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The Effects Of Carbon Monoxide On The Chromosomal Puffing Patterns Of The Salivary Glands Of The Drosophila melanogaster

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THE EFFECTS OF CARBON MONOXIDE
ON THE CHROMOSOMAL PUFFING PATTERNS
OF THE SALIVARY GLANDS OF THE DROSOPHILA MELANOGASTER

Submitted, in partial fulfillment of the
Requirements for Graduation with Honors,
to the Department of Biology,
Carroll College, Helena, Montana

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March 30, 1979
This thesis for honors has been approved for the Department of Biology, March 30, 1979.

Dr. James J. Manion, Advisor

Dr. John A. Christenson

Sister Miriam Clare
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**Erasable Cond**  
**Cotton Fiber**
We wish to thank our thesis advisor, Dr. James J. Manion, for his advice and support. We also wish to thank our readers, Dr. John A. Christenson and Sister Miriam Clare, for their cooperation. We are indebted to Karen Streets of Shodair Children's Hospital of Helena, Montana, for her advice, and to the hospital for the use of photographic equipment. Finally, we greatly appreciate all the help we received from many of our fellow students who generously gave their time and understanding support.
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ABSTRACT

The 110 hour larval stage puffing patterns are described for *Drosophila melanogaster* larvae treated with carbon monoxide. A comparison of our findings is made with Michael Ashburner's control animals. We find that the carbon monoxide treated larvae portray no great induction of puffing when compared to Ashburner's controls. Also the various difficulties encountered the their possible solutions are discussed.
I. INTRODUCTION

In 1933 Emil Heitz and Hans Bauer of the University of Hamburg were the first to recognize the giant polytene chromosomes found in well-differentiated metabolically-active organs of the Dipteran flies. Since their discovery, these giant chromosomes have been the subject of intensive investigation. The polytene chromosomes are composed of a characteristic sequence of rings or bands that are considered to be the material equivalent of the conceptual Mendelian genes. These bands have served as landmarks in the making of maps and reference systems of the giant chromosomes by Painter (1934); Bridges (1935, 1938), and others. Of the Dipteran order, the Drosophila melanogaster fruit flies have received the greatest attention; the most detailed maps worked out about them. It was obvious during these studies that there are localized areas of increased diameter in the chromosomes, referred to as puffs or Balbiani rings.

In 1952 Beerman showed that these puffs are not random in occurrence but are often specific both to tissues and to developmental stages. It was proposed that puffs are the visible expression of sites of genetic activity at the chromosome level, and this has been confirmed.

Detailed studies on the patterns of puffing activity in the
salivary gland chromosomes of Drosophila melanogaster have been carried out, most notably by Becker (1959, 1962) and Ashburner (1967, 1969, 1970). These studies relate the appearance and size of puffs to developmental time tables, concentrating on the larval and prepupal stages of the life cycle. Also, the effects of various experimental environmental treatments on puffing activity has been the subject of much research with the hope of learning how different environmental factors influence puffing and thus illuminate at least some of the forces that control the behavior of genes.

The first detailed study of abnormal puffing patterns as a result of experimental treatment was by Kroeger (1960), although previously Beerman (1952) had noted the peculiarities in behavior of puffs after cold treatment, and Becker (1959) the appearance of novel puffs after treatment of isolated salivary glands. Subsequently Ritossa (1962, 1963, 1964); Berendes and Holt (1964); Berendes (1965, 1968); Berendes, van Breugel and Holt (1965); and van Breugel (1966) analyzed the puffing reactions to a variety of experimental treatments - especially temperature shocks and anoxia. Beerman and Clever (1964) and Richards (1976 a, b) gave evidence that hormones may control the activity of some puffs. Also, Ashburner (1970 presented evidence that high temperature shocks and nitrogen anoxia induces puffing.
The purpose of the present study is to determine if carbon monoxide influences chromosomal puffing in *Drosophila melanogaster* larvae; if it does, how this transpires. We plan to determine if there is an overt genetic response to a high carbon monoxide environment. If novel puff formation is induced, we propose to illustrate where and when the new puffing activity occurs.

