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$CuSO_4$ Resistance in *Drosophila melanogaster*

III. Various Changes of Characters accompanied with Acquisition of Resistance to Copper[1)]

By

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The present author reported in the first report (YANAGISHIMA and SUZUKI, 1959a) that the larvae of *Drosophila melanogaster*, whose parent flies had been reared in the sublethal copper medium, i.e., 0.5 mM $CuSO_4$-containing medium (they are called the Cu-strain in this report), could acquire a resistance to 4 mM $CuSO_4$-containing medium, in which about 50% of the normal larvae could emerge. The Cu-strain showed higher pupation and emergence rates in 4 mM $CuSO_4$-containing medium than the control (normal) strain, of which larvae had no contact with copper; and the former developed in 4 mM $CuSO_4$ medium faster than the latter.

In the second report (YANAGISHIMA and SUZUKI, 1959b), the present author examined whether any difference in the copper resistance acquired was found among the Cu-strains different in their own careers in the experience of 0.5 mM $CuSO_4$-containing medium during the larval stages. Of course, *Drosophila melanogaster* has three instar stages, and at first it was examined whether any difference in the copper resistance could be found between the larvae that had been reared in 0.5 mM $CuSO_4$ medium during one of the three larval stages and those that had been reared during all of the larval stages. The result was that the copper resistance can be acquired during the larval period in 0.5 mM $CuSO_4$-containing medium irrespective of the length of time in this medium. Furthermore, no significant difference in oviposition number could be found among the flies that had had different careers there.

Number of eggs laid by the Cu-strain was not different from that laid by the control strain. The Cu-strain, however, showed higher hatching rate than the control strain, and the difference was significant. Further resistance experiments were performed using various flies of different careers, of which ancestors being reared in 0.5 mM $CuSO_4$ medium or in 4 mM $CuSO_4$ medium, and some were continued rearing in these media, while others were returned

1) Contributions from the Adaptive Variation Research Group, No. 55.

to the normal medium. The results of these experiments showed that the resistance to copper could be acquired during the life of only one generation in the sublethal (0.5 mM) $CuSO_4$ medium and the change seemed to be induced by the adaptive processes.

In the present report, which is the third one of this series, the author wishes to show the alterations of various characters, such as behaviors, longevity, sensitivity to enzyme inhibitors, and so on, evoked in accompany with the induction of the resistance to copper.

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Experiments

1. *Fundamental method of experiment.*

Experiments were performed with *Drosophila melanogaster* Oregon RS strain, derived from the standard stock culture in the Genetics Laboratory of Zoological Institute, Kyoto University.

The control medium was PEARL's synthetic medium, 15 ml of which was put in a 50 ml culture bottle. The copper-containing medium was prepared in a culture bottle, mixing PEARL's synthetic medium with 0.5 mM of $CuSO_4$ immediately after boiling. Just before the employment, two drops of yeast suspension, which had been prepared by suspending 10 mg of dry yeast in 20 ml of distilled water, were added.

Twenty flies, 10 males and 10 females which had emerged two days before were put in a oviposition tube, 3 cm in diameter and 12 cm in length, with cotton plugs at both ends. Then a slide glass with PEARL's synthetic medium on it, on which yeast suspension had been dropped, was inserted into the tube, so that the flies could deposit eggs on it. Usually several of these tubes were used at the same time. The slide glass was renewed every day and the newly hatched larvae were put by 20 individuals into a culture bottle. The renewal of the slide glass was performed at a definite time of a day and only the larvae which had hatched within 2 hours after that time were used. Then the adults emerged from these bottles were counted every 24 hours, and measured the emergence rates. The culture bottle was put in an incubator at a constant temperature of $25° \pm 0.1°C$.

2. *Body length.*

Method: The first instars of the normal strain were transferred to the medium containing 0.5 mM of $CuSO_4$. The adult flies emerged from this

copper-containing medium were transferred to the oviposition tube in which the slide glass having the normal medium on it was inserted. The larvae hatched from these eggs were denoted as the larvae of the Cu-strain.

The larvae of the Cu-strain and those of the control strain were both put onto the "test copper medium", which contained $CuSO_4$ in 4 mM density, or onto the normal medium, and the body lengths of adults emerged from these media were measured and the differences were examined statistically.

The measurements of body length were performed as to those flies one day old after emergence, male and female separately.

Results: The results of the measurement are shown in Table 1. In the case of male flies, the Cu-strain was larger than the control strain when reared on the control medium (δ $0.05>\alpha>0.02$, φ $0.6>\alpha>0.5$). In the test medium, no difference was found between the two strains, regardless of sex (δ $0.7>\alpha>0.6$, φ $0.4>\alpha>0.3$).

Table 1. Body lengths of the flies of the control and Cu-strains cultured on the control medium and the medium containing 4 mM $CuSO_4$.

Test medium	Control strain						Cu-strain					
	♂			♀			♂			♀		
	Number of adult	Mean body length	Standard deviation	Number of adult	Mean body length	Standard deviation	Number of adult	Mean body length	Standard deviation	Number of adult	Mean body length	Standard deviation
		mm			mm			mm			mm	
Control	78	2.69	0.10	65	3.01	0.12	89	2.72	0.11	80	3.10	0.14
$CuSO_4$	92	2.60	0.10	140	3.00	0.12	104	2.59	0.08	80	3.22	0.17

Examinations of differences between two strains (t-test).

	♂	♀
Control	$0.05>\alpha>0.02$*	$0.60>\alpha>0.50$
$CuSO_4$	$0.70>\alpha>0.60$	$0.40>\alpha>0.30$

* Significant.

3. *Longeviiy.*

In order to find whether any differences in ecological characters existed between the Cu- and normal strains, the longevity test was performed at the start.

a. *On the agar medium (PEARL's medium).*

Method: Some applications of the method used in the oviposition test mentioned in the first report of this series were made to the longevity test. The oviposition tube was a glass cylinder of 3 cm in diameter and 12 cm in length with cotton plugs in both ends. The flies of about 12 hours after

emergence were narcotized and transferred into the oviposition tube. One tube contained 5 female and 5 male flies. A slide glass, with PEARL's synthetic medium on it, on which yeast suspension had been dropped, was inserted into the tube. The slide glass was renewed every day and at the same time the living flies were counted till all the flies in the tube died away. Here the longevity means the average length of the life of adult flies, that is to say, from the time of the flies' emergence till their death.

