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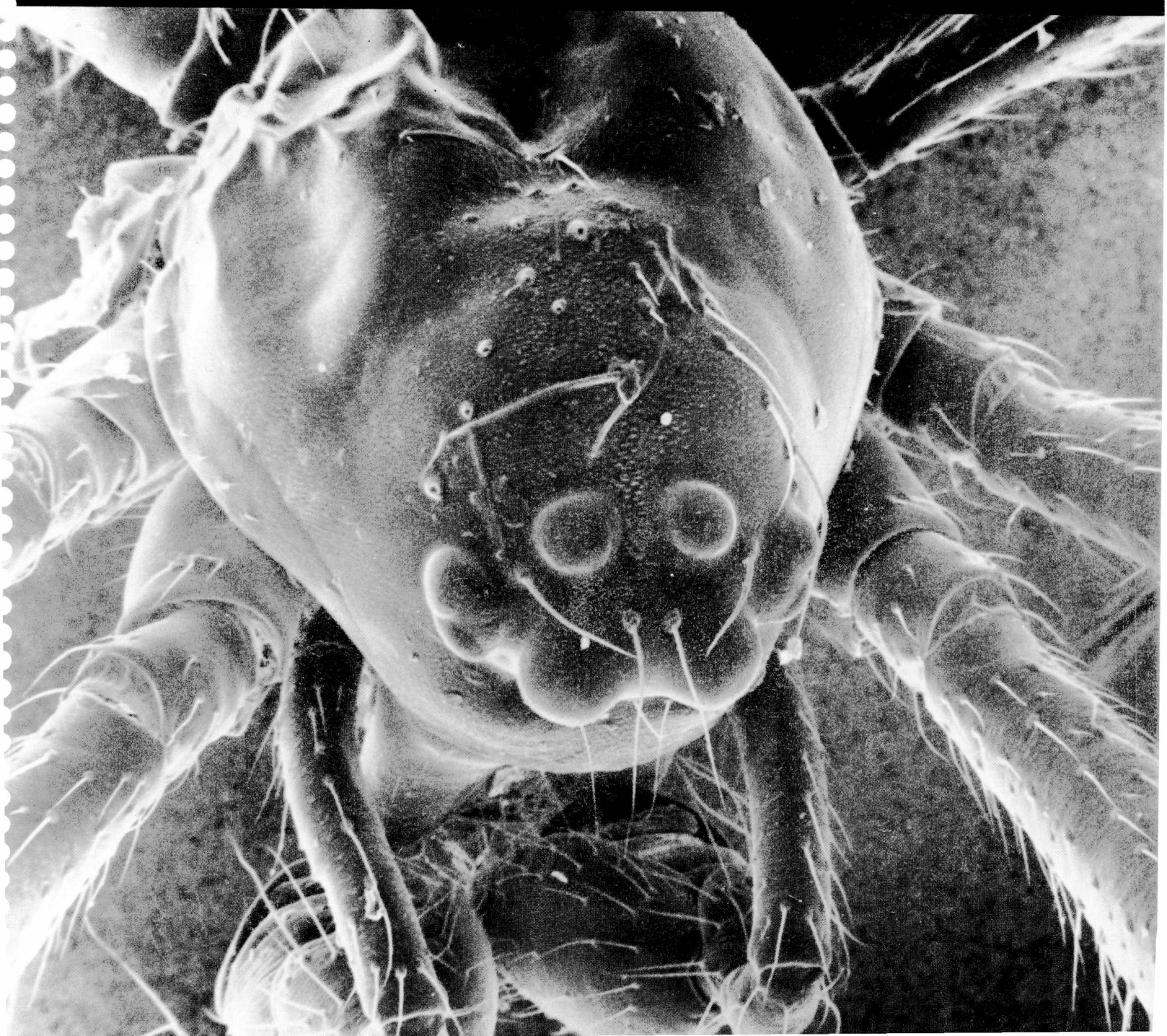

Rhodes College Science Journal

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Cover Photo: Spider SEM by Cynthia Uphoff

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PREFACE

The Rhodes College Science Journal is a student-edited, annual publication which recognizes the scientific achievements of Rhodes students. Founded seven years ago as a scholarly forum for student research and scientific ideas, the journal aims to maintain and stimulate the tradition of independent study. We hope that in reading the journal, other students will be encouraged to pursue scientific investigations and research.

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AN ELECTROPHORETIC STUDY OF GERMAN COCKROACH ESTERASES: THEIR DISTRIBUTION IN BODILY TISSUES AND INHIBITION DUE TO DURSBAN INSECTICIDE

JENNIFER BISHOP

Research supported by a Howard Hughes Medical Institute Grant

ABSTRACT

Esterase allozymes of a resistant strain of the German cockroach, Blattella germanica, were analyzed using polyacrylamide gel electrophoresis. Hemolymph, frass, gut, and fat body tissues were observed to be sources of unique esterases. Inhibition of these esterases due to Dursban incubation of the gels occurred in varying degrees. It is hypothesized that the site or source of pesticide degradation is the cockroach gut.

INTRODUCTION

The problem of insect resistance to pesticides is not a modern one. Observations of chemical resistance in insect species were made as early as 1887, and Melander is credited with the first publication on this subject in 1914 (Perry,1964). The cockroach, notorious for being one of the most repugnant household pests, has been the focus of many scientific studies dealing with insect resistance. Recently, attention has been drawn to the cockroach's resistance to particular types of insecticides, such as organophosphate compounds which have widespread use in commercial pest control. One of the principal questions that has been pursued in this area of research is the examination of the specific biological mechanism of resistance within non-susceptible strains of the cockroach. Although several hypotheses exist that give possible explanations for resisistance in the insect, the precise interaction of organophosphate insecticides with the roach's physiochemical processes is not known. It is believed that roach esterases may play a role in cockroach resistance, but further research must be undergone to give substantial support for this hypothesis.

