.

In the screen for dominant temperature-sensitive paralysis using a type I-b device, a female which occasionally shuddered was noticed in the lower vial. The behaviour was found to be inherited as a sex-linked dominant; consequently, the mutant chromosome was balanced over the multiply inverted chromosome, FM6.

While anaesthetised under ether, the shuddering flies scissored their wings and shook their legs in quick vigorous spasms. In order to localise the genetic site responsible for the behaviour under ether, females were crossed to flies carrying the following markers (followed by their symbols and genetic location): yellow - y (0.0), crossveinless - cv (13.7), vermilion - v (33.0), forked - f (56.7), carnation - car (62.5). Heterozygous females were testcrossed and all male and female progeny were scored. The shuddering males were extremely weak and uncoordinated and survived only for one to three days under good culture conditions. In order to reduce the severe effects of crowding and competition on these flies, single females were mated to three males in shell vials and were serially transferred to new vials each day for 10 days before being discarded.

This mutant will hereafter be referred to as Shuddering or Shu.

The effects of monovalent cations on shuddering behaviour.

The possibility that lithium ions might suppress shuddering behaviour was suggested by their therapeutic effects upon nervous disorders in guinea pigs and man (Cade, 1949). The similarities between shuddering and these disorders will be discussed later. Ammonium ions were also tested because the aberrant leg shaking of neurological

mutants of Drosophila under ether anaesthesia was reversibly suppressed by ammonia (Kaplan, personal communication).

Four pairs of Shu/FM6 flies were placed in quarter pint bottles (1 pair per bottle) containing various concentrations of LiCl, KCl, NaCl or NH_4Cl, and twelve pairs were placed on standard media. The number of jerks and shudders produced by each pair of flies during one minute was recorded daily. Shudders lasting for about three seconds or more were counted as two shudders. Prior to each observation, the bottle cap was replaced with a transparent plastic petri plate. Following this disruptive manipulation, the bottle was left undisturbed. Under these conditions, flies treated with 0.32 M to 0.35 M LiCl ceased shuddering after three and four days of treatment; however, the mortality rate was high. Consequently, a second series of experiments was performed to test the capacity of LiCl to suppress shuddering induced by mechanical stimulation, and to determine whether or not the flies would continue to live, if they were returned to standard media at critical times.

Groups of 5 to 40 Shu/FM6 flies were placed in quarter pint milk bottles on standard media and media containing LiCl, NaCl and NH_4Cl at 0.2M, 0.32M and 0.33M concentrations. Every 12 or 24 hours, flies in each bottle were shaken down repeatedly. By one and a half days of age, Shu/FM6 flies kept on standard media would inevitably give a shuddering response to this type of mechanical stimulation. Two types of responses were recorded: "no shuddering" or "slight shuddering". The latter class was defined by a few slight jerks seen within a group. Two groups of 40 flies were transferred from 0.32M LiCl to standard media after three days. The rest of the flies were returned to standard media

after six days or at various earlier times when the first deaths occurred in each bottle.

Bottles of media which were not used immediately were stored in plastic bags to reduce desiccation and consequent changes in salt concentration. The flies were usually transferred to new media every three days in order to minimise exposure of the flies to desiccated and microbially infected media. Virgin females were used in most experiments to obviate changes in the behaviour of adults on media which had become softened and dampened by developing larvae. In cultures containing many larvae, Oregon-R flies walked slowly with a slight side to side wobble. The same behaviour was accentuated in Shu/FM6 flies in which the wobble frequently became a more vigorous shudder.

Observation of a temperature-sensitive paralytic mutant.

A male whose paralysis was temperature-dependent was isolated from an $\hat{X}\hat{X}/Y$ mother using a type II screening device. Upon crossing the male to $\hat{X}\hat{X}/Y$ females, all male offspring showed temperature-sensitive paralysis. Females heterozygous for the mutation and FM6 were unaffected by temperature, thereby showing the mutation to be recessive. Homozygous females were also paralysed at 29°C.

Males carrying the mutation were crossed to y cv v f car and their F_1 daughters were testcrossed at 22°C. The F_2 progeny were scored for the visible markers and the males were screened for paralysis at 29°C. The mutation was called paralytic temperature-sensitive and designated as $para^{ts}$.

Preliminary observations of the effects of temperature upon the

behaviour of $\underline{para^{ts}}$ were made by simply transferring the flies to vials which had been preconditioned to various temperatures.

In order to examine behaviour at constant temperatures, the flies were placed into twin observation chambers which were built into a transparent temperature-controlled water bath (Figure 7)*. The temperature in the chambers was maintained by constantly exchanging water between the observation bath and a Blue M constant temperature bath. The temperature was monitored with a Yellow Springs Instrument Company general purpose thermistor probe and registered on a Rustrak recorder. Generally 5 to 10 $\underline{para^{ts}}$ and an equal number of Oregon-R flies were observed over a two hour period following transfer of the flies to the observation chamber. Most experiments required one person to monitor behaviour continuously while another recorded the changes in behaviour with temperature and time.

A second type of chamber was constructed in order to observe the flies while the temperature was being changed (Figure 8). The flies were placed in the bottom of three chambers which were formed by stacking four 3.5cm plastic petri plates on top of each other. The chambers were sealed together with plastic insulation tape. A 1mm air hole was made in each of the upper three plates, each hole having a staggered position, with respect to its vertical axis, in relation to the other two. A thermistor wrapped in insulation tape above the sensing resistor was seated snugly into the lower chamber. By partially submerging this assembly in a water bath, the temperature of the bottom chamber was quickly altered and equilibrated within the variations of the bath itself.

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* This apparatus was designed and built by Tom Grigliatti.

FIGURE 7

Twin water-heated chambers for comparing behaviour at constant temperatures.

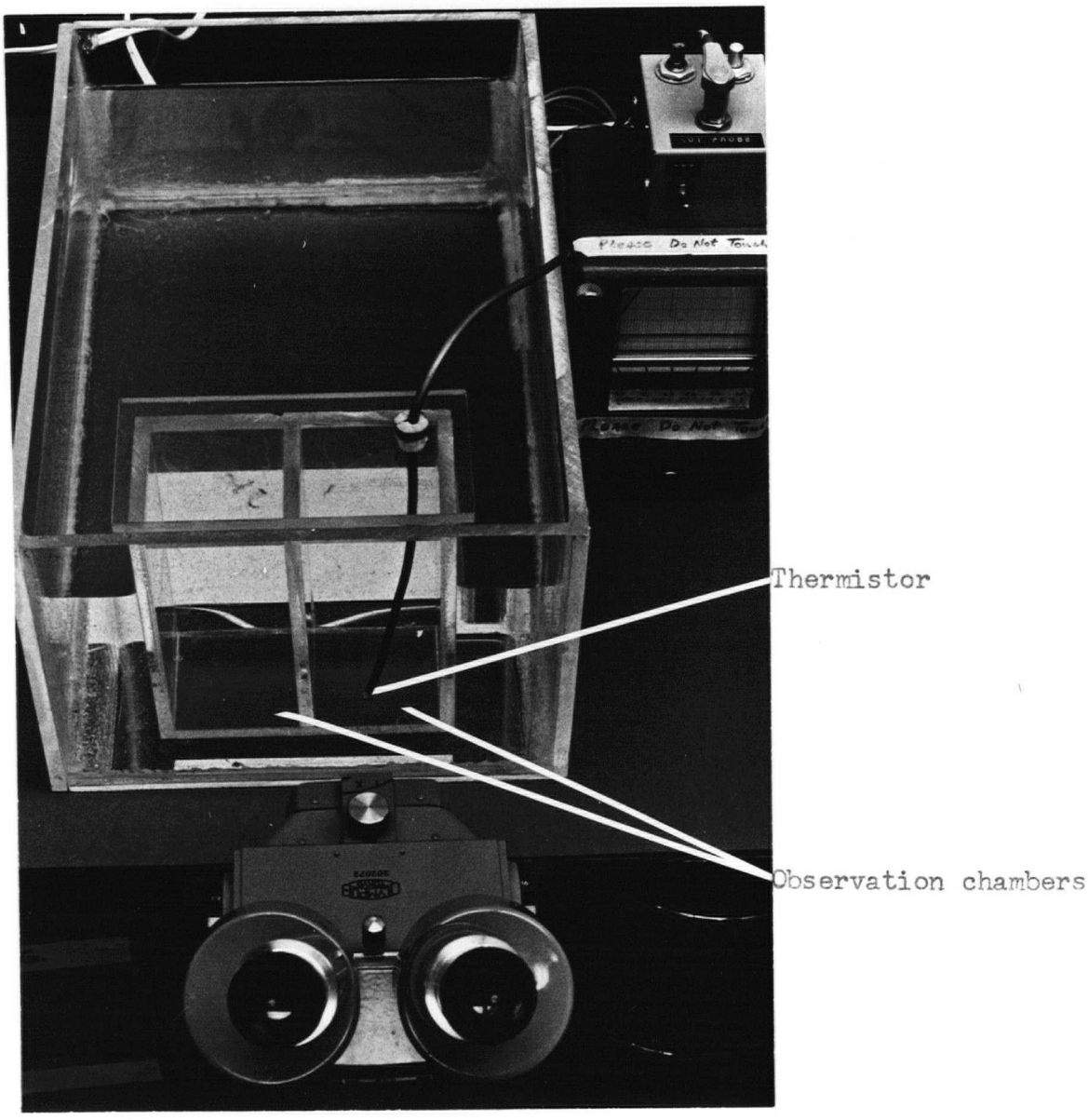
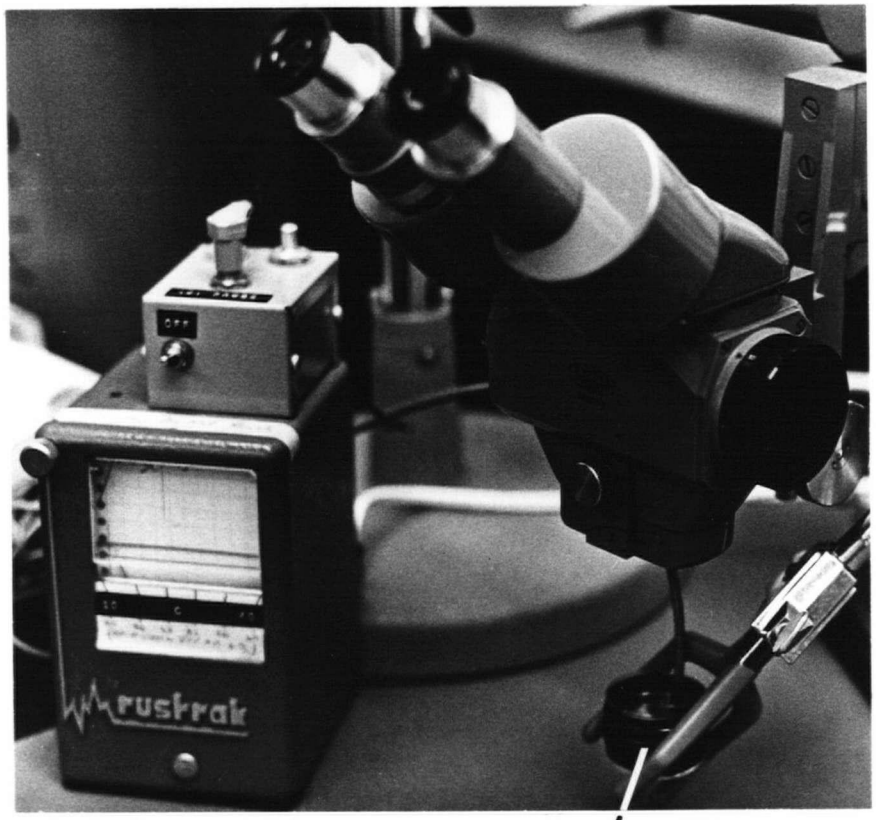
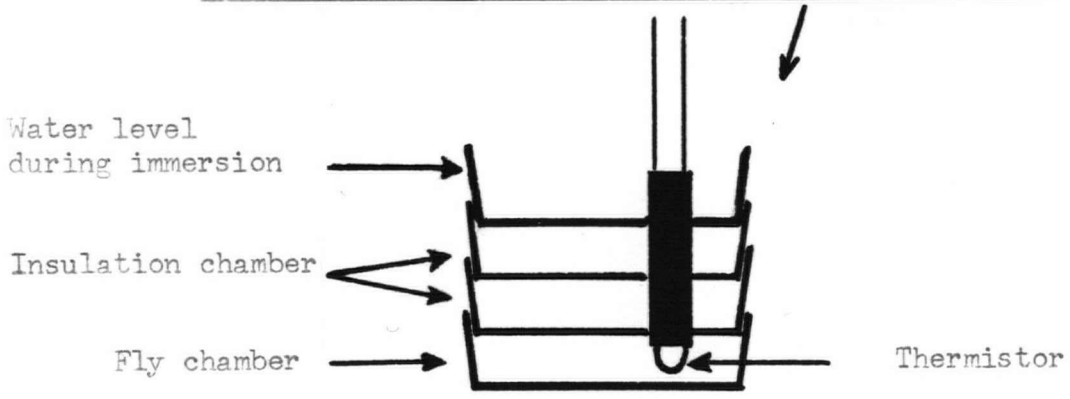


