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Behavioral Effects of Temperature Sensitive Mutations Affecting Metabolism of cAMP in *Drosophila melanogaster*

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SAVVATEEVA, E. V. AND N. G. KAMYSHEV. *Behavioral effects of temperature sensitive mutations affecting metabolism of cAMP in* Drosophila melanogaster. PHARMAC. BIOCHEM. BEHAV. 14(5) 603–611, 1981.—Temperature-sensitive mutations affecting metabolism of cAMP were obtained in *Drosophila melanogaster* to elucidate the possible involvement of cAMP in behavior. Temperature-dependent hypersensitivity to theophylline, propranolol and dihydroergotoxin following treatment with ethylmethanesulfonate was used to screen for such mutations in the X-chromosome. Biochemical analysis of cAMP content and activity of phosphodiesterase revealed two mutants with increased content of cAMP, 2 mutants with low activity of phosphodiesterase and 1 mutant with high activity of the enzyme. Locomotor activity of the ts-mutants correlated with cAMP content, increasing at 29°C in mutants with an enlarged amount of cAMP and in mutants with low activity of phosphodiesterase and decreasing in the mutants with high activity of the enzyme. The latter mutant also failed to learn to avoid shock-associated odorant. One of the mutants with increased content of cAMP, but insensitive to propranolol, displayed better learning ability than the wild type. The learning performance of the mutants is interpreted proceeding from the metabolism of cyclic nucleotides in cholinergic and dopaminergic structures of the brain.

Drosophila melanogaster ts-Mutants X-chromosome cAMP Cyclic nucleotide phosphodiesterase Locomotor activity Learning

IT is evident that *Drosophila melanogaster* has substantial advantages in a study of genetic determination of behavior, due to its well studied hereditary mechanics which make possible the genetic analysis of its behavior and its nervous system. Since mutations can disturb functioning in any part of the nervous system—receptor, CNS and effector—one of the approaches of behavioral genetics study implies induction and selection of mutations changing a behavioral performance, and the subsequent elucidation of the mechanism of action of a mutation on behavior [2]. Another approach implies induction and selection of mutations which disturb functions of the nervous system (for instance, enzymochemistry).

It is generally accepted that cyclic AMP plays a role as a universal regulator of cell functions, especially in the nervous system, and mediates effects of a variety of hormones and neurotransmitters on the genetic apparatus of target cells [12,13]. Thus, selection of mutations which disturb metabolism of cAMP could be helpful in the elucidation of its role in behavioral processes.

Since such mutations have not yet been available in higher organisms (besides tissue cultures [3]), the work reported in the present paper was designed to induce the mutations in *Drosophila melanogaster* and to study their effects on behavior, namely on locomotor activity and learning. Bearing in mind the extremely important role of cAMP in cell functions and probable lethality resulting from any change in cAMP metabolism, it was reasonable to obtain temperature-sensitive (ts) mutations, which could exert their effects in a certain restrictive temperature, e.g., 29°C.

METHOD

Subjects and Mutagenesis

Normal *Drosophila melanogaster* of the Canton-Special (CS) wild type strain were maintained on yeast-raisins medium. Four hundred males were treated with ethylmethanesulfonate in the gas phase according to Schwarzman [25] and mated to virgin females C(1)DX so that each F_1 progeny male carried a treated X-chromosome received from his father. One thousand individual F_1 males were mated to C(1)DX females [17], producing a stock in which the males carried identical, potentially mutant, X-chromosomes. To screen for ts-mutations affecting cAMP metabolism, the flies

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TABLE 1
CHARACTERISTICS OF SELECTED ts-MUTANTS

Stock	Sensitivity to an inhibitor	Activity of Phosphodiesterase nmol/mg Protein/min Mean		Content of cAMP pmol/mg Protein Mean	
		Males	Females	Males	Females
Control	—	1.14 (4)	0.90 (4)	4.8 (4)	3.0 (5)
66	theophylline	0.62* (4)	1.32 (5)	4.5 (3)	5.5 (3)
155	propranolol	1.59 (5)	1.29 (5)	6.8* (4)	5.4 (4)
398	unspecific ts-lethality	1.63* (4)	0.94 (4)	6.3 (3)	5.2 (4)
622	—	1.12 (4)	1.04 (4)	6.8* (4)	5.9 (4)
980	propranolol	0.56* (4)	1.11 (4)	5.4 (3)	4.6 (2)

*$p \leq 0.05$ Wilcoxon criterion; in parentheses—number of tests.