A secondary purpose in doing this thesis is to become more aware of the problems involved in original experimental research, and to gain first-hand experience in the important field of scientific research.

Our thesis was initiated at the end of our junior year when Ross Ronish and Wade Lillegard suggested we continue their ill-fated project. Actual work began several weeks before school commenced in August, 1978. We gathered materials obtainable from Carroll and ordered the remainder. In addition, we commenced our literature review and drew up a protocol. Actual experimentation began the first week of the spring semester of 1979, work having been delayed the fall semester due to medical school interviews and other activities. Our experimentation and analysis of results accelerated from approximately January 8, 1979, until March 13, 1979. As of this point we anticipate further work to verify various suggestions of chromosomal analysis methods that will be included in an addendum to this paper.
II. MATERIALS AND METHODS

The majority of materials were obtained from Carolina Biological supply, and the Biology or Chemistry Departments of Carroll College.

A. Stock

Because Michael Ashburner was so successful with the "wild type" Oregon-R strain of Drosophila melanogaster, we utilized it in our experimentation. As ascertained from Ashburner's writings, this Drosophila strain is homozygous for two deficiencies: (1) terminal area of right arm of chromosome 2, (2) another terminal deficiency of the left arm of chromosome 2.

The flies were maintained at 25°C ± 1°C in culture vials containing 4-24 Instant Drosophila Medium (Blue). Depending upon the number of flies needed for experimentation, a varying number of cultures was easily maintained. We maintained eight culture vials to insure more than adequate numbers of flies for egg laying purposes.

B. Anesthetizing

We utilized the Drosophila Anesthetizer with ether available from Carolina Biological Supply. Included with the anesthetizer were clear instructions as to the proper and efficient use of the
device. The anesthetizer was utilized at various stages which will be indicated later in this paper. It enabled effective immobilization of the flies so that we could expedite sorting and handling.

C. *Drosophila* Life Cycle

An integral part of this experiment was the synchronization of the *Drosophila* larvae. At least a cursory knowledge of the *Drosophila* life cycle is needed in order to understand this synchronization. We adapted the terminology utilized by Ashburner. Of particular interest during the approximate ten-day cycle were the "Ashburner" larva, prepupa, and pupa stages.

In order to understand Ashburner's terminology we had to familiarize ourselves with another life cycle scheme. Under this scheme, there are four distinct phases: (1) egg, (2) larva, (3) pupa, (4) adult. Approximately twenty-four hours after egg laying, the larva hatches and subsequently molts twice. Three instars, stages of larval development, correspond to the periods of larval development before and after the two molts. The pupa stage follows the last larval stage, third instar stage, followed by the adult.

The scheme followed by Ashburner adds an additional stage, the prepupa, to the above scheme. A developing fly is termed a larva up to the time of the eversion of the anterior spiracles and a prepupa (white puparium formation) from the eversion of the
anterior spiracles to the time of head eversion at which time it is termed a pupa. Essentially, the addendum prepupa stage immediately follows the third instar, thus being a substage of the pupa phase of the other scheme.

Our experimentation dealt primarily with the third larval instar stage. Samples of larvae were collected and dissected at 110, 115, and 120 hours following the midpoint of egg collection; these will be explained subsequently. An important time reference point as used by Ashburner was 120.6 ± 2.8 hours from midpoint of egg collection, also referred to as white puparium formation.

D. Culturing and Synchronization

This stage of the experiment is vital in that it insures collection and dissection of larvae of the same approximate developmental stage. The synchronization method that follows was derived primarily from Ashburner with some alterations, and will be presented in a general fashion (important steps and timing with respect to egg collection midpoint) and a more specific fashion (actual days and times of steps as derived from our notes). The purpose of such a dual presentation is to emphasize both the importance of synchronization and the complexity of the timing involved.

