ON THE RESISTIBILITY OF DROSOPHILA TO SODIUM CHLORIDE
I. STRAIN DIFFERENCE AND HERITABILITY
IN *D. MELANOGASTER*

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IT has been suggested that larvae of *Drosophila* generally do not require an addition of sodium chloride to the culture media (LOEB 1915; RUBINSTEIN, BURLAKOWA and LWOWA 1935, 1936; KALMUS 1943; KING 1953). Of course this does not mean that they are able to grow in the absence of the sodium salts in the media, because sodium salts are always present in yeasts used for the diet or are included as impurities even in synthetic media. It is generally believed on the other hand that the presence of sodium chloride in excess in the diet exerts an inhibitory effect upon the larval growth and causes eventual death. Recently, IMAIZUMI (unpublished) found that a strain of *D. melanogaster* long reared in our laboratory is highly resistant to the presence of sodium chloride in the culture medium. The author has performed a series of experiments on the resistibility and the requirement of *Drosophila* for sodium chloride from the genetical and physiological standpoint. The present paper deals with the results of the experiments, in which the strain differences of *D. melanogaster* in the resistibility and the heritability of this character are examined.

MATERIALS AND METHODS

Four strains of brown (*bw*) used were *bw*(Kyoto), *bw*(Pasadena), *bw^d* and *bw^{2b}*; of these *bw*(Kyoto) has been reared continuously in our laboratory for many years, and the others were obtained in September 1958 through the courtesy of the California Institute of Technology. Besides the *bw* strains, five wild stocks of Oregon and Canton, namely Ore-RS, Ore-RS(G.23), Ore-R, Ore-R(G.266) and Canton-S were used. Eight mutant strains, sepia (*se*), clot (*cl*), white (*w*), Henna-recessive (*Hn^{rs}*), vermilion (*v*), *bar-3* (Ives), vestigial (*vg*), and dumpy (*dp*) were also tested. Of these strains, isogenic lines Ore-RS(G.23) and Ore-R(G.266) have been inbred in our laboratory by brother-sister pair matings for 23 and 266 generations respectively before being used. The other strains have been maintained by massmating for many years.

Stocks of these strains have been raised continuously at $25 \pm 1^\circ\text{C}$ and under a favorable population density in bottles, (content 180 cc) which contained culture medium consisting of agar 2 g, sugar 6 g, koji (*Aspergillus oryzae* on moist rice) 14 g, KH_2PO_4 0.1 g, water 100 ml; several drops of a thick suspension of yeast added before use.

In order to conduct the present experiments, it was necessary to minimize the NaCl content in the medium. So, instead of the regular culture medium, a semi-synthetic medium according to the following formula was used as the standard: Merck's agar powder 2 g; sugar 10 g; Oriental's dry yeast 10 g; tartaric acid 0.4 g; *n*-butyl benzoate 1 ml (10% solution in 70% ethyl alcohol); and deionized water 100 ml. Butyl benzoate was used to inhibit the growth of molds; tartaric acid was added to the agar solution at temperatures below 70°C to avoid hydrolysis of agar; yeast was killed by boiling.

All experiments were performed at 25°C. To test for resistibility and to select resistant individuals, the NaCl medium was prepared by adding NaCl at a concentration of 1.0 M to the standard medium. At concentrations higher than 1.6 M the medium desiccated soon giving unfavorable effects on the life of flies, and at concentrations lower than 0.3 M strain differences in resistibility were not clearly manifested under the conditions of the present experiments.

Experiment 1: Strain differences in resistibility

Emergence rate test: Twelve mutant and five wild-type strains described above were used in this experiment. Eggs from populations of ten pairs of flies 4–7 days old were transferred to the culture medium within three hours after being laid. Groups of 50 eggs each were put on a glass plate, 2.5 cm × 7.5 cm, which had been covered with 6 g of either the NaCl medium or the standard medium. The plate was introduced into a glass tube 12 cm in length and 3 cm in diameter. A piece of moist filter paper was provided in each tube to give suitable moisture, and the tube was closed with cotton and kept at 25±1°C. Under the experimental conditions described above, the number of emerging adults was counted.