FIGURE 8

A semi-immersible chamber for observing behaviour during temperature changes.





The quickest temperature changes were produced by moving the chamber from one bath to another. More gradual temperature increases were achieved by immersing the chamber in a Haake F.S. constant temperature bath and raising the bath temperature electrically.

Observations were made through a dissecting microscope with sub-stage illumination by light reflected from the bottom of the bath. Overhead light could not be used because the reflections obscured the view, and the heat radiation was sufficiently high to immobilise $\underline{para}^{ts}$ flies.

Because the flies were anaesthetised with ether in order to place them in the chamber, and because the chamber temperature was altered by handling during its assembly, the flies were maintained for an hour at 22°C following their recovery from the anaesthetic.

Interactions between $para^{ts}$ and neurological mutations.

Under ether anaesthesia, the sex-linked mutants Hyperkinetic^{1P}-$\underline{HK}^{1P}$ (30.9 ± .6), Hyperkinetic^{2T} - $\underline{HK}^{2T}$ (30.4 ± .7) and Shaker^{5} - $\underline{Sh}^{5}$ (58.2 ± .6) shake their legs vigorously and rapidly (Kaplan and Trout, 1969). In the case of $\underline{Sh}^{5}$, the leg movements are accompanied by repeated scissoring of the wings. These tremulous flies were called "neurological mutants" by Kaplan and Trout (1969) and will hereafter be generalised by the abbreviation Neuro.

Ikeda and Kaplan (1970a) demonstrated that the characteristic rapid leg shaking of $\underline{HK}^{1P}$ originated as impulse bursts in the three paired motor areas of the thoracic ganglion. The rhythmic bursts were truly endogenous and received no contributions from the cephalic, sensory or neuromuscular systems. Studies of bilateral thoracic mosaics

containing $\underline{HK^{1P}}/+$ * and $\underline{HK^{1P}}/0$ tissues (Ikeda and Kaplan, 1970b) indicated that the electrophysiology of $\underline{HK^{1P}}/0$ and $\underline{HK^{1P}}/+$ tissues and the associated behaviour was bilaterally autonomous within the thorax and also independent of the rest of the body. The interactions of Neuro and $\underline{para^{ts}}$ mutations could be best analysed in Neuro $\underline{para^{ts}}/Y$ males.

Single Neuro +/+ $\underline{para^{ts}}$ females were placed with wild type males for 7 days at 22°C and then discarded (Figure 9, step 1). The male progeny were scored for temperature-sensitive paralysis at 29°C and either-induced leg shaking at 22°C and 29°C. The phenotypes were compared with Neuro, $\underline{para^{ts}}$ and Oregon-R stocks. Newly arising phenotypes which exhibited characteristics of both Neuro and $\underline{para^{ts}}$ were designated Neuro $\underline{para^{ts}}/Y$. The genetic integrity of the phenotype was tested by crossing each male to females carrying attached-X chromosomes and scoring for the same phenotype in male progeny (Figure 9, step 2). The double mutation condition of the chromosomes could be verified by re-isolating the single mutations by recombination. Therefore, single Neuro $\underline{para^{ts}}/++$ females derived from each of the original putative recombinant double mutant males were crossed to Oregon-R males (step 4). The male progeny were then scored for the occurrence of $\underline{para^{ts}}$ and Neuro behaviour separately.

Interaction of $\underline{para^{ts}}$ and Minutes.

The possibility that the mutation M(1)0, located at 56.6, might be a deletion which would span the paralytic region, prompted a test for pseudodominance of $\underline{para^{ts}}$ when heterozygous with M(1)0. A partial paralysis distinct from that of $\underline{para^{ts}}$ homozygotes was observed in

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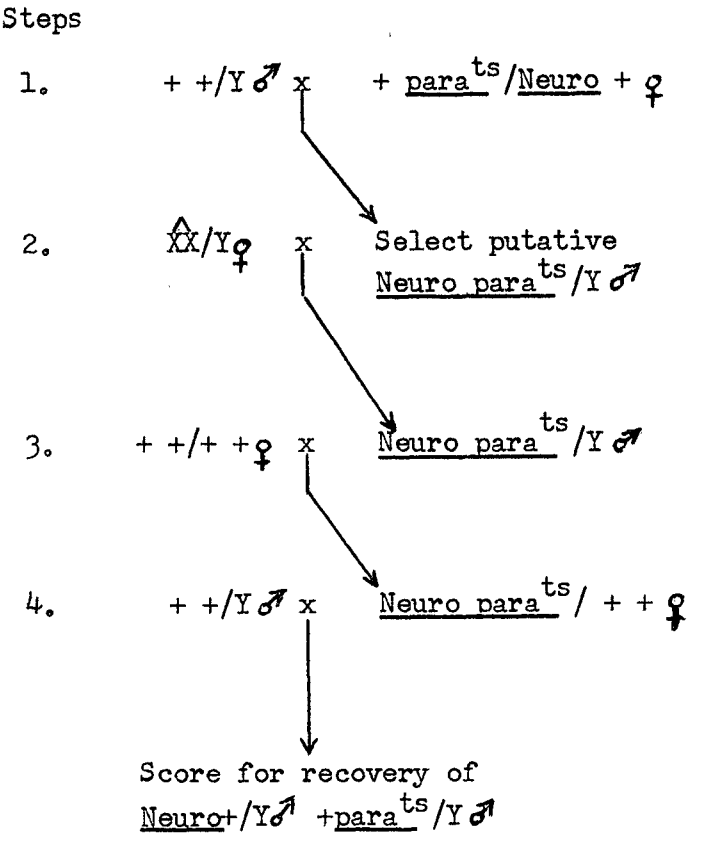
* For all practical purposes HK^{1P} can be regarded as recessive.

FIGURE 9

Protocol for generating and testing Neuro para^{ts}/Y males.



M(1)0 f/para^{ts} females, but not in f/para^{ts} controls. This suggested that Minute mutations in general might affect the expression of para^{ts}. Male para^{ts} flies were crossed to single females of the following genotypes: M(1)0 f/FM6, M(2)e^{S}/SM5; M(2)S^{7}/SM5; M(3)w^{124}/In(3R)C, e 1(3)e; M(3)w^{B}/In(3R)C, e 1(3)e. Five matings of each type were made on two separate occasions. The progeny were raised to 22°C, transferred directly into 29°C vials and observed for any change in behaviour.

RESULTS

Shuddering: phenotype and map position

There were no obvious behavioural differences between Shu/FM6 and Shu/+ flies in either the "conscious" or anaesthetised states. Since the Shuddering mutation was conveniently maintained in Shu/FM6 females, they were the main subjects of study. A four day old Shu/FM6 fly shuddered on an average of 8 times a minute, although individuals varied from less than 1 to more than 50 times a minute. The shuddering phenotype was produced by a rapid sequence of leg jerks which caused the fly to lunge in various directions. The resultant "shudder" lasted for a fraction of a second to about four seconds, and varied in intensity from a slight jerk to a shudder which was strong enough to throw the fly on its back. Although shuddering occurred spontaneously, it could also be elicited by gently moving a bottle containing Shu/FM6 flies which had previously been left undisturbed.

Drosophila can often be induced to fly when they are lifted from a surface by means of a toothpick glued to the dorsal side of the thorax. Under this tethered flight condition, Shu/FM6 flies vibrated their legs continuously. In rare instances, Oregon-R flies also vibrated their legs, but this movement was inevitably stopped during flight.