from each such stock were permitted to develop from egg on two sets of selective media, containing inhibitors of cyclic nucleotide phosphodiesterase (PDE)–(theophylline, 0.2 ml of 0.2% solution per 1 ml of medium) and of β- and α-adrenergic receptors, which are believed to be in a close functional relationship with adenylate cyclase [4,11] (propranolol (Obsidan ISIS Chemie Zwikau), 3 mg per 1 ml of medium; dihydroergotoxin (Spofa), 0.2 ml of 0.1% solution per 1 ml of medium). One set was placed in 22°C, another in 29°C and the relative survival of males developed in both temperatures was compared. Complete absence or strong reduction in males surviving at 29°C on any of the selective media indicated a mutation. Control stock was the progeny of C-S males and C(1)DX females.

Biochemical Tests

Potentially mutant 5 day old males from those stocks that satisfied the selection criterion were subjected to biochemical measurements of cAMP content according to Gilman [9] using a cAMP assay kit (Amersham), and of cyclic nucleotide PDE activity according to Cheung [5]. In the latter case snake venom was used as a source of 5'nucleotidase and incubation lasted for 20 min in 37°C. Inorganic P was determined according to Doose [6]. Protein determinations were according to Bandet and Giddey [1].

Behavioral Procedures

All procedures were carried out in the permissive temperature of 25°C and in the restrictive temperature of 29°C.

Locomotor activity (LA) in a new surrounding was measured according to Luchnikova [18]. Males were placed in a set of 6–7 empty vials, 10 males in each, and a number of moving flies was scored each 5 min during a 30 min test in 25°C and during a 60 min test in 29°C. In the latter case the results of each half hour were analyzed separately to distinguish the most temperature sensitive period. The tests were performed from 1 to 5 days after hatching. The flies were maintained at 22°C between tests.

The learning test. The paradigm was similar to that of Quinn *et al.* [22]. The apparatus consisted of two sliding Plexiglas holders accepting Plexiglas tubes, the start tube on the lower holder and 5 tubes on the upper one. Among these 5 tubes 1 was a "rest tube" with holes at the far end. Tubes 2 through 5 contained copper grids etched on flexible backing rolled up to fit inside the tubes with connecting tabs folded out. The first two tubes served for training, and two others for testing.

Odorants (0.25% menthol and 0.5% benzaldehyde) were spread over the grids in 0.2 ml of solution in absolute ethanol, allowing 5 min for evaporation. The shock voltage was 90 V (50 Hz). For training, 10 flies were placed in the start tube and the apparatus was laid horizontally in front of a horizontal daylight lamp. Positively phototactic flies were prompted to move toward the light and to enter a proper tube. The schedule was as follows: 60 sec in the rest tube; 30 sec in the tube with the first odorant associated with the electric shock; 60 sec in the rest tube, 30 sec in the tube with the second odorant and again 60 sec in the rest tube. After 3 training runs the flies were tested for learning in two test tubes, containing the same odorants on fresh grids (with no voltage applied) and the number of flies in the start tube (i.e., avoiding shock-associated and control odorants) was recorded. To control for odor bias, a reciprocal experiment with a fresh group of flies of the same genotype was performed, voltage being applied to the tube with the second odorant. For each half-experiment the fraction of flies avoiding the shock-associated odorant minus the fraction of flies avoiding the control odorant was determined. The learning index Λ was defined as the average of the two values. $\Lambda = 1$ represents perfect learning; $\Lambda = 0$ indicates no learning.

The ability of flies to sense the odorants on the grids was measured in the choice-chamber apparatus according to Dudai *et al.* [7].

The excitability of the neuromuscular apparatus (an indirect characteristic of a sensitivity to electroshock) was measured in 0-hour prepupae by the method described elsewhere [23].

RESULTS

Biochemical Characteristics of ts-Mutants

While being tested on selective media, males of 4 stocks from 1,000 demonstrted temperature-dependent lethality