The results are listed in Table 1, in which resistibility is indicated as the proportion of emergence rate in the NaCl medium tests to that in the standard medium tests. As illustrated in the table, the resistibility to NaCl varies in different strains and there seems to exist no correlation between the resistibility and the phenotype of the strain. Although both strains of *bw*(Kyoto) and *bw*(Pasadena) carry the identical *bw* gene, there was a considerable difference between the two strains in resistibility. Similarly, differences in resistibility were found between *bw*(Kyoto) and *bw^p* and *bw^{zb}*. Furthermore, the resistibility varied among the five wild-type strains. It is remarkable that Ore-R(G.266) and Ore-RS(G.23) differ from the original strains, Ore-R and Ore-RS, in their resistibilities. Of the 17 strains tested, resistibility was very low in 16 strains: less than 0.1 in 11 strains, and between 0.2 and 0.3 in five strains. The *bw*(Kyoto) strain exhibited a striking contrast to the other strains showing a high resistibility, in which about one half of the eggs grew to imagoes in the NaCl medium, and the resistibility is found to be approximately 0.7.

Emergence rates of *bw*(Kyoto), *se* and Ore-RS on the culture media containing various concentrations of NaCl were examined. As shown in Figure 1, the maximum rate for *bw*(Kyoto) occurred at 0.5 M NaCl, while in Ore-RS and *se* the rate decreased as the concentration of NaCl increased. On the standard medium,

TABLE 1
Emergence rates, resistibilities and sex ratios in strains of D. melanogaster in the presence of NaCl in the culture medium at a concentration of 1.0 M

Strain	Date of test	No. of eggs tested In standard medium	Emergence rate (percent) In NaCl medium	Resistibility	Sex ratio In standard medium	Sex ratio In NaCl medium
<i>bw</i> (Kyoto)	Nov., 1957-Dec., 1958	1000	71.6±4.58	0.69±0.039	95.1	78.9
<i>bw</i> (Pasadena)	Nov., 1958-Jan., 1959	500	88.6±1.40	0.31±0.050	86.1	46.8
Ore-R	Dec., 1958-Oct., 1959	500	89.8±0.02	0.27±0.007	104.1	90.6
Ore-RS (G.23)	June, 1959	750	88.1±5.81	0.23±0.015	92.7	76.7
<i>bw^p</i>	May, 1959-Oct., 1959	500	90.0±2.80	0.21±0.010	90.7	75.5
<i>se</i>	June, 1957-Mar., 1958	750	80.3±2.41	0.18±0.010	98.0	92.9
Ore-R (G.266)	Dec., 1958	800	74.1±2.65	0.10±0.007	103.8	128.0
Canton-S	Dec., 1957-Nov., 1959	750	89.0±0.60	0.08±0.016	96.9	133.3
<i>w</i>	Nov., 1957-Jan., 1960	800	86.0±4.57	0.06±0.015	90.8	81.8
<i>dp</i>	Dec., 1959-Mar., 1960	750	89.4±3.65	0.05±0.008	94.0	68.4
<i>v</i>	May, 1959-Jan., 1960	1000	91.8±3.44	0.04±0.014	95.2	57.9
<i>bw^{ab}</i>	Nov., 1958-Dec., 1959	750	89.9±4.43	0.04±0.012	84.2	70.6
<i>bar-3</i>	Dec., 1959-Mar., 1960	750	90.6±1.40	0.04±0.007	95.9	56.3
Ore-RS	June, 1957-Dec., 1958	750	80.7±4.46	0.04±0.012	86.2	228.6
<i>cl</i>	Nov., 1957-May, 1958	750	75.1±1.61	0.03±0.009	90.2	35.7
<i>vg</i>	Dec., 1959-June, 1960	750	88.6±2.05	0.01±0.007	90.5	60.0
<i>Hn^{r3}</i>	Oct., 1957-May, 1958	750	90.9±2.17	0.01±0.006	79.5	100.0

FIGURE 1.—Graph showing emergence rates in three strains of *D. melanogaster* in the culture media containing various concentrations of NaCl.

Ore-RS and *se* strains, both being strongly susceptible to NaCl, showed the highest emergence rate, of about 80 percent. This rate was higher than that of the *bw* (Kyoto) strain by ten percent. At 0.5 M of NaCl, however, the rate dropped to 40 percent in the Ore-RS and *se* strains respectively, while the rate of the *bw* (Kyoto) strain rose to about 95 percent. At 1.3 M, no emerged adult was found in Ore-RS and few in *se*, but the rate of the *bw* (Kyoto) strain remained at one half of the maximum.