When Shu/FM6 flies were anaesthetised with ether, they remained still for only a brief period before shaking their legs vigorously and rapidly. The shaking was generated in the tibiae and tarsae while the femurs remained relatively still. The intensity of the shaking was

spasmodic, unlike the continuous tremor of the legs in tethered flight. Eventually wing scissoring began, accompanied by movements of the halteres, antennae and head. These later movements did not normally begin simultaneously, but they were often observed to be in synchrony with one another. Very similar movements were seen in etherised flies which were decapitated. When these headless flies recovered from ether, they righted themselves and then shuddered occasionally, although the shuddering seemed infrequent when compared with whole flies. None of these patterns of behaviour was observed in whole or decapitated FM6 or Oregon-R flies.

Shu/Y males were extremely shaky. They were thrown onto their backs frequently. Under ether they shook their legs more vigorously than the heterozygotes. When they were able to walk, the males usually dragged their wings which were shrivelled and held laterally, drooping towards the tips.

Shuddering mapped at 55.1 ± 0.4*. This position was calculated by the relative position of the mutation observed between v and f and placed into the book distance. The book value for the v to f distance is 23.7 units whereas the observed distance was 19.6 units. The behaviour of recombinants after recovery from ether indicated that shuddering, leg shaking and wing scissoring were not separable by crossing over and therefore were phenotypic manifestations of the same mutation.

Effects of monovalent cations on shuddering behaviour

Table 1 summarises the results of two experiments which tested

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* The 95% confidence limits were calculated by the method of Stevens (1942).

TABLE 1

Frequency of spontaneous shuddering in Shu/FM6 flies after different salt treatments. Frequency is expressed as shudders per fly per minute. Where death has occurred, the survivors are presented in brackets as a fraction of the original number of flies.

		Experiment 1				Experiment 2			
Age in days		0-2	1-3	2-4		1-1 1/2	2-2 1/2	3-3 1/2	4-4 1/2
Days on treatment		0	1	2		1	2	3	4
Salt	Molarity				Molarity				
	0.0	7.1	13.1	12.4	0.0	2	4.7	6.8	8.1
LiCl	0.12	4.4	7.5	11.4	0.32	0.4(7/8)	0.5(6/8)	0.0(6/8)	0.0(6/8)
	0.18	7.3	12.5	9.1	0.33	0.5(6/8)	5.3(6/8)	0.0(6/8)	1.0(2/8)
	0.24	7.0	11.5	17.8	0.34	0.0(7/8)	0.0(4/8)	0.0(4/8)	(0/8)
	0.30	6.1(7/8)	5.6(7/8)	9.7(7/8)	0.35	0.1(6/8)	1.0(5/8)	0.0(5/8)	0.0(4/8)
	0.36	5.5(7/8)	2.2(5/8)	0.0(5/8)	0.36	0.8	3.2	1.6	0.0(5/8)
NaCl	0.12	9.7	15.8	13.0	0.32	1.6	4.5	6.3	8.9
	0.18	10.1	14.9	9.9	0.33	4.0	7.7	8.8	8.8
	0.24	7.9	7.9	12.1	0.34	2.6	8.8	10.3	11.1
	0.30	9.9	13.5	15.3	0.35	1.5	4.3	10.1	10.9
	0.36	12.3	12.4	12.3(6/8)	0.35	3.2	6.0	9.3	9.0
NH_4Cl	0.12	8.8	9.4	0.8	0.32	3.7	12.6	6.8	9.0
	0.18	3.5	14.9	5.5	0.33	1.1	6.9	5.9	9.0
	0.24	12.4	7.9	7.3	0.34	4.3	5.0	4.9	3.9
	0.30	12.1	13.5	14.5	0.35	2.6	2.8	3.6	3.8
	0.36	9.8	12.4	4.0	0.36	2.6	8.6	1.8	5.8
KCl	0.32					5.3	2.1	0.5	4.4
	0.33					3.2	3.1	3.1	5.0(7/8)
	0.34					3.6	0.8	5.9	5.8
	0.35					1.7	0.4	3.4	2.0
	0.36					1.0	0.9	2.3	1.9

the effects of various salts on spontaneous shuddering in undisturbed Shu/FM6 flies. The experiments differed with respect to the ages of the flies and the times at which observations were made relative to the beginning of treatments. The shuddering frequency of flies placed on standard media and NaCl media increased with time. No clear trends were seen in flies placed on NH_4Cl and KCl. 0.32M to 0.35M LiCl seemed to suppress shuddering after three or four days of treatment, however many flies died during this time.

A second set of experiments tested the capacity of LiCl to suppress shuddering induced by mechanical stimulation and attempted to increase survival by returning the flies to standard media at various critical times. The number of flies placed on each treatment together with the number which subsequently died is presented in Table 2. The results of the treatments which suppressed shuddering are presented in Figure 10. Although many flies died on 0.32M and 0.33M LiCl, the number of survivors (102 out of 131) did not change from day 6 to day 13. The tests clearly indicated a reduction or complete suppression of shuddering after three days of treatment with 0.32M or 0.33M LiCl. The previous data indicated that even under less disturbing conditions, untreated Shu/FM6 flies of an age comparable to the treated series would be shuddering at an average rate of 7 times a minute. Shuddering was also suppressed in a group of 5 flies placed on 0.2M LiCl and 5 flies on 0.33M NaCl. Shuddering could not be elicited from the latter group when they were first placed on the treated media. This nonresponsive behaviour was occasionally seen in very young flies. Shuddering was easily elicited on subsequent days. Therefore, the initial observation probably did not reflect an effect of the NaCl.

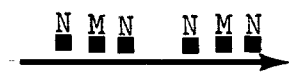
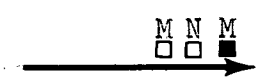
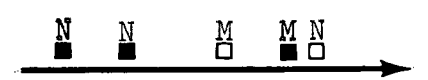
TABLE 2

Total numbers of flies tested for shuddering induced by mechanical stimulation after different salt treatments.

	0.2M	0.32M	0.33M
LiCl	25	66 (15 died)	65 (14 died)
NaCl	5 (1 died)	5	5
NH_4Cl	10	10	10
Standard Media 45			

FIGURE 10

Reduction of shuddering in Shu/FM6 flies subjected to mechanical stimulation. White squares indicate slight shuddering, black squares no shuddering. Arrows indicate duration of treatment. Survivors are presented as a fraction of the original sample size. M and N signify observations made at midnight and noon, respectively.

Age of flies	# Observations per 24 hours	Survivors/ flies tested	Treatment	Time in days 0 1 2 3 4 5 6
0-1	1	29/40	.32M LiCl	
0-1	1	30/40	.33M LiCl	
1/2 - 1	2	4/6	.32M LiCl	
1/2 - 1	2	4/5	.33M LiCl	
1 - 1 1/2	2	9/10	.32M LiCl	
1 - 1 1/2	2	8/10	.33M LiCl	
1 - 1 1/2	2	9/10	.32M LiCl	
1 - 1 1/2	2	9/10	.33M LiCl	
1/2 - 1	2	5/5	.2 M LiCl	
1/2 - 1	2	5/5	.33M NaCl	

Lethargic behaviour and a fine tremor which are symptoms of toxic levels of lithium in rats and men (Schou, 1958, 1959), were occasionally seen in Shu/FM6 individuals and were usually followed by death. However, most flies in which shuddering had been suppressed showed neither of these symptoms. In fact, they appeared to be more active than untreated Shu/FM6 flies of the same age.

Isolation of $para^{ts}$

An estimate of the number of flies screened for sex-linked recessive or autosomal dominant temperature-sensitive paralysis was obtained by counting the number of flies in one or two bottles out of every thirty screened. The results are summarised in Table 3.

Of the estimated 250,000 flies screened, 293 were immobilised at 29°C. 200 of these were found to be dead. It is not known whether death occurred before, during or after the screening process. Of the 93 flies which recovered mobility at 22°C, 34 were fertile. One fly was found to transmit heritable temperature-sensitive paralysis. The sex-linked mutation, $para^{ts}$, mapped at 53.9 (Suzuki et al., 1971).

It is thought that a much larger number of flies was screened for dominant temperature-sensitive paralysis, although estimates of this number were not made. The chances of finding the dominant mutation may have been altered by the use of two types of screening devices rather than one. For these reasons, a valid comparison of the success of the two screening procedures cannot be made.

Effects of different temperatures on $para^{ts}$ flies

At 22°C, $para^{ts}$ flies exhibited normal walking, climbing and

TABLE 3

Estimated number of flies screened for sex-linked recessive and autosomal dominant temperature-sensitive paralysis.

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

flying abilities. When shaken into vials which had been preheated to 29°C, they became paralysed within 5 seconds. Upon returning to 22°C, they regained very active mobility within 2 or 3 seconds. Paralysis and recovery could be induced repeatedly in this way with no apparent harm to the flies. When kept at 29°C for prolonged periods, $\underline{para^{ts}}$ flies did not remain paralysed. After 30 minutes, most flies were able to get to their feet. At still later times, they were able to walk and climb. However, these flies never exhibited the same degree of coordination and activity that they had shown at 22°C. At any time, a shift to 22°C would result in an immediate recovery of wild type activity.

The mutation did not appear to affect visceral muscles. After a shift from 22°C to 29°C, heart pumping could still be seen through the dorsal abdominal cuticle of $\underline{para^{ts}}$ flies. After being transferred from a 22°C to a 29°C Drosophila Ringers solution (Ephrussi and Beadle, 1936), the $\underline{para^{ts}}$ ejaculatory duct also continued to pulsate.

None of the larval instars was affected by transfer from a 22°C culture to Drosophila Ringers solutions kept at 29°C. However, a comparative study of the development of $\underline{para^{ts}}$/Y and XX,$\underline{para^{ts+}}$/Y siblings indicated that the number of successful eclosions by the males was normal when pupae raised at 29°C were shifted to 22°C prior to eclosion, but greatly reduced when pupae were maintained at 29°C (Suzuki et al., 1971).