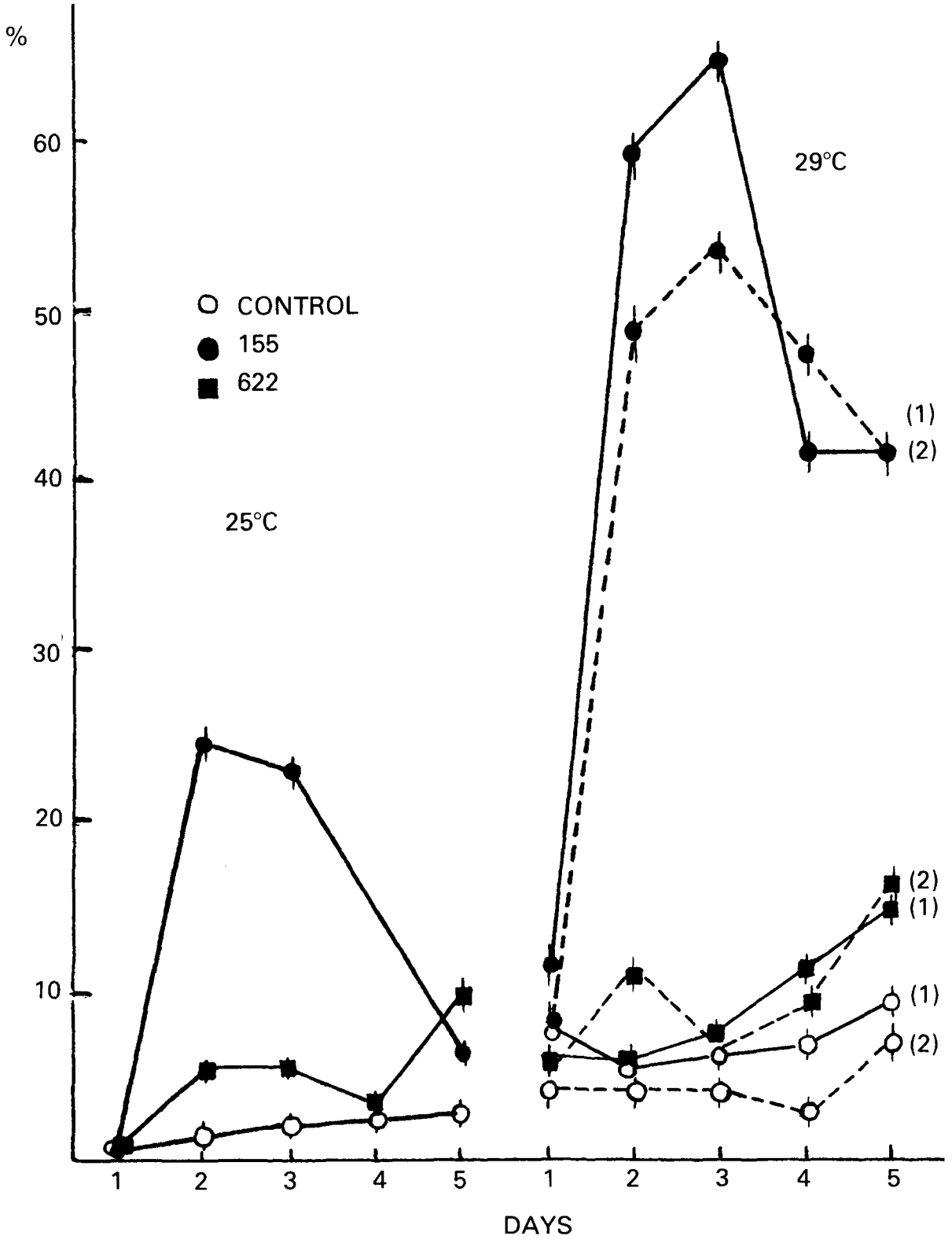

FIG. 1. Locomotor activity (percent of moving files) in ontogenesis of ts-mutants with an increased content of cAMP. Solid line—the period of maximal temperature sensitivity at 29°C.