Results: It is clear from the present results shown in Fig. 1 and Table 2 that female flies could live longer than males in both the Cu- and normal strains, and the flies of the Cu-strain could live longer than the normal strain, without distinction of the sex. All these differences are statistically significant ($0.001 > \alpha$). It is of great interest that longer longevity is accompanied with stronger resistance to copper.

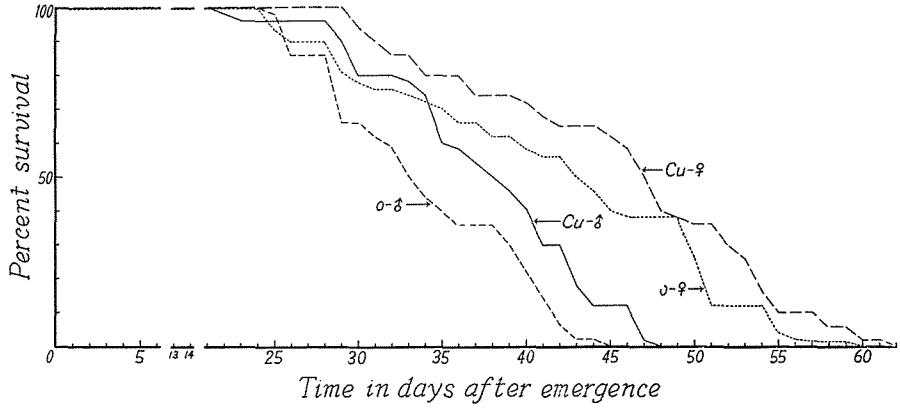

Fig. 1. Results of the longevity test on PEARL's synthetic medium.
O: flies of the control strain. Cu: flies of the Cu-strain.

Table 2. Results of the longevity test on PEARL's synthetic medium.

Strain	♂			♀		
	Number of flies	Mean length of life	Standard deviation	Number of flies	Mean length of life	Standard deviation
		day			day	
Control	50	32.64	5.83	50	36.00	6.82
Cu-strain	50	37.80	5.95	50	45.72	7.28

Examinations of differences between two strains (t-test).

♂ $0.001 > \alpha^*$
♀ $0.001 > \alpha^*$

* Significant.

b. *On liquid media containing copper.*

In order to know more about their longevity, another experiment was carried out, using sucrose solution containing copper. As mentioned by POULSON (1950a, b, 1955) and POULSON *et al.* (1951, 1952, 1958a, b), copper absorption of flies depends on concentrations of copper in culture medium and this phenomenon may have some effects on longevity of the flies. Experiment was made by using the methods used by HASSETT (1948), OHSAWA and TSUKUDA (1956) and SANG (1956).

Material and Method: The flies, about 12 hours old after emergence, were anesthetized with ether and divided into male and female groups. In this way, 10 flies were tested at once.

Test bottles were the same as those used by OHSAWA and TSUKUDA (1956), as shown in Fig. 2. A J-shaped glass tube which was 5 mm in diameter and 120 mm in length was inserted through cotton plug. The upper end of the J-shaped tube was plugged with cotton tightly, to avoid evaporation of water. Rolled filter paper being infiltrated with test solution was inserted into the other end of the tube. 0.1 M of sucrose solution supplemented with or without 4 mM $CuSO_4$ were used. After having removed the cotton plug of the upper end, 1.5 ml of the test solution was carefully introduced into the tube with a pipette. The test solution was added every day and it was changed for new one every 3 days. In such a test bottle, 10 flies were put in and living individuals were counted every day. Rearing temperature was 25°±0.1°C as usual.

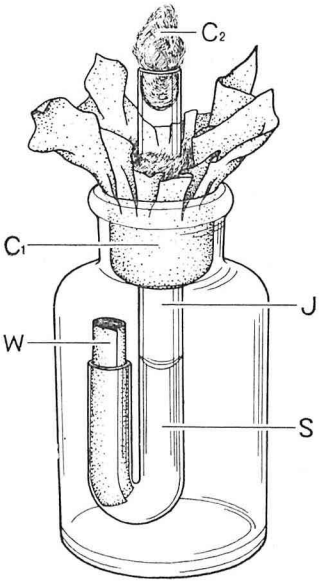

Fig. 2. Experimental equipment used in the longevity test with sucrose solution. C1 and C2: Cotton plugs. J: J-shaped tube containing a sucrose solution (S). W: Wick made by rolling a strip of filter paper.

Results: The experimental results are shown in Tables 3 and 4, and Figs. 3 and 4.

We can see from the results that the flies of the Cu-strain could live longer than those of the normal strain on the control solution (sucrose 0.1 M), without regard to sex. The differences are statistically highly significant (♂: $0.05 > \alpha > 0.02$, ♀: $0.01 > \alpha > 0.001$). On the contrary, the normal strain could live longer than the Cu-strain on the test solution with copper. These differences are also statistically significant (♂, ♀: $0.001 > \alpha$). There was also significant difference in longevity, between male and female flies on the copper-containing test solution in both cases of the Cu- and the normal strains. It must be noticed here that the flies of the Cu-strain could live longer than the normal

Table 3. Results of the longevity test performed by supplying simple 0.1M sucrose solution.

Strain	♂			♀		
	Number of flies	Mean length of life	Standard deviation	Number of flies	Mean length of life	Standard deviation
		day			day	
Control	60	24.98	3.87	60	23.70	3.80
Cu-strain	70	26.27	2.76	70	26.22	7.71

Examinations of differences between two strains (t-test).

♂ $0.05 > \alpha > 0.02$*

♀ $0.01 > \alpha > 0.001$*

* Significant.

Table 4. Results of the longevity test performed by supplying sucrose solution containing $CuSO_4$ in 4mM.

Strain	♂			♀		
	Number of flies	Mean length of life	Standard deviation	Number of flies	Mean length of life	Standard deviation
		day			day	
Control	210	5.20	1.45	200	6.52	1.70
Cu-strain	250	4.75	1.73	260	6.15	1.57

Examinations of differences between two strains (t-test).

♂ $0.001 > \alpha$*

♀ $0.001 > \alpha$*

* Significant.