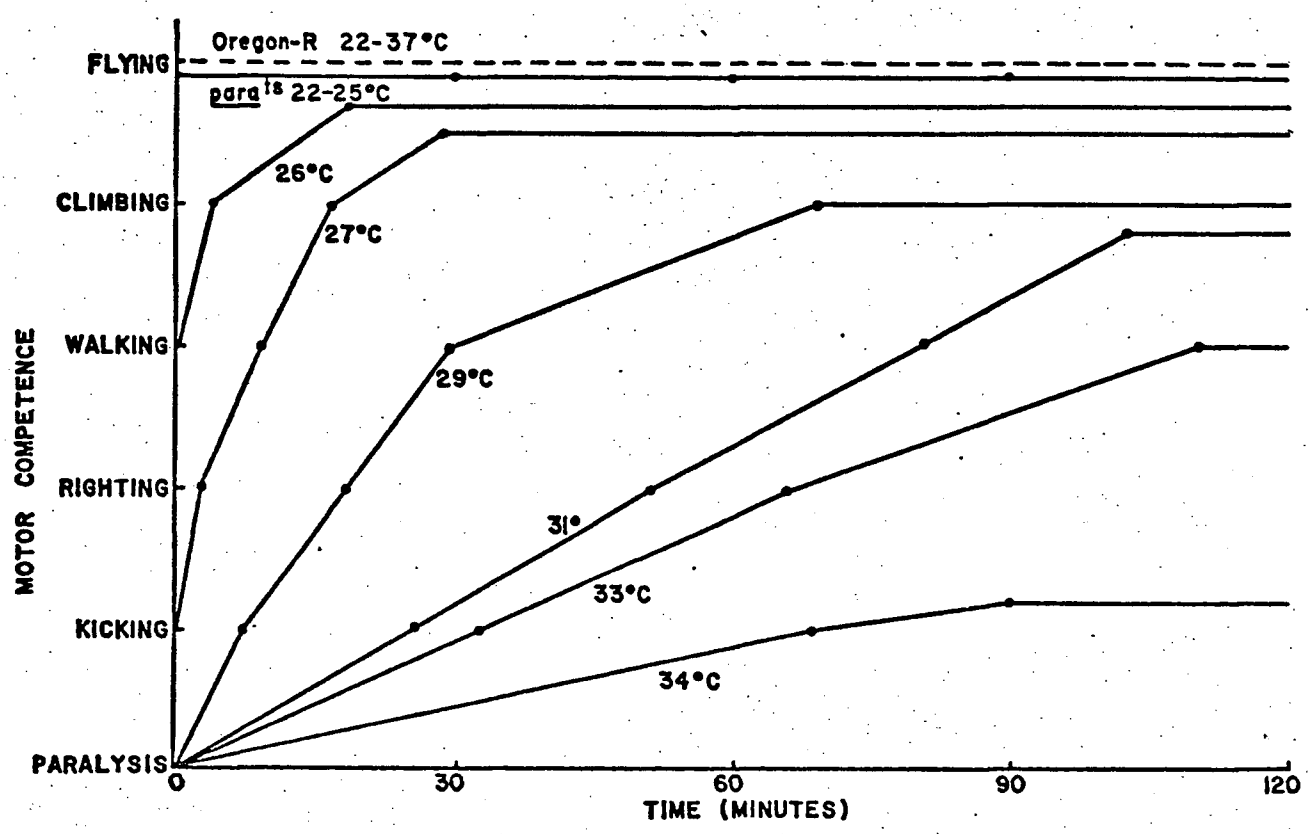
The behavioural responses to increased temperature were studied in flies which had been raised at 22°C by shaking flies from 22°C vials into separate water-heated chambers. Observations of changes in behaviour were made on 20 of each sex of $\underline{para^{ts}}$ and Oregon-R flies at one degree intervals over a 22°C to 35°C range. Behaviour was monitored

continuously for the first hour and then once every 10 minutes over the second hour.

For purposes of general comparison, specific types of behaviour were classified in the following order of increased motor competence: paralysis, leg shaking, self-righting to a standing position, walking, climbing, flying. Figure 11 is a graph of these motor activities, exhibited by at least 50% of the flies, plotted on an arbitrary scale against time. Recovery rates were far from uniform within a group; initiation of the same activities being separated by as much as 50 minutes in different flies. Although the average mobility clearly improved with time, the recovery of each fly was not always progressive. Flies which had demonstrated good walking and climbing abilities would occasionally revert to a very weak stance for several minutes. Even within a single fly, recovery did not appear to be uniformly progressive. Often one or two legs on one side of the body would move while the legs on the opposite side remained still. Differences in the extension of the legs frequently caused the fly to list while standing and pitch and roll while walking. The debilitating effects of progressively higher temperatures were manifested in both the extent of the initial response and the length of time taken to recover further activity at various temperatures. $\underline{para^{ts}}$ flies were noticeably debilitated by shifts to the 26°C to 28°C range. At higher temperatures they were completely paralysed. At 29°C, $\underline{para^{ts}}$ flies were able to climb after 70 minutes; at 31°C, 105 minutes were needed to regain the same activity. After two hours at any temperature up to 33°C, the flies were able to recover mobility upon returning to 22°C. However, a two hour exposure to 34°C resulted in 90% of the flies dying. Oregon-R flies were unaffected by

FIGURE 11

Recovery of motor competence with time after increases in temperature.



two hour exposures to temperatures ranging from 22°C to 37°C.

A list of the types of movements observed during recovery at immobilising temperatures is presented in roughly chronological order in Table 4. All of the movements listed were rarely seen in a single fly. However, they were crude indicators of levels of recovery.

Although 22°C had been chosen as the permissive temperature for screening and most shift experiments, it was subsequently discovered that *para*^{ts} flies raised at 17°C became slow or immobile when transferred to 22°C. Upon returning to 17°C, they became active within a few seconds. Thus it appeared that the *para*^{ts} mutation had rendered the fly sensitive to temperature increases over at least a 17°C to 29°C range.

The experiments which followed the discovery of 22°C sensitivity were designed to test a simple model which had been used to explain paralysis and recovery phenomena. For heuristic purposes, *para*^{ts} might be assumed to produce an enzyme whose catalytic efficiency was an inverse function of temperature (i.e., Km is a direct function of temperature) and whose reaction product permitted mobility of the fly when the product was formed at a sufficient rate. Given equal substrate concentrations, the rate of product formation would diminish at high temperatures and motor activity would consequently diminish also. However, the reduced efficiency of the enzyme would also cause the substrate concentration to increase if the substrate were supplied to the enzyme at a sufficient rate. If the substrate level then became high enough to overcome the increased Km of the enzyme, product formation might again reach a rate which would permit mobility. This hypothesis is presented as an example of a basic concept, namely a "damming up"

TABLE 4

Types of movement which may be seen during recovery from temperature-induced paralysis. The list is in roughly chronological order.

1.	Paralysis (legs contracted).
2.	Extension and contraction of the femurs.
3.	Tarsal twitching.
4.	Extension and contraction of the tibias.
5.	Slow movements of the wings laterally or dorsally and ventrally.
6.	Ventral flexing of the posterior abdomen. Movements of the genitalia. Excretion. Head nodding.
7.	Leg extension followed by pawing movements.
8.	Righting.
9.	Walking.
10.	Preening.
11.	Climbing.
12.	Flying.

effect. The accumulation of a neural transmittor might have fit the observations as well, if this process were more clearly understood.

If the notion of damming up substrate were correct, flies conditioned at 17°C with a relatively low substrate concentration, would be expected to take longer to recover mobility at 29°C than flies conditioned to 22°C. Accordingly, 44 $\underline{para^{ts}}$ flies (0 to 3 days of age) which had been raised at 22°C were divided into two equal groups. One group was left at 22°C, while the other was placed at 17°C for three days. 22°C and 17°C groups of $\underline{para^{ts}}$ flies were then simultaneously transferred to 29.5°C. A detailed comparison was made of two groups of 3 flies while the remaining two groups of 19 flies were observed more casually for the recovery of basic parameters of mobility, such as standing, walking and climbing. By all measured criteria, 17°C-conditioned flies recovered more slowly than flies conditioned to 22°C. The difference is illustrated by the graph shown in Figure 12 in which the numbers of flies able to stand is plotted against time. Only after more than an hour do the two curves approach each other.

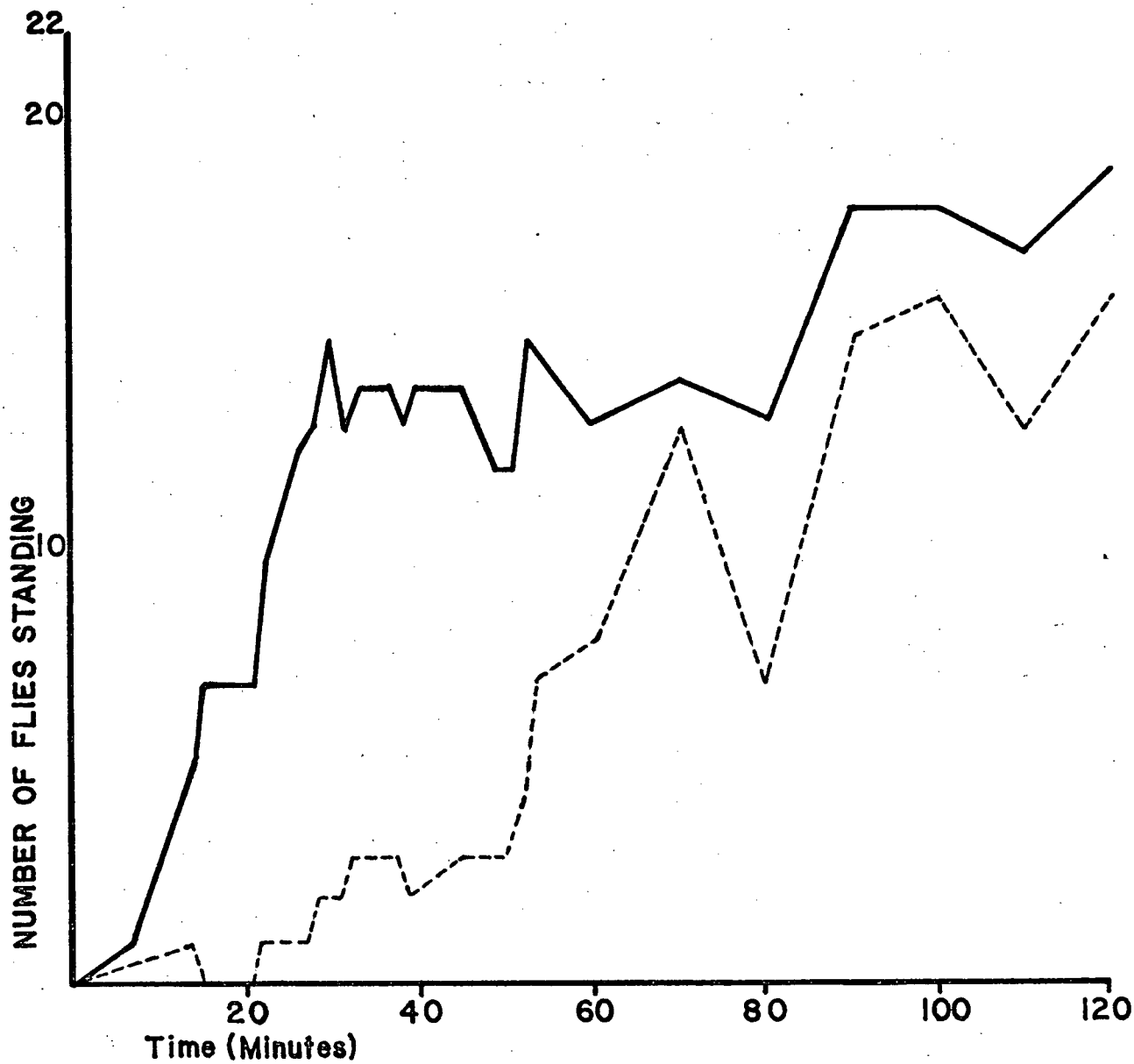
Although these experiments support the "substrate accumulation" model, they also suggest that the time taken for recovery is inversely related to the temperature shock.* While temperature shock is an intuitively understandable term, for purposes of clarity it shall be defined here as increase in body temperature per unit of time. Thus its magnitude at any instant is a direct function of the difference in temperature between the fly's body and its environment.