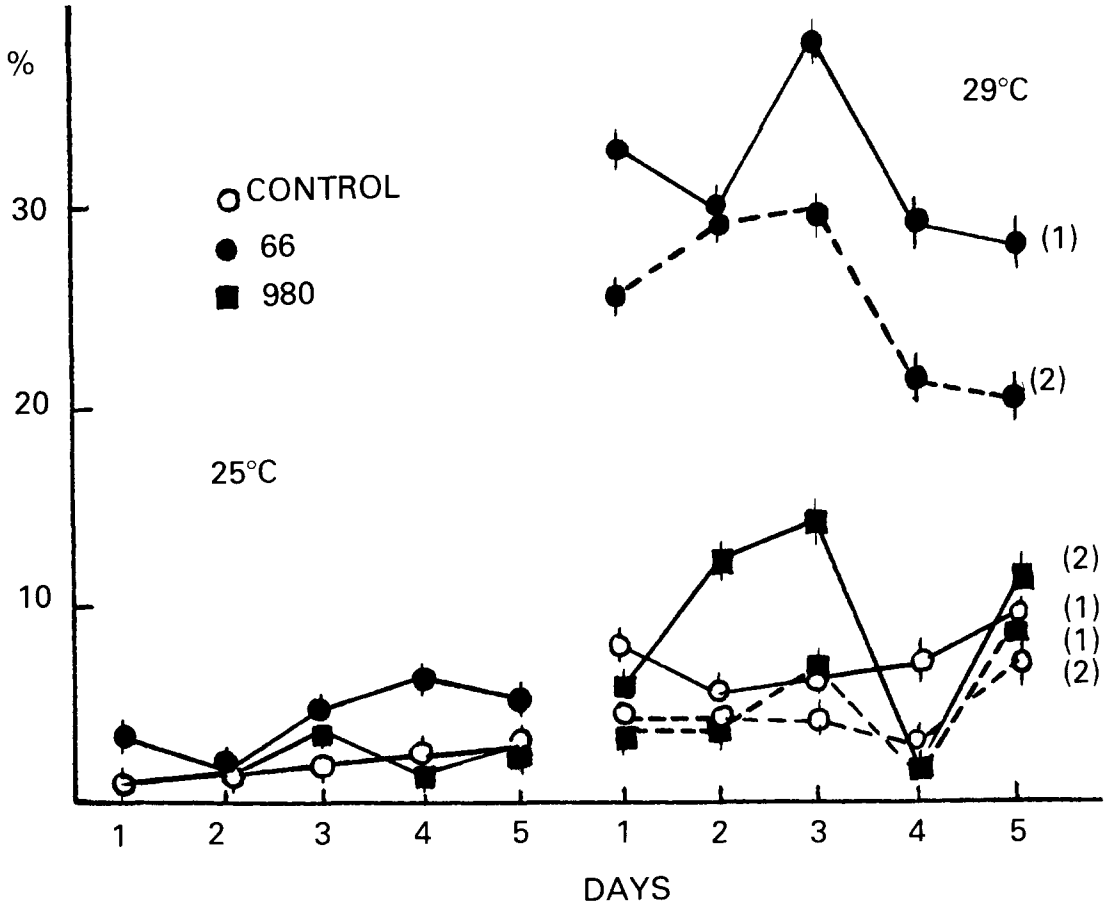

FIG. 2. Locomotor activity in ontogenesis of ts-mutants with lowered activity of cyclic nucleotide phosphodiesterase.

during development from egg in 29°C (Table 1). Two stocks appeared to be sensitive to propranolol: males from stock 155 could not survive in 29°C, while males from stock 980 were significantly reduced in number. Stock 66 was sensitive to theophylline. Males from stock 398 showed ts-lethality irrespective of the inhibitors added. All these stocks, together with stock 622 which did not demonstrate sensitivity to any selective media, were subjected to the biochemical analysis (Table 1).

Males from stock 66, hypersensitive to theophylline, had lower activity of cyclic nucleotide PDE than control males. Males from stock 155 hypersensitive to propanolol had an increased amount of cAMP. Since the activity of their PDE did not differ from that of the control, it could be indicative of an increased activity of the adenylate cyclase. Males from stock 398 had significantly more active PDE. Males from stock 622, insensitive to the inhibitors used, had an increased amount of cAMP and at the same time normal activity of PDE.

To obtain additional evidence that ts-sensitivity to inhibitors in males results from the mutational changes in the activities of the enzymes and that the mutations are located on the X-chromosome and not in autosomes, females from the same stocks were also subjected to the biochemical analysis. No biochemical changes similar to that of the corresponding males were found, though females from all mutant stocks had slightly more cAMP than control females. Thus, all the following experiments were carried out on males.

Locomotor Activity of ts-Mutants

The only visible peculiarity of behavior, differing from that of the wild type flies, was in ts-mutants 66 with lowered activity of PDE: in both permissive and restrictive temperatures they had incoordinated movements, impaired geo- and phototaxis and flight ability, though their wings are quite normal. Since other mutants do not exhibit any visible alterations in behavior it seemed reasonable to measure their locomotor activity (LA), one of the most informative characteristics of Drosophila behavior.