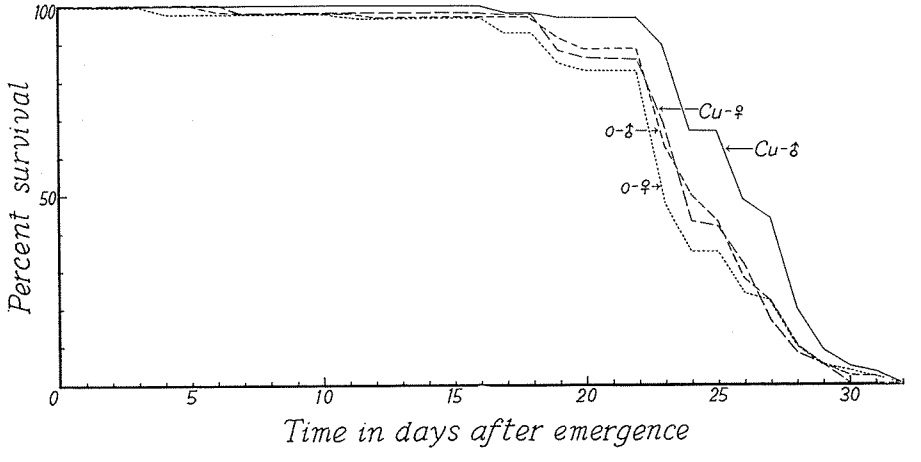

Fig. 3. Results of the longevity test performed by supplying simple 0.1 M sucrose solution.

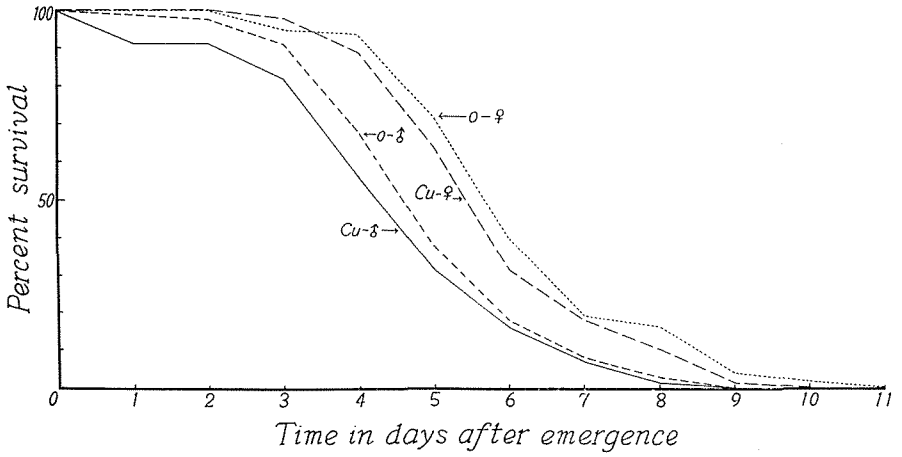

Fig. 4. Results of the longevity test performed by supplying 0.1 M sucrose solution containing $CuSO_4$ in 4 mM.

strain on both 0.1 M of sucrose solution and PEARL's synthetic medium, when they were not supplied with copper, whereas the Cu-strain showed shorter longevity on 0.1 M sucrose solution containing 4 mM of copper than the control strain. The reason of this peculiar phenomenon will be discussed later on.

The same type of the experiment was made, using 2 mM of $CuSO_4$. The results are shown in Fig. 5 and Table 5, from which we can see that, in the Cu-strain, the mean life duration was 13.60 days for males and 13.50 days for females, but in the control strain, it was 11.21 days for male and 12.00 days for female flies.

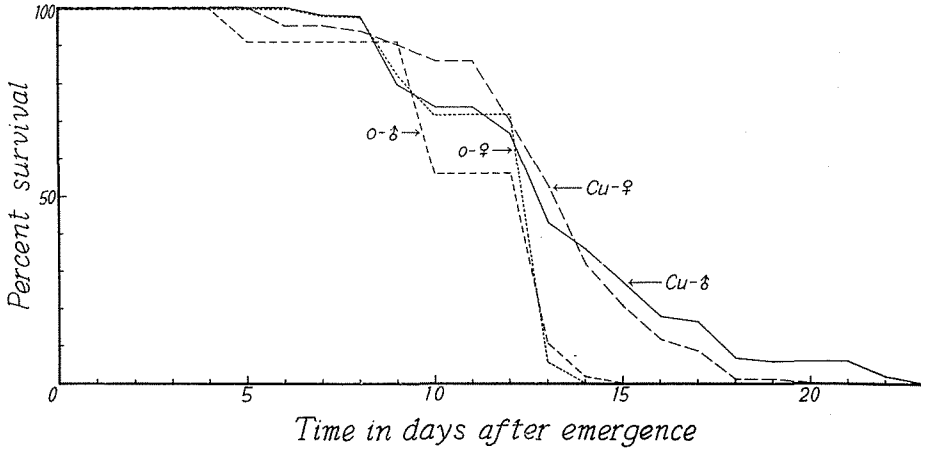

Fig. 5. Results of the longevity test performed by supplying 0.1 M sucrose solution containing $CuSO_4$ in 2 mM.

Table 5. Results of the longevity test performed by supplying sucrose solution containing $CuSO_4$ in 2 mM.

Strain	♂			♀		
	Number of flies	Mean length of life	Standard deviation	Number of flies	Mean length of life	Standard deviation
		day			day	
Control	50	11.21	8.19	50	12.00	1.74
Cu-strain	100	13.60	4.01	110	13.50	2.73

Examinations of differences between two strains (t-test).

♂ $0.001 > \alpha$*

♀ $0.01 > \alpha > 0.001$*

* Significant.

It is clear that the Cu-strain can live longer than the normal strain on 0.1 M sucrose solution containing 2 mM of $CuSO_4$, which is inconsistent with the result when 4 mM of $CuSO_4$ is contained. On the other hand, we know that the Cu-strain can live longer than the control strain in PEARL's medium containing 4 mM of $CuSO_4$. What is the reason of this inconsistent phenomenon? The author considers that nutrient conditions of culturing media are, at least, concerned with this phenomenon, i.e., PEARL's medium is far richer than the sucrose solution, and it is probable that the degrees of copper absorption or of resistance to copper are different among these different media.

4. *Behavior-Photokinesis.*

Photokinesis experiment (activity test) was performed in order to know if there were any differences in behavior between the Cu-strain and the normal one.

Material and method: The following preparation was made before the experiment, irrespective of the flies of the Cu- and the normal strains.

The flies one day old after emergence were narcotized with ether, and were divided into male and female groups. These groups were transferred separately into small tubes (1.5 cm in diameter and 9 cm in length) containing 2 ml of PEARL's synthetic medium, 10 flies to one tube. Ten tubes containing 10 male flies each and another 10 tubes containing 10 female flies each were used in one test. The activity test was carried on in the daytime (9–15 o'clock) from 2 to 3 days after emergence. The reason why this photokinesis test was carried on in the daytime was that the flies had the daily rhythmic change in activity and it was most stable in the daytime.