Adult flies were collected from the culture vials, anesthetized,
and then placed within petri dishes containing 4-24 blue medium for egg collection. As determined by Ronish and Lillegard, approximately twenty flies per dish was optimum with 24 petri dishes being utilized. After a four day period, to insure the laying of adequate numbers of eggs, egg collection commenced (Monday, 4:40 p.m. - 5:30 p.m., midpoint 5:00 p.m.) being confined to a maximum one hour period. Eggs were placed in petri dishes containing a medium of 3% agar to which 5% ethyl acetate had been added. Approximately 10 to 12 plates were employed to accommodate the 10 to 12 people needed to insure optimum collection. A high density of eggs per plate was found best suited for more convenient subsequent larval collection. This number of people was found necessary also for larvae collection and larvae dissection. The eggs were best collected by using a dissecting microscope and a sharp dissecting needle.

Twenty-two hours after the midpoint of egg collection (Tuesday, 3:00 p.m.) all first instar larvae were removed and destroyed as rapidly as possible (approximately 15 to 30 minutes). Two hours later (Tuesday, 5:00 p.m.) those larvae that had hatched subsequent to the destruction of the first instar larvae were collected and placed in petri dishes containing yeast-glucose medium {30 grams agar, 100 grams D-glucose, 100 grams dried viable yeast, 6 ml of 10% mold inhibitor; all within 1 liter of H₂O - Ashburner). Upon the advice of
Ronish, a minimal amount of medium was used in each plate to insure easier larval collection, and ten larvae were placed in each dish. This number was chose to insure adequate nutrition which enhanced normal chromosome maturation.

Approximately fifty dishes were prepared for the collection of larvae. Of these, thirty-seven dishes were utilized, thereby creating the possibility of approximately 370 samples. This number was collected to facilitate adequate sample sizes. As ascertained from the work of Ashburner, Ronish, and Lillegard, 20 to 30 samples per control and carbon monoxide group would be adequate material. Hence, for the 110, 115, and 120 hour groups, a minimum number of about 160 to 240 flies would be needed and comfortably supplied by the potential 370.

E. Dissection

After the required incubation times, as determined from midpoint of egg collection, larvae were dissected at 110, 115, and 120 hours (Saturday, 7:00 a.m., 12:00 noon, 5:00 p.m.). Dissection was accomplished using a dissecting microscope and two dissecting needles. The larvae were placed in a petri dish containing a hypotonic solution of sodium chloride (7% NaCl). One needle was placed at the approximate midpoint of the larva and the other just behind the mouth parts. Pulling both needles apart gently and smoothly, the
head with trailing salivary glands was removed. The glands were then separated and placed in fixative (3:1, ethanol:propionic acid) where the glands could be stored indefinitely at 0° C. At no time were the glands allowed to dry out. Dissection was terminated after a one-half hour interval (Saturday, 7:30 a.m., 12:30 p.m., 5:30 p.m.). This was the procedure followed for the control groups.

The experimental group, carbon monoxide treated, was handled in the same way except for the gassing procedure. As an example, the 110 (7:00 a.m.) group was handled as follows: forty-five minutes before dissection (6:15 a.m.) the larvae arbitrarily chosen for gassing were placed in a jar with a removable lid. The lid contained appropriate inlet and outlet glass tubing for introducing carbon monoxide and exhaust. The lid was sealed to the jar with stopcock grease. The carbon monoxide, approximately 99% pure, was contained in a tank with a regulator. The carbon monoxide was run into the jar for an adequate time (approximately 15 minutes) to completely "flush" the jar. All tubing was then clamped to obtain a seal. At the end of the forty-five minute period, the larvae were removed and dissected coordinately with the controls.

A problem arose at this point in the experimentation. Our supply of carbon monoxide ran out, probably because of inadequate filling by Airco of Vancouver. For the 115 and 120 hour groups, we improvised
and produced carbon monoxide in the following manner: carbon dioxide was forced through a heated glass cylinder containing carbon. This method we derived from *Chemical Principles and Properties* by Michell J. Sienko and Robert A. Plane.

**F. Staining**

The glands were removed from the fixative and placed in a drop of stain for varying time lengths (approximately five minutes for acetocarmine). Untimed larvae were used to determine the best stain. Four different stains were tried with varying degrees of success. Acetocarmine from the Carroll College Microbiology stock yielded the best results, with good banding contrast. This stain was utilized in our final work with the actual experimental chromosome material. Giemsa stain, with and without trypsin treatment of the glands, was tried with no successful results. Produced were blue masses of material with no visible chromosomes. The stain suggested by Ronish and Lillegard (1:6 mixture of 1% carmine to 1% orcein, both in ethanol, and diluted with 50% proprionic acid 1:1) was also unsatisfactory. The fourth stain, carmine, also provided unsatisfactory results.

