THE EFFECT OF COPPER AND IRON SALTS AND TRYPTOPHAN ON HEAD ABNORMALITIES AND MELANOTIC TUMORS IN DIFFERENT STOCKS OF *DROSOPHILA MELANOGASTER*¹

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COPPER and iron compounds and tryptophan were added to the food medium and injected into larval and adult fruit flies, *Drosophila melanogaster*, in a series of experiments designed to test the effect of different chemicals on specific gene action. Salts of heavy metals are known to alter phenotypes (GOLDSCHMIDT and PITERNICK 1957; RAPOPORT 1939; SANG and McDONALD 1954), and under certain conditions they act as mutagenic agents (HERSKOWITZ 1951; LAW 1938). Tryptophan was shown in a previous study (SIMMONS and GARDNER 1958) to be related to the proportion of flies with head abnormalities. Effects of copper and iron salts and tryptophan on the expression of head abnormalities, melanotic tumors, and general mortality of flies are reported.

MATERIALS AND METHODS

Two melanotic tumor stocks, *tu*^{50j} and *tu*¹⁵⁰, two stocks with structural abnormalities in the head region identified in previous reports as tumorous head stocks, *tu-h* and *w tu-h* (tumorous head with white eyes), and two wild inbred stocks, Canton and Stephenville, were used in the experiments.

The *tu*^{50j} stock was obtained in the summer of 1958 from W. J. BURDETTE at the University of Utah and maintained through inbreeding and selection in our laboratory. When the flies were kept at room temperature, between five and ten percent of the adults had melanotic tumors. The *tu*¹⁵⁰ stocks had been inbred and selected for melanotic tumors over a period of ten years at Utah State University. While the experiments reported here were in progress, some seven to 14 percent of the adult flies had melanotic tumors which occurred most commonly in the abdomen but occasionally in the thorax. The tumors were colorless in early larvae and became pigmented as the larvae grew older. In the adults the tumors appeared as encapsulated masses of black pigment.

The *tu-h* phenotype and genetic mechanism have been described (GARDNER 1948; NEWBY 1949; GARDNER and WOOLF 1949; GARDNER and STOTT 1951; GARDNER and RATTY 1952). Some 86 percent of abnormal flies occurred in cultures maintained at 25°C during the period in which these experiments were in

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progress. In the *w tu-h* stock, the proportion of flies with head abnormalities was somewhat lower, ranging from 60 to 75 percent. Both wild stocks used in these experiments have been inbred for many years in our laboratory and are known to be free from major modifiers or alleles of the tumorous head genes. (A different Stephenville stock was found to give different results and was postulated (GARDNER and WOOLF 1949) to carry an allele of one of the tumorous head genes.)

The standard medium (corn meal, agar, molasses, with live yeast added) was used throughout the study. Aliquots of the experimental additives were introduced to measured quantities of hot medium and distributed in the medium by thorough stirring. Each chemical being tested was added to the live yeast suspension as well as to the basic medium. Pilot experiments were conducted to determine tolerances of the different *Drosophila* stocks to various concentrations of additives.

By making use of laying cages (SIMMONS and GARDNER 1958) through which parent flies could be transferred to a series of cultures for oviposition, the same individuals were used to produce eggs for an entire experiment. First instar larvae were collected by flooding (with water) the medium upon which the eggs were previously deposited. Ten larvae were inoculated into each of several six-inch test tubes containing slants of the medium appropriately prepared with additives for treatment. Tubes were maintained at $25^{\circ} \pm 1^{\circ}\text{C}$ with a relative humidity of 45 ± 5 percent. Larvae reaching the pupal stage and pupae becoming adults were recorded, and percentages for treatments were calculated. Proportions of adults expressing melanotic tumors or head abnormalities were determined. Concentration levels of copper sulfate (CuSO_4) were 0, 160, 320, 640, 960, 1280, and 1600 $\mu\text{g}/\text{ml}$, and those of iron ammonium sulfate ($\text{Fe}(\text{NH}_4)(\text{SO}_4)_2$) were 0, 660, 1320, 1980, 2640, and 3300 $\mu\text{g}/\text{ml}$.