The test apparatus is shown in Fig. 6. Flies to be tested were introduced from side arm (1.5 cm in diameter and 9 cm in length) to main glass tube (3.5 cm in diameter, 40 cm in length). A glass plug with some perforations, through which air could enter but flies could not, was plugged to the side arm. Three mark lines are ticked at intervals of 10 cm on outside of the main test

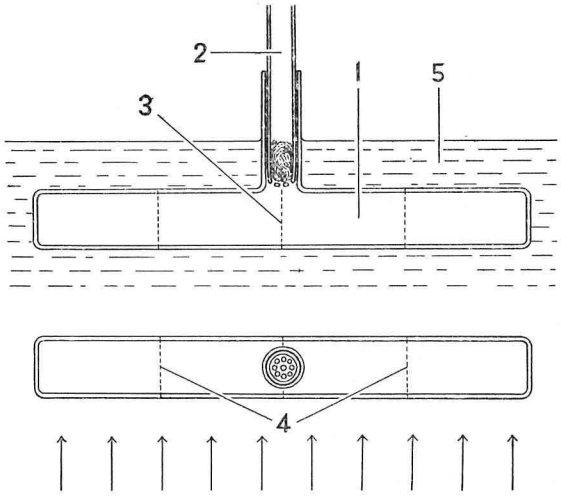

Fig. 6. Schematic representation of the experimental equipment for the activity test. Upper figure: Side view. Lower figure: Upside view. 1: Test tube of 3.5 cm in diameter and 40 cm in length. 2: Side arm of the test tube (diameter 1.5 cm, length 9 cm). 3, 4: Three quarterly lines marked on the test tube. 5: Thermostat with water of 25°C. Arrows show illumination beams of diffused light.

Table 6. Results of the activity test (photokinesis experiment). Each figure is the averages of five consecutive counting using the same materials.

Control strain		Cu-strain	
♂	♀	♂	♀
1.26	1.24	1.72	0.58
1.06	2.76	2.10	1.08
0.94	0.82	2.16	2.16
2.16	2.38	3.26	2.32
2.82	2.22	2.74	1.88
2.06	1.98	3.08	2.28
0.52	0.08	2.40	0.02
0.38	0.50	0.14	0.66
0.74	0.52	1.48	0.12
Average 1.32	1.38	2.11	1.23

Examinations of differences between two strains (F-test).

♂ $0.05 > \alpha > 0.02$*

♀ $\alpha > 0.20$

* Significant.

tube. This test tube was submerged in a thermostat of 25°C in the dark, and after 20 minutes of adaptation in the dark, a diffused light was switched on. Illumination on the test tube was about 550 lux. The number of flies which crossed the mark lines during 1 minute was counted and this counting was repeated five times. The mean value of this five countings was taken as an index of photokinesis.

Results: As shown in Table 6, photokinesis index was greater in male flies of the Cu-strain than the control strain ($0.05>\alpha>0.02$), but in the case of female flies, there was no difference between the Cu- and normal strains ($\alpha>0.20$). It is very interesting that there is a great difference in the photokinesis index between the male and the female flies of the Cu-strain. Thus, copper caused not only the resistance to copper but also the change in moving activity.

5. *Effects of rearing temperature.*

There were significant differences not only in longevity but also in photokinesis between the copper-adapted strain (Cu-strain) and the normal one. Here it can be said that general physiological activities must have also changed in the copper-adapted strain.

Rearing temperature is known to have much effect on the physiological activities of flies, so that the effects of temperature on acquisition of resistance were studied. Although most other experiments were carried on at 25°±0.1°C, the present tests were made, in one case, under the combination of 20°C and 25°C, and in the other case, under the combination of 25°C and 28°C.

Method: a) *In the case of combining 20°C and 25°C.* As shown in Fig. 7 schematically, as soon as the flies of the normal strain had emerged, 20 male and the same number of female flies were transferred into an oviposition tube. These tubes were divided into two groups, of which one was placed in an incubator of 20°C and the other was placed in that of 25°C (these were the control).

Twenty 1st instars which had hatched in these tubes were transferred to a culture bottle containing PEARL's synthetic medium. Ten culture bottles were prepared for each group, and these bottles were cultured at 20°C and 25°C respectively. The adult flies emerged from these bottles (Fig. 7, I and II) were transferred to oviposition tubes. Twenty larvae hatched from these eggs were transferred to a bottle, in which 15 ml of 4 mM $CuSO_4$-containing PEARL's medium was put, to test the emergence rate.

In this case, the following four kinds of combinations were made:

(i) All treatments were done at 20°C through the experiment (Fig. 7, I and IV).

(ii) The parents were cultured at 20°C (Fig. 7, I), but both oviposition and hatching were done at 25°C and the hatched larvae were cultured at 25°C in 4 mM $CuSO_4$-medium (Fig. 7, VI).

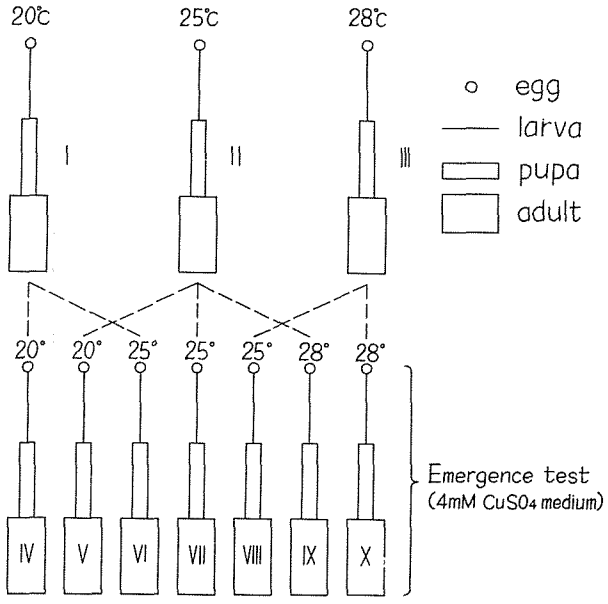

Fig. 7. Procedures of the experiment to test the effect of rearing temperature on the acquisition of resistance to copper. I-X: adult flies, which were subjected to different rearing temperatures in their careers.

(iii) The parents were cultured at 25°C (II), but the treatments thereafter (including oviposition) were all made at 20°C (V).