**G. Slide Preparation/Chromosome Spreading**

As with the determination of the most effective stain, untimed experimentations were necessary to discover the most effective chromosome spreading technique. Untimed larvae salivary glands were
dissected, fixed, and subjected to various procedures over approximately seven weeks. We had barely adequate results. The following is a listing of a few of the many techniques tried. The last listed is the one we eventually adopted.

(1) This technique was suggested in the Carolina *Drosophila* Manual. Stain; place cover slip; and try various squashing pressures with blunt instruments or the ball of your thumb. We obtained little spreading with this procedure.

(2) Karen Streets of Shodair Children’s Hospital suggested using heated (with Bunson burner) or cooled slides (with dry ice or refrigerator) and dropping the glands from a height of approximately one to two feet to enhance spreading. We tried virtually all possible combinations of these suggestions but again had little spreading. One item to note is that Karen Streets deals with human chromosomes and karyotypes; these were procedures performed with human materials, not *Drosophila*.

(3) Dr. James J. Manion suggested the most successful procedure, the one we eventually utilized with our experimental material. The glands were removed from the fixative and placed in stain and then the cover slip was applied. Next, the slides were heated mildly with a flame and then squashed with a slight lateral motion of the cover slip. The slight lateral motion tended to enhance spreading.
Before finally adopting this procedure for our experimental material, we applied it to the various procedures mentioned earlier with no better results.

Further, both of us tried contacting outside sources concerning *Drosophila* salivary gland chromosome spreading. Michael Ashburner is located in England; Beerman and Clever, experts on chromosome puffs, are also in Europe. These persons' locations made contact out of the question. Dr. Manion suggested contacting some "West Coast" authorities. Overtures were made at the University of Oregon but to no avail. While in Chicago one of us contacted the Genetics Department at the University of Chicago. A *Drosophila* geneticist there, Dr. Throckmorton, stated that our problem of spreading was the result of inappropriate fixative. However, all of our material had been fixed; there seemed to be no way of reversing the fixative's ill effects.

At this point the decision as to whether to prepare permanent slides or temporary slides was made. We found that the albumin solution applied to future permanent slides (*Drosophila* Guide by Demerec and Kaufmann) tended to reduce spreading greatly. Therefore we abandoned the permanent slide route. Instead we prepared temporary slides by applying clear fingernail polish around the edge of the cover slip to prevent drying.
During the preparation of slides of the 110 hour controls for photography, we placed the slides at 0°C for storage. At this temperature, assumed to be safe due to storage of the glands in fixative at 0°C., the chromosomes were destroyed. However, 110 hour controls are well documented in Ashburner's works. The 110 hour carbon monoxide slide group was subsequently placed for storage in the chilling section of a refrigerator with favorable results.

H. Photography

We next took our 110 hour carbon monoxide slides to Shodair Children's Hospital where we utilized their photographic equipment to record the chromosome spreads on film. The slides were photographed at 1000x magnification with a 35 mm camera. A green light filter was used to enhance banding contrast. Development and blow-ups were achieved, using Shodair's developing equipment. One must be careful during photography that the slides do not dry out due to the intensity of heat produced by the lighting equipment of the microscope.

The photographs were then examined to determine the effect, if any, of carbon monoxide on puffing patterns.
III. RESULTS

Microscopic observation and the examination of over forty photographs revealed no great induction of puffs in the 110 hour carbon monoxide treated group in comparison to Ashburner's 110 hour controls. Several of the photographs are shown in Plates I through IV. In Plate I the puffed area marked by the X is probably puff 58 D, E which is one of the two largest autosomal puffs normally found in the 110 hour group according to Ashburner (1967). The banding pattern is not well defined but the outline of the chromosome indicates it is the distal end of the arm 2R. The puff is located in the correct position and approximates the correct size. On Plate II the distal end of arm 3L can be identified due to shape and banding pattern. Plates III and IV show other photographs with other sections of unidentified chromosome. In these and all other photographs examined, no novel puffs were identified.

