ABSTRACT

Clifton M. Carey. DNA-INDUCED TRANSFORMATION IN A MULTICELLULAR ORGANISM; DROSOPHILA MELANOGASTER. (Under the direction of Dr. Patricia Daugherty) Department of Biology, July 1980.

Fourteen transformed individuals were observed when homozygous sepia Drosophila melanogaster eggs were exposed to deoxyribonucleic acid (DNA) extracted from the wild type D. melanogaster. Eggs were exposed to a DNA-enriched medium and allowed to develop into adults in this medium. Inheritance of the acquired trait was studied, but not demonstrated. Control experiments indicated that the genetic code of the wild type DNA was responsible for the transformation.

The DNA used in these experiments was extracted from adult flies following a procedure developed by this author. The DNA extracted was of high purity, large molecular weight, and high transforming activity.

DNA-INDUCED TRANSFORMATION IN A MULTICELLULAR ORGANISM; *DROSOPHILA MELANOGASTER*

A Thesis

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Clifton M. Carey

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DNA-INDUCED TRANSFORMATION IN A MULTICELLULAR ORGANSM; *DROSOPHILA MELANOGASTER*

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INTRODUCTION

"Transformation is the name given to genetic recombination brought about by the introduction of purified chromosomes (DNA)" (Watson, 1970). The process of transformation was first discovered in 1928 by Griffith when he observed the transformation of rough to smooth pneumococcal types when exposed to the DNA of the smooth pneumococcal variety.

It was not until 1944 that Avery, et al., discovered the chemical nature of the substance which induces transformation in pneumococcal types. Since that time, many researchers have been able to show that purified deoxyribonucleic acid (DNA) can induce transformations in unicellular organisms: (McCarty, 1946; Zinder and Lederburg, 1952; Taylor, 1958; Ravin, 1961; Ottolenghi and Hotchkiss, 1962); and many more.

The mechanism for extracellular DNA to be introduced into the intracellular gene pool is still under investigation and is subject to discussion between researchers. Fox and Hotchkiss (1957) have found DNA receptor sites on the cell wall of Staphylococcus. The functioning of these sites requires a mixture of amino acids and glucose; it is independent of cellular growth, and is completely inhibited by Chloromycetin, a specific inhibitor of protein synthesis in Staphylococci. Strauss (1970) has reported that a rate limiting, energy dependent step is required to transport DNA into the cell. Tichy and Landman (1969) found that cells without mesosomes cannot transform. Harris and Barr (1971) reported that extracellular DNA was incorporated

in single strands of approximately 2,000 nucleotides. Once transported into the cell, several things could happen to these strands: (1) the donor strands "re-anneal," (2) the host cell degrades (metabolizes) the strands, (3) the single strand of the donor anneals with an opening in the host DNA, or (4) the donor strand is partially metabolized, and the remaining portion then anneals with an opening in the host DNA. Harris and Barr also showed that the donor DNA is rapidly membrane bound, presumably on a mesosome, where it can remain for long periods of time. Arwert and Venema (1973) reported that integration of donor DNA into the recipient genome occurred within 20 minutes at 30°C Celsius. They also reported that approximately 75% of the DNA which was initially absorbed was ultimately released back into the medium. In this case, the molecular weight of the donor DNA was $5\text{-}8 \times 10^7$ and when released it was 9×10^6. Seto and Tomaz (1974) have found that divalent cations (Ca^{+2}, Mg^{+2}, etc.) are required for the DNA to be transported into the cell.

Fox and Yoon (1966) were the first to report transformation in a multicellular (eukaryotic) organism. They reported that 10 out of 11 loci which they studied exhibit transformation in Drosophila melanogaster. Nawa and Yamada (1968) reported successful transformation of red-eyed Ephestia with DNA from the wild type (black-eyed). Ottolenghi-Nightingale (1969) reported DNA induced melanin production in skin cells of albino mice. Hess (1969) reported successful transformation in higher plants. In the latter two cases, a lowered frequency of transformation was reported when DNase treated preparations were used.

RNase did not alter the frequency of transformation, indicating that only DNA material could induce a transformation.

More researchers have reported successful transformation in various multicellular organisms: Ledoux, et al., (1974) used bacteria DNA to correct a thiamineless condition in Arabidopsis thaliana; Nawa, et al., (1975) reported transformation in Capsicum annum L.; Devilly and Houghton (1977) observed transformation in Gloeocapsa alpicola; Shutko, et al., (1979) have caused incomplete transformation of rat cells with a fragment of adenovirus 12 DNA. Present studies appear to center on using a virus as an injector of DNA materials into the host; thus, eliminating the unrealiable and unknown steps involved in the active transport of DNA across the cellular membrane.

Of importance to this thesis are the works specifically dealing with D. melanogaster. Fox and Yoon (1966) first reported transformation in D. melanogaster. Their method involved collecting the egg as the female laid it, in an "ovitron." They then soaked the egg in a DNA solution, for seven to eighteen hours, and allowed it to develop in normal culture medium. Upon emergence, they observed mosaic patterns in eye color, indicating a non-complete transformation. In 1970, Fox and Yoon established transformed stocks in which the acquired phenotype, in mosaic form, was found in some individuals through seven successive generations. Here they presented the exosome theory of DNA incorporation in a eukaryotic genome. The exosome theory states that the added genetic material is not directly annealed into the host's genome as reported for prokaryotic organisms (Harris and Barr, 1971), but rather,

it is formed into a very small, extra chromosome called an exosome. Theoretically, this exosome is replicated during cell division in the same manner as the host's original chromosomes. But since all of the cells of the eukaryote do not contain this exosome, mosaic patterns are observed.