(iv) All the treatments were performed at 25°C (II and VII). Of course, all the tests mentioned above were made with both the normal and the Cu-strains in parallel at the same time, and 10 bottles were used at each set. The experiments were repeated thrice in all.

b) *In the case of combining 25°C and 28°C.* As shown schematically in Fig. 7, two kinds of rearing temperature, 25°C and 28°C, were used. Other procedures were similar to the previous experiments. In this case, the experiments were repeated twice.

Results: a) The results of the cases of combining 20°C and 25°C are shown in Table 7. The emergence rates of the Cu-strain were always higher than those of the normal strain. The differences were demonstrated as statistically significant, except the case of VII, as shown in Table 7.

In almost all cases, there was no significant difference in acquisition pattern of copper resistance among various temperature treatments. This means that there was no significant correlation between rearing temperatures at which the tests had been made, i.e., two kinds of the rearing temperatures, 20°C and 25°C, had no effects on copper resistance. The only one exceptional case

Table 7. Effects of rearing temperatures (20°C and 25°C) on the acquisition of resistance to copper. IV, V, VI and VII indicate the flies, whose careers are shown in Fig. 7.

	IV						V					
	Cu-strain			Control			Cu-strain			Control		
	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%
1	200 (10)	124	62.0	200 (10)	98	49.0	200 (10)	133	66.5	200 (10)	107	53.5
2	200 (10)	141	70.5	200 (10)	112	56.0	200 (10)	130	65.0	200 (10)	114	57.0
3	200 (10)	139	69.5	200 (10)	114	57.0	200 (10)	125	62.5	200 (10)	113	56.5
Total	600 (30)	404	67.3	600 (30)	324	54.0	600 (30)	388	64.6	600 (30)	334	55.6

	VI						VII					
	Cu-strain			Control			Cu-strain			Control		
	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%
1	200 (10)	138	69.0	200 (10)	90	45.0	200 (10)	131	65.5	200 (10)	117	58.5
2	200 (10)	137	68.5	200 (10)	114	57.0	200 (10)	129	64.5	200 (10)	121	60.5
3	200 (10)	129	64.5	200 (10)	106	53.0	200 (10)	127	63.5	200 (10)	117	58.5
Total	600 (30)	404	67.3	600 (30)	310	51.6	600 (30)	387	64.5	600 (30)	355	59.1

Examinations of differences in emergence (χ^2-test).

Between Cu- and control strains		Between two treatments		
			Cu-strain	Control
IV	$0.001 > \alpha$*	VI : IV	≒	$0.50 > \alpha > 0.30$
VI	$0.001 > \alpha$*	IV : V	$0.10 > \alpha > 0.05$	$0.70 > \alpha > 0.50$
V	$0.01 > \alpha > 0.001$*	IV : VII	$0.50 > \alpha > 0.30$	$0.10 > \alpha > 0.05$
VII	$0.10 > \alpha > 0.05$	VI : V	$0.10 > \alpha > 0.05$	$0.20 > \alpha > 0.10$
		VI : VII	$0.50 > \alpha > 0.30$	$0.02 > \alpha > 0.01$*
		V : VII	≒	$0.30 > \alpha > 0.20$

* Significant.

having statistically significant difference is seen between VI and VII of the normal strain, of which resistance test was made at 25°C, but the parental careers of both flies were different, the former being reared at 20°C and the latter at 25°C.

b) The results of the cases of combining 25°C and 28°C are shown in Table 8. When we compare their emergence rates with the Cu-strain and the

normal strain, there were no statistically significant differences in VII, VIII and IX, but statistically significant differences were found in X. Even in the former 3 cases, the actual results show that the emergence rates of the Cu-strains were higher than those of the normal strains.

In the next place, there are remarkable effects when the treatments of temperature are different. This is a phenomenon fairly contrasted with the former combination of rearing temperatures (20°C and 25°C). For example, VII flies that were trained to copper at 25°C showed higher emergence rate than VIII flies trained at 28°C, and the same tendency was found between IX and X. When the parental temperature careers are the same, however, no

Table 8. Effects of rearing temperatures (25°C and 28°C) on the acquisition of resistance to copper. Other explanations see Table 7.

	VII						VIII					
	Cu-strain			Control			Cu-strain			Control		
	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%
1	200 (10)	131	65.5	200 (10)	117	58.5	200 (10)	117	58.5	200 (10)	93	46.5
2	200 (10)	129	64.5	200 (10)	121	60.5	200 (10)	113	56.5	200 (10)	110	55.0
Total	400 (20)	260	65.0	400 (20)	238	59.5	400 (20)	230	57.5	400 (20)	203	50.7
	IX						X					
	Cu-strain			Control			Cu-strain			Control		
	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%	No. of larvae	Emerged No.	%
1	200 (10)	135	67.5	200 (10)	119	59.5	200 (10)	112	56.0	200 (10)	82	41.0
2	200 (10)	131	65.5	200 (10)	120	60.0	200 (10)	110	55.0	200 (10)	97	48.5
Total	400 (20)	266	66.5	400 (20)	239	59.7	400 (20)	222	55.5	400 (20)	179	40.7

Examinations of differences in emergence (χ^2-test).

Between Cu- and control strains

VII	$0.20 > \alpha > 0.10$
IX	$0.10 > \alpha > 0.05$
VIII	$0.10 > \alpha > 0.05$
X	$0.01 > \alpha$*

*Significant.

Between two treatments

	Cu-strain	control
VII : IX	$0.80 > \alpha > 0.70$	≒
VII : VIII	$0.05 > \alpha > 0.02$*	$0.02 > \alpha > 0.01$*
VII : X	$0.01 > \alpha > 0.001$*	$0.001 > \alpha$*
IX : VIII	$0.02 > \alpha > 0.01$*	$0.02 > \alpha > 0.01$*
IX : X	$0.01 > \alpha > 0.001$*	$0.001 > \alpha$*
VIII : X	$0.70 > \alpha > 0.50$	$0.20 > \alpha > 0.10$

significant difference in the copper resistance is found between the Cu- and normal strains, irrespective of F_1's temperature careers in the test medium. This is clear between VII and IX or between VIII and X (refer to Table 8).

After all, when the effects due to 25°C and 28°C are compared, the parental careers have a decided influence, 28°C having a worse effect on the resistant character to copper of F_1 generation, irrespective of the rearing temperature (temperature at the time of the emergence test) of F_1 generation.