The purpose of this study was to acquire information on the distribution of the cockroach's esterases within its bodily tissues, and on the effects that an organophosphate insecticide has upon these esterases. The primary questions that have arisen center around the link that has been established between esterase activity and resistance in strains of *Blattella germanica*, the German cockroach, and whether esterases are directly responsible for resistance, and, if so, how they convey this insecticide resistance in the insect (Jones and Bancroft, 1983, unpublished; Burrow, 1989, unpublished).

An esterase is a hydrolase which, through the addition of water, cleaves an ester bond resulting in the formation of an acid and an alcohol (Palmer, 1985). The active ingredient in many pesticides contain ester bonds, and, therefore, it is logical to question the role of esterases in insect resistance (Burrow,1989).

MATERIALS AND METHODS

Four laboratory strains of *Blattella germanica* with varying degrees of susceptibility to Dursban L.O., an organophosphate insectide, were available for this research. Of the four strains, two were determined to be resistant, M.S.U. and Texas A&M, while the two others, Hazard WARF and Johnsons Wax, were identified as susceptible. In order for a strain to be labeled as "resistant" to the insecticide, a significantly higher dose of Dursban was required to kill fifty-percent of the population. This dosage requirement was determined through lethal dosage (LD50) work done by Jones and Bancroft (1983) and Domon and Holden (1989, unpublished). Because I was seeking an understanding of the role of esterase action in roach resistance, the use of the MSU strain ,which proved to be the

most highly resistant of the four, seemed to be an appropriate choice to work with for a pesticide inhibition study. Dursban L.O. is a trademarked product of DOW Chemical Company containing the active ingredient, organophosphorothioate chlorpyrifos (DOW Chemicals U.S.A., 1989).

Polyacrylamide gel electrophoresis (PAGE), using a Hoefer Vertical Slab Gel Electrophoretic Unit, the SE 600, was the method chosen to analyze cockroach esterases in homogenized tissues. Gel polymerization was initiated by ammonium persulfate and accelerated by TEMED . The gels were cast with a 7 1/2% running gel, a 5% stacking gel, and a 1.5 mm comb that produced fifteen sample wells. Recipes and casting procedures for the gels were obtained from Burrow (1989). A constant current was set at thirty amperes for a single slab and sixty amperes for two slabs, while the voltage was allowed to fluctuate. The unit was left to run for approximately two hours and forty-five minutes or until it was visibly evident that the samples had run off the bottom of the gels. It was typical for the voltage to climb as high as 280 volts over this time period. The initial voltages during the runs were normally close to 175 volts.

Electrophoretic runs consisted of a control and an experimental gel. The control gel was stained with alpha-naphthyl acetate and Fast Blue RR for seventeen minutes and then stored in 7% glacial acetic acid. The experimental gel was incubated for two minutes in .1% Dursban, thoroughly rinsed with distilled water, and then stained with the alpha-naphthyl acetate and Fast Blue RR for seventeen minutes. It was also stored in glacial acetic acid.

Gels were analyzed by placing them on a Porta Trace Light Table, and making band measurements used to determine RM (relative migration) values. Each RM value was computed by dividing the distance of the band from the top of the running gel (origin) by the distance of the standard from the origin. A porcine liver esterase standard was used in every run. When the same homogenized sample was repeatedly used in different wells of the same run, the bands with the best resolutions were measured. Photographs were taken of runs in which the bands were dark and distinct enough to appear on film. A Kodak Polaroid Camera was used and set at ss 125 with the f-stop at 32.

Runs were performed with pooled samples of MSU nymphs. Glass homogenizers were utilized for roach whole body homogenizations. Insects were weighed individually and ground in Peacock buffer, which was added in specific amounts according to Burrow's Weight Scale for Homogenate Dilutions (Burrow, 1989). The liquid homogenates were microcentrifuged for five minutes, and, if the samples needed to be pooled, the supernatants were poured together and then re-microcentrifuged for two minutes. This homogenate was mixed with tracking dye on parafilm and micropipetted into the gel wells.

After noting patterns in the inhibition of certain esterase bands on gels of the early whole body sample runs, an attempt to categorize the specific esterase bands according to their tissue sources was made. It was hoped that first, by identifying unique bands from different sources, and then analyzing the inhibition effects of Dursban on these bands, that a certain tissue or esterase could be identified as the possible cause of insecticide resistance. Two tissues, the gut and fat body, were chosen to be analyzed due to the ease of their dissection and their probability of containg high esterase activity (Cook and Forgash, 1965).

Samples of hemolymph and frass were also run on the gels. Hemolymph is thought to contain esterases and to play the role of moving the chlorpyrifos through the body of the roach. Frass, the solid excretion of the cockroach, has been previously used as an indicator of a specific esterase in bollweevil experiments (Jones and Bancroft, 1977), and was therefore believed to offer some sort of esterase profile of the insect. It is worthy to mention that the bulk of the research done by Pam McQuillen involved using the frass and/or the hemolymph of individual roaches to determine if these sources could be reliable indicators of the esterase activity in the whole bodies of the roaches.