The results of determination of LA in mutants with the increased content of cAMP are plotted in Fig. 1, with lowered activity of PDE in Fig. 2 and in mutants with the increased activity of PDE in Fig. 3. LA of the control stock was rather low at 25°C, but increased at 29°C, probably due to nonspecific temperature activation, which was indicated by the significant decline in LA in the second half of the 1

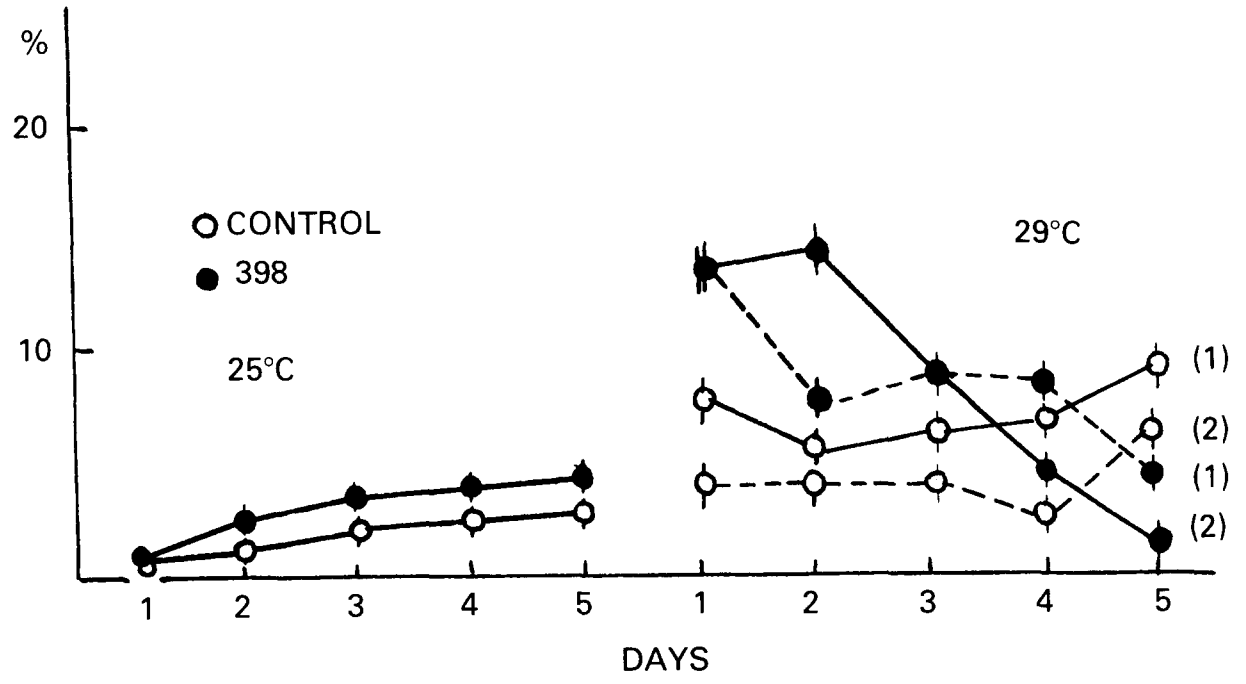

FIG. 3. Locomotor activity of ts-mutant with the increased activity of cyclic nucleotide phosphodiesterase.

hour test. LA of ts-mutant 155 (Fig. 1) rapidly increased in both temperatures to the 2nd day of imago life, its magnitudes at 29°C being almost 3 times as large as its own level in 25°C and 15 times as large as the corresponding control values (second half of the test). The differences between the levels of LA of ts-mutant 622 and control were similar at both temperatures.

LA of ts-mutants 66 and 980 with low activity of PDE (Fig. 2) at 29°C, compared to that in 25°C, underwent, respectively, 8-fold and 4-fold increases.

LA in ontogenesis of ts-mutant 398 with high activity of PDE suffered a drastic decline at 29°C, its level on the 5th day being 2.5 times lower than its own level at 25°C and 4 times lower than the corresponding values of the control at 29°C.

Thus, the significant increase in LA was observed in those cases where temperature-dependent increases in the content of cAMP had to occur either in the mutants with an originally increased amount of cAMP, or in mutants with lowered activity of PDE. On the contrary, a decrease in cAMP content, which had to take place at 29°C in the organism of ts-mutant 398 as a result of the high activity of PDE, led to the decrease in LA.

Learning

Learning procedures were also carried out both at 25°C and 29°C. In the last case flies were transferred to 29°C 30 min before the experiment. The results are presented in Table 2 and in Fig. 4.

ts-mutant 66 failed to learn in both temperatures, being incapable of training in the apparatus due to an impaired phototaxis (Fig. 4). ts-mutant 155, as well as ts-mutant 980, had normal learning ability despite the temperature. ts-mutant 398 had a higher learning ability than control flies in permissive temperature and failed to learn at 29°C. ts-mutant 622 succeeded in learning at 29°C if compared to control flies.