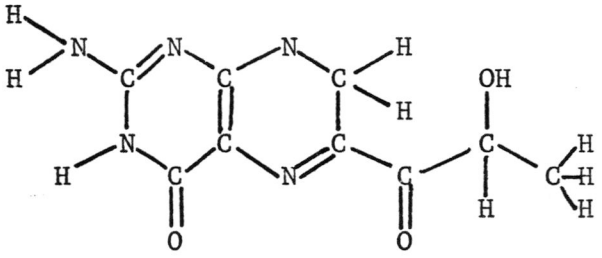
It should also be noted that the gonads must also have this exosome in order for the trait to become inheritable. Fox, et al., (1970) repeated the previous experiments on a larger scale and found no whole body transformants (all transformants were mosaics); even transformants of subsequent generations were mosaics. They concluded that the mechanism of transmission of genetic information in the process of transformation cannot be by the conventional model of integration as seen in prokaryotes. They postulated that the introduced genetic material associated itself with the homologous chromosome segments, but are never integrated into the linear structure of the chromosome. Further evidence indicated that the introduced genetic information mapped at the site of the target locus and that the original chromosomal information was still present at that site (Fox et al., 1971a). Cytological alterations in the salivary gland cells were studied, and chromosome aberrations were observed. Thread-like structures were found lying to the side, or off the tip of the chromosome with fine threads connected to the chromosome (Fox and Valencia, 1975).

The exosome theory, however, does not describe all eukaryotic transformations. Germeraad (1976) utilized a method of microinjection described by Okada, et al., (1974). Wild type DNA material was

injected into multinucleate host embryos of vermilion and brown-eyed mutants. Whole body transformants were observed in the vermilion experiments, and in the subsequent stable transformed lines. The original trait (vermilion) was not observed. That is, in subsequent generations the transformed trait (wild type) could not be recombined away from the original vermilion alleles, indicating linear integration of introduced DNA into the original chromosomal structure of the host. Germeraad also reported that other experiments involving the vermilion and rudimentary mutants resulted in the unstable expression and/or transmission of the acquired traits. Stable lines could not be produced in six to eight generations. There was no patchiness in the eyes which would be expected if the low activity was due to mosaicism in expression. This could be because the introduced genetic material does not intimately associate with the target locus, but rather remains unintegrated and is irregularly reproduced, expressed, or transmitted (Germeraad, 1977). However, this does not necessarily support or invalidate the exosome model. Most probably, the integration model and the exosome model both describe transformation and partial transformation of stable whole body and stable mosaic strains respectively. The method of introduction of the DNA material into the host may make the difference between whole body transformation and partial, or mosaic, transformation. This is because the injected DNA material would be of greater molecular weight than the transported DNA; and, therefore, it would be more active in a multinucleate host than the smaller less concentrated fragments of the transported DNA material.

Some goals of this project include: (1) to generate transformants in the sepia mutant D. melanogaster by introduction of wild type DNA; and (2) to determine inheritability of the introduced trait by trying to generate successive generations.

Eukaryotic transformation was studied in this project using the sepia mutant of D. melanogaster as the host subject. The sepia mutant is an autosomal recessive trait in which the eye has a sepia color (dark brown); see Plate #1. The gene for sepia is located on the third chromosome, at locus number 26 (Lindsley and Grell, 1972). The sepia eye color is present at eclosion, darkens with age and becomes almost black in five to seven days. Pigmentation of the ocelli is consistent and normal (Lindsley and Grell, 1972). The sepia color is a combination of brown and yellow pigments, isosepiapterin and sepiapterin. The structure of sepiapterin has been found by Forrest and Nawa (1962) through NMR studies to be:



Note that isosepiapterin was found to be similar.

It has been shown that the only difference between the wild type and the sepia mutant is in the eye color pigments. Chromatograms made from the thorax and abdomen regions do not differ (Hadorn and Mitchell, 1951). Chromatograms also show that the heterozygous wild type can be differentiated from the homozygous wild type. The eye pigments of

of heterozygous flies have trace amounts of the yellow sepiapterin pigments and isozanthopterin, in addition to the red pigments found in homozygous wild type flies (Lindsley and Grell, 1972).

The sepia mutant was ideal for these experiments because of the very close similarities to the wild type. Any lessening of the very dark eye color after allowing the fly to age five days could be easily observed. Furthermore, the gene for sepia eye color is autosomal and mono-allelic (Lindsley and Grell, 1972). Thus, only the most simple of transformations need take place to become phenotypic.

Of importance to any transformation study are the characteristics of the DNA introduced to the host. Important factors include relative purity, average molecular weight, transforming activity and any possible noxious chemical byproduct(s) which could be associated with the isolated DNA. Consideration of the ease, speed, safety, and cost of the extraction procedure is also of importance. There are three basic DNA extraction techniques described: the Phenolic Method (Kirby, 1957; Saito and Miura, 1963; Tartof, 1973), the Enzymatic Isolation Methods (Marmur, 1961; Lambert and Laird, 1971; Fox, et al., 1975), and the Salting-Out Methods (Sevag, et al., 1939; Meade, 1964). Many of these techniques overlap in some ways such as Phenolic procedures utilizing enzymes in some steps, etc. The procedure developed for this project was unique. This procedure was a combination of the Salting-Out Methods and Enzymatic Methods, with modification made by this author. The resulting technique was relatively simple and yields high purity, large molecular weight, high activity DNA. The Phenolic Methods were avoided because of possible deleterious effects of phenolic

residues in the DNA on the host.

MATERIALS AND METHODS

DNA Extraction

The technique used in these experiments for extracting DNA from the adult Drosophila melanogaster was unique. It consisted of a combination of the Enzymatic Isolation Methods (Marmur, 1961) and the Salting-Out Methods described by Sevag, et al., (1938), with a few modifications by this author. Other DNA extraction methods have been described; however, the following method was chosen because of its yield of high purity, large molecular weight and high activity DNA through a relatively simple procedure. The following is a brief description of the biochemistry involved in the technique.

To release DNA and other macromolecules in soluble form, the adult flies were homogenized in a saline citrate and ethylenediaminetetraacetic acid (EDTA) solution. The saline citrate protects the cellular structure of the cells by maintaining an osmotically stable environment. The EDTA protects the cell membrane from attacks by lysozyme.

The cells were then lysed by using a strong solution of sodium dodecyl sulfate (SDS) which ruptures the cell membranes. The lysis step is carried out slowly and at $0^{o}C$ to minimize any proteolytic and nucleolytic enzyme activity. The macromolecules (DNA, RNA, and proteins) are precipitated slowly by layering absolute ethanol at $0^{o}C$ over the homogenate, and gently stirring.

Treatment of the precipitate with sodium perchlorate ($NaClO_4$) denatures the protein content. The nucleic acid content (DNA and RNA)

can then be separated from the denatured proteins by precipitation with absolute ethanol.