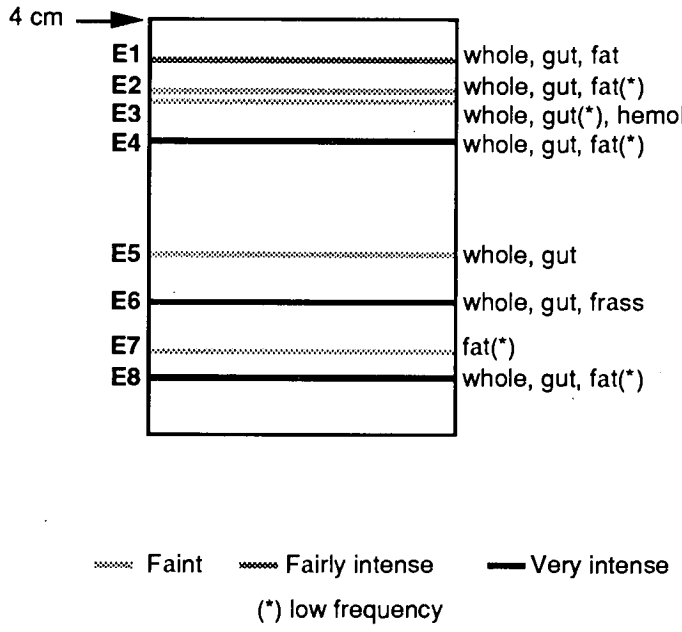
The techniques of Cook and Forgash (1965) were used as guidelines in homogenizing the gut and fat body tissues, and methods of collecting hemolymph and frass were originated in the laboratory. The mixture preparations of all of these four types of samples to be run on the gels were adjusted on a trial and error basis as the results were produced and the experiment progressed. The gut tissue and frass were all pooled samples which were weighed and then homogenized in Peacock buffer according to Burrow's Weight Scale. The samples were microcentrifuged for two minutes, and the liquid was mixed with tracking dye on parafilm. Hemolymph was collected according to a method developed by McQuillen (1989, unpublished), and pooled in a Peacock buffer mixture on parafilm. Fat bodies were

collected, placed on depression slides, ground with a glass stirring rod, and then mixed with tracking dye.

RESULTS

All successful electrophoretic runs produced zymograms which contained two definite regions of esterase allozymes. The slower moving esterases were said to be in the "upper region" and the faster moving esterases were found in the "lower region" Many of the bands were very blurred or so faint that accurate measurement was not possible. RM values and migration distances of certain bands were not easily comparable between runs, and comparisons were done mostly by looking at the esterase pattern relationships between the whole body and the other tissues. The following composite zymogram (Figure 1) is a hypothetical model of the overall esterase pattern suggested by the results of all of the MSU nymphal runs. To the right of each band is written the hypothesized tissue or source from which this esterase originated. The migration distances are approximate from the origin. No definite values could be given for individual bands because the results were of such wide variation. The esterase bands were numbered in order to make discussion easier. Intensities of the bands were also recorded.

FIGURE I



Upper Region

Esterases 1-4: These results were somewhat difficult to interpret because of the close proximity of the bands. The gut seems to contribute the majority of the esterases seen in this region, along with some contributions by the fat body. These allozymes were extremely inhibited by Dursban. They exhibited a fifty to one-hundred percent loss of intensity after pesticide incubation.

Lower Region-

Esterase 5: It is possible that this is an allozyme that occurs in low frequency in MSU nymphs. Burrow found several cockroach esterases which had a low frequency of appearance in electrophoretic runs (1989).

Esterase 6: This very intense band seemed to originate solely from the gut. It is logical that this allozyme also appeared in the roach's frass which is the final destination of many chemicals which travel through the insect's alimentary tract. More electrophoretic research needs to be performed to

acquire information on esterases which are found in frass. Esterase 6 was inhibited fifty-percent or less from the Dursban.
Esterase 7: This allozyme band was observed in the fat tissue, but did not appear on the whole body profile runs. It appeared as a faint band which was inhibited up to seventy-percent by Dursban.
Esterase 8: This is an intense band seen in gut and fat tissue runs and was also reflected in the whole body results. Dursban inhibited it by fifty-percent or less. This band was slightly less intense than Esterase 6.

DISCUSSION

The electrophoretic results clearly suggest that individual tissues within the cockroach are the source of specific and unique esterases. The organophosphate insecticide, Dursban, inhibited certain esterases in varying degrees. Together, these results lend a clue as to how the pesticide may physiochemically react in vivo in resistant and susceptible strains of Blattella germanica.

The modes of entry and action of organophosphate pesticides for the cockroach are not known. Dursban L.O., termed as a contact insecticide, is generally assumed to enter the roach by means of absorption through its cuticle. Along with this assumption occurs the idea that the hemolymph begins the transportation process of the pesticide through the insect's body. Cornwell (1968) suggested a different hypothesis in writing that liquid pesticides which dry on surfaces (one type of contact insecticide) are picked up by the insect on its cuticle and then ingested via the mouth during grooming. If this were true, then the pesticide would be directly entering the alimentary tract. Several facts about the cuticle layer could easily lend support to the idea that Dursban L.O. may be ingested during grooming, rather than absorbed through the cuticle. The roach cuticle is water-proofed by a waxy layer, and Dursban L.O., being a water emulsified formulation (Dow Chemicals, U.S.A.), might have problems permeating this barrier. The cuticle seems to be more easily penetrated by oil-based compounds which have a more rapid toxic action (Cornwell, 1968). The main reason why the mode of entry became of great interest was the idea of determining the exact site of the detoxification or degradation of the pesticide in the body of the cockroach. If the mode of entry of the pesticide were known, one might be able to hypothesize the pesticide's pathway through the roach, and this could lend clues as to the most likely sites of detoxification in the insect.

During analysis of the zymograms, certain bands seeemed to stand out more than others. The two intense esterase bands consistently observed in the lower region, Esterases 6 and 8, were of special interest. Of all of the allozymes, these esterases were the least inhibited by Dursban incubation. It could be hypothesized that these esterases degrade the pesticide to a sub-lethal level in the roach without depleting their own supply of esterases to a lethal level. Esterases 6 and 8 were both observed to be present in the gut tissue, and this led to the belief that the site of pesticide degradation was in the gut. This could also support Cornwell's statement of pesticide ingestion, although the chemical would not necessarily have to enter via the mouth in order to reach the gut.

Several studies have been done which suggest that the gut, or gastric esterases, are involved in organophosphate inhibition action. Calhoun (1960) treated American cockroaches with tri-orthyl-tolyl phosphate (TOCP) and noted that symptoms of severe gastric malfunction were observed after only twenty-four hours, although there was no sign of significant neural impairment even after several weeks. Cook, Nelson, and Hipps (1969) stated that it was not unreasonable to postulate that these enzymes were involved in the action of TOCP on gastric function, knowing the cockroach contains a large complement of esterases (Cook and Forgash, 1965).