The sex ratio of emerged adults seems to be influenced by addition of NaCl to the medium, at least at a concentration of 1.0 M (Table 1). Of the 17 strains tested, 14 strains showed a considerable decrease in number of emerged males as compared with females. On the other hand the ratio of the male exceeded that of the female in the remaining four strains. In the latter strains, however, emerged adults were so few in number as to make the ratios unreliable. This problem will be considered in a separate communication.

The time required for the development is considerably prolonged in the NaCl medium. Under normal conditions at 25°C, the time lapse from hatching to emergence is about 190 hours as reported by BODENSTEIN (1950). With 1.0 M NaCl, it takes at least 220 hours to reach the adult stage. In some cases it took 293 hours, i.e. one and a half times as long as the time in the control.

On a culture medium containing a high concentration of NaBO₂ or NaF, emerging adults often show such phenocopies as eyeless, rough and antennaless, or they produce melanotic tumors (SANG and McDONALD 1954). In the present experiment using NaCl such abnormalities were not found. Adults that emerge on NaCl

medium are somewhat small as compared with flies grown on standard medium, but they are fertile and able to produce progenies quite normal in every respect.

Detailed observations on the effect of the NaCl medium to the developmental processes will be described in a subsequent paper.

Adult mortality test: Two mutant strains *bw* (Kyoto), *se* and a wild-type strain Ore-RS were used in this experiment. Flies to be tested were restricted to those of 5–7 days old. About 50 flies, males and females at a ratio of nearly 1:1, were introduced into a milk bottle containing 50 cc of the 1.0 M NaCl medium. The numbers of flies which had died were counted at intervals of 12 hours.

The results obtained were analyzed according to BLISS's method (BLISS 1934, 1937) which had been designed for judging the effect of insecticides. Probit values were obtained from the proportion of dead individuals at the end of each feeding period against the initial number of flies and were plotted in graphic form. Based on this graph, the time at 50 percent mortality (LT-50) and the standard deviation (S) were calculated.

As illustrated in Figure 2, these probit values fall nearly on a straight line. On

FIGURE 2.—Resistibilities in three strains of *D. melanogaster* to NaCl. Graph showing the probit values of mortalities of adults at each feeding period in the culture medium containing 1.0 M NaCl.

the NaCl medium, about one half of the flies in the Ore-RS strain died within 24 hours from initiation of the experiment, while in the *se* and *bw* (Kyoto) strains the mortalities within 24 hours were less than 25 percent and five percent respectively. Striking contrasts are afforded at the end of the 60th hour in that there were observed no survivors in Ore-RS and only four percent in *se* while 30 percent of the *bw* (Kyoto) tested were still alive. Even in the resistant *bw* (Kyoto)

strain, however, no survivor was found on the NaCl medium after 96 hours. In contrast, there hardly appears in any strain any fly mortally weakened during the course of this experiment in the standard medium. As presented in Table 2,

TABLE 2

Resistibilities and sex ratios of adults in three strains of D. melanogaster to NaCl. In the standard medium hardly any fly in any one of the strains died within 96 hours from initiation of the experiment

Strain	No. of flies tested ♂ ♀		LT-50 (hours)	S	Sex ratio of survivors at LT-50 ♂ : ♀
<i>bw</i> (Kyoto)	1374	1299	51.5	16.95	77.6:100
<i>se</i>	1440	1455	36.0	14.08	84.8:100
Ore-RS	1393	1317	26.5	10.75	61.8:100

LT-50 in the NaCl medium was 26.5 hours for Ore-RS, 36.0 hours for *se*, and 51.5 hours for *bw* (Kyoto). From these results, it is clear that flies of the *bw* (Kyoto) strain are most resistant to NaCl among the three strains, and that the *se* strain is more resistant than Ore-RS. These facts are well in accord with the resistibilities of these strains as manifested in the emergence rate test mentioned above.