6. *Uptake of copper.*

From the experimental results mentioned above, we saw that the Cu-strain was different from the control strain in many respects, such as behavior and longevity. In these circumstances, it is important to know about the uptake of copper by flies in order to make clear the reason why the above-mentioned changes have taken place in the Cu-strain.

a. *Chemical analysis.*

Total copper content in adult flies was measured by employing colorimetric method. The flies of both strains to be used for analyzing their copper contents were cultured in culture bottles, each containing 30 ml of PEARL's medium with 4 mM of $CuSO_4$. One hundred 1st instar larvae which had hatched in oviposition tubes were put in an above-mentioned culture bottle at 25°C. The flies which had emerged in these bottles were transferred to other culture bottles containing PEARL's medium without copper, in order to make them free from the copper attached to the body surface or accumulated in the digestive organ. After four days of culture on PEARL's medium, the adult flies were narcotized and washed with water for 1 hour, followed by drying in an oven at 85°C. The dried flies were made into ashes with dry combustion method and then copper was analyzed with colorimetric method. As organic reagents, *p*-dimethylaminobenzylidenerhodanin used in KOLTHOFF's method (1930) and dithizone used in MEHURIN's method (1935) and FISHER's method (1934) were used.

However, the copper contents shown by these methods were so small, irrespective of the Cu- or normal strains, that the comparison between these two strains was impossible.

In the next test, analysis was made by using a photoelectric spectrophotometer. The flies were prepared in the same way as mentioned above. Materials were decomposed with wet combustion method according to MILLER and MILLER (1948). Copper analysis was made SANDELL's method (1950), using sodium diethyldithiocarbaminate as reagent. As shown in Table 9, the copper content of the flies increased when they were reared in the copper containing medium, irrespective of the Cu-strain and the control strain, and thus the difference between them was not seen.

b. *Histochemical analysis.*

As chemical analysis did not bring about sufficient result, cytochemical method was used in order to know the difference in copper uptake by the two

Table 9. Copper contents (μg/dry weight) of the Cu-strain and control strain grown up in 4mM $CuSO_4$ containing PEARL's medium. Copper content was determined by SANDELL's method. In comparison, the copper content of the control strain when fed by PEARL's normal medium is cited.

Grown up in 4mM $CuSO_4$ medium		Grown up in control medium
Control strain	Cu-strain	Control strain
1.698	1.637	0.518
1.845	1.962	0.911
1.902	2.193	
2.581	2.589	
1.989	2.095	0.714 (Average)

strains. The experimental materials were obtained by the same procedure as mentioned above, but in this case, fixation was made with absolute alcohol instead of washing in water. Reagents used were potassium ferrocyanide and *p*-dimethylaminobenzylidenerhodanin after OKAMOTO and UTAMURA's method (1938, 1939) and sodium diethyldithiocarbaminate after KAMAMOTO's (1938) and WATERHOUSE's methods (1945). Although about ten thousand of preparates were made and observed, it was impossible to detect copper distribution in fly tissues by these methods.

After all, the author could not find any difference in copper content between the two strains by the chemical analyses. In order to know the copper content in detail, an autoradiographic method will be the best of all as shown by POULSON and his co-workers (1950, 1952, 1955), so the application of this method will be a future task.

7. *Sensitivity to enzyme inhibitors.*

It can be assumed that some physiological changes in metabolisms must have taken place within the bodies of flies of the Cu-strain. In order to detect this point, the following enzyme inhibitor test was attempted. $CH_2I \cdot COOH$ and NaF were used as inhibitors of glycolysis, 2, 4-dinitrophenol as uncoupling agent, and As_2O_3 and *p*-chloromercuribenzoate as SH-inhibitors.

Method: The concentrations of the inhibitors and the numbers of test bottles are shown in Tables 10-15. The 1st instar larvae of the Cu-strain and the control strain were transferred to test bottles containing PEARL's synthetic medium with various kinds of inhibitors and the numbers of adult flies emerged were counted, male and female separately.

In the case of 2, 4-dinitrophenol 70% ethyl alcohol was used as solvent, so the same quantity of alcohol was added to the control culture for blank test.

Results: The experimental results are shown in Tables 10-15 and Fig. 8.

a) *CH_2I·COOH.* As shown in Table 10, both strains showed 5–98% of emergence rates on the culture media containing 0.05–75$\times 10^{-5}$ Mol of CH_2I·COOH.

It is noticeable that at almost all concentrations, the Cu-strain showed lower emergence rate than the control strain, and the difference as a whole was statistically significant.

Table 10. Emergence rates of the two strains on the media containing CH_2I·COOH. Figures in parentheses show numbers of rearing bottles.

Concentration $\times 10^{-5}$ Mol	Control strain			Cu-strain		
	No. of larvae	Emerged		No. of larvae	Emerged	
		No.	% to larvae		No.	% to larvae
0.05	360 (18)	354	98.3	360 (18)	353	98.0
0.1	400 (20)	380	95.0	400 (20)	392	98.0
0.2	360 (18)	324	90.0	360 (18)	324	90.0
5.0	400 (20)	370	92.5			
10	400 (20)	358	89.5			
15	360 (18)	313	86.9	360 (18)	300	83.3
20	360 (18)	268	74.4	360 (18)	219	60.8
25	360 (18)	274	76.1	360 (18)	231	64.1
40	400 (20)	234	58.5	400 (20)	204	51.0
50	360 (18)	189	52.5	360 (18)	166	46.1
75	360 (18)	18	5.0	360 (18)	0	0
Total	4120 (206)	3082		3600 (180)		

Examinations of differences between two strains (χ^2-test).
$0.01 > \alpha > 0.001$* Significant.

b) *NaF.* The results are shown in Table 11. The Cu-strain showed lower emergence rate than the control strain, in the concentration range of 25–500$\times 10^{-5}$ Mol, and the difference was statistically significant.

c) *2, 4-dinitrophenol.* When only alcohol, used as solvent of 2, 4-dinitrophenol stock solution, was added to PEARL's medium, no effect on emergence rates of both strains was observed (see Table 12), whereas when 2, 4-dinitrophenol was used, as shown in Table 13, the Cu-strain showed lower emergence rate than the control strain, and this difference was statistically highly significant.

d) p-*chloromercuribenzoate* (*P.C.M.B.*). The experimental results are shown in Table 14. The emergence test was performed at the concentrations from 0.75 to 30$\times 10^{-5}$ Mol. The tendency that the Cu-strain showed lower emergence rate was clear, but the statistical examination failed to show this difference was significant.

Table 11. Emergence rates of the two strains on the media containing NaF. Figures in parentheses show numbers of rearing bottles.