---

* I am grateful to Dr. Hans Stich for raising this important point.

FIGURE 12

The number of *para*^{ts} flies, preconditioned at 22°C (solid line) and 17°C (dashes) which were able to stand after being transferred to 29.5°C.



If the temperature were raised at different rates over the same interval, and within a short enough time to induce paralysis, the rates of recovery should distinguish between the validities of the two arguments. According to the "shock hypothesis", the faster rise in temperature should prolong recovery. On the other hand, the quicker arrival at the high temperature should cause the substrate to accumulate at its maximum rate from an earlier time, thus shortening the time needed for recovery by the "accumulation hypothesis".

The effect of temperature shock was studied in four groups of $\underline{para}^{ts}$ flies, each group being comprised of 5 males and 5 females, 0 to 2 days old. Observations of the number of flies able to stand were made once a minute during and following each temperature rise. When the temperature was raised from (22.2°- 22.4°C) to (27.0°- 27.6°C) within a 14 (Figure 13 a) and 6 minute (Figure 13b) interval, the response was dramatically different. The same effects can be seen in Figures 14a and 14b, although this comparison may not be valid since the faster temperature rise was also greater by one degree.

These experiments suggest that $\underline{para}^{ts}$ flies are sensitive not only to temperature, but also to temperature shock. The effect of shock was seen in the fly's initial response and in its rate of recovery. The results contradict a "substrate accumulation hypothesis".

The responses of $\underline{para}^{ts}$ flies in the above experiments strongly suggest that the monitored temperatures were underestimates of the actual chamber temperatures. The source of error was probably heat loss from the thermistor resistor by conduction along 1 cm. of probe wire. Fortunately the significance of these experiments is not changed by this error.

FIGURE 13

The number of <u>para</u>^{ts} flies able to stand when temperatures were changed from (22.2° - 22.4°C) to (27.0° - 27.6°C) within (a) 14 minutes, (b) 6 minutes. Solid line - number of flies able to stand; dashes - temperature.

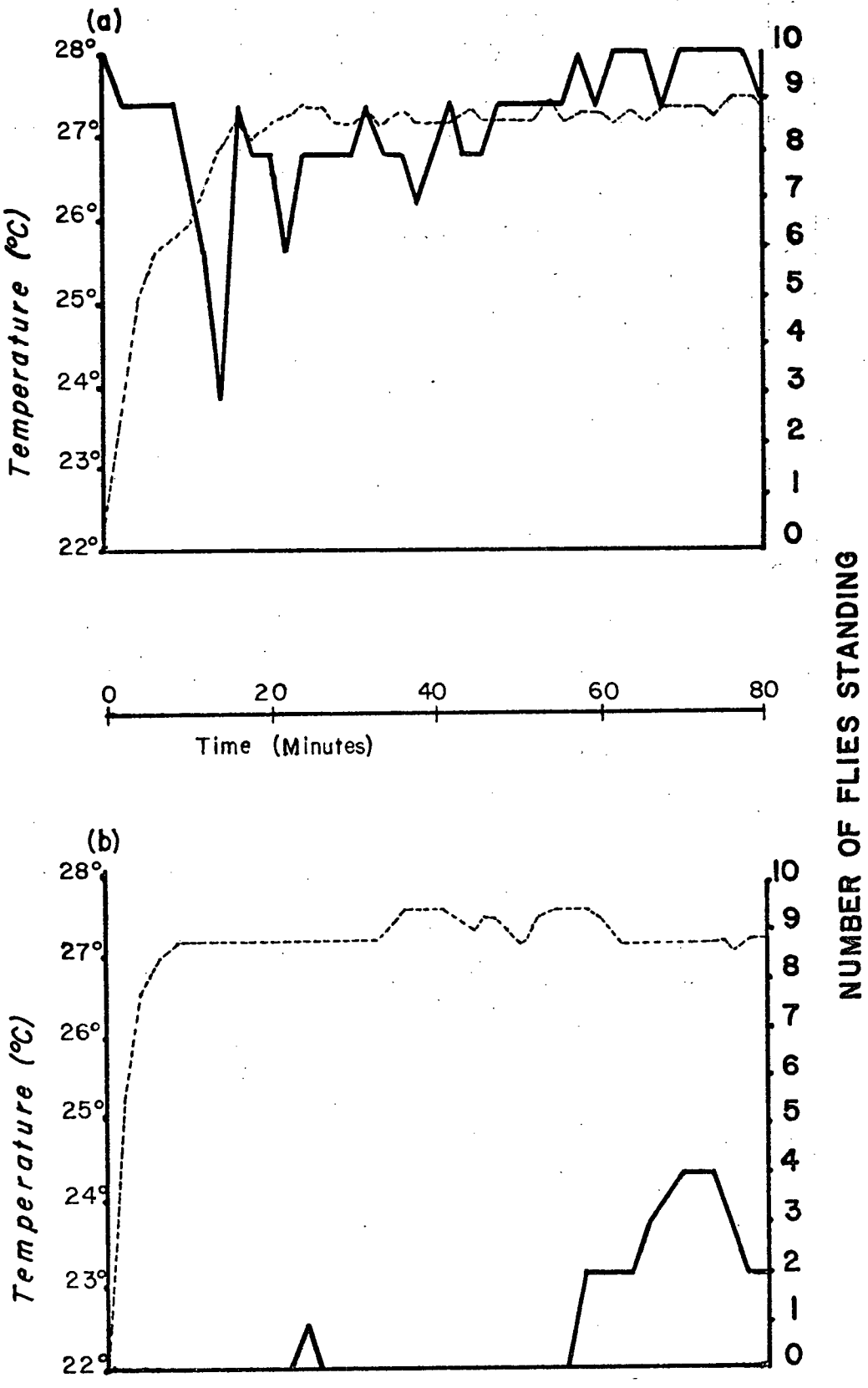
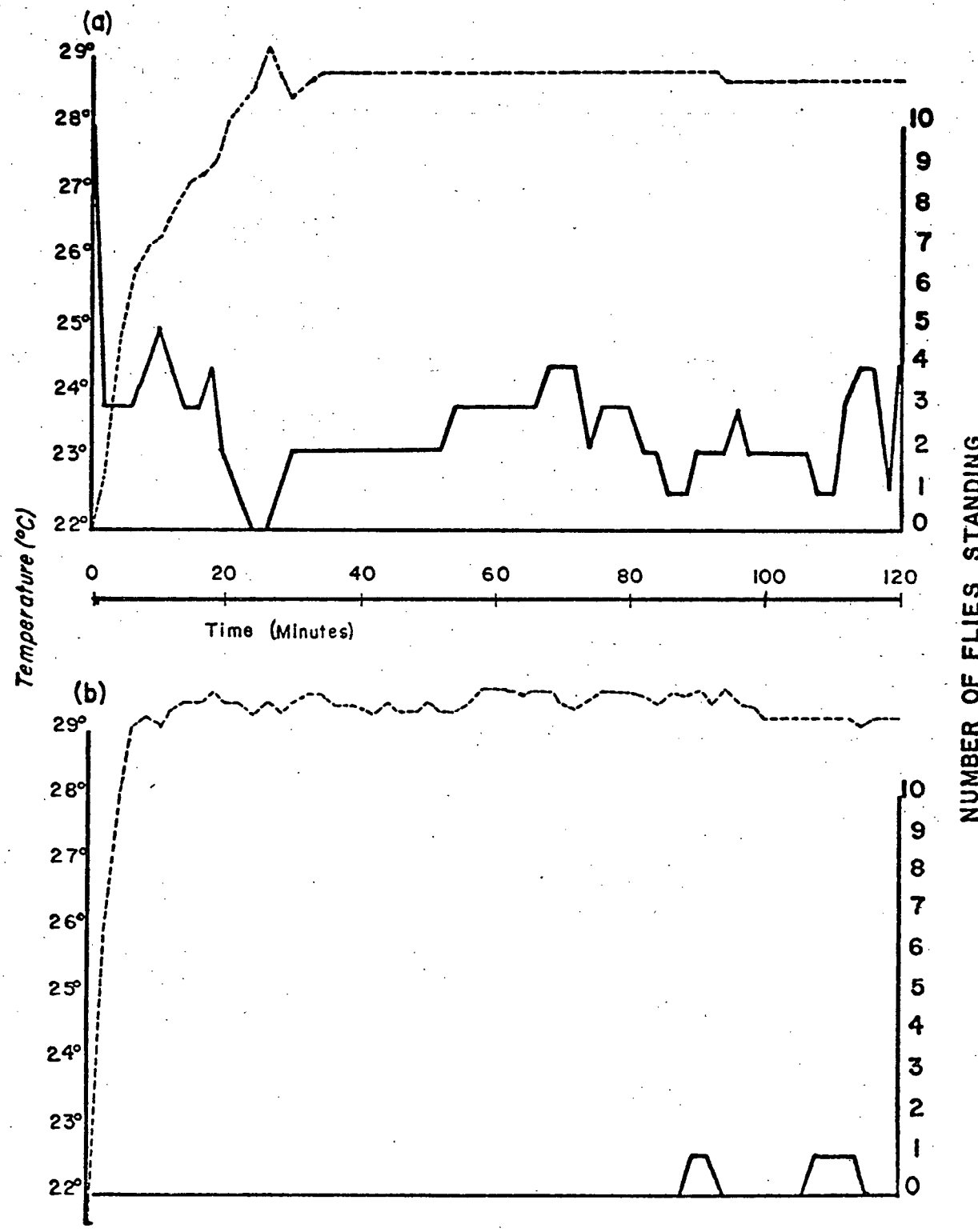


FIGURE 14

The number of para^{ts} flies able to stand when temperatures were changed from (21.6° - 22°C) to (28.5° - 29.5°C) within (a) 24 minutes, (b) 6 minutes. Solid line - number of flies standing; dashes - temperature.