The sex ratio of *D. melanogaster* is generally believed to be 90–95 males against 100 females in normal cultures. As far as these strains are concerned, the sex ratio of survivors at LT-50 is shifted considerably from that of the control by an addition of 1.0 M NaCl to the medium. The ratio of female to male is increased (Table 2). These results would suggest that in the adult stage the female is more resistant to NaCl than the male.

Experiment 2: Increase of resistibility to NaCl by selection

In order to determine whether the resistibility to NaCl is a hereditary character, the *bw* (Kyoto) strain which revealed the highest resistibility (0.69) in the preceding experiment, was reared through successive generations on 1.0 M NaCl medium. At the initiation of the test, 500 eggs laid by populations of ten pairs of flies randomly chosen were put on the 1.0 M NaCl medium and left to grow to imagoes under the same culture conditions as used in experiment 1. The emerged adults were counted and the rate calculated. Under these conditions much fewer eggs were laid than under normal culture conditions. To obtain a sufficient number of eggs for continuation of the selection experiment, the survivors were transferred to the standard medium at the end of the 24th hour after emergence. In successive generations, eggs laid on this standard medium were transferred to 1.0 M NaCl medium and reared as in the first generation. Thus resistant individuals were selected from generation to generation using the NaCl medium. The emergence rate on the standard medium was tested in some generations to permit comparisons with that on the NaCl medium. While continuing the selection along the line of generations with the NaCl medium (line A in Figure 3), two lines

The emergence rates in the three lines are illustrated in Figure 3. In the initial generation, the emergence rate was 50.2 percent in the NaCl medium and 68.0

percent in the standard medium. In the generations of line A, the rate in NaCl medium rose gradually to 91.6 percent in the ninth generation, i.e. higher than in the standard medium. It remained almost unchanged in subsequent generations, at least up to the 25th generation. These facts would indicate that selection pressure is very effective in raising the resistibility to the maximum attained after nine generations. The rate in the standard medium also increased but more slowly through the generations in this line. In the 25th generation the rate was 78.0 percent, being higher than that in the initial generation by ten percent. The emergence rates both in the NaCl and in the standard medium ascended with the succession of generations. The experiment is being continued in order to examine whether the resistibility is remaining at its high level in subsequent generations.

Line B, started from the eggs produced by flies of the third generation in line A, was raised on the standard medium. In line A the emergence rate in the NaCl medium had at that generation been raised to 68.3 percent, while no remarkable change had occurred yet with regard to the rate in the standard medium. The resistibility to NaCl in line B decreased from generation to generation and returned to the level of the initial strain within eight generations. Then it remained unchanged through subsequent generations at least up to the 12th generation. The emergence rate in the standard medium, on the other hand, was maintained practically at the initial level through these generations. The experiment using line B was discontinued at the 12th generation.

Line C was derived from the ninth generation of line A. It exhibited a striking contrast to line B with regard to the stability of the emergence rates in NaCl and in standard medium. In the ninth generation of line A, the emergence rates in the NaCl medium and in the standard medium were as high as the maximum rates of the line. Through 13 successive generations of line C, these rates persisted with only slight fluctuations. It should be noted that the high rate was kept nearly unchanged at least through 13 generations, notwithstanding the fact that flies in this line had not been exposed to the NaCl medium but had been reared exclusively on standard medium. From the results of the selection experiment, it is concluded that lines C and A were stabilized with regard to the resistibility to NaCl. Another noteworthy fact is that the emergence rate in the standard medium remained always lower than that in the NaCl medium by about ten percent.

DISCUSSION

The role of NaCl in the diet of *Drosophila* is not well understood. LOEB (1908) was able to raise larvae of *D. melanogaster* in the absence of NaCl except as impurities in the chemicals used. This experiment of LOEB was confirmed by RUBINSTEIN, LWOVA and BURLAKOWA (1935, 1936). KALMUS (1943) also showed that *D. melanogaster* could be reared without addition of NaCl to the diet. SANG's synthetic culture medium (1956) contained only a trace of NaCl. These data would suggest that *D. melanogaster* hardly require, if at all, the presence of NaCl in the diet. A large amount of NaCl is definitely deleterious.