If a single allozyme is indeed the key factor in cockroach pesticide resistance, it is not known if the cockroach is resistant to pesticides because it has an overabundance of an esterase common to all strains of cockroaches or if a unique esterase is responsible for resistance. Mouches et al. (1986) hypothesized that an amplification of an esterase gene was responsible for the resistance in *Culex quinquefasiatus*, yet both qualitative and quantitative differences in esterase content have been observed in insects (Dauterman and Hodges, 1978; Burrow, 1989). Georghiou stated that enzyme modifications play a very important role in resistance to insecticides (1978). Studies have also suggested that there may be more than one resistant mechanism in insects (Oppenorth, 1959), so it is not known how applicable different insect studies are in the research of the resistance which has

developed in a specific species.

Although these research results do not illustrate a precise role of esterases in cockroach resistance, they are of significant value in the journey towards the understanding of the biochemical mechanisms which control insect resistance. This study has shown that hemolymph, frass, the fat body, and the gut tissue have individually distinctive patterns of esterases in the MSU nymph, and several esterases are present in more than one tissue. The two intense bands, Esterases 6 and 8, of the lower region consistently displayed less inhibition due to Dursban incubation than other esterase bands. From these results, I would hypothesize that the site of degradation or detoxification of Dursban is located in the gut, but further research must be undergone in order to more accurately depict the distribution of esterases in the German cockroach and the inhibitory effects that Dursban has upon these esterases.

ACKNOWLEDGEMENTS

I would like to acknowledge Josh McCanless, Steve Domon, and William Holden for their previous research efforts which introduced me to the puzzling question of roach resistance, and Jennifer Burrow for her wonderfully informative paper which served as a guideline throughout my research. I would like to thank Keith Robinson and Pam McQuillen, my fellow summer researchers, for their ever-present enthusiasm and willingness to help and Trey White for his aid in graphic design. Finally, I would like to thank Dr. B.R. Jones for his continual patience and encouragement.

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A SYSTEMATIC STUDY OF THE SUBFAMILIAL CLASSIFICATION OF THE RUBIACEAE OF SHELBY COUNTY, TENNESSEE

WANDA BROYLES

Research funded by the PEW Midstates Science and Mathematics Consortium's
Summer Fellowship Program (1989)

ABSTRACT

I have studied morphological characters of the following species: Cephalanthus occidentalis, Diodia teres, Galium aparine, G. circaezans, G. obtusum, G. pilosum, G. triflorum, G. uniflorum, Hedyotis caerulea, H. crassifolia, Mitchella repens, Oldenlandia uniflora, Sherardia arvensis, and Spermacoce glabra. These species' characters were used to represent their genera. Calyces, corollas, leaves, seeds, and fruits were extracted from herbarium sheets or collected from plants growing wild. They were observed and photographed under an ISI-MiniSEM. The flavonoids of Galium, Sherardia, and Hedyotis were analized. From my observations and the flavonoid analysis results, a binomial data table was established for the eight genera where 0=common and 1=advanced for several characters. This table was sorted by computer programs into the most parsimonious trees. The computer generated trees were then compared to the subfamilial classification given in Bentham and Hooker's Genera Plantarum.

INTRODUCTION

Cephalanthus, Diodia, Galium, Hedyotis, Mitchella, Oldenlandia, Sherardia, and Spermacoce are the only genera of the Rubiaceae family located in Tennessee. (Wells and Sharp). According to Bentham and Hooker's Genera Plantarum (1837), these genera are divided into two series and five tribes (**See Table 1**). This classification was tested by Bremekamp (1966) who requested that even more research be done. Due to availability only the eight genera listed above could be studied. Oldenlandia is kept as a genus (instead of Hedyotis) for my own curiosity to test its being united with Hedyotis. Also, Genera Plantarum was written before the union of the two.

PROCEDURE

Collection of Specimens

Most plant specimens were collected from the herbaria of Memphis State University and Rhodes College. The remaining specimens were collected on the campus of Rhodes College from the Rubiaceae members growing wild there.

Flavonoid Analysis

Leaves were dried, crushed, and soaked in 85% methanol for at least two days. The leaf parts were then filtered out and the solution was concentrated by evaporating much of the methanol and water. Chromatagrams were dotted with the solution. One edge of each chromatagram was placed in TBA until capillary action and gravity separated the flavonoids horizontally. The adjacent edge was immersed in 15% acetic acid which separated the flavonoids vertically. The Rf values were measured. The separate flavonoids were cut out and resoaked in 100% methanol for 24 hours. The solutions were then run through columns of glass wool, sand, and sephadex in order to remove all paper material. The flavonoid solutions were then re-concentrated and spectra were run. The spectra graphs determined that Galium and Sherardia have flavonols (peak on or after wavelength 350), while Hedyotis has flavones (peak before wavelength 350).

SEM Photography

Plant parts were stored in FAA until they could be prepared for observation. The plant tissues were run through solutions with increasing percentages of ethyl alcohol. They were then submerged in 100% acetone and critical point dried. The tissues were then mounted on stubs and sputter coated.

Another process was tried in which tissue was dipped in hexamethyl-disilizane (HMDS) instead of being critical point dried. HMDS caused considerable collapse of cells.

Photographs were taken on an ISI-MiniSEM. During my research period I learned how to operate the SEM sufficiently by myself. Photos were used to observe minute morphological characters.

Analysis of Data

A binomial data table was created using morphological characters and the flavonoid results (**See Table 2**). In this table 0=common and 1=advanced. The data was analized by three computer programs: Sokal, Penny, and Dollo. The order of the data can inhibit the resulting trees; therefore, four different orders, allowing each genus to be at the beginning, middle, and end, were run through each program. The trees created with the least number of inversions will be compared to Bentham and Hooker's classification.

RESULTS

Of all the trees created three took 19 reversions and one took only 18. All other trees required 34-37 reversions. Since these trees used the least number of inversions they will be used for comparison with Genera Plantarum. All four of these trees were created by the Dollo program which is based on one simple idea: parsimony. The trees are arranged so that characters evolve only once (if possible with the given data). The trees are pictured in **Figure 1a-d**.