Ability of the Mutants to Sense the Odorants

The behavior of the mutants in the choice-chamber apparatus (Table 3) did not correlate with their learning performance: the decrease in sensitivity to benzaldehyde of ts-mutant 66 could not contribute to their failure to train in the apparatus for learning; the decreased sensitivity at 29°C to the same odorant of ts-mutant 155 and to both odorants of ts-mutant 980 had no influence on their learning ability, and finally, decreased sensitivity to menthol of ts-mutant 622 was observed in both temperatures, though at 29°C it was the best in learning. Thus, different learning abilities of the mutants could not be ascribed to their ability to sense odorants.

Thresholds of Neuromuscular Excitability

Since neuromuscular excitability had to be measured in 0-hour prepupae at 29°C as well, larvae were allowed to stay at 29°C for two hours before pupation. No difference between thresholds to long-duration stimuli (Reobase) of control and ts-mutants were observed in both temperatures, though there was the decrease in the threshold of ts-mutants 155, 622 and 980 at 29°C (Table 4). Thus, this characteristic considered as an indirect measure of sensitivity to the electroshock could not be helpful in interpreting the different learning ability, but is to some extent valuable as an additional characteristic of the mutants themselves. It is attrac-

TABLE 2

LEARINING INDICES OF ts-MUTANTS IN PERMISSIVE AND RESTRICTIVE TEMPERATURE

Stock	(Mean ± SE) 25°C	Number of Tests	(Mean ± SE) 29°C	Number of Tests
Control	0.25 ± 0.037	4	0.34 ± 0.030	4
66	−0.05 ± 0.035*	4	−0.06 ± 0.062*	3
155	0.24 ± 0.008	3	0.30 ± 0.031	10
398	0.46 ± 0.022*	3	0.02 ± 0.041*	5
622	0.25 ± 0.030	3	0.45 ± 0.026*	6
980	0.28 ± 0.033	5	0.33 ± 0.068	5

*$p \leq 0.05$ Student's criterion.

TABLE 3

ABILITY TO SENSE ODORANTS IN CHOICE-CHAMBER APPARATUS. FRACTION OF FLIES IN A TUBE WITH ODORANT (MEAN ± S.E.)

Stock	Menthol		Benzaldehyde	
	22°	29°	22°	29°
Control	0.27 ± 0.084	0.28 ± 0.031	0.20 ± 0.019	0.20 ± 0.037
66	0.26 ± 0.032	0.27 ± 0.043	0.34 ± 0.040*	0.33 ± 0.038*
155	0.26 ± 0.061	0.37 ± 0.064	0.17 ± 0.024	0.28 ± 0.030†
398	0.39 ± 0.057	0.32 ± 0.041	0.21 ± 0.023	0.28 ± 0.052
622	0.47 ± 0.043*	0.39 ± 0.027*	0.29 ± 0.008	0.19 ± 0.042
980	0.19 ± 0.057	0.37 ± 0.031†	0.15 ± 0.031	0.32 ± 0.026†

$p \leq 0.05$ Student's and Burn's criterions.
*Difference between a stock and the control.
†Intrastrain difference at two temperatures.

tive to suppose that the rise of the excitability at 29°C is mediated via a system of cAMP, since it occurs in ts-mutants 155 and 622 with the increased content of cAMP and in ts-mutant 980 with a low activity of PDE, but this assumption is too tentative since the rise in the excitability does not exceed the control level at 29°C and is absent in mutant 66.

DISCUSSION

Keeping in mind rather simple and formal reasons, one could expect that a hypersensitivity to an inhibitor of the two enzymes of cAMP metabolism would be indicative of a mutation which impair either enzyme of cAMP synthesis, or the enzyme of its degradation. For instance, the hypersensitivity to theophylline could be indicative of a decrease in activity of phosphodiesterase or of a reciprocal increase in activity of adenylate cyclase. In the first case a concentration of theophylline having no effect on the survival of normal flies, would be large enough to inhibit mutationally impaired PDE and to cause an imbalance in the "collaboration" of the two enzymes and the resultant lethality. In the second case an imbalance is avoided only when the inhibitor is absent, and inhibition of normal PDE could lead to lethality due to an abnormally active adenylate cyclase. These considerations

TABLE 4

THRESHOLDS OF NEUROMUSCULAR EXCITABILITY IN VOLTS TO ELECTRIC STIMULI 50 msec MEASURED IN ts-MUTANTS IN 0-HOUR PREPUPAE

Stock	25° (Mean ± SE)	n	29° (Mean ± SE)	n
Control	4.0 ± 0.08	22	3.8 ± 0.10	21
66	4.0 ± 0.08	19	4.0 ± 0.14	12
155	4.6 ± 0.12	27	4.0 ± 0.16*	16
398	5.1 ± 0.21	21	3.7 ± 0.10	22
622	4.3 ± 0.12	18	3.9 ± 0.10*	21
980	4.3 ± 0.09	27	3.8 ± 0.10*	15

*$p \leq 0.05$ Burns and Student criterion, intrastrain difference.