Thus, in the recent work by KING (1953), who studied the effects of alkali metal ions on the development of *D. melanogaster*, a highly inbred Canton-S strain did not grow in a culture medium containing 0.5 M NaCl. This result seems to be in accord with the general belief that an excess of sodium chloride in the diet causes inhibition of growth and eventual death. An exception to this is the recent result of WADDINGTON (1959), in which Ore-K showed emergence rate of about 50 percent in a culture medium containing six percent NaCl (about 1 M), the rate being as high as the level of *bw* (Kyoto) strain tested by the writer.

Sodium salts are generally known to affect osmotic regulation of living matters. Data given by WIGGLESWORTH (1938) show the average osmotic pressure of insect haemolymph to be approximately equivalent to 0.18 M NaCl. In larvae of Diptera, it is equivalent to 0.23 M NaCl at a maximum and 0.10 M at a minimum. Taking these data into account, the concentration of NaCl in the culture medium of the present work would be several times as high as that isosmotic with *Drosophila* haemolymph. The NaCl medium may exert some hyperosmotic effects upon the internal organs when taken into the digestive tract, and afford a desiccating environment to *Drosophila* through its life span. Recently, CROGHAN and LOCKWOOD (1960) reported that the haemolymph of larvae of Ore-K developing in a medium containing seven percent NaCl was markedly hypotonic to the medium and that there was only a small rise of the NaCl concentration in the haemolymph. WADDINGTON (1959) reported that the anal papilla of the fly resistant to such high concentration of NaCl is larger in size than that of the susceptible one. The function of this organ is not known exactly but is supposed to be concerned with osmotic regulation. In the present material, however, no morphological changes were noticed.

In the present work the resistibility to NaCl attained by the strain selected through continual application of the NaCl medium is higher than among any other known strains. The selection experiment proves the character to be hereditary. The attainment of this resistibility would be deduced to have been the consequence of selection pressure exerted through successive generations in the presence of NaCl at a high concentration in the culture medium rather than due to the result of a built-up tolerance in individuals following successive NaCl intake. Therefore, the name *srb* (sodium chloride resistant brown) is given to the selected strain which is stable with regard to the high resistibility to NaCl. The raised resistibility of the strain is maintained at nearly constant level through many subsequent generations, reared on normal culture medium which does not contain a detectable amount of NaCl. In addition to the increase in resistibility to NaCl, emergence rate in the standard medium also rises slowly but steadily in line A. One may assume that some selection pressure toward higher emergence rate is active in the standard medium.

It is very interesting that the presence of high concentrations of NaCl in the medium is found to furnish a more favorable environment to both the *bw* (Kyoto) and *srb* strains than does the medium without NaCl. From this fact alone, however, it cannot be concluded that these strains have a requirement for NaCl. To

solve the problem whether the high resistibility to NaCl in these strains is related to such a requirement further work is necessary.

To elucidate the nature of hereditary resistibility of *Drosophila* to NaCl additional experiments are under way.

SUMMARY

(1) The present paper deals with various phenomena revealed by many strains of *Drosophila melanogaster* under the presence of NaCl added to the culture medium.

(2) The resistibility to NaCl varies in different strains, and there is no correlation between resistance and the mutant markers or lack of markers (Visibles) in the various strains.

(3) Only about one to 28 percent of the eggs from most strains grew to imagoes in a medium containing 1.0 M NaCl. The *bw*(Kyoto) strain exhibited a striking contrast to other strains by its high resistibility, in which about one half of the eggs grew to imagoes in the medium containing 1.0 M NaCl. The most remarkable difference is seen in the adult mortality test: within 60 hours on the medium, all adults of the *se* and Ore-RS strains had died, while 30 percent of *bw*(Kyoto) survived.

(4) The high resistibility is not a general character of different *bw* strains.

(5) The sex ratio of emerged adults in both resistant and susceptible strains seems to be influenced, at least at a concentration of 1.0 M NaCl.

(6) The resistibility to NaCl is a hereditary character. By raising the *bw* (Kyoto) strain through successive generations on 1.0 M NaCl medium, it was possible to raise the emergence rate gradually to 91.6 percent at the ninth generation after which it remained almost unchanged, at least up to the 25th generation. The increased resistibility of the strain was maintained through many subsequent generations at nearly the same level even after culture on normal NaCl "free" medium.

Throughout the text "emergence rate" does not mean number emerging per unit time but a simple percent emergence.

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