To check the results of the feeding experiments, chemicals were introduced directly into larvae and adults by injection. Some 25 larvae or 20 adult flies were anesthetized and placed at one time on a 1×3 inch microslide covered with scotch tape (sticky side up) for each operation. Slides were placed on the stage of a dissecting microscope, and chemicals were introduced with a microsyringe calibrated to deliver the same quantity in each injection. Operations were observed under a magnification of $150\times$. After injection the specimens were quickly removed from the slide, washed with Ringer's solution, allowed to recover from etherization in a moist chamber, and transferred to sterile dishes containing the standard medium. Mortality of larvae and adults was calculated and compared with that of the controls. Proportions of adults with abnormal expressions were tabulated.

EXPERIMENTAL RESULTS

Preliminary experiments with copper sulfate showed that concentrations of 160 $\mu\text{g}/\text{ml}$ and 320 $\mu\text{g}/\text{ml}$ were slightly toxic, 640 $\mu\text{g}/\text{ml}$ was highly toxic, and no flies survived the 960 $\mu\text{g}/\text{ml}$ level. A differential mortality occurred among the treated stocks; tumorous head flies being most susceptible. In these stocks the

controls with no additive showed a 60 to 70 percent mortality. At levels of 160 $\mu\text{g/ml}$ and 320 $\mu\text{g/ml}$, 70 to 80 percent did not survive, and all flies died at the 640 $\mu\text{g/ml}$ level. The melanotic tumor strain, *tu¹⁵⁰*, was intermediate, and *tu^{50j}* was quite similar to the wild Stephenville stock. Canton showed the lowest mortality at all treatment levels. Percentages of larvae, based on samples of 200, which failed to reach the adult stage are summarized in Figure 1. Results from the two *tu-h* stocks were not statistically different from each other but different from those of all others, whereas the two melanotic stocks were different from each other. Mortality occurred in both larval and pupal stages. In all cultures to which copper sulfate was added the developmental period was prolonged. A

FIGURE 1.—Percentages of larvae which failed to reach adult stage when different levels of copper sulfate were added to food medium.

minimum of 14 days was required for the flies raised in a culture with excess copper to reach the adult stage compared with ten days for the controls. There was no statistically significant change in the expression of melanotic tumors or tumorous head expressions which could be attributed to the copper treatment.

A phenotypic change was observed when copper sulfate was added to the medium of the melanotic tumor strain, *tu*¹⁵⁰. In the culture to which 320 µg/ml of copper sulfate was added, 13 females and 12 males, from a total of 148 progeny, expressed eye abnormalities similar to some expressions of the mutant, eyeless. Among the 182 progeny from the culture with 160 µg/ml, one female and five males were eyeless. No such expression was observed among several thousand untreated flies of this stock. Flies from no other stock have responded in this way to copper treatment.

Upon recognition of the unexpected eye abnormalities in the copper treated *tu*¹⁵⁰ stock, eight pair matings were set up with *tu*¹⁵⁰ parents. Six were treated with 320 µg/ml, and two were maintained as controls in the same environment but without copper sulfate added. Following eclosion, adult flies with the eyeless phenotype were found in two of the six experimental cultures, whereas no abnormal flies were found in the control cultures.

The induced eyeless flies were inbred in cultures without copper sulfate added to the medium. Within four generations 98 ± 2 percent of the flies were eyeless. One of the new eyeless lines, symbolized tentatively *ey'*, was maintained and has now been selected and inbred for two years. Nearly 100 percent of the flies in this stock express the eyeless trait. Some expressions in the new *ey'* stock are illustrated in Figure 2. When *ey'* flies were outcrossed to wild type, Stephenville, soon after the stock was established, eight from a total of 242 F₁ progeny had slight eye abnormalities. In the F₂, 54 of the total 269, or 20 percent, had eye abnormalities, many of which were more severe than those observed in the F₁. Studies designed to investigate the genetic mechanism and the location of the gene or genes involved are in progress.