Because RNA and DNA are structurally similar, the separation of the two is difficult. The separation was achieved by denaturing the RNA content with two RNA specific nucleolytic enzymes, RNase A, and RNase T_1 (Takadiastase). (DNA could likewise be denatured by the DNA specific enzyme, DNase, if one were trying to isolate RNA instead of DNA.) After the RNA has been denatured, the DNA can be separated by precipitating with absolute ethanol. To inactivate any trace amounts of DNase, which may be a naturally occurring contaminant of the RNase A or RNase T_1 used above, the resulting DNA precipitate was dissolved in a saline citrate solution. The citrate ions act as an inhibitor to DNase, and along with refrigeration, the denaturization of the DNA extract is virtually stopped.

Due to sheer forces from the physical handling during the extraction procedure, low molecular weight amino acids and nucleic acids may be present in the final DNA sample. To purify the DNA, dialysis was required to separate the large molecular weight DNA from the free nucleic acids and amino acids. The result was high purity, high molecular weight DNA.

The resulting DNA can be characterized by measurement of its hyperchromic shift, and by the 280/260 nm absorbance ratio found with an ultraviolet spectrophotometer. Comparison to Salmon Testes DNA (prepared by Carolina Biological Supply) as the standard gives the actual concentration of the extracted DNA.

The following is a list of chemicals and materials required for the DNA extraction procedure.

Required chemical solutions

Absolute Ethanol (ETOH)

15-20g Adult D. melanogaster, homozygous

Chloroform:Isoamyl alcohol, 24:1 mixture, by volume

0.10 M Ethylenediaminetetraacetic Acid (EDTA)

RNase A, 1 mg/ml - Carolina Biological Supply, Burlington, N. C.

RNase T_1, 1 mg/ml - Carolina Biological Supply, Burlington, N. C.

0.15 M Sodium chloride (NaCl)

0.015 M Sodium citrate ($Na_3C_6H_5O_7$)

0.15 M Sodium citrate ($Na_3C_6H_5O_7$)

25% Sodium dodecyl sulfate (SDS)

5.0 M Sodium perchlorate ($NaClO_4$)

Required apparatus

Beakers, sterile

Boiling water bath (100°C)

Centrifuge tubes, sterile

Centrifuge capable of 10,000 x g

Dialysis Membrane

Flasks, sterile

Glass pipet, sterile

Glass rod, sterile

Ice water bath (0°C)

Magnetic stirrer

Potter-Elvehjum homogenizer

Pyrex wool, sterile

Stoppers, sterile

Thermometer (-20 to 100°C)

Ultraviolet spectrophotometer at 260, and 280 nm

Vortex mixer

Procedure: DNA Isolation Technique

Part 1 - Release of DNA and other macromolecules in soluble form by osmotic destruction of the cellular membrane.

1. Homogenize a known amount of frozen adult flies with a Potter-Elvehjem Homogenizer in four volumes of 0°C 0.15 M sodium citrate and 0.1 M ethylenediaminetetraacetic acid (EDTA) solution.

2. Add 5 ml per g of fly of 25% sodium dodecyl sulfate (SDS) and stir slowly with a magnetic stirrer at 0°C for 45 minutes. This should result in a viscous soapy solution. Note: The chitinous membranes of the adult fly body cannot be ruptured with this method.

3. Filter the solution through pre-washed, and autoclaved pyrex wool. Wash the filtrate with 10 to 20 ml of EDTA solution.

4. In an ice bath, precipitate the macromolecules by slowly layering two volumes of absolute ethanol (ETOH) at 0°C over the filtrate and gently stirring with a glass rod.

5. After precipitation has been completed, collect the precipitant by centrifugation at 10,000g for 10 minutes at 0°C.

6. Discard the supernatant and wash the precipitant with abosolute ETOH at 0°C.

7. Collect the precipitant in one or two tubes with the washings, and recentrifuge. Discard the supernatant. This precipitate can be stored in absolute ETOH at -20°C if necessary.

Part 2 - Separation of the nucleic acids from proteins by denaturing the protein fraction with sodium perchlorate.

1. Dissolve the precipitant from Part #1 in 15 ml of 5.0 M sodium perchlorate ($NaClO_4$) and 21 ml of chloroform-isoamyl alcohol (24:1) solution. Cover and mix on a Vortex mixer or shake vigorously until a white emulsion is obtained, approximately five minutes.

2. Transfer to centrifuge tubes and centrifuge at 10,000g for 20 minutes at 0°C.

3. Carefully collect the supernatant by pipet. Discard the middle layer (denatured protein) and the bottom layer (chloroform).

4. In an ice bath, precipitate the supernatant by gently layering two volumes of 0°C absolute ETOH and gently stirring with a glass rod.

5. Collect the precipitate by centrifugation at 10,000g for 10 minutes at 0°C.

6. If any denatured protein was present (middle layer), repeat Steps 1 through 5 until no middle layer is present in Step #3. The final precipitate can be stored in absolute ETOH at -20°C if necessary.

Part 3 - Separation of DNA from RNA by denaturization of RNA with RNase A and RNase T_1.

1. Dissolve the precipitant from Part #2 in 9.0 ml 0.015 M saline citrate solution. Add 0.25 ml RNase A solution. (See

Preparation of RNase Solution at the end of this section.)

2. Incubate at 37°C for 15 to 30 minutes, gently shake periodically.

3. Add 1.0 ml of 0.15 M saline citrate. Mix vigorously with 10 ml chloroform-isoamyl alcohol (24:1) solution until a white emulsion is obtained.

4. Centrifuge at 10,000g for 20 minutes at 0°C.

5. Collect the supernatant. Discard the middle layer (denatured RNA) and the bottom layer (chloroform).

6. Precipitate the supernatant by slowly layering 0°C absolute ETOH and gently stirring with a glass rod.

7. Collect the precipitate by centrifugation at 10,000g for 10 minutes at 0°C.

8. Dissolve the precipitate in 9.0 ml 0.015 M saline citrate solution. Add 0.25 ml RNase T_1 solution. (See Preparation of RNase Solution at the end of this section.)

9. Repeat above Steps 2 through 7.

10. If any denatured RNA was present, repeat above Steps 1 through 9.

11. Repeat Part #2 to remove any residual proteins (the RNases).

Preparation of RNase Solution: Dissolve 1 mg/ml RNase (A or T_1) in 0.15 M sodium chloride (NaCl) solution. Gently heat in boiling water bath five to ten minutes. Heating inactivates any DNase which may be present. Cool slowly and store at 0°C or less (Saito and Miura, 1963).