The histograms presented in Figures 1 through 7 refer to Ashburner's 110 hour control groups and indicate the normal puffing pattern. A close look at Ashburner's chromosome control histograms 1 through 7 readily reveals the basis for our first statement of our results. The autosomal puffs (Histograms 1-4) and X-chromosome puffs (Histograms 6-7) are minimal in number and mean size during the 110 hour stage. Only one of the 5 chromosome arms, the X-chromosome arm, shows any
great degree of puffing. Histograms 5A and 5B graphically portray the lack of puffing both in mean size and number in the autosomal chromosomes. The 110 hour period for autosomal chromosomes exhibits the least number of puffs and the smallest puffs by comparison to all other testing periods shown. Hence, through our photographic and microscopic observations of minimal puffing in the 110 hour carbon monoxide treated chromosomes, we concluded, as previously stated, that no great induction of puffs occurred in carbon monoxide treated 110 hour chromosomes.

The 115 and 120 hour groups were discarded as data sources due to uncertainty of the purity of the carbon monoxide produced and the possible presence of chromosome affecting impurities as a result of the improvised method of carbon monoxide generation.
IV. DISCUSSION

It is our contention that carbon monoxide treatment does not influence normal puffing patterns in salivary gland chromosomes of Drosophila larvae. However, the results obtained were far from adequate to conclusively state that carbon monoxide influences puffing in any way. Although the results tend to indicate that there is no great induction of puffs, it is possible that some puffing was induced which was not obvious to us. Unfortunately, all chromosomes in the prepared smears had some areas of entanglement which prevented clear observation and identification of puffs if present. This also prevented mapping localization and identification of all puffs normally present according to Ashburner's work. Therefore, it was impossible to determine if the normal puffing pattern was inhibited.

The results obtained were only from the 110 hour group, which normally shows only slight puffing activity. It is possible that in the 115 and 120 hour groups, which usually show greater puffing activity, that the carbon monoxide may have had a more marked effect on the normal puffing pattern either through induction or inhibition of puffing.

One question that may be asked at this point is: In what fashion, if any, could carbon monoxide influence puffing? It is
possible that carbon monoxide like other substances may act directly on the genetic material by stimulating the chromosomes to greater or lesser activity in the production of enzymes that may enhance the cell's ability to adapt. The chromosomes may respond in a high degree with the induction of puffs or possibly in a more subtle fashion in which no puffing is observed. Another possibility would be that carbon monoxide somehow blocks normal metabolism in some way and this stimulates a genetic response by puffing or without puffing. Still another alternative is that carbon monoxide blocks the normal activity at the chromosome level resulting in inhibition of puffing or inhibition of chromosome activity other than puffing (possibly by blocking enzyme action). Unfortunately, our results are of little aid in deciphering this problem.

Wolsky (1937) has shown that carbon monoxide strongly inhibits oxygen consumption in Drosophila melanogaster pupae. This would produce conditions of anoxia. Indeed, after being subjected to the carbon monoxide treatment, our larvae displayed symptoms of severe anoxia showing very little movement. Anoxia has been shown to induce abnormal puffing patterns with the appearance of novel puffs (Ashburner, 1970). Thus, one might expect the induction of puffing from this rather indirect reasoning alone. However, the anoxia produced in Ashburner's studies utilized nitrogen and not carbon monoxide.
Probably the greatest benefit from our work was the recognition of several problems, and their possible avoidance, involved in using *Drosophila melanogaster* salivary gland polytene chromosomes for study.

One major problem encountered, and never completely overcome, and which more than anything else limited our results, was the difficulty involved in preparing smears which had all arms well spread for easy identification and analysis. A possible solution to the problem was recognized too late for our benefit. This involved the fixative employed. It was suggested by Ashburner that a 3:1 ethanol to propionic acid mixture be used as a fixative. Dr. Throckmorton, *Drosophila* geneticist from the University of Chicago, indicated that this might cause the chromosomes to clump and stick together preventing easy spreading. Instead, a 1:1:1 lactic acid, acetic acid, water mixture was suggested and might be tried in future work. Bridges (1935) obtained adequate results using acetocarmine, both as a fixative and a stain, and this might be more efficient.