Series

Only one of the four trees placed Series A and Series C together. The remaining three intermix the two series together.

Tribes

The Galieae tribe held true with four out of four trees pairing Sherardia and Galium together. One major change occured. Diodia also paired with the Galieae in all four trees. Diodia is a member of Spermacoceae according to Genera Plantarum.

The Hedyotideae, Hedyotis and Oldenlandia, were paired in three trees.

Naucleeae, Cephalanthus, occured alone.

The Spermacoceae, Diodia and Spermacoce, did not pair up in any tree. However, Mitchella (Anthospermeae) and Spermacoce paired in three as did Hedyotis and Spermacoce.

DISCUSSION

Before the results can be discussed, one must consider the effects of using so few species to represent a genus and the validity of a computer derived tree. No doubt more species would better represent their genera and greatly increase the correctness of this study. In the next semester I hope to obtain the parts of more species from around the world. As for the computer generated trees, I believe that computers can do a great job of compiling data. The only flaw is that not all possible orders of the eight genera could be run due to the incredible amount of time that would take.

According to the results, a few of Bentham and Hooker's placements must be questioned. Cephalanthus appears to be least related to the other genera and not a member of Series A. Diodia and Spermacoce show no evidence of being in the same tribe. Diodia instead paired with the Galieae. The idea of series must be revamped as the genera do not pair in a way that forms two distinct groups.

With these findings I hope to do more extensive research which will form a more valid and parsimonious classification for these and other genera.

ACKNOWLEDGEMENTS

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Table 1

	SERIES	TRIBE
Cephalanthus	A	Naucleeae
Hedyotis	A	Hedyotideae
Oldenlandia	A	Hedyotideae
Mitchella	C	Anthospermeae
Diodia	C	Spermacoceae
Spermacoce	C	Spermacoceae
Galium	C	Galieae
Sherardia	C	Galieae

Table 2

	Cephalanthus	Hedyotis	Oldenlandia	Mitchella	Diodia	Spermacoce	Galium	Sherardia
flavonols	?	1	?	?	?	?	0	0
no hairs on corolla	?	0	0	1	0	?	0	0
papillae on corolla	?	0	0	1	0	?	1	1
leaves < 3 cm	1	0	0	0	0	1	0	0
hairs on leaf	1	0	0	1	0	1	0	0
no uncinate hairs on fruit	0	0	0	0	0	0	1	0
not craterform seed	0	1	?	0	0	0	1	0
smooth seed surface	0	1	?	0	0	1	1	0
not large cotyledons	1	0	?	0	0	0	0	0
no rough seed surface	0	1	?	0	0	1	1	0
not > 2 seed/fruit	0	1	1	1	0	0	0	0
no hairs on fruit	1	1	0	1	0	0	0	0
no papillae on fruit	0	1	0	0	0	0	0	0
no straight hair on fruit	0	0	1	0	1	1	0	1
no hair on calyx	0	0	1	0	1	0	1	1
no straight hairs on calyx	0	0	1	0	1	0	0	1
no uncinate hairs on calyx	0	0	0	0	0	0	1	0
raphides present	1	0	0	1	0	0	0	0
not 4-6 whorled leaves	0	0	0	0	0	0	1	1
no hairs on leaf surface	0	0	0	0	1	0	1	1
long flower tube	?	0	1	0	0	?	1	0
no papillae on leaf	0	0	0	0	0	1	0	0

0=common and true 1=advanced ?=unknown

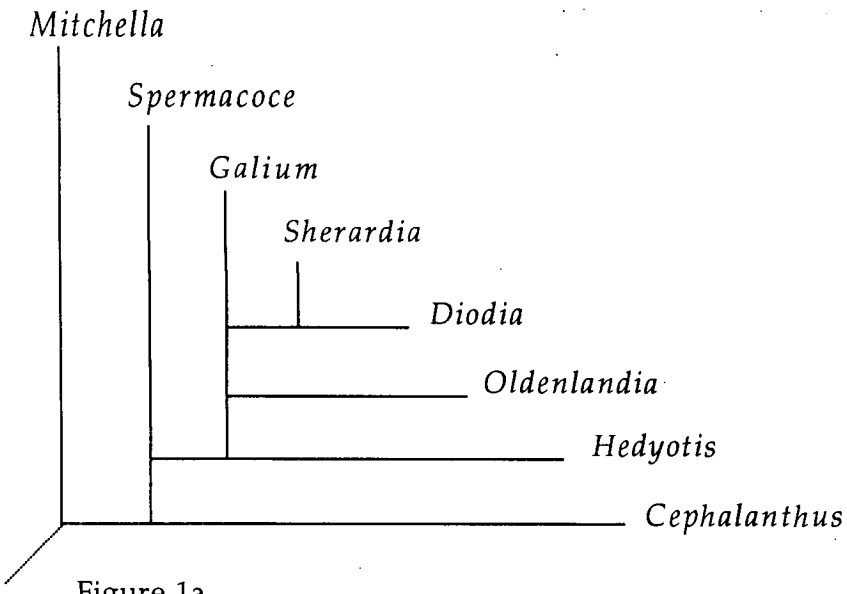

Figure 1a.