Part 4 - Separation of large molecular weight DNA from small molecular weight nucleic acids and amino acids by dialysis.

1. Dissolve the precipitate from Part #3 in 18 ml 0.015 M saline citrate solution. Add 2.0 ml 0.15 M saline citrate solution, and gently mix.

2. Place the resulting solution in dialysis bags.

3. Dialyze twice against a solution of 900 ml 0.015 M saline citrate and 100 ml 0.15 M saline citrate for 24 hours each at 4°C.

4. Store until use in the saline citrate solution at 0°C. For long-term storage, precipitate with absolute ETOH and store at 0°C as a solid with a covering layer of absolute ETOH to protect the DNA from oxidation.

Part 5 - Ultraviolet characterization of DNA by measurement of the hyperchromic shift, and spectrophotometric determination of the DNA concentration.

1. Prepare a blank solution of 90 ml 0.015 M saline citrate and 10 ml 0.15 M saline citrate.

2. Dilute a known concentration of dialyzed standard DNA solution with the blank solution to obtain an absorbance of less than 1.0 at 260 nm.

3. Dilute the sample DNA solutions by the same amount as was necessary to dilute the standard in Step #2. Treat all samples and standards the same.

4. Record the optical density of the DNA solutions at 260 nm and 280 nm.

5. Melt the DNA in a boiling water bath for five to ten minutes.

6. Rapidly cool the melted DNA in an ice bath.

7. When at room temperature, record the optical density of the DNA solutions at 260 nm and 280 nm.

8. The ratio of the optical densities of 280/260 is proportional to the protein content of the DNA sample.

9. If any protein is found to be present, then repeat Part #2.

10. The DNA concentration can be measured by comparison of the optical density values at 260 nm and 280 nm to standard calibration curves for DNA. The hyperchromic shift is calculated from the change in the 280/260 ratios found between the thermally denatured (melted) DNA and the original DNA.

Culture and Exposure Experiments

Throughout the complete project, each culture vial contained 15 ml of Drosophila medium, Carolina Biological Supply Formula 4-24. A 15.0 ml aliquot of 4 ug/ml DNA-saline citrate solution was added to each DNA-enriched vial, totaling 60 ug DNA per vial. 15.0 ml of 0.015 M saline citrate solution was added to the dry medium in the saline-control experiments. 15.0 ml of distilled water was added in the water-control experiments. Adult flies used in these experiments were inspected and found to be normal sepia mutants, or normal wild type flies. Experiments and controls were performed in triplicate with three pairs of homozygous adult flies (3 male, 3 female) per vial.

The following is a list of chemicals and materials required for the Culture and Exposure experiments.

Required chemical solutions

Adult D. melanogaster, homozygous sepia and wild type

Carbon dioxide gas (CO_2)

4 ug/ml DNA-saline citrate from DNA extraction methods

Ethyl Ether

0.015 M saline citrate ($Na_3C_6H_5O_7$)

Salmon Testes DNA - Carolina Biological Supply, Burlington, N. C.

Distilled water

Required apparatus

Culture vials

Dissecting microscope, with photographic attachments

Drosophila medium - Carolina Biological Supply Formula 4-24
Burlington, N. C.

Ektachrome film, 35 mm fine grain

Etherizer

Microspatula

Tweezers

8 to 13 day exposure experiments: The adult flies were allowed to eat, breed, and lay eggs in the various experimental or control environments. After eight days, but before any of the F_1 progeny had emerged, the adult flies were cleared from the culture vials and inspected for any observable phenotypic changes which may have developed due to the ingestion of the experimental medium.

Upon emergence, the F_1 progeny was cleared from the culture vials daily for the next five days. All nontransformed F_1 progeny were counted and placed in fresh culture vials containing normal culture

medium. All putative transformants were selected and placed in fresh culture vials with normal culture medium. With the exception of the wild type cultures from the control experiments, selection of potential transformants was made solely on the visual (microscopic) criterion of red, or redness in the otherwise sepia eye color. Because the sepia eye color does not fully develop in the first days of emergence from the pupa case (Lindsley and Grell, 1972), the young, adult flies were "aged" for five days to allow for complete eye color development. At the end of the aging period, all flies were re-inspected and counted.

During the "aging" process, the F_1 generation bred and laid eggs in the fresh culture vials. The adult (F_1 generation) was cleared from these vials at the end of the five-day aging period. The resulting F_2 generation emerged in a few days and was carefully inspected for the transformed trait, the presence of which would indicate inheritability of the trait.

Table #1 indicates the experimental and control culture conditions to which the flies were exposed for the 8 to 13 day experiments. Some of the transformed flies were photographed; see Plates 1 thru 4. Some of the other transformed flies were used in paper chromatography experiments to determine which pigments were present in the transformed eye. (See page 22 for the procedure, Table #6, and Figure #1 for the results of the paper chromatography experiments.)

4-hour limited exposure experiments: Because the time required for ingested food material to pass through the system of an adult fruit fly is approximately four to five hours (King and Wilson, 1955), a

series of experiments were conducted where flies were exposed to DNA-enriched media for four hours then moved to fresh medium-containing culture vials. The culture vial preparation was the same as for the 8 to 13 day exposure experiments.

After the flies had been exposed to the DNA-enriched medium for four hours, they were transferred to vials containing normal culture medium. These flies were inspected daily for seven days to observe any effects of their exposure. They were then cleared from the holding vials. No offspring had emerged at this time. When the offspring emerged, they were also checked for any eye color mutations which may have occurred. Table #2 indicates the experimental conditions to which the flies were exposed for the four-hour exposure experiments.

These four-hour exposure experiments were conducted to determine if the allo-DNA affects the egg while in the parent, or whether it affects the egg or later stages only after development begins in the enriched culture medium.

TABLE 1

EXPERIMENTAL AND CONTROL CULTURE CONDITIONS TO WHICH STOCKS OF D. MELANOGASTER WERE EXPOSED FOR 8 TO 13 DAYS

Culture #	Phenotype	Additive to Culture Medium
1	Sepia	Wild Type DNA
2	Sepia	Sepia DNA
3	Sepia	Salmon Testes DNA
4	Sepia	Saline Solution
5	Sepia	Water
6	Wild Type	Saline Solution
7	Wild Type	Water

Culture #1 - Sepia flies were exposed to the allo-DNA extracted from the wild type mutant. This was the experimental culture expected to develop dominant (wild type) phenotype.