When 0.001 M copper sulfate was injected into the bodies of adult male and female flies, and the injected flies were mated, four of 501 progeny had eye abnormalities. When the injection-induced eyeless flies were mated among themselves, the trait was established and maintained in nearly all of the individuals generation after generation. The phenotype of the injection induced flies was similar to that induced in the feeding experiments. None of the 456 progeny of the controls injected with Ringer's solution showed the eyeless trait.

Relative viability of the six experimental *Drosophila* stocks in response to equally spaced concentration levels of iron ammonium sulfate ($\text{Fe}(\text{NH}_4)(\text{SO}_4)_2$) is illustrated graphically in Figure 3. The numerical results are summarized in Table 1. Percentages of eclosion are based on samples of 200 larvae. Analysis of variance showed a significant difference between the wild stocks (Stephenville and Canton) compared with the other four stocks tested. The tumorous head stocks were different from the melanotic tumor stocks in mortality response to the iron treatment. No difference was detected between the two wild stocks nor between the two tumorous head stocks, but a significant difference was observed

FIGURE 2.—Expressions of eyeless phenotype in inbred lines resulting from copper treatment: 1. Side view of normal head. 2. Eye indented on anterior margin by bridge of bristle-bearing tissue. 3. Moderate symmetrical decrease in eye size with inward depression in contour of eye. 4,5. Eyes with odd-shaped segments split off. 6,7,9. Small segments of eye with facets bulging out as though on a stalk. 8. Dorsal view of head showing extra antennae. 10. Eyes segmented into isolated sections. 11. Both eyes completely missing (eyeless) and one antenna missing. 12. One eye missing, one palpus duplicated.

between the two melanotic tumor strains. Variations between treatments were also significant. Total mortality of all groups increased as the iron compound was increased. The curve of response exhibited a linear trend within the range of the treatment levels.

The iron compound, like copper sulfate, prolonged the developmental period. Eclosion began in 15 days after the eggs were laid compared with 14 days for

FIGURE 3.—Percentage of larvae which failed to reach adult stage when different levels of iron ammonium sulfate were added to food medium.

TABLE 1
Percentages of flies to reach adult stage grouped by treatment with iron ammonium sulfate

Stock	3300 µg/ml	2640 µg/ml	Treatment 1980 µg/ml	1320 µg/ml	660 µg/ml	Control
Canton	52	54	65	69	74	64
Stephenville	57	62	68	67	64	62
tu-h	18	28	22	31	27	39
w tu-h	7	15	19	34	36	34
tu ¹⁵⁰	36	51	57	53	45	49
tu ^{50j}	19	23	24	39	50	61
Treatment means	31.5	38.8	42.5	48.8	49.3	51.5

copper treatment and ten for the controls. A significant decrease in tumorous head expression and an increase in melanotic tumors were associated with the increased concentration of iron in the medium. This observation was verified in larger experiments, and analyses were made to distinguish the variation caused by the differential toxic effect on the flies and the direct effect of the treatment. A high correlation of 0.76 ($r(0.05) = 0.576$) was found between the decrease

of tumorous head expression and mortality. The flies expressing the tumorous head trait in the *tu-h* cultures were apparently more susceptible to the iron compound than those which were wild type in appearance.

This same question arose in a previous study (SIMMONS and GARDNER 1958) in which tryptophan was added to the medium of tumorous head flies, and the proportion of flies expressing the trait was decreased. Results of recent experiments (Table 2) have shown that the decrease is due to a differential toxicity response between flies expressing the trait and those appearing as wild type, rather than a direct effect on the genetic-physiological mechanism of the developing flies. The difference in the percentage of abnormal flies (27 percent) was not significantly different from the differential percentage (24 percent) in survival.