Concentration $\times 10^{-5}$ Mol	Control strain			Cu-strain		
	No. of larvae	Emerged		No. of larvae	Emerged	
		No.	% to larvae		No.	% to larvae
25	420 (21)	381	90.7	420 (21)	375	89.2
50	420 (21)	411	97.8	420 (21)	365	86.9
100	360 (18)	315	87.5	360 (18)	301	83.6
200	420 (21)	273	65.0	420 (21)	258	61.4
300	420 (21)	155	36.9	420 (21)	126	30.0
400	320 (16)	48	15.0	320 (16)	20	6.2
500	420 (21)	0	0	420 (21)	0	0
Total	2780 (139)	1583		2780 (139)	1445	

Examinations of differences between two strains (χ^2-test).
$0.05 > \alpha > 0.02$* Significant.

Table 12. Emergence rates of the two strains on the media containing alcohol (blank test of 2, 4-dinitrophenol).

Concentration $\times 10^{-5}$ Mol	Control strain			Cu-strain		
	No. of larvae	Emerged		No. of larvae	Emerged	
		No.	% to larvae		No.	% to larvae
1.0	140 (7)	136	97.1	140 (7)	138	98.5
2.5	140 (7)	132	94.2	140 (7)	134	95.7
5.0	140 (7)	125	89.2	140 (7)	130	92.8
10	140 (7)	131	93.5	140 (7)	129	92.1
15	140 (7)	124	88.5	140 (7)	134	95.7
20	140 (7)	132	94.2	140 (7)	134	95.7
30	140 (7)	129	92.1	140 (7)	129	92.1
50	140 (7)	130	92.8	140 (7)	135	96.4
Total	1120 (56)	1039		1120 (56)	1063	

Examinations of differences between two strains (χ^2-test).
$\alpha > 0.99$ Non significant.

Table 13. Emergence rates of the two strains on the media containing 2, 4-dinitrophenol.

Concentration $\times 10^{-5}$ Mol	Control strain			Cu-strain		
	No. of larvae	Emerged		No. of larvae	Emerged	
		No.	% to larvae		No.	% to larvae
1.0	420 (21)	396	94.2	420 (21)	375	89.2
2.5	420 (21)	360	85.7	420 (21)	354	84.2
5.0	420 (21)	396	94.2	420 (21)	321	76.4
10	360 (18)	327	90.8	360 (18)	311	86.3
15	420 (21)	282	67.1	420 (21)	273	65.0
20	420 (21)	204	48.5	420 (21)	159	37.8
30	420 (21)	135	32.1	420 (21)	90	21.4
50	420 (21)	0	0	420 (21)	0	0
Total	3300 (165)	2100		3300 (165)	1883	

Examinations of differences between two strains (χ^2-test).
$0.001 > \alpha^*$ Highly significant.

Table 14. Emergence rates of the two strains on the media containing P.C.M.B.

Concentration $\times 10^{-5}$ Mol	Control strain			Cu-strain		
	No. of larvae	Emerged		No. of larvae	Emerged	
		No.	% to larvae		No.	% to larvae
0.75	280 (14)	256	91.4	280 (14)	227	81.0
1.0	500 (25)	454	90.8	500 (25)	400	80.0
1.5	280 (14)	228	81.4	280 (14)	214	76.4
20	280 (14)	30	10.7	280 (14)	16	5.7
25	280 (14)	20	7.1	280 (14)	14	5.0
30	280 (14)	0	0	280 (14)	0	0
Total	2080 (104)	988		1900 (95)	871	

Examinations of differences between two strains (χ^2-test).
$0.50 > \alpha > 0.30$ Non significant.

Table 15. Emergence rates of the two strains on the media containing As_2O_3.

Concentration $\times 10^{-3}$ Mol	Control strain					Cu-strain				
		Pupated		Emerged			Pupated		Emerged	
	No. of larvae	No.	% to larvae	No.	% to larvae	No. of larvae	No.	% to larvae	No.	% to larvae
0.03	280 (14)			246	87.8	280 (14)			270	96.4
0.05	280 (14)			270	96.4	280 (14)			250	89.2
0.1	280 (14)			256	91.4	280 (14)			270	96.4
0.3	280 (14)			254	90.7	280 (14)			256	91.4
0.5	280 (14)			248	88.5	280 (14)			236	84.2
1.0	280 (14)			268	95.7	280 (14)			240	85.7
3.0	280 (14)			252	90.0	280 (14)			240	85.7
5.0	280 (14)	213	76.0	192	68.5	280 (14)	202	72.1	173	61.7
6.0	440 (22)	268	60.9	132	30.0	440 (22)	254	57.7	78	17.7
7.0	280 (14)	56	20.0	7	2.5	280 (14)	4	1.4	2	0.7
10	280 (14)	0	0	0	0	280 (14)	0	0	0	0
Total	3240(162)			2125		3240(162)			2015	

Examinations of differences between two strains (χ^2-test).
$0.05 > \alpha > 0.02$* Significant.

e) *As_2O_3*. The results are shown in Table 15. Concentrations used were $0.03\text{-}10.0 \times 10^{-3}$ Mol. The Cu-strain showed lower emergence rate than the control, and this difference was statistically significant[2].

From the experimental results mentioned above, it can be said that the Cu-strain showed usually lower emergence rate than the control strain on the media containing inhibitors. These results clearly show that the Cu-strain differs from the control strain in some physiological characters concerning with metabolism.

Discussion

As described above, the offspring of the flies that had spent their larval stage on the 0.5 mM $CuSO_4$-containing medium showed not only the definite increase in resistance to copper but also various changes in morphological, physiological and ecological characters.

1. The reports on the change of size or weight of the body of the resistant strain of insects to various toxic agents have been given by GREIFF (1943), PIMENTEL *et al.* (1951), BOCHNIG (1956) and SUZUKI and TÔYAMA (1956). Accord-

2) It is very interesting that among the flies emerged from As_2O_3-containing medium, irrespective of the Cu- or the control strains, those with curled wings and of dark colour were very frequently found.

ing to the reports of PIMENTEL *et al.* (1951) and SUZUKI and TÔYAMA (1956), there is no difference in body size between DDT-resistant housefly and susceptible one. On the other hand, BOCHNIG (1956) showed that, in housefly, the body weight of the DDT-tolerant strain was heavier than that of the non-tolerant strain. In *Drosophila melanogaster*, GREIFF (1943) has said that the body weight of imago in $ZnSO_4$-selected line is smaller than that of the control line.