Culture #2 - Sepia flies were exposed to the iso-DNA extracted from sepia mutant flies. This was a control culture to determine whether or not any by-product of the DNA extraction technique would affect the phenotype of the offspring.

Culture #3 - Sepia flies were exposed to medium containing Salmon Testes DNA. This was a control to determine the chemical effects of DNA on the developing larvae and exposed adults.

Culture #4 - Sepia flies were exposed to the saline citrate solution used to dissolve the various DNAs used in cultures 1 thru 3 above.

DNAs used in Cultures 1 thru 3 above. Ultraviolet characterization of this solution showed no protein or DNA content. This control culture was set up to determine the environmental effects of saline citrate on exposed adults and their progeny.

Culture #5 - Sepia flies were placed in a vial containing normal medium as a base line control for the entire project. This was done to determine whether any deleterious factors affected the survival rate of Cultures 1 thru 4 above.

Culture #6 - Wild type flies were exposed to the same saline citrate solution as in Culture #4. This was done to determine whether the saline solution affected the wild type adults or offspring.

Culture #7 - Wild type flies were exposed to normal medium conditions for a comparison to the results of Culture #6.

TABLE 2

EXPERIMENTAL AND CONTROL CULTURE CONDITIONS TO WHICH STOCKS OF D. MELANOGASTER WERE EXPOSED FOR FOUR HOURS

Culture #	Phenotype	Additive to Culture Medium
8	Sepia	Wild Type DNA
9	Sepia	Sepia DNA
10	Sepia	Salmon Testes DNA

Culture #8 - Sepia flies were exposed to the allo-DNA extracted from the wild type mutant. This was done to determine if any transformants would be generated as a result of the parents' ingestion of

the DNA-enriched medium. Any transformants would have had to have been transformed by DNA ingested by the parent flies.

Culture #9 - Sepia flies were exposed to the iso-DNA extracted from the sepia mutant. This was a control to determine whether any by-product of the DNA extraction method ingested by the parent would cause transformations in the offspring.

Culture #10 - Sepia flies were exposed to Salmon Testes DNA. This was a control culture to determine whether any transformants could be generated by the chemical effects of DNA on the parents.

Paper Chromatography of Whole Eyes

Eye pigments were separated using a paper chromatography technique described by Hadorn and Mitchell (1951). A set of three aged flies were chromatographed from the F_1 generation in each Culture #1 thru #7, except Culture #6, which was not chromatographed. See Figure #1 and Table #6 for paper chromatography results.

The following is a list of chemicals and materials required for the paper chromatography of whole eyes from D. melanogaster samples from the culture and exposure experiments.

Required chemical solutions

Adult D. melanogaster, three of each type to be chromatographed

Concentrated Ammonium Hydroxide (NH_4OH)

N-Propanol (C_3H_7OH)

Distilled water (H_2O)

Required apparatus

Glass rod

Jar with lid, twelve inches high

Pencil

Stapler

Ultraviolet lamp (254 nm and 350 nm)

Wax paper

Whatman #1 Filter Paper, 8 x 11

Paper chromatography procedure:

1. Draw a pencil base line one inch from the bottom of a large (8 x 11) piece of Whatman #1 filter paper. Make six equidistant marks on this line.

2. Prepare the chromatography solution. The chromatography solution is composed of 60 ml N-Propanol, 24 ml distilled water, and 6 ml Concentrated ammonium hydroxide. Allow the mixture to stand covered for 30 minutes to equilibrate.

3. With an underlying piece of wax paper to avoid fluid loss, smash three whole fly heads on each mark.

4. Staple the chromatography paper into a non-overlapping cylinder, place into the jar containing the chromatography solution, and cover.

5. Allow the solvent front to rise to within one inch of the top edge of the chromatography paper, then remove from the jar.

6. Air dry the chromatograph. The resultant spots can be observed under ultraviolet light at 254 nm and 350 nm.

RESULTS AND DISCUSSION

DNA Extraction Procedure

Through ultraviolet characterization, the DNA extracted was found to be of high purity, and high concentration; See Table #3. The high relative viscosity indicated a high-average molecular weight.

TABLE 3

COMPOSITION AND CONCENTRATION OF EXTRACTED DNA

Mutant Type	Initial Weight of Adult Flies Extracted (g)	Concentration of Extracted DNA (mg/ml)	Concentration of Protein Fraction	Percent DNA
Wild	7.0	0.03	0.01	75%
Sepia	7.2	0.05	0.01	83%

Culture and Exposure Experiments

Fourteen out of a total of 210 offspring showed the acquired phenotype of redness in the omatidia. The results of these experiments indicate that *wild type* DNA does affect the eye color of the *sepia* mutant; See Table #4 and Plates #1 thru 4. The markedly reduced number of offspring of the *wild type* DNA enriched, as compared with the *sepia* DNA-enriched, and all other control cultures could also indicate that the lowered survivability was due to the introduction of the allo-DNA (*wild type*).

TABLE 4

NUMBER OF PUTATIVE TRANSFORMANTS IN THE FIRST GENERATION FROM THE DNA EXPOSURE EXPERIMENTS

Culture #	Phenotype	Medium Additive	#Putative Mutants	#Normal Offspring
1	Se	Wild DNA	14	196
2	Se	Sepia DNA	3	497
3	Se	Salmon DNA	0	525
4	Se	Saline	0	508
5	Se	Water	0	373
6	++	Saline	0	455
7	++	Water	0	540
8	Se	Wild DNA	0	30
9	Se	Sepia DNA	0	19
10	Se	Salmon	0	28

The above results indicate that the saline citrate and the salmon testes DNA had no transforming effect on the developing or adult fly. The effect of the iso-DNA (sepia) could be due to fragments of genetic material having an effect on the host. Fox and Yoon (1966) also reported a "low order general mutagenic effect" observed as a slight increase of transformation for the test loci among flies treated with homologous DNA. In this experiment, the frequency of these transformants is important, which is much less than the frequency of transformants observed in the allo-DNA (wild type). The lack of transformants from the four-hour exposure

experiments indicates that the DNA did not enter the egg while the egg was still in the parent fly. The four-hour exposure experiments also showed that the DNA ingested by the parent was not responsible for any transformations observed in the 8 to 13 day exposure experiments.