The culture medium in which the tryptophan experiments were conducted was examined under magnification to locate the dead organisms and to determine the developmental stage when death occurred. The dead eggs, larvae, and pupae which could be located were classified as follows:

Developmental stage	Number located	Percent of total
Egg	48	17
First instar larva	30	11
Second instar larva	54	19
Third instar larva	106	38
Pupa	40	14

It was observed that many third instar larvae seemed unable to pupate. The larvae grew to a very large size, and dark melanotic bodies appeared throughout their abdomens.

Melanotic tumor incidence was progressively increased with increased levels of the iron compound. A correlation of 0.67 ($r(0.05) = 0.576$) was found between mortality and melanotic tumor incidence. Whether the wild appearing flies were more susceptible to iron treatment, or the additive influenced melanotic tumor production, has not been determined. In the previous study (SIMMONS and GARDNER 1958) 0.6 percent tryptophan added to the medium was shown to induce melanotic bodies in about eight percent of the flies in one wild stock (Stephenville). Other studies (GLASS and PLAINE 1955; KANEHISA 1955; MITTLER 1952; PLAINE and GLASS 1955) have shown that tryptophan increases the expression

TABLE 2
Comparison of the expression and survival rate of tumorous head with and without 0.5 percent L-tryptophan added

Level	No. eggs	Female		Male		Total		Percent abn.	Percent survival*
		Abn.	+	Abn.	+	Abn.	+		
Control	1,000	260	40	300	80	560	120	82	68
0.5 percent L-tryptophan	1,000	120	113	120	87	240	200	55	44
Difference								27	24

* Number eclosing adults/total number of eggs.

of melanotic tumors in tumor bearing stocks. Transition metals have been related to the metabolism of tryptophan (KIKKAWA, OGITA, and FUJITO 1955), a known precursor of melanotic tumor pigment. This indicates that the observed variation in incidence of melanotic tumors results from a difference in pigmentation, which makes tumors recognizable rather than an actual induction of tumors by the iron.

DISCUSSION

The most intriguing problem arising from this study is the relation between treatment with copper sulfate and the new eyeless phenotype induced in the *tu¹⁵⁰* stock. Three hypotheses are being considered and tested: (1) an allele for eyeless was segregating in the *tu¹⁵⁰* stock but was not expressed because modifiers had accumulated favoring the wild type, normal eye. Copper sulfate in the medium interacted in such a way that those flies with the genetic background most favorable to the eyeless expression showed a slight but visible abnormality. When these flies were selected and mated among themselves, modifiers favorable to the expression were accumulated with the eyeless allele, bringing about the expression independently, i.e., not requiring copper sulfate. (2) Genetic assimilation similar to that described by WADDINGTON (1957). (3) Copper sulfate influenced some controlling element and brought about a directed change at the eyeless locus.

The penetrance and expressivity of the mutant eyeless are known to decrease when eyeless flies are mass cultured in the laboratory over long periods of time (SPOFFORD 1956). Apparently modifiers acting against the abnormality are selected, and flies carrying *ey* alleles eventually become almost, if not completely, indistinguishable from wild type. No such allele is known to have been introduced into the *tu¹⁵⁰* stock in our laboratory, and during the period of several years while this stock has been carried in our laboratory the eyeless phenotype has never been observed in untreated flies.

WADDINGTON (1957) treated wild type *Drosophila* pupae with heat and obtained aberrant flies, some of which lacked posterior crossveins in their wings. Crossveinless flies were selected and heat treatment was continued generation after generation. After about 14 generations the heat treatment was discontinued, but some of the flies continued to express the crossveinless characteristic. Stocks were built up from these flies which eventually had a high percentage of crossveinless individuals. Similar experiments were conducted with ether treatment. A phenotype resembling the mutant *bithorax* was selected, and after 28 generations it became established, i.e., assimilated, even though the ether treatment was discontinued. The eyeless phenotype in the present study became visibly distinguishable in the first generation of copper sulfate treatment. Only four generations were required to build up a stock with nearly 100 percent eyeless individuals.