At the present investigation, the body length of males of the Cu-strain, when cultured in the control medium, became larger than the control strain. But when cultured in the 4 mM $CuSO_4$ containing medium, there was no difference between the two strains. Namely, the body size or weight is clearly the function of environmental conditions and the inconsistent results of above investigators may have their reason in this point.

2. One of the most obvious differences found between the Cu-strain and the control strain was that of longevity. The Cu-strain could live longer than the control when they were cultured on the copper-free medium or on the 2 mM $CuSO_4$-containing medium, but the results were reversed on the 4 mM $CuSO_4$-containing medium. The supposition that this phenomenon was caused by the different ability of copper absorption between the two strains has already been referred to.

In both strains, female flies could live longer than male ones, regardless of media. PEARL and PARKER (1924) described that female flies can live longer than male ones, regardless of food supply. While GREIFF (1940, 1943) has shown that in the case of wild-type, male flies can live longer than female ones, when starved. The results obtained by the present author agree very well with the data given by PEARL and PARKER (1924).

3. It has already been reported that strains resistant to insecticides have different behavior patterns from those of susceptible strains (THOMSON, 1948; BARBER and SCHMITT, 1949; KING and GRAHAM, 1949; BRUCE and DECKER, 1950; HADAWAY, 1950; MORRISON, 1950; BUSVINE, 1951; and CHADWICK, 1955). DDT-resistant housefly is less active than the susceptible one and the former shows such behavior as avoiding DDT-treated surface.

In the results obtained by the present author, photokinesis index was greater in male flies of the Cu-strain than the control strain, but in the case of female flies, there was no difference between the Cu-strain and the normal strain. It is very interesting to know the relations between the resistant mechanism and such change in the behavior, but it is yet to be shown in future.

4. As for copper metabolism of *Drosophila*, there is a series of POULSON and his co-worker's research (POULSON, 1950, 1955; POULSON *et al.*, 1951, 1952, 1955, 1958). According to POULSON (1950), the midgut epithelium of *Drosophila* can accumulate mineral elements selectively. As for copper accumulation in the body of *Drosophila*, he performed some experiments, using sodium diethyldithiocarbaminate and fluorescence microscopy, and found that the accumulated copper in the fly's body was involved in porphyrins to be detoxicated. Recently,

POULSON and BOWEN (1951) have studied the uptake, distribution and excretion of copper, using Cu^{64}, mixed in culture media on which the larvae of 4 species of *Drosophila* were cultured. It was found by them that in some cases copper uptake of larvae depends upon concentrations of copper in the culture medium, but in other cases, there is no relation between the amount of copper accumulated in body and the copper concentration in the culture medium. Two pathways are considered to be possible for the flies to uptake copper. One is that flies uptake copper directly from media in the form of ion, and another is that flies uptake copper *via* yeast cells to which copper has been bound (POULSON *et al.*, 1952).

The author has performed colorimetric analysis of copper contained in the body of the Cu-strain in order to ascertain those data mentioned above. Copper content was measured with DUBOSCQ's colorimeter, using *p*-dimethylaminobenzylidenerhodanin and dithizon as reagents, but no significant difference in copper content between the Cu-strain and the normal strain was found. The same result was also obtained, using a photoelectric spectrophotometer. Furthermore, the author could not detect copper in tissues, using the histochemical method after OKAMOTO and UTAMURA (1938, 1939) and WATERHOUSE (1945). After all the present author could not find any difference in copper content between the Cu-strain and the normal strain; nevertheless, the results of the present research may indicate that there is some difference in the copper uptake between the two strains.

5. It was found that the Cu-strain was different from the normal strain in many respects such as physiological activities and behavior patterns. The above mentioned changes that had taken place within the Cu-strain were all thought to have been very much affected by temperature. Contrary to the expectation, when the normal flies were made to acquire the copper resistance by training on the sublethal copper medium, the author could not find out any different modes of results among three different rearing temperatures, 20°, 25° and 28°C. In other words, whenever the flies were reared by 0.5 mM $CuSO_4$-containing medium, they gained the resistance to copper irrespective of rearing temperatures.

However, one point must be noted here. That is, the high rearing temperature (28°C) of parent flies has a bad influence on the degree of resistance to copper of F_1 flies, irrespective of the rearing temperatures of F_1 generation. The reason for this phenomenon is not clear at present.

6. Then, the author attempted to inquire more deeply into the physiological conditions of the resistant flies, and has studied some biochemical activities, using various kinds of enzyme inhibitors. The results indicate that the Cu-strain is more susceptible than the control strain to all the enzyme inhibitors tested, including those of oxidative phosphorylation and glycolysis together with SH-inhibitors. It is clear that the metabolic activities of the Cu-strain were fairly changed.

After all, it can be postulated from the results mentioned above that the total physiological activities of cells of the Cu-strain have been higher than those of the control strain.

Summary

When *Drosophila melanogaster* Oregon RS strain was cultured on the medium containing 0.5 mM of $CuSO_4$, which was sublethal dose to the fly, the larvae of the next generation became tolerant to copper. This copper resistant strain was denoted as the Cu-strain by the present author.

The main results described in this paper are as follows:

1. The body length of males of the Cu-strain, when cultured in the normal PEARL's medium, was found larger than the control strain. But when cultured in the test medium containing 4 mM $CuSO_4$, no difference was found between the two strains.

2. The Cu-strain could survive longer than the control strain on the normal medium and the medium containing a small amount of copper (2 mM); but it was shorter on the medium containing copper in higher concentration (4 mM).

3. The photokinesis was tested, in order to compare one of the ecological characters between the two strains. In the case of male flies, the Cu-strain showed significantly higher activities than the control strain, but in the case of female flies, there was no significant difference in the activities between the two strains.

4. Effects of rearing temperatures on the copper resistance were tested, changing from 25° to 28°C or to 20°C. In all these temperatures, the Cu-strain showed higher emergence rates than the control strain when they were cultured in the 4 mM $CuSO_4$-containing media. The emergence rates at 20° and 25°C were higher than that at 28°C.

5. To know the reason why the above mentioned differences occurred, the copper content in the body of the two strains was measured, using a photoelectric spectrophotometer, but no difference could be found between the two strains when they were grown up on the medium containing $CuSO_4$ in 4 mM.

6. In order to know the differences of biochemical activities between the two strains, the effects of enzyme inhibitors on emergence rates were tested. The Cu-strain was more sensitive than the control strain to all of the enzyme inhibitors tested.

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