Second generations developed in the aging vials which contained normal culture medium. Observation of these were made to determine the inheritability of the putative mutant phenotype. See Table #5. The results can be interpreted to indicate that the gonads were not affected by the introduced DNA. If transformation had occurred in the gonads, then the wild type trait (redness in the sepia eye) would be present in the F_2 generation as a dominant trait over the sepia trait.

TABLE 5

RESULTS OF THE SECOND GENERATION
CROSSES MADE IN NORMAL CULTURE MEDIUM

Culture # of F_1 Generation	Parents	#Mutants Found (F_2)	#Normal Offspring
1	Se(+) x Se[1]	0	133
2	Se x Se	0	315
3	Se x Se	0	343
4	Se x Se	0	214
5	Se x Se	0	194
6	++ x ++	0	281
7	++ x ++	0	191

[1]Se(+) are the putative transformants from Culture #1.

Paper Chromatography of Whole Eyes

The results of the paper chromatography experiments show that the putative transformants did not have any isosepiapterin and only a little sepiapterin. Normal wild type flies also had no isosepiapterin and little sepiapterin. The sepia fly would normally have significant amounts of these pigments. These results indicate that the putative transformants were changed phenotypically from the normal sepia; See Table #6 and Figure #1.

TABLE 6

RESULTS OF THE PAPER CHROMATOGRAPHY OF THE WHOLE EYES FROM THE CULTURE AND EXPOSURE EXPERIMENTS

Pigment	Wild Type	Adults from Sepia Culture Exposed to Medium Enriched with: Water	Saline	++ DNA	Se DNA	Salmon DNA
Isosepiapterin	0	+	+	0	+	+
Biopterin	+	+	+	+	+	+
2-amino-4-hydroxy pteridine	+	+	+	+	+	+
Sepiapterin	Little	+	+	Little	+	+
Xanthopterin	+	+	+	+	+	+
Isoxanthopterin	+	+	+	+	+	+
Drosopterins	+	+	+	+	+	+

Note: + means that the pigment was present, and 0 means that the pigment was not present.

FIGURE 1

PAPER CHROMATOGRAPH OF D. MELANOGASTER EYE PIGMENTS

Circles outlined in solid lines were those spots visible at 254 nm; those outlined in broken lines were those spots visible only 350 nm and not 254 nm.

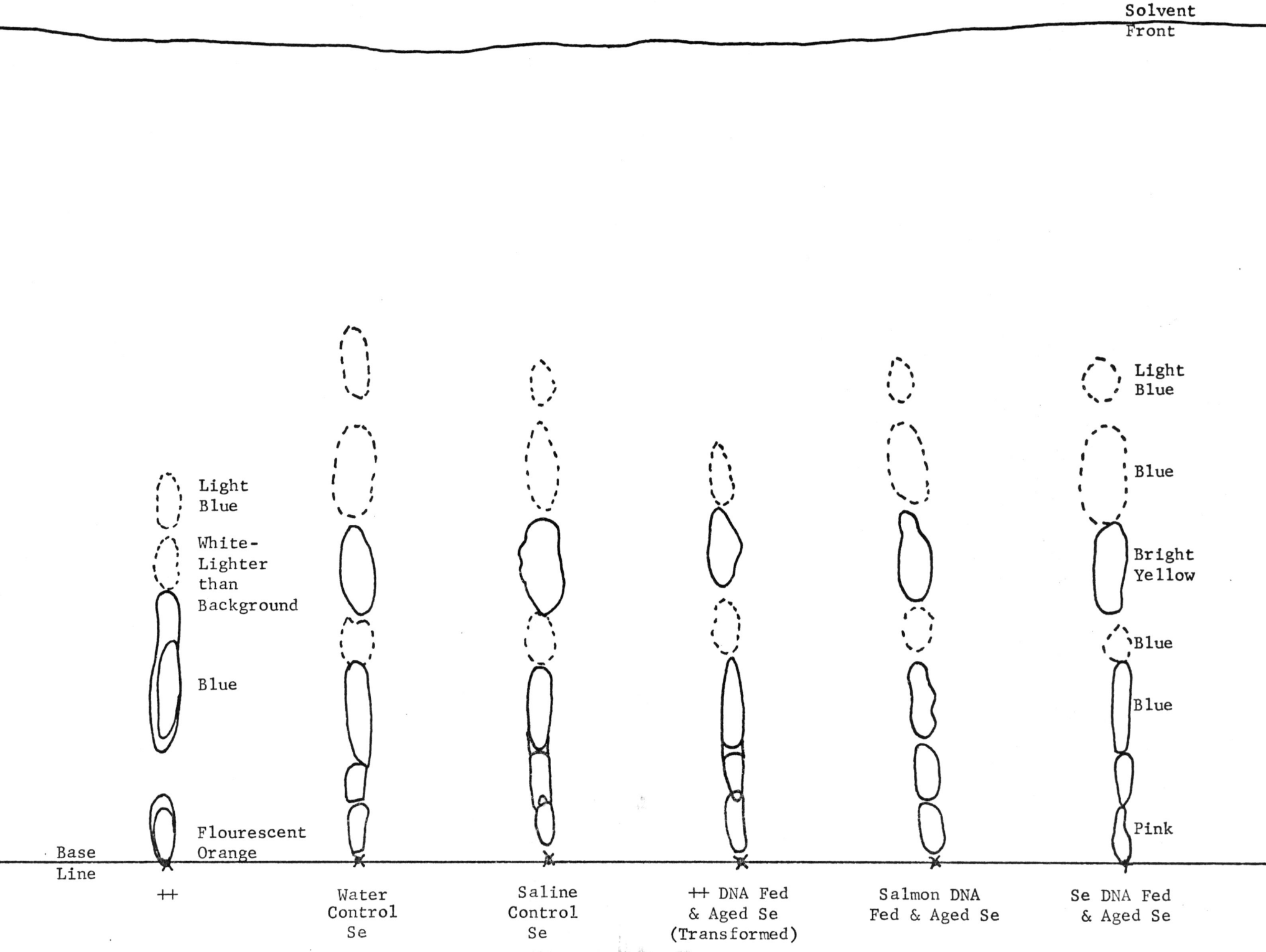

Solvent Front
Light Blue
White-Lighter than Background
Blue
Flourescent Orange
Base Line
++
Water Control Se
Saline Control Se
++ DNA Fed & Aged Se (Transformed)
Salmon DNA Fed & Aged Se
Se DNA Fed & Aged Se
Light Blue
Blue
Bright Yellow
Blue
Blue
Pink