Interactions between $para^{ts}$ and neurological mutants

Putative Neuro $para^{ts}$/Y males exhibiting the characteristics of both mutations were recovered from Neuro/$para^{ts}$ females. The standard positions of the Neuro mutations can be seen in Figure 15. The number of HK $para^{ts}$/Y recombinants was less than expected, while the number of $para^{ts}$ Sh^{5}/Y males agreed well with theoretical recombination distances (Table 5). These discrepancies probably reflect differences in survival under crowded conditions.

The double mutant nature of the phenotypically selected recombinants was confirmed in all but two cases. In both cases, the exceptions resulted from classification of HK^{1P}/Y males as HK^{1P} $para^{ts}$/Y. Each double mutant was verified by reisolating single mutant chromosomes by recombination (Table 6).

The response of Neuro $para^{ts}$/Y males to a 22°C to 29°C shift was a quick cessation of all movement whether induced by ether anaesthetisation or not. All movements could be restored at 22°C and stopped again at 29°C within a few seconds.

HK^{1P}/Y flies were also somewhat sensitive to shifts from 22°C to 29°C. Ether-induced leg shaking would cease within 15 seconds to a minute of exposure to 29°C. Unlike the legs of HK^{1P} $para^{ts}$/Y, these legs began to shake again after about a minute. Leg shaking could also be reinitiated by returning the HK^{1P}/Y flies to 22°C, but could not be stopped again by returning them to 29°C. It should also be emphasised that the sensitivity of HK^{1P}/Y flies to temperature could usually, but not always, be demonstrated. In short, the response of HK^{1P}/Y differed from that of HK^{1P} $para^{ts}$/Y by being slow, unreliable, unsustained and nonrepeatable.

FIGURE 15

Genetic locations of Hk, para^{ts} and Sh^{5} on the X chromosome.

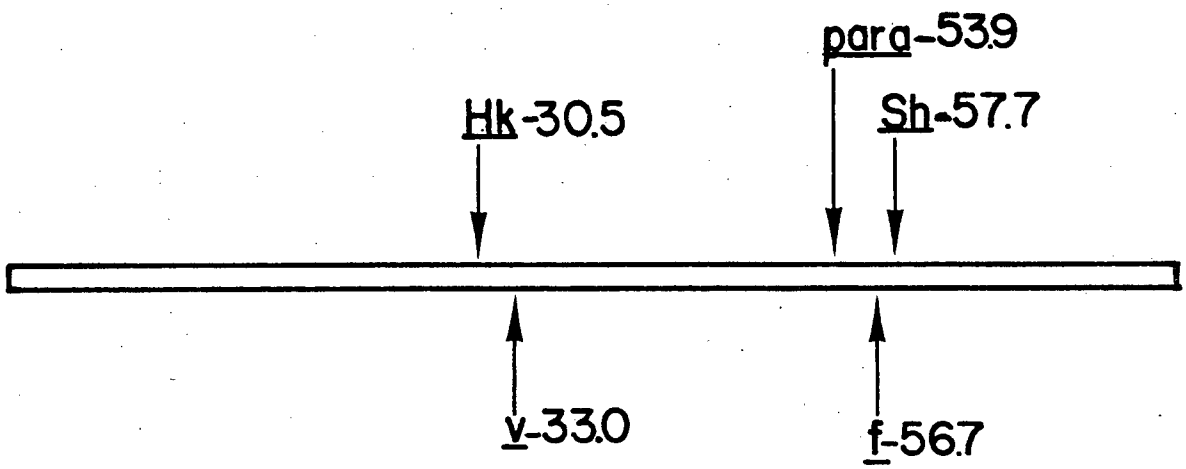


TABLE 5

Recombination data for *para*^{ts} and different *Neuro* mutants.

Maternal parent	# female progeny	Male progeny recovered		% Crossing over
		Non-crossovers	Crossovers	
+ *para*^{ts}/*HK*^{1P}+	523	*HK*^{1P} + 261	+ + 49	14.2 (23.4)*
		+ *para*^{ts} 102	*HK*^{1P} *para*^{ts} 11	
+ *para*^{ts}/*HK*^{2T}+	421	*HK*^{2T} + 176	+ + 45	17.9 (23.9)
		+ *para*^{ts} 108	*HK*^{2T} *para*^{ts} 17	
para^{ts}+/+ *Sh*^{5}	590	+ *Sh*^{5} 154	+ + 4	3.2 (3.8)
		para^{ts} + 116	*para*^{ts} *Sh*^{5} 5	

* figure in bracket is standard distance.

TABLE 6

Reisolation of single mutation chromosomes by recombination of double mutants.

Maternal parent	# female progeny	Male progeny recovered				% Crossing over
		Non-crossovers		Crossovers		
$\underline{HK^{1P}para^{ts}}/++$	911	$\underline{HK^{1P}}$ $\underline{para^{ts}}$	210	$\underline{HK^{1P}}$ +	92	24.4 (23.4)*
		+ +	268	+ $\underline{para^{ts}}$	62	
$\underline{HK^{2T}para^{ts}}/++$	2,261	$\underline{HK^{2T}}$ $\underline{para^{ts}}$	489	$\underline{HK^{2T}}$ +	224	23.5 (23.4)
		+ +	735	+ $\underline{para^{ts}}$	152	
$\underline{para^{ts}}$ $\underline{Sh^{5}}/++$	706	$\underline{para^{ts}}$ $\underline{Sh^{5}}$	193	+ $\underline{Sh^{5}}$	18	5.1 (3.8)
		+ +	314	$\underline{para^{ts}}$ +	7	

* figure in bracket is standard distance.

The behaviour of $\underline{HK^{1P}}$/Y and $\underline{HK^{1P}para^{ts}}$/Y flies were similar at 22°C. Both showed a kinetogenic response (jumping in response to hand movements) and both shook their legs vigorously under ether anaesthesia.

$\underline{HK^{2T}para^{ts}}$/Y flies did not show a kinetogenic response to hand movements at 22°C. Under ether, leg shaking was very subdued when compared with $\underline{HK^{2T}}$/Y and in many cases, was reduced to a rapid tremor of the tarsae. At 22°C, $\underline{Sh^{5}para^{ts}}$/Y males exhibited the characteristic leg shaking seen in $\underline{Sh^{5}}$/Y mutants under ether anaesthesia, but the wings did not scissor.

The vigorous leg shaking of $\underline{HK^{2T}}$/Y and the wing scissoring of $\underline{Sh^{5}}$/Y were restored when the $\underline{para^{ts}}$ mutation was removed from the respective $\underline{Neuro\ para^{ts}}$ chromosomes by recombination.

Interaction between $para^{ts}$ and Minutes

Of the Minutes tested in combination with $\underline{para^{ts}}$, only M(1)0 interacted to produce a change in behaviour following a 22°C to 29°C shift. $\underline{M(1)0\ f/para^{ts}}$ females assumed stationary positions on the floor and walls of the observation chamber for 3 minutes or more while FM6/Y and $\underline{FM6/para^{ts}}$ controls remained highly active. Unlike $\underline{para^{ts}}$ homozygotes, $\underline{M(1)0\ f/para^{ts}}$ flies were able to remain on their feet after the rise in temperature, although the stances of the flies seemed weak and the first movements were extremely slow. The recovery of normal walking and climbing activity took about two hours. As with $\underline{para^{ts}}$ flies, mobility could be quickly and completely restored by returning the flies to 22°C.

DISCUSSION

The search for inherited temperature-sensitive paralysis has led to the discovery of two sex-linked mutations which affect motor activity; *para*^{ts} -(53.9) and *Shu* -(55.1). A cross of *Shu*/*para*^{ts} females by *para*^{ts}/Y males failed to yield wild types or double mutant recombinants among 2,256 progeny. Therefore, the *Shu* and *para*^{ts} mutations may be much closer than first estimates had indicated.

A third mutation, *M(1)0* -56.5), appeared to interact with *para*^{ts} to produce an altered paralytic phenotype in *para*^{ts}/*M(1)0* females. However, a direct comparison of such females with *para*^{ts} flies has not yet been made. Furthermore, the *M(1)0*-bearing chromosome was not studied genetically or cytologically. Thus, it is not known whether a pre-existing *para* allele or deletion of the *para*$^{+}$ locus exists on the chromosome.

Until further information is obtained on the genetic and functional relationships of the *Shu* and *para*^{ts} mutations, an understanding of shuddering and paralysis can only be inferred by phenotypic symptoms and the alteration of their expression by genetic, chemical and physical manipulation.

Shu/*FM6* flies are similar to the homozygous neurological mutants, *HK*^{1P}, *HK*^{2T} and *Sh*^{5}, in that they all exhibit abnormally vigorous and rapid leg shaking under ether anaesthesia. The behaviour of *HK*^{1P}/+ and *HK*^{2T}/+ females was found to be very difficult to distinguish from either Canton-S or Oregon-R wild type females. The leg shaking of *Sh*^{5}/+ was subdued compared with the homozygous females, but not completely suppressed. Comparisons were drawn between the homozygous neurological

mutants ($\underline{HK}^{1P}$, $\underline{HK}^{2T}$ and $\underline{Sh}^{5}$) and $\underline{Shu/FM6}$ females because of their phenotypic closeness; albeit the genetic comparison is inconsistant.