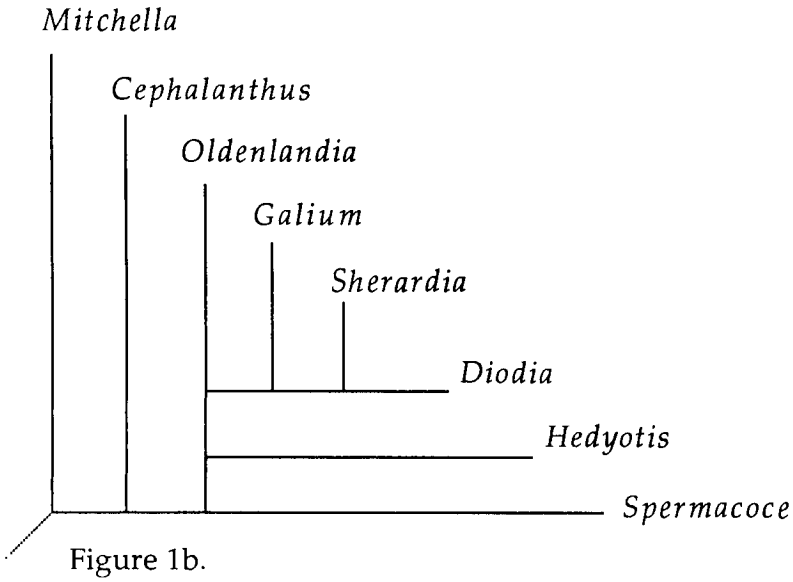

Figure 1b.

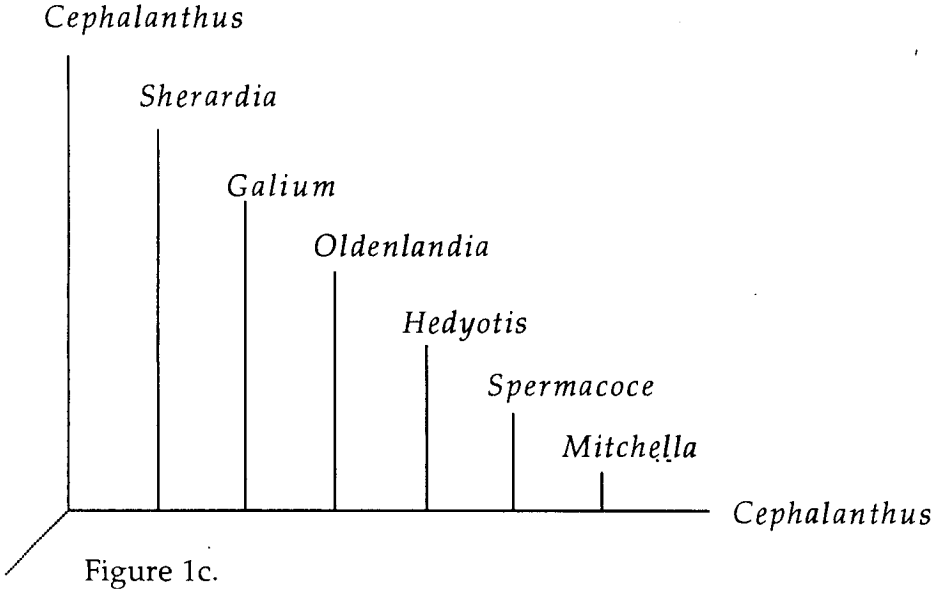

Figure 1c.

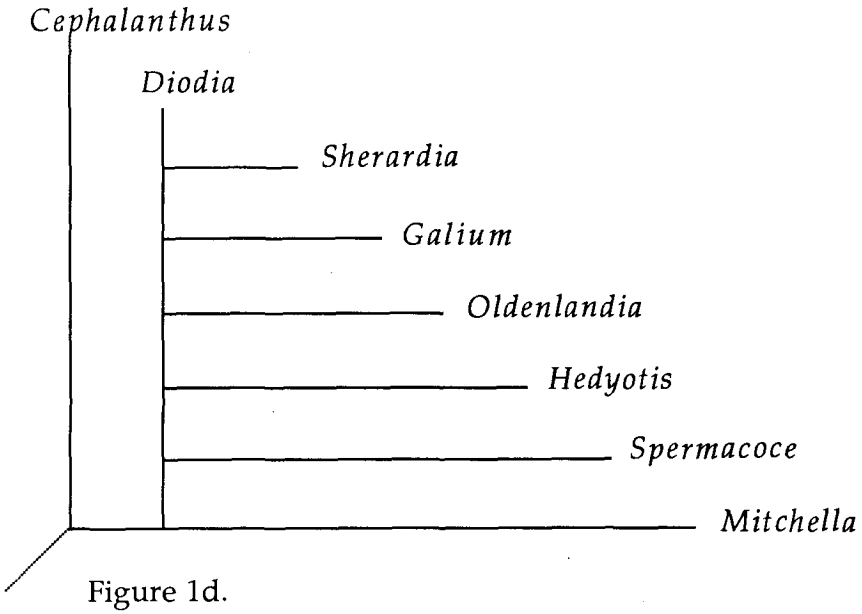
Cephalanthus
Diodia
Sherardia
Galium
Oldenlandia
Hedyotis
Spermacoce
Mitchella

Figure 1d.

THE STRUCTURAL BASIS OF AN ALTERED PATTERN OF DESENSITIZATION IN A MUTANT FORM OF THE TURKEY ERYTHROCYTE BETA-ADRENERGIC RECEPTOR

Mary Chervenak

ABSTRACT

This research is concerned with the structural basis of an altered pattern of desensitization in a mutant form of the turkey erythrocyte beta-adrenergic receptor. Unlike mammalian receptors, avian receptors typically do not demonstrate a significant inhibition of function known as desensitization when exposed to an excess of agonist. A clone isolated from cDNA of the turkey beta-adrenergic receptor, however, has been found to behave like a mammalian receptor when in the presence of an agonist. This alteration in receptor function can be explained in part by the size of the receptor protein. The mutant protein is nearly 10 kDa shorter that the protein expressed by the wild type DNA. Because the mutant turkey receptor is comparable in size to a normal mammalian (hamster) receptor, both of which desensitize, it has been surmised that the missing portion of the mutant turkey receptor DNA is linked with the avian receptor proteins, the sequence of the DNA responsible for the desensitization capabilities of an avian receptor can be determined.