PLATE 1

Normal *Sepia* Phenotype

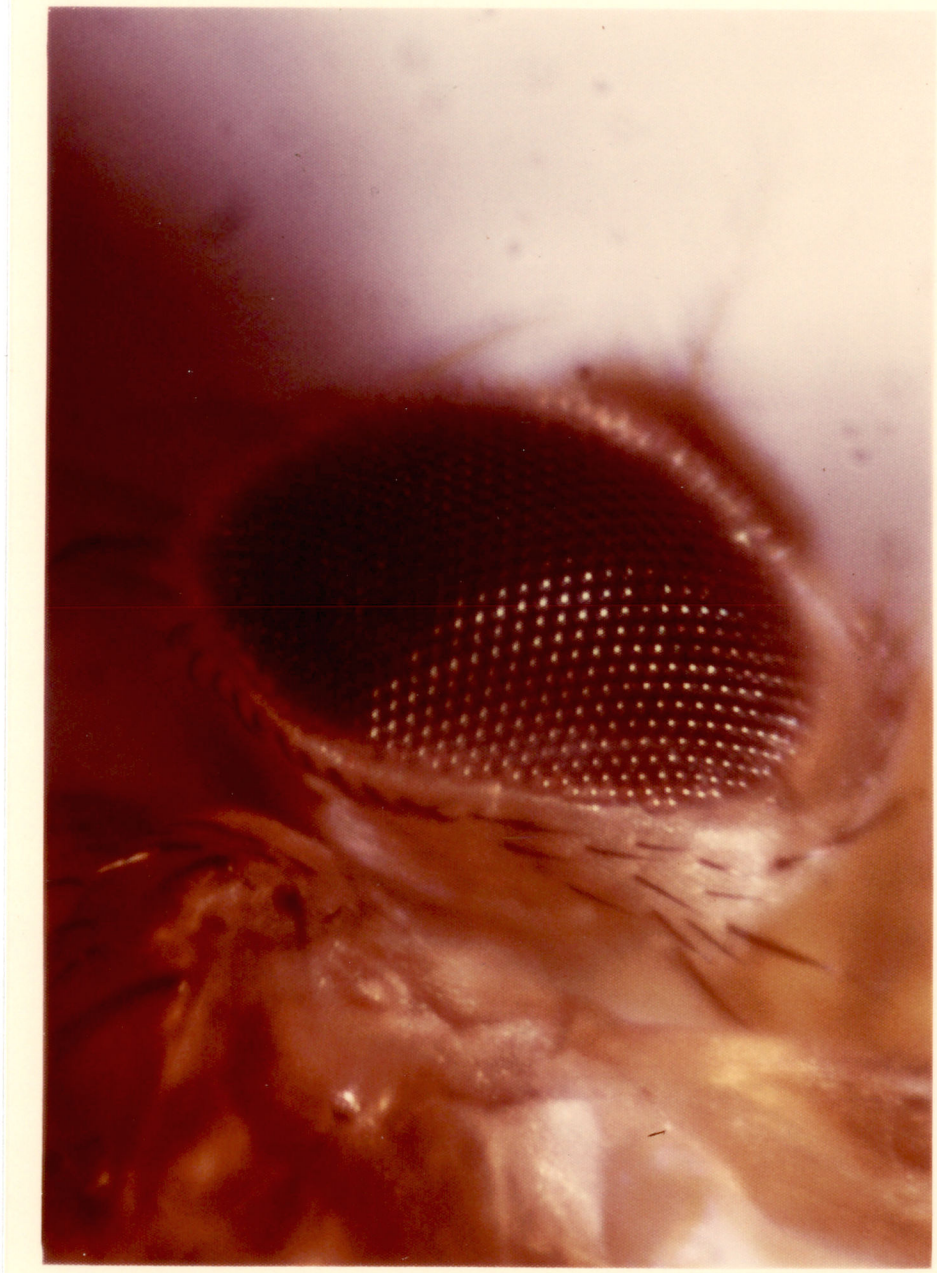


PLATE 2

Normal Wild Type Phenotype

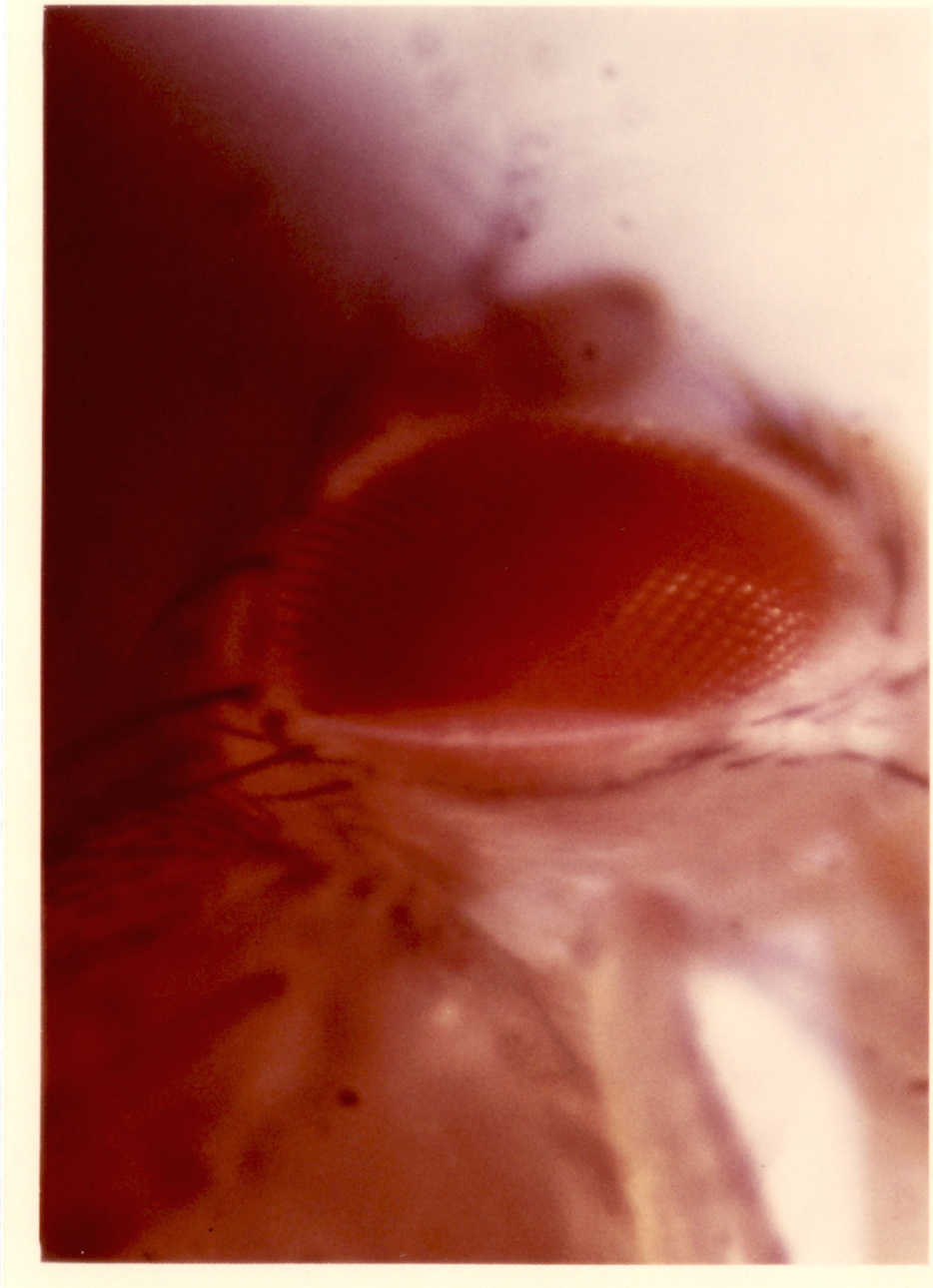


PLATE 3

Comparison of the Wild Type - Upper Left
Normal Sepia - Upper Right
Transformed Sepia - Lower Center

PLATE 4

Comparison of Four Different Transformed Sepia

Note the varying degrees of transformation. All four flies were from the sepia x sepia Culture #1, made in wild type DNA enriched medium.

The results of the experiment indicate that wild type DNA does affect the eye color of sepia D. melanogaster by lightening the characteristic dark brown (sepia) color of the normal sepia mutant to varying degrees of red-brown colors. The paper chromatography results indicated that the red-brown pigments of the transformed phenotype were very similar to the pigments found in the wild type phenotype. This phenotype, however, was not inheritable, as demonstrated by the lack of this phenotype in the second generation offspring.

The lack of the transformed phenotype in the second generation does not indicate that the process of transformation did not take place in these experiments. It does indicate that the cells of the gonads did not contain any of the introduced DNA, so that the acquired trait could not be passed on to following generations. These results are consistent with the results of Fox and Yoon (1966) who found that some transformed strains did not pass the acquired trait on to subsequent generations.

The results of the study may be interpreted in terms of either the exosome theory or the integration theory of the mechanism of transformation. The exosome theory states that the introduced DNA will align itself with its corresponding locus. The exosome must be replicated to be passed on to subsequent generations of flies (Fox and Yoon, 1970). In this experiment, an exosome could have been present and replicated in the cells which give rise to the ommatidia producing a phenotypically whole body, rather than a mosaic (patchy) transformant. Since the acquired trait does not pass on to subsequent generations, the

transformation must be defined as a mosaic transformation because the gonad cells did not acquire and replicate the exosome.

The integration theory of transformation states that the introduced DNA directly anneals with the homologous locus in the cellular genome (Harris and Barr, 1971). In this experiment, the introduced DNA could have annealed only in the cells which give rise to the ommatidia and not into the genome of the gonad cells. This possibility exists because the cells which give rise to the ommatidia differientiate at a different time than do the cells which give rise to the gonads (Demerec, 1950). The resulting phenotype would be a consistent (not patchy) red-brown eye color in the transformed fly. The acquired trait would not pass on to subsequent generations because the gonads did not contain the introduced DNA.