The movements of $\underline{Shu/FM6}$ and $\underline{Sh}^{5}$ flies under ether are similar in that they both scissor their wings and halteres as well as nodding their heads and extending their antennae. However, when $\underline{Shu/FM6}$ and $\underline{Sh}^{5}$ were etherised simultaneously and examined together, $\underline{Sh}^{5}$ flies were found to begin shaking their legs much sooner than $\underline{Shu/FM6}$ flies did. The wing scissoring of $\underline{Sh}^{5}$ flies involved shorter, quicker movements. It was also noted that the leg shaking movements of $\underline{Sh}^{5}$ flies involved all segments of leg while the movements of $\underline{Shu/FM6}$ flies were mainly confined to the tibia and tarsus. Thus, $\underline{Sh}^{5}$ and $\underline{Shu/FM6}$ were phenotypically distinguishable.

$\underline{Sh}^{5}$, $\underline{HK}^{1P}$ and $\underline{HK}^{2T}$ flies jump in response to the visual stimulus of a hand movement or to mechanical agitation (Kaplan and Trout, 1969). However, $\underline{Shu/FM6}$ flies responded only to mechanical stimulation and did so by shuddering while standing in one spot. Individual $\underline{Shu/FM6}$ females continued to shudder periodically, even when left for long periods in undisturbed isolation.

The pattern of rapid leg shaking in $\underline{HK}^{1P}$ flies has been demonstrated to result from rapid bursts of neural activity (Ikeda and Kaplan, 1970a). The fact that the movements of the wings, halteres, head and antennae of $\underline{Sh}^{5}$ flies and $\underline{Shu/FM6}$ females were usually in phase with one another also suggests they were elicited by a common neurogenic activity rather than independent myogenic impulses.

The possibility that lithium might suppress the shuddering behaviour of $\underline{Shu/FM6}$ flies was suggested by its therapeutic effects on analogous disorders in guinea pig and man (Cade, 1949). The analogies drawn

between flies and man were crude. Yet the variety of behavioural disorders which this ion had been shown to suppress suggested that it acted upon a common factor which might even be found in flies.

Cade (1949) discovered that lithium salts were able to suppress the severe convulsant effect of intraperitoneal urea injections upon guinea pigs. He also discovered that the same treatment seemed to eliminate the expression of chronic and recurrent mania in man. According to Van der Velde (1970) subsequent studies have involved more than 3,000 patients. Critical evidence for the successful treatment of mania has been supplied by Maggs (1963). Strong evidence for its prophylactic action against the recurrence of depression in manic depressive psychoses has also been presented (Baasturp and Schou, 1967; Goodwin et al., 1969). Shou (1959; 1963) noted that unlike other psychotherapeutic drugs, lithium did not suppress normal mental, emotional or physical activity. Thus, the ion seemed to act upon the disorder per se.

The similarity between the shuddering behaviour of Shu/FM6 flies and urea-induced clonic convulsions in guinea pigs is fairly obvious. Both are sudden, vigorous, uncontrolled motor activities. The syndromes of recurrent mania and manic depression can also be compared with the shuddering of Shu/FM6 in that they are both characterised by rises and falls in motor activity (Kraepelin, 1906; Ariety, 1959); albeit the rise in activity of the fly is more sudden and uncontrolled. In man the rises in motor activity are accompanied by a loss of control over the large repitoire of behaviour which is characteristic of his species. Thus he becomes verbose, intrusive and occasionally destructive (Kraepelin, 1906; Ariety, 1959). Manic and depressed states may occur

repeatedly (Lundquist, 1945) and often cannot be attributed to environmental stress (Hudgens et al., 1967). The shuddering of Shu/FM6 flies is also recurrent and sponeous although the shuddering response can also be elicited by mechanical stimulation.

Among the chloride salts tested, LiCl demonstrated a unique capacity to supress both induced and spontaneous shuddering activity in Shu/FM6 flies. However, it was not possible to sustain doses which would supress the increased nervous activity of older flies without killing them. It is interesting to note that older people are also less likely to respond to lithium therapy (Van der Velde, 1970).

The observed toxicity of lithium in flies suggests that the supression of shuddering might be a pathological prelude to death. This was ruled out by the survival of lithium-treated flies which had ceased to shudder for one or more days, when they were placed on standard medium. Most flies in which shuddering had been suppressed exhibited wild type levels of activity and showed no toxic symptoms.

The attempt to suppress shuddering in all of the treated flies probably required the use of salt concentrations which exceeded the tolerance of some individuals in the sample. The uptake, excretion and and sensitivities to lithium have been found to differ among individuals in both man and animals (Schou, 1958, 1959; Weischer, 1969). Consequently, during lithium therapy in man, the dosage must be controlled while blood levels and behaviour are monitored (Schou, 1959). Shuddering was suppressed in a group of five flies treated with a relatively low dose of LiCl (0.2M) (Figure 10) which had no effect on survival. However, higher concentrations would have exceeded the therapeutic level and may have killed the flies.

Lithium has also been found to supress induced aggression in rats, mice and golden hamsters as well as the natural aggression of male Siamese fighting fish (Weischer, 1969; Sheard, 1970). Once again, these treatments did not suppress normal levels of activity. These experiments were probably suggested by aggressive behaviour which is often displayed in the manic state. At this time it does not seem profitable to examine the validity of this comparison or the analogies which have been drawn between flies and man. On the other hand, the aetiological significance of these experiments cannot be ignored. The results suggest that among the mechanisms which give rise to these various forms of behaviour in such widely separated organisms, there may be one which is common to them all. It follows that future investigations into the causes of shuddering behaviour in Drosophila might benefit from information concerning the effects of lithium on manic depressive biochemistry in man and related biochemistry in other animals.

Before reviewing the biochemistry of these psychoses, it is important to distinguish at least two inherited disorders. Manic depression appears to be transmitted as a sex-linked, dominant mutation with partial penetrance. Its linkage with colour blindness and the Xg^a blood group has been demonstrated (Reich *et al.*, 1969; Winokur and Tanna, 1969). Manic depressive males have fathers who are not affected whereas 63% of their mothers are affected (Winokur *et al.*, 1969). First degree relatives of manic depressive probands often experience only depression. Another disorder in which both probands and their relatives show only depression appears not to be sex-linked (Winokur and Pitts, 1965; Winokur *et al.*, 1969).

Lithium has been shown to act against mania and manic depression. While lithium seems to relieve some types of recurrent depression (Baastrup and Schou, 1967; Gooodwin et al., 1969) its use as a general treatment of depressed patients has been questioned (Van der Velde, 1970).

The discovery, by Coppen et al., (1965) that lithium treatment caused a drop in intracellular sodium, gained significance in view of the later discovery that intracellular sodium was related to mood in the following way: Normal Na^+ < Depressive Na^+ < Manic Na^+ (Coppen et al., 1966). It is likely that the change in intracellular sodium concentration occurs in nerve cells as well as other tissues. Lithium has been shown to compete sucessfully with sodium for the "early" influx channels in the formation of action potentials (Meves and Chandler, 1965; Cole, 1968). At the same time, the active removal of lithium from the cell occurs more slowly than sodium (Araki et al.,1965).

The linking of depression to low levels of seratonin and nonadrenaline, and of mania to high levels of these biogenic amines has largely been inferred from the effects of reserpine, imipramine and the manoamine oxidase inhibitors on mood and body biochemistry (Bunney and Davis, 1965; Schildkraut, 1965).

Lithium ions have been shown to accelerate the uptake of noradrenaline into preparations of synaptosomes in vitro (Colburn et al., 1967). Uptake of seratonin into the red blood cells of manic deppressive patients is also increased by lithium (Murphey et al., 1970). Katz et al., (1968) discovered that lithium seemed to reduce the rate at which noradrenaline and seratonin were released from electrically-stimulated brain slices. These results may indicate that lithium increases the

efficiency of the reuptake mechanism for biogenic amines.

Schandberg et al., (1967) found that lithium accelerated norepinephine catabolism within a few hours of treatment. However, Bliss and Ailion, (1970) maintained that after two weeks of lithium treatment no change in noradrenaline metabolism could be detected.

It is known that intracellular levels of adenosine 3'5'-cyclic monophosphate (cyclic-AMP) rise in response to the catecholamines and seratonin in many target tissues (Sutherland, 1968). Therefore, it is not too surprising to find that cyclic-AMP is excreted at higher than normal rates in manic patients and lower than normal rates in depressed patients (Paul et al., 1971a). The transition from the depressed to the manic state is also marked by a transitory increase in cyclic-AMP excretion which is even greater than that of the manic state (Paul et al., 1971b). These authors have also shown that levels of cyclic-AMP follow the direction of clinical change brought about by lithium. Thus cyclic-AMP dropped in patients recovering from mania and rose in patients recovering from depression (Paul et al., 1971a).

This finding tends to broaden rather than confine the problem, since cyclic-AMP is a common intermediate in hormal control (Sutherland, et al., 1968). Investigations of two other systems in which cyclic-AMP is involved -- glycolycis (Sutherland and Robinson, 1966) and steroid production (Sutherland et al., 1968) -- have not yet revealed clear chemical relationships with manic depressive disorders (Coppen, 1967; Rubin, 1967; Van der Velde, 1969; Heninger and Meuller, 1970).

Thus far, the mechanism upon which lithium operates to produce its effect has not been discovered. To this end it may be useful to compare the biochemistry of manic depressive patients with that of Shuddering

flies.

Whereas the patterns of behaviour seen in Shuddering flies suggested a nervous disorder, the temperature-induced cessation of all behaviour patterns in $\underline{para}^{ts}$ flies could have any number of possible bases for the effect. The requisite insight into the tissue specificity of the mutation was gained by examining genetic mosaic individuals derived from $\underline{y\ para}^{ts}/\underline{In(1)w}^{vC},++$ zygotes (Suzuki et al., 1971). Loss of the somatically unstable ring X chromosome, $\underline{In(1)w}^{vC}$ (Hinton, 1955) produced individuals which carried both $\underline{y\ para}^{ts}/++$ and $\underline{y\ para}^{ts}/0$ cells. Tissue derived from X/0 cells could be recognized externally by the expression of the recessive cuticular mutation, y.

It was discovered that flies with yellow legs and wild type bodies were able to move their legs back and forth at high temperature, but were unable to coordinate or position their legs properly. Flies with yellow thoraces contracted their legs in response to a temperature rise, whereas flies with yellow heads assumed a normal stance but were unable to walk. Flies with yellow abdomens were unaffected by temperature. Bilateral, mosaic flies moved their wild type legs at high temperature, but were unable to move their yellow legs. The results of these experiments indicate that the $\underline{para}^{ts}$ mutation probably has a direct effect upon the nervous system which is not mediated through a freely circulating factor. Furthermore, the presence of the mutation in different parts of the nervous system appears to lead to behaviourally distinct consequences. The observation that the recovery of mobility in different parts of the body of $\underline{para}^{ts}$ flies at high temperature often seems to occur at different rates may also indicate the gene's autonomy.