DISCUSSION

The beta-adrenergic receptor, located on the cell surface, is a receptor for epinephrine and norepinephrine. The receptor activates adenylate cyclase by regulating the function of the GTP-binding regulatory protein G_s. In catalyzing the binding of GTP to the regulatory protein, the receptor activates G_s enabling it to bind to adenylate cyclase and stimulate adenylate cyclase activity. Adenylate cyclase is responsible for the production of cAMP, which monitors both cell-specific and basic housekeeping functions (Figure 1). This account, however, is concerned less with the binding specifics of the receptor and more with the receptor's ability to desensitize during continuous stimulation.

When a biological signaling system is exposed to agonist, the system becomes increasingly less responsive over a period of time (Figure 2). This loss of function, or desensitization, can be expressed by the system in a number of ways. One common method of desensitization involves the disabling, destruction, or relocation of receptors. The apparent loss of receptors on the cell surface is known as down regulation, but avian erythrocyte receptors, such as the turkey erythrocyte beta-adrenergic receptor, do not down regulate.

Dr. John Perkins, a dean and research professor at the University of Texas, Southwestern Medical Center, was interested in the desensitization patterns of mammalian and avian receptors. A year ago, while performing comparative desensitization experiments with mammalian and avian erythrocyte receptors, Dr. Perkins noticed the previously mentioned observation: mammalian receptors do desensitize; avian receptor do not. Using previously cloned cDNA from the turkey erythrocyte beta-adrenergic receptor, he transfected the DNA into L-cells and selected several clones. Nine of these clones behaved nornally, that is, they did not desensitize, but the tenth clone down-regulated like a mammalian receptor.

The proteins resulting from expression of both the normal and the mutant receptor DNA were sized on an SDS gel. The notable difference between the normal and the mutant turkey beta-adrenergic receptor proteins is that the mutant receptor protein is approximately 20 kDa shorter that the normal receptor protein. This fact is effectively illustrated by the electrophoretic mobility of the proteins on the SDS gel. A protein expressed by normal beta-adrenergic receptor DNA L-cells is about 50 kDa

while the protein expressed by mutant DNA migrates within the range of 33 to 35 kDa.

Because the protein expressed by the mutant DNA was considerably shorter than expected , one or several alterations must have taken place within the cDNA coding for the receptor. To understand these structural changes, a brief description of the G-protein binding receptor is necessary. A G-protein binding receptor spans the bilayer membrane seven times, with the N-terminus outside the cell and the C-terminus inside the cytoplasm (Figure 3). The possible sites for alteration within the DNA of the protein include the N-terminus, the internal section, and the C-terminus. Because the N-terminus consists of no more than 40 amino acids, and is therefore very short, a significant deletion is not likely to occur. The N-terminus is also located outside of the membrane and is not much involved with desensitization capabilities. Although the internal region covers a major portion of the protein, a large deletion in this region is again unlikely. With every mutation, a 66% chance exists that the mutation will cause a destructive frameshift that deactivates the receptor. The mutant receptor, however, is active, which is evidenced by the desensitization capabilities. The final region of the protein, the C-terminus, is a reasonable guess at the place where the deletion may have occurred. The C-terminus has been implicated in other experiments as being at least partially responsible for desensitization, and because the receptor desensitizes, a C-terminus deletion seems to be a reasonable explanation for the mutation.

Two possibilities have been proposed as explanations for the change within the C-terminus (Figure 4). The first possibility considered was a point mutation causing a STOP codon insertion with the DNA coding for the receptor protein. This mutation would result in the premature termination of the protein as it is being translated, so the protein would be shorter and lacking its C-terminus. The second possibility considered was the idea that the receptor DNA may not have been fully incorporate into the host cell genome during the cloning process. During translation, this flaw in recombination would result in read-through into the L cell genome (the host genome) until a random STOP codon is recognized. The protein produced from the incorrectly incorporated DNA would be shortened and without its C-terminus. In place of the C-terminus would be a series of random amino acids read from the host genome that have nothing to do with the receptor protein. This string of "junk" amino acids is terminated only when a STOP codon appears in the host genome code.

A relatively new technological development, the polymerase chain reaction (PCR), was used to explore the nature of the receptor DNA in both the normal and the mutant forms. By amplifying the C-terminus region with the two bracketing primers, a great deal of information about the region could be gained. The PCR technique is an *in vitro* method for enzymatic amplification of a specific DNA sequence. A pair of non-complimentary sense/anti-sense primers that flank the region to be amplified are used. These primers anneal to opposing strands of DNA. Because an excess of primer is present throughout the reaction, the formation of the primer-template complex will be more probable than the reassociation of the two DNA strands when the temperature is lowered after denaturation. The PCR process has three steps that proceed cyclically: 1. The denaturation of the double-stranded DNA at 94 degrees Celcius, 2. The annealing of the primers to the template at 37 Celcius, and 3. The extension of the primers using a heat-stable DNA polymerase at 72 Celcius (Figure 5). The newly created DNA is again denatured at a high melting temperature, the primers reannealed, and the cycle repeats. With each cycle, some strands that are synthesized extend beyond the region contained within the primers, but generally this "long product" is unimportant because the amount of "short product" is overwhelming. DNA is accumulated exponentially, thereby producing microgram quantities from a minimal amount of starting material. The PCR experimentation regarding the beta-adrenergic receptor provided sufficient preliminary data to allow for the assessment of possible alteration within the DNA coding for the mutant receptor.

The PCR stradegy involved using two pairs of primers made up against the wild-type receptor: two located in the DNA near the C_terminus of the protein and the two internal primers. The first experiment used primers PRC1 and PRC2, which fall within the region of DNA corresponding to the C-terminus of the protein (Figure 6). The DNA of untransfected cells (the DNA of cells that supposedly contain no vestige of the receptor) and of cells expressing mutant or wild-type receptor was amplified with the two primers. The DNA was transferred to a nitrocellulose filter, and the filter was hybridized with an end-labeled probe. The significant feature of the resulting autoradiogram (Figure 7) is that no band appears in the lane containing the mutant receptor DNA (LSVt22), as expected,

because the normal; receptor should have a C-terminus. This suggests that the region that codes for the C-terminus in the mutant has somehow been changed or eliminated because nothing was amplified during PCR. There was however two problems with the experiment. A double band appeared in both the lane containing the DNA from the untransfected cells and the lane containing the DNA from the wild type cells. There are 453 basepairs between primers PCR1 and PCR2. The upper band corresponds to 430 basepairs and the lower band corresponds to 380 basepairs. The band appearing in the "untransfected lane" are not bright, but they are of the same molecular weight as the bands in the wild type lane. No good explanation, barring cross-contamination of the DNA samples prior to amplification, exists for the appearance of either the double band or of the presence of these double bands in the lane containing untransfected DNA.