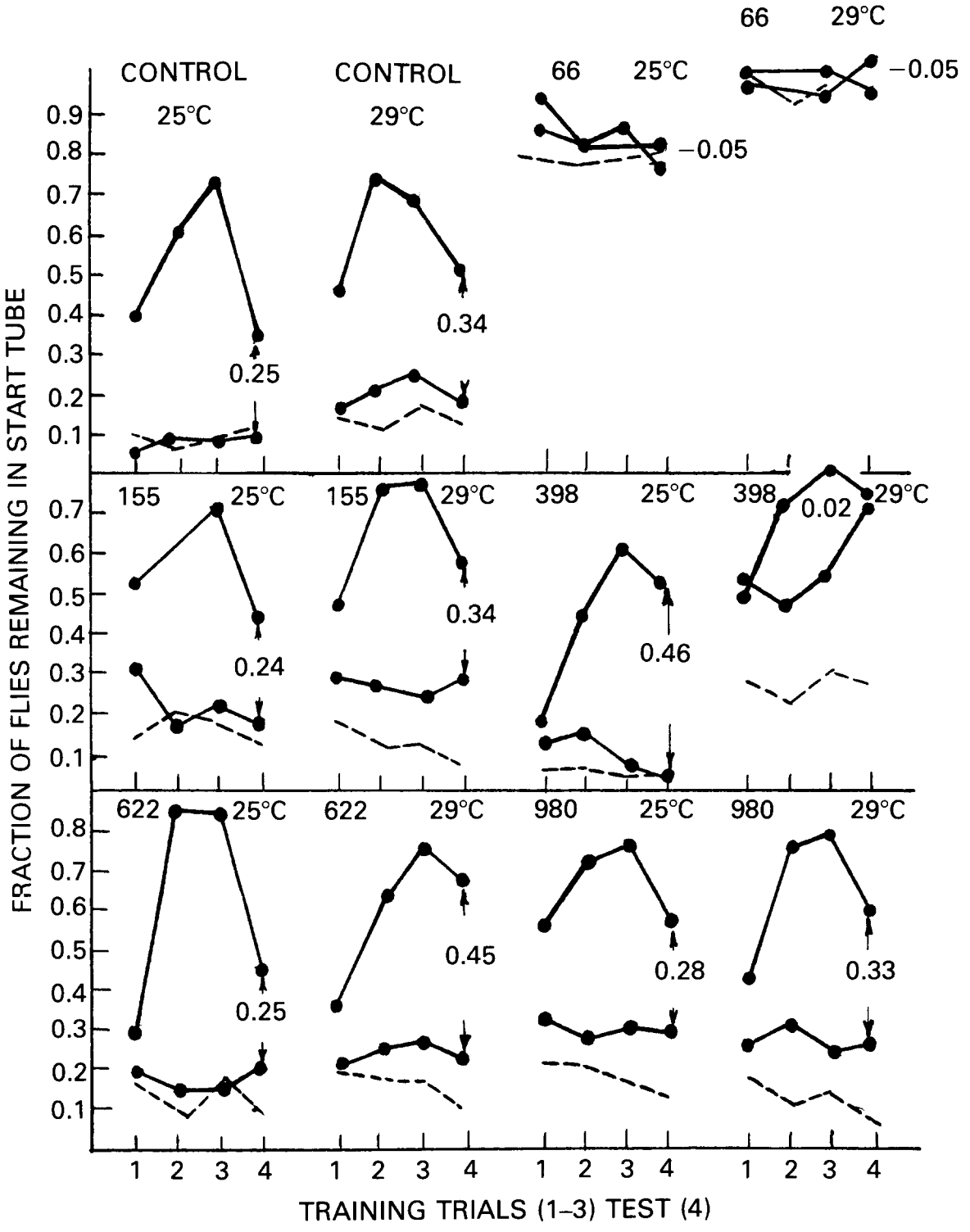

FIG. 4. Behavior of normal and mutant flies during training and testing. Fraction of flies in start tube after 30 sec is shown for the three training trials and for the test. Each point represents the mean. Upper solid line—the fraction of flies avoiding the shock-associated odorant; lower solid line—the fraction of flies avoiding control odorant; dotted line—the fraction of flies avoiding rest tube.

appeared to be true only for ts-mutant 66, hypersensitive to theophylline and having low activity of PDE. In the case of ts-mutant 155, hypersensitive to propranolol, we observed an enlarged content of cAMP due to probably more active adenylate cyclase, instead of an expected loss in its activity. Moreover, this is in contradiction to the well-known mode of action of propranolol [8,11]. However, it should be kept in mind that adenylate cyclase is a rather complicated system itself, with an unknown number of protein componets and with an unknown mode of interrelations betwen them and the β-adrenergic receptors as well [20]. So it is not surprising that a mutation impairing a component of the system would lead to unpredictable effects.