SUMMARY AND CONCLUSIONS

Fourteen putative transformed D. melanogaster were observed in the first generation offspring when sepia parent flies were allowed to breed and lay eggs in a medium enriched with wild type DNA. The transformation observed was a redness in the otherwise brown eyes of the sepia mutant which persisted after five days of aging. Paper chromatography of the whole eyes showed that isosepiapterin and sepiapterin, pigments characteristic of the sepia mutant, were absent in the transformed fly. Control experiments disproved the possibility of environmentally-induced phenotypic changes.

The DNA extraction procedure used in these experiments was unique. It was a combination of the Salting-Out Methods and Enzymatic Isolation Methods, with some modifications made by this author. The procedure proved to be relatively simple and yielded high purity, large molecular weight, and high activity DNA.

The DNA material was introduced to the flies by allowing eggs to be laid in a DNA-enriched medium. The eggs developed in this medium until the adult flies emerged from the pupa cases. Adult flies exposed to the DNA-enriched medium showed no changes in phenotypic characteristics. Eggs laid by the adult flies which had ingested the DNA-enriched medium developed into normal, non-transformed adults if they were allowed to develop in normal culture medium. The transformation process took place only when the egg (or larva) was exposed to the DNA material outside of the parent.

The putative transformed flies were segregated and allowed to breed and lay eggs in normal culture medium. The resulting progeny had no transformed individuals. This fact does not disprove that transformation took place in these experiments. It showed that the cells of the gonads did not contain the added DNA material, or that the added DNA material did not replicate so that it could be passed on to following generations.

The presence of transformed offspring in these experiments show that transformation in a multicellular organism (D. melanogaster) is possible. Inheritability of the transformed trait depends on introducing the DNA into the genome of the gonads, and the introduced DNA must also replicate.

Expression of the transformed trait depends on introducing the DNA material into the genome of the cells which give rise to the ommatidia, and that DNA must be able to transcribe mRNA. In these experiments, it appears that transformation of the cells which give rise to the ommatidia took place, but that transformation of cells of the gonads did not occur.

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