Since no other stocks have responded in this way to copper treatment, there must be a particular genetic background already established in *tu¹⁵⁰* or an instability which makes possible a genetic change. Copper sulfate may stimulate some controlling element to bring about a directed chemical change at the eyeless gene locus. RAPOPORT (1939) showed this locus to be relatively unstable, but there is

no precedent for a chemically directed response even in a particular stock.

Many chemical agents are now known to induce mutations, but there is no record of a particular chemical inducing a specific mutation and leaving the other genetic material unaffected. Mutations have been found to occur more or less at random, i.e., one locus is as likely to mutate as another. In the present study the eyeless phenotype first occurred following the introduction of copper sulfate into the food medium. When the chemical was injected directly into *tu*¹⁵⁰ parent flies a few progeny were eyeless. In each case eyeless flies were selected, and nearly 100 percent expressed the trait within four generations of inbreeding.

Copper sulfate has been identified as a mutagenic agent (HERSKOWITZ 1951). LAW (1938) introduced this chemical into larvae of *Drosophila melanogaster* by injection and also into fertilized eggs by diffusion from the medium, and found it to induce lethal mutations at a rate greater than one percent. The induced lethals, however, were not genetically identical, i.e., they did not involve the same gene locus. KURODA and TAMURA (1956) have shown that copper ions added to a synthetic medium culturing abdominal melanotic tissue will influence tumor proliferation. KANEHISA and POULSON (1958) have described a nucleo-cytoplasmic interaction in the control of copper accumulation in *Drosophila melanogaster*. POULSON, BROWN, HILSE, and RUBINSON (1952) have studied copper metabolism in four species of *Drosophila*. RAPOPORT (1939) found a number of chemicals, mostly heavy metals, that produce phenocopies (nonhereditary copies of mutant phenotypes) when added to the food medium of *Drosophila*. The chemical agents were highly specific in the type of phenotype produced. SANG and McDONALD (1954) were able to direct to some extent the production of specific phenocopies in *Drosophila* when they induced eyeless expression by treatment with sodium borate. GOLDSCHMIDT and PITERNICK (1957) directed the production of phenocopies with different boron salts. The present expression of eyeless must be associated with a permanent change in the genetic material or a recombination effect rather than a phenocopy.

SUMMARY

Copper sulfate and iron ammonium sulfate at different concentrations were added to the food medium of six *Drosophila melanogaster* stocks (*tu*^{50j}, *tu*¹⁵⁰, *tu-h*, *w tu-h*, Stephenville, and Canton). Copper sulfate treatment produced no significant change in the expression of melanotic tumors or tumorous head phenotype. Increased copper sulfate levels to 960 µg/ml were accompanied by a linear increase in mortality in both larval and pupal stages, increased duration of larval development, and differences in mortality among tested stocks. A phenotype resembling that of the mutant, eyeless, was induced in flies from the *tu*¹⁵⁰ stock by copper sulfate treatment. Flies showing the new trait were selected and inbred. A new stock in which nearly 100 percent of the flies showed the new eyeless phenotype was developed and maintained. Iron ammonium sulfate treatment at levels to 3300 µg/ml increased the incidence of melanotic tumors in the *tu*^{50j} and *tu*¹⁵⁰ stocks but decreased the proportion of tumorous head flies in the *tu-h* and *w tu-h*. Increased levels of the iron compound were accompanied by a linear increase in mortality, increased duration of larval development in the melanotic

tumor and tumorous head stocks, and differences in mortality among the tested stocks. The decrease in expression of tumorous head abnormalities when tryptophan was added to the food medium (SIMMONS and GARDNER 1958) was attributed to differential toxicity response rather than the genetic-physiological mechanism of the developing flies.