The nonspecific (with respect to inhibitors) ts-lethality of ts-mutant 398 with high activity of PDE could be understood, assuming that cyclic nucleotide PDE of *Drosophila* is capable of hydrolysing both cAMP and cGMP. The recent findings of Kiger and Golanthy [15] who attempted to localize the genes for cAMP PDE and cGMP PDE by means of local aneuploidy throughout the whole genome of *Drosophila* and thus revealed the structural gene for cAMP PDE in the 3D4 region of the X-chromosome and no such gene for cGMP PDE, do not exclude among the others the interpretation that there is one enzyme in *Drosophila* which hydrolyzes both cyclic nucleotides.

Our preliminary experiments indicate that this is likely, since there are no additive effects in PDE activity when the enzyme is allowed to hydrolyze the mixture of both substrates present in saturating equimolar amounts. Under these conditions PDE of ts-mutant 398 appeared to be more active than the normal one for each substrate and their mixture.

ts-mutant 622, insensitive to the inhibitors used, nevertheless has the enlarged content of cAMP, probably due to more active adenylate cyclase, which is sensitive to other agonists than presumably noradrenaline-sensitive adenylate cyclase of ts-mutant 155, selected on the β-adrenergic blocker propranolol.

More difficult to understand is why ts-mutant 980, sensitive to propranolol, has PDE with low activity, since it is believed that propranolol does not influence PDE activity [16].

Considering the involvement of cAMP in behavioral processes, one should proceed from the functional distribution of neurotransmitters in the nervous system whose effects are known to be mediated via cAMP.

It is known that noradrenaline and adrenaline are found in the vegetative nervous system of insects where it is believed to innervate muscles [14,21].

Tunnicliff and co-workers [26] have shown that *Drosophila* strains, selected on high and low locomotor activity, have respectively high and low levels of noradrenaline if compared to an intermediate level in flies with normal locomotor activity. Since cAMP is a second messenger for this catecholamine it is not surprising that locomotor activity is formed on the basis of biochemical events in the nervous system involving cAMP.

Concerning learning ability, it should be noted first of all that the procedure worked out by Benzer's group proved to be highly reproducible, since the learning index measured in normal flies in the two laboratories appeared to be the same. The learning index of the mutant dunce [7] and that of our ts-mutant 398 at 29°C are of the same order. The possibility of finding mutants with better learning ability than that of the wild type was brought in question by Médioni [19]. However now this possibility is demonstrated.

Since there are no correlations between learning ability and the ability to sense odorants, and neuromuscular excitability and locomotor activity as well (e.g., the learning ability through the whole period of ontogenesis tested in the ts-mutant 155 was the same despite the drastic changes in its locomotor activity), one can assume that the differences observed result from changes in functions of an associative part of the CNS, rather than from that of receptor or effector parts of the nervous system.

It is known that the predominant neurotransmitter in the CNS of insects is acetylcholine [14,21]. Its effects are mediated via cyclic GMP [10]. As for catecholamines, only dopamine [15] is present in the CNS, while noradrenaline and adrenaline are almost completely absent [15]. Based on this evidence, it can be assumed that ts-mutant 398 with a high activity of PDE fail to learn at 29°C probably because of greater hydrolysis of cGMP in cholinergic structures of the brain.

The high learning ability of ts-mutant 622 possibly results from an increase in the content of cAMP in dopaminergic structures of the brain. The suggestion that the increased amount of cAMP in this mutant is a result of high activity of dopamine-sensitive adenylate cyclase is supported by the fact that ts-mutant 622, contrary to ts-mutant 155, is not sensitive to the β-blocker propranolol. Insensitivity to β-agonists and antagonists is argued to be a distinctive property of dopamine-sensitive adenylate cyclase [24]. This suggestion helps to understand this only case when the increased cAMP content did not lead to an increase in locomotor activity. Besides, as it has been shown [26] dopamine content is the highest in the strains of *Drosophila* with low locomtor activity. Studies intended to check out the suggestions made are in progress.

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