Another problem encountered involved use of albuminized slides in preparing permanent smears. This was suggested by Demerec and Kaufmann (1960), Ronish and Lillegard (1978), and several others. From our experience, smearing on the sticky albuminized surfaces produced only macerated rolls of tissue. The best smears were on clean glass slides with slight pressure on the cover slip as previously described.
Drosophila has served as an important tool in examination of the complex relationship existing between the genetic material and the environment. Much research has been done in an attempt to shed light on this relationship. Our work with carbon monoxide was an attempt to contribute to the understanding of this relationship.

Drosophila chromosome analysis requires skill, patience, much work and some luck. The results presented here are far from conclusive. Avoidance of the many pitfalls herein identified should enable future researchers to obtain more detailed and comprehensive data.

Although we obtained little significant data, this thesis did make us aware of the difficulties involved in chromosome work, and created an appreciation of the trials and tribulations generally involved in research work.
Histograms showing the changes in mean size of chromosome arm 2L puffs during the late third instar larval and prepupal stages. The vertical line at 0 h. indicates the time of puparium formation and that at 12 h. the time of pupation.

HISTOGRAM 1
Histograms showing the changes in mean size of chromosome arm 2R puffs during the late third instar larval and prepupal stages

HISTOGRAM 2
Histograms showing the changes in mean puff size of chromosome arm 3L puffs, during the late third instar larval and prepupal stages.
Histograms showing the changes in mean size of chromosome arm 3R puffs during the late third instar larval and prepupal stages

HISTOGRAM 4
5A. The change in mean size of autosomal puffs during larval and prepupal development

HISTOGRAM 5A

5B. The change in the number of puffs present on the salivary gland autosomes during larval and prepupal development

HISTOGRAM 5B
Histograms showing the changes in mean size of X chromosome puffs in male D. melanogaster larvae (110-120 hrs) and prepupae (0-12 hrs)

HISTOGRAM 6
Histograms showing the changes in mean size of X chromosome puffs in female D. melanogaster larvae and prepupae.
PLATE I.

Chromosomal spread of 110 hour larva - Carbon Monoxide exposed
(The black X marks puff 58 D, E on arm 2R.)
PLATE II.

Chromosomal spread of 110 hour larva - Carbon Monoxide exposed
(Distal section of arm 3L is identifiable in these two photographs. It is marked by a black arrow.)
PLATE III.

Chromosomal spread of 110 hour larva - Carbon Monoxide exposed
PLATE IV.

Chromosomal spread of 110 hour larva - Carbon Monoxide exposed
LITERATURE CITED


Beerman, W.: Chromomerenkonsanz und spezifische modifiktionon der chromosomenstruktur in der entwicklung und orgondifferenzierung von *Chironomus tentans*. Chromosoma (Berl.) 5, 139-198 (1952).


* Articles read
LITERATURE CONSULTED


ADDENDUM

As discussed previously, the major factor limiting our results was an inability to obtain chromosome smears with arms well separated for clear examination of puffing activity. Dr. Throckmorton of the University of Chicago attributed this problem to the fixative employed. He suggested that instead of using a 3:1 mixture of ethanol to propionic acid as suggested by Ashburner, a 1:1:1 mixture of acetic acid, lactic acid, and water might be tried. Also, Bridges, in one of his papers, suggested using acetocarmine for 20 minutes to one hour in the fixing process.

Each of these fixing techniques was employed in the preparation of 10 chromosome smears and the results were compared to those obtained using 3:1 ethanol to propionic acid fixative. The acetic acid, lactic acid and water fixative produced slightly better spreading results with one very good spread observed. The acetocarmine fixative failed to reveal any noticeable improvement in chromosome spreading. This fixative produced well-stained chromosomes but the nuclear membrane remained intact, preventing good spreading of the chromosome arms.

In conclusion, it is our belief that in future Drosophila melanogaster chromosome work, best results would be obtained using the 1:1:1 acetic acid, lactic acid, water fixative suggested by Dr. Throckmorton.