LITERATURE CITED

- GARDNER, E. J., 1948 Head tumors in *Drosophila melanogaster*. Utah Acad. Sci. Proc. **25**: 164.
- GARDNER, E. J., and F. J. RATTY, 1952 Penetrance and expressivity of tumorous head in *Drosophila melanogaster* and relative viability of flies carrying tumorous head genes. Genetics **37**: 49-61.
- GARDNER, E. J., and G. H. STOTT, 1951 Genes producing a maternal effect and modifiers of tumorous head in "wild" and tumor-bearing stocks of *Drosophila melanogaster*. Genetics **36**: 72-83.
- GARDNER, E. J., and C. M. WOOLF, 1949 Maternal effect involved in the inheritance of abnormal growths in the head region of *Drosophila melanogaster*. Genetics **34**: 573-585.
- GLASS, H. B., and H. L. PLAINE, 1955 Genetic control of tryptophan metabolism in *Drosophila*. pp. 940-944. *Amino Acid Metabolism*. Edited by W. D. McELROY and H. B. GLASS. Johns Hopkins Press, Baltimore, Maryland.
- GOLDSCHMIDT, R. B., and L. PITERNICK, 1957 The genetic background of chemically induced phenocopies in *Drosophila*. J. Exptl. Zool. **136**: 201-228.
- HERSKOWITZ, I. H., 1951 A list of chemical substances studied for effects on *Drosophila* with a bibliography. Am. Naturalist **85**: 181-199.
- KANEHISA, T., 1955 A relation between the incidence of melanotic tumors and the tryptophan metabolism in *Drosophila melanogaster*. Japan J. Genet. **30**: 170-171.
- KANEHISA, T., and D. F. POULSON, 1958 Nucleo-cytoplasmic interaction in the control of metal accumulation in two strains of *Drosophila melanogaster* and their heterozygotes. Proc. 10th Intern. Congr. Genet. **2**: 140-141.
- KIKKAWA, H., Z. OGITA, and S. FUJITO, 1955 Nature of pigments derived from tyrosine and tryptophan in animals. Science **121**: 43-47.
- KURODA, Y., and S. TAMURA, 1956 The tissue culture of tumors in *Drosophila melanogaster*. III. The effects of Cu-ion upon the melanotic growth of tumors. Zool. Mag. (Tokyo) **65**: 11-15.
- LAW, L. W., 1938 The effects of chemicals on the lethal mutation rate in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S. **24**: 546-550.
- MITTLER, S., 1952 Influence of amino acids upon incidence of tumors in the *tu^{50j}* stock of *Drosophila melanogaster*. Science **116**: 657-659.
- NEWBY, W. W., 1949 Abnormal growths on the head of *Drosophila melanogaster*. J. Morphol. **85**: 177-196.
- PLAINE, H. L., and H. B. GLASS, 1955 Influence of tryptophan and related compounds upon the action of a specific gene and the induction of melanotic tumors in *Drosophila melanogaster*. J. Genet. **53**: 224-261.
- POULSON, D. F., V. T. BROWN, R. M. HILSE, and A. C. RUBINSON, 1952 The copper metabolism of *Drosophila*. Proc. Natl. Acad. Sci. U.S. **38**: 912-921.
- RAPOPORT, J. A., 1939 Specific morphoses in *Drosophila* induced by chemical compounds. Bull. Biol. Med. Exp. S.S.S.R., Moscow **7**: 415-417.
- SANG, J. H., and J. M. McDONALD, 1954 Production of phenocopies in *Drosophila* using salts particularly sodium metaborate. J. Genet. **52**: 392-412.
- SIMMONS, J. R., and E. J. GARDNER, 1958 The effect of tryptophan on the penetrance of tumorous head in crosses among selected stocks of *Drosophila melanogaster*. Genetics **43**: 164-171.
- SPOFFORD, J. B., 1956 The relation between expressivity and selection against eyeless in *Drosophila melanogaster*. Genetics **41**: 938-959.
- WADDINGTON, C. H., 1957 *The Strategy of the Genes*. George Allen and Unwin, London, England.