In addition to walking and climbing activities, tethered flight was

also found to be sensitive to temperature change (Suzuki et al., 1971). Flight could be quickly initiated or stopped by exposure to 22°C and 29°C, respectively.

The fact that the temperature sensitivity of $\underline{HK^{1P}\ para^{ts}/y}$ is characteristic of $\underline{para^{ts}}$ rather than $\underline{HK^{1P}}$ suggests that $\underline{para^{ts}}$ directly or indirectly supresses the initiation or subsequent conduction of impulses burst from the thoracic ganglion. The temperature-dependence of $\underline{HK^{2T}para^{ts}}$/Y males supports this contention since the expression of the $\underline{HK^{2T}}$ allele was not subject to temperature change.

Some of the mosaic flies who had mutant tissue around one eye were observed to follow a helical path at 29°C when climbing up the inside surface of a cylinder (Suzuki et al., 1971). Since the mutant eye was facing upward during the climb it was thought that vision in that eye might be impaired. These mosaic individuals showed a normal optomotor response (i.e., they turned in the direction of moving stripes) at 22°C and 29°C. When paint, which had been shown to effectively shut out light, was used to cover the wild type eye and ocelli, the flies still showed a normal optomotor response at 22°C, but behaved ambiguously at 29°C. Normal changes in potential of the surface of the eye in response to stimulation by light (Hotta and Benzer, 1969) were recorded for $\underline{para^{ts}}$ at both 22°C and 30°C.*

Studies of microbial systems have indicated that a temperature-induced loss of function may be caused by the thermal instability of a particular molecule. The instability can be caused by a single amino acid change within a protein (Jockusch, 1964, 1966) or a single base change

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* The electroretnagrams were obtained for us through the kindness of Drs. Yoshiki Hotta and Seymour Benzer.

within a transfer RNA species (Smith et al., 1970). The capacity of some mutant molecules to function normally depends upon the temperature at the time of their synthesis and assembly, but does not depend on subsequent temperatures (Edgar and Lielausis, 1964; Sadler and Novick, 1964). The functioning of other mutant molecules is temperature-dependent subsequent to syntheses (Maas and Davis, 1952; Igarashi, 1969; Sadler and Novick, 1965).

The speed of response of $\underline{para}^{ts}$ flies to temperature change precludes the possibility of much macromolecular synthesis and suggests that alterations in behaviour are mediated through a pre-existing thermolabile product. Although mutant proteins have often been sighted as the basis of temperature sensitivity, the nature of the thermolabile factor in $\underline{para}^{ts}$ flies cannot be assumed.

The sensitivity of a mutant function to a particular temperature change is often an exaggeration of the wild type sensitivity to the same change. Guthrie et al., (1969) attributed the enhanced recovery of ribosomal assembly defects among cold-sensitive mutants to the normal sensitivity of this process to cold. The sensitivity of catalase activity (Igarashi, 1969) and the half life of pantothenate synthesising enzyme (Horowitz and Fling, 1952) to increased temperature is grossly exaggerated in the mutant condition. O'Donnovan and Ingraham (1965) discovered that a mutant phosphoribosyl ATP pyrophosphoralase in E. coli which had a greatly increased sensitivity to feedback inhibition by histidine. The additional sensitivity of the mutant enzyme to inhibition at low temperatures was about the same as that of the wild type enzyme, but the consequences to the growth of the mutant were much greater.

The process of screening for heat-sensitive mutants may tend to

select systems which are already somewhat sensitive to heat prior to mutation. If the phenomenon of temperature-sensitive paralysis is viewed in this way, the demonstrated sensitivity of the central nervous system to increased temperatures acquires a special interest. Kerkut and Taylor (1958) observed that the maximum impulse frequency emitted by an isolated thoracic or abdominal cockroach ganglion occurred at the temperature at which the animal had been preconditioned and fell off gradually above and below that temperature. These readings were obtained by changing the temperature of the ganglion slowly and permitting time for the frequency to reach a steady state. A rapid temperature increase of 16°C caused impulses to cease almost immediately. After eleven seconds of inactivity, the impulses suddenly resumed at a higher frequency than before. The same effect was achieved by a 13°C increase in crayfish abdomenal ganglia and a 3°C increase in slug pedal ganglia (Kerkut and Taylor, 1958). In all these systems, quick drops in temperature induced transient increases in impulse frequency. These transient responses to temperature were characteristic of some, but not all central nervous elements (Kerkut and Taylor, 1958). Nevertheless, the speed of reaction of the ganglia which control mobility to cold and heat shock was very reminiscent of $\underline{para}^{ts}$ behaviour.

The biochemical limits which define an organism's temperature range as well as its means of acclimatising to a new temperature have been extensively studied. In their review of this subject, Hochachka and Somero (1971) have stressed the importance of enzyme substrate affinity, as implied by low Km values, in determining the range of immediately exploitable temperatures. Adaptation to a new range may require <u>de novo</u> synthesis of an enzyme whose lowest Km values extend

over the new range. Thus, when Rainbow trout were transferred from 17°C to 2°C they were immobilized. After a few weeks, a return of mobility was accompanied by the appearance of a new low temperature acetylcholenesterase and the disappearance of a high temperature form (Baldwin and Hochachka, 1970). It appears that this is a simple example of a common phenomenon (Hochachka and Somero, 1971).

Long term adaptation is also accompanied by ionic changes (Heiniche and Houston, 1965) and alterations in the phospholipid species of the central nervous system (Roots, 1968). Preliminary tests of short and long term acclimation of *para*^{ts} flies to high temperature suggested the existence of short and long term mechanisms for regaining mobility (Suzuki *et al.*, 1971). The short term advantage appears to be quickly lost after a brief shift down followed by a return to high temperature. The long term gain is not so easily lost and may, therefore, be accomplished by an acclimation process such as *de novo* synthesis of a new factor to replace or aid the *para*^{ts} factor.

An organism's insensitivity to temperature need not always depend on the insensitivity of systems within it. Hochachka and Samero (1971) have named five mechanisms which are probably required to balance the sensitivity of fructose diphosphatase to AMP inhibition at low temperatures. By mutation this kind of balance could easily be upset (O'Donnovan and Ingraham, 1965).

The positions and nature of the temperature-sensitive elements of the *para*^{ts} nervous system are yet unknown and must await the results of electrophysiological investigation.*

The discovery of the dramatic rise in acetylcholenesterase and

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* These studies are being conducted by Dr. Kazuo Ikeda.

choleneacetyltransferase activities in Drosophila melanogaster during adult metamorphosis (Dewhurst et al., 1970) provides a plausible explanation for the adult-specific function of the $para^{ts}$ mutation. However, the activities of these enzymes were not found to be temperature-sensitive in $para^{ts}$ flies.*

Tunnicliff et al. (1970) discovered that dopamine concentrations are below normal in an active strain of Drosophila melanogaster and above normal in an inactive strain. The opposite was true for noradrenaline. Thus, noradrenaline has become one of the candidates for biochemical study in both $para^{ts}$ and Shuddering flies. However, if altered levels of noradrenaline were discovered in these flies, the problem of primary cause would still remain.

The investigation of primary causes of behavioural defects is best carried out in single mutant systems. The selective recovery of single gene mutants affecting the motor system is feasible and has, in this instance, yielded two mutants of general neurological interest.

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* This biochemical analysis was conducted by Dr. Linda Hall.

SUMMARY

With the intention of recovering mutations affecting neural and/or muscular systems, a search for a temperature-sensitive mutant among Drosophila melanogaster adults was conducted. Mechanical screening devices for the separation of active from immobilised flies were constructed. Two mating schemes were used to recover chromosomes from males treated with ethyl methanesulphonate. In one scheme the treated males were crossed with wild type females so that only dominant mutations would be detected in the F_1 generation. In the second scheme, the males were crossed to attached-X ($\hat{X}\hat{X}$/Y) females, thus permitting the detection of sex-linked recessive or autosomal dominant mutations. The F_1 generations were raised to adulthood at 22°C and then screened for individuals which were immobilised at 29°C.

From approximately 250,000 progeny of $\hat{X}\hat{X}$/Y females, one male which exhibited heritable temperature-sensitive paralysis was recovered. The mutation (symbolised as $para^{ts}$ for paralytic-temperature-sensitive) was found to be recessive and sex-linked, mapping genetically at 53.9.

$para^{ts}$ flies which had been raised at 22°C were paralysed within 5 seconds of exposure to 29°C and recovered full mobility within a few seconds of exposure to 22°C. Sensitivity to temperature change was detected only in adult flies.

Prolonged exposure of $para^{ts}$ flies to 29°C resulted in a gradual recovery of mobility. In general, the length of the recovery time was directly related to the magnitude and rate of the temperature rise.

The movements of etherised and non-etherised $\underline{HK^{1P}para^{ts}}$/Y, $\underline{HK^{2T}para^{ts}}$/Y and $\underline{para^{ts}Sh^{5}}$/Y males were subject to the same temperature-sensitive paralysis that $\underline{para^{ts}}$ flies exhibited. The observation that leg shaking of $\underline{HK^{1P}para^{ts}}$/Y males was rapidly supressed and reinitiated by temperature change suggests that $\underline{para^{ts}}$ may have directly or indirectly supressed the initiation or subsequent conduction of impulse bursts which have been shown to cause the characteristic leg shaking of $\underline{HK^{1P}}$ flies.

In the search for a dominant mutation causing temperature-sensitive paralysis a fly carrying a dominant mutation which caused periodic shuddering was discovered. The sex-linked mutation, called Shuddering (Shu), mapped genetically at 55.1. It was possible to suppress the shuddering behavior by feeding the flies media containing LiCl but not NaCl, KCl, or NH_4Cl. The significance of these observations has been considered in the light of other effects of lithium ions upon the biochemistry, physiology and behaviour of man and other animals.

Under ether anaesthesia Shu flies shook their legs rapidly and vigorously. Abnormal movements of the wings, halteres, head and antennae were also observed. These movements were usually in phase with each other suggesting that they were under neural control and did not result from independent myogenic activities.

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