As a control to confirm the reliability of the initial DNA prep, the PCR amplification experiment was repeated using the two internal primers PCR4 and OL373 (Figure 6). On the autoradiogram (Figure 8), bands appear in the lane containing the LSVt22 mutant DNA and in the lane containing the positive control (In this case, the positive control was a plasmid). This suggested that the DNA of the mutant and the wild type receptors shared similarities, and therefore, no random internal deletions had occurred in the DNA of the mutant receptor. Again, however, the inexplicable double bands appear in both lanes.

Although the data in inconclusive, enough preliminary information was collected to continue experimentation with the LSVt22 mutant receptor. From the PCR results, the conclusion that the mutant DNA had been somehow rearranged and shortened upon acceptance into the host cell genome was drawn. Because the actual size of the mutant receptor was unknown and the importance of the genomic tail to the capabilities of the receptor was yet unassessed, the next experimental concern was to determine the sequence of the DNA coding for the mutant protein. To decifer the sequence of the DNA, and to determine exactly what pieces of code were missing and changed within the DNA, a genomic library was constructed. Because the turkey erythrocyte beta-adrenergic receptor has a well-characterized sequence of DNA, the construction of a total genomic library is not required; a size-selected genomic library is smaller, more manageable, and therefore more efficient. A normal receptor is 1.8 kilobases long with an expression region of approximately 1.5 kilobases. The mutant receptor is shorter than the normal receptor, and so its expression region falls within the expression region of the normal receptor. An ncoI restriction site exists directly before the initiation codon of the receptor. By cutting with pairs of nucleases that do not cut within the receptor, one enzyme, when coupled with ncoI, should give a suitable sized fragment with a minimal amount random genetic code. Because the size of the DNA fragment is limited and characterized in this fashion, the chances of finding a positive clone for sequencing purposes are markedly improved.

Although this project was concluded without successfully sequencing the code for the mutant receptor, the data collected supports the conclusion that a significant change has occurred in the mutant receptor DNA, a change which is probably the result of some form of translocation of the DNA upon incorporation into the host genome. With further experimentation, the sequence of the mutant turkey beta-adrenergic receptor will be unraveled and the questions concerning the mutant receptor's ability to desensitize can be answered.

A STUDY OF SMALL SCALE CORONAL STRUCTURE

JENNIFER GAINES

Summer research performed at the High Altitude Observatory,
a division of the National Center for Atmospheric Research
under the supervision of Dr. Robert M. MacQueen

ABSTRACT

Since there is obvious large scale structure and there is evidence for extremely small microscale structure in the solar corona, a search was made for optical evidence of fine coronal structure smaller than that seen with the naked eye in eclipse photographs. Negatives of photographs made at eclipses were digitized, and Fourier transforms were applied to those scans. These negatives were unique in that they were made using radially-graded neutral density filters . Preliminary examination of these transforms indicates evidence for real coronal structure above noise in the several arc-second spatial range.

INTRODUCTION

The corona is the highest layer of the sun's atmosphere; starting above the chromosphere, it spans a volume from near the sun's surface to as far as the earth. The corona is extremely hot (between one and two million degrees Kelvin); because of the intense heat, most of the elements in the corona become highly or completely ionized, creating a reservoir of free electrons, in motions constrained by the magnetic field lines of the sun. Thus, in a broad sense, one could say that when one observes the corona, a virtual "map" of the sun's field lines is displayed. Understanding the structure of the solar corona aids in understanding how and why the sun's magnetic field evolves and changes in space and time.

The corona itself does not radiate visible light. The part of the sun we see regularly is the photosphere, a lower, cooler layer of the sun's atmosphere that is optically thick and hence luminous. The corona can only be seen during an eclipse, or with a special telescope called a coronagraph, which creates an artificial eclipse. The visible corona is caused by Thompson scattering of photons by the coronal electrons. The light of the corona, taken as a whole, is only one millionth of the brightness of the visible solar disk.

With casual examination, one can see quite a bit of structure in the corona, most notably streamers and polar plumes (see figure 1). These structures, of angular size as much as several to ten arc minutes in the sky, are known as "large scale structures", and have been described as long as man has viewed eclipses. Closer visual examination of coronal images reveals finer scale structures, typically less than one arc minute of angular subtense. There are hints of fine, diffuse rays of even smaller scale, especially visible at the solar poles. The goal of the present study is to determine, in as quantitative a manner as possible, the nature of the small scale structures, e.g. those fine structures of scale much less than an arc minute of angular subtense.

It should be noted that from radio techniques there is proof for coronal structure on an extremely small scale, called "microstructure" (down to the sub arc second range). This is achieved by studying an effect known as interplanetary scintillation. Just as the stars we see "twinkle", so do radio sources "twinkle" when observed through the corona. This is caused by the motion of extremely small structures of electrons streaming away from the sun in the solar wind. By observing this radio scintillation it is possible to determine the size of the structure that caused it, (Harmon, 1975) and current evidence suggests that structures as small as 0.03 arc second may be present in the corona.

Thus we have visible evidence for large scale structure and evidence through radio observation for extremely small microstructure. What we hope to locate is visible evidence for fine structure down to the several arc second range. The best place to search for such evidence will be in the lower part of the corona, where it is brightest; the eye can pick up evidence of structure in this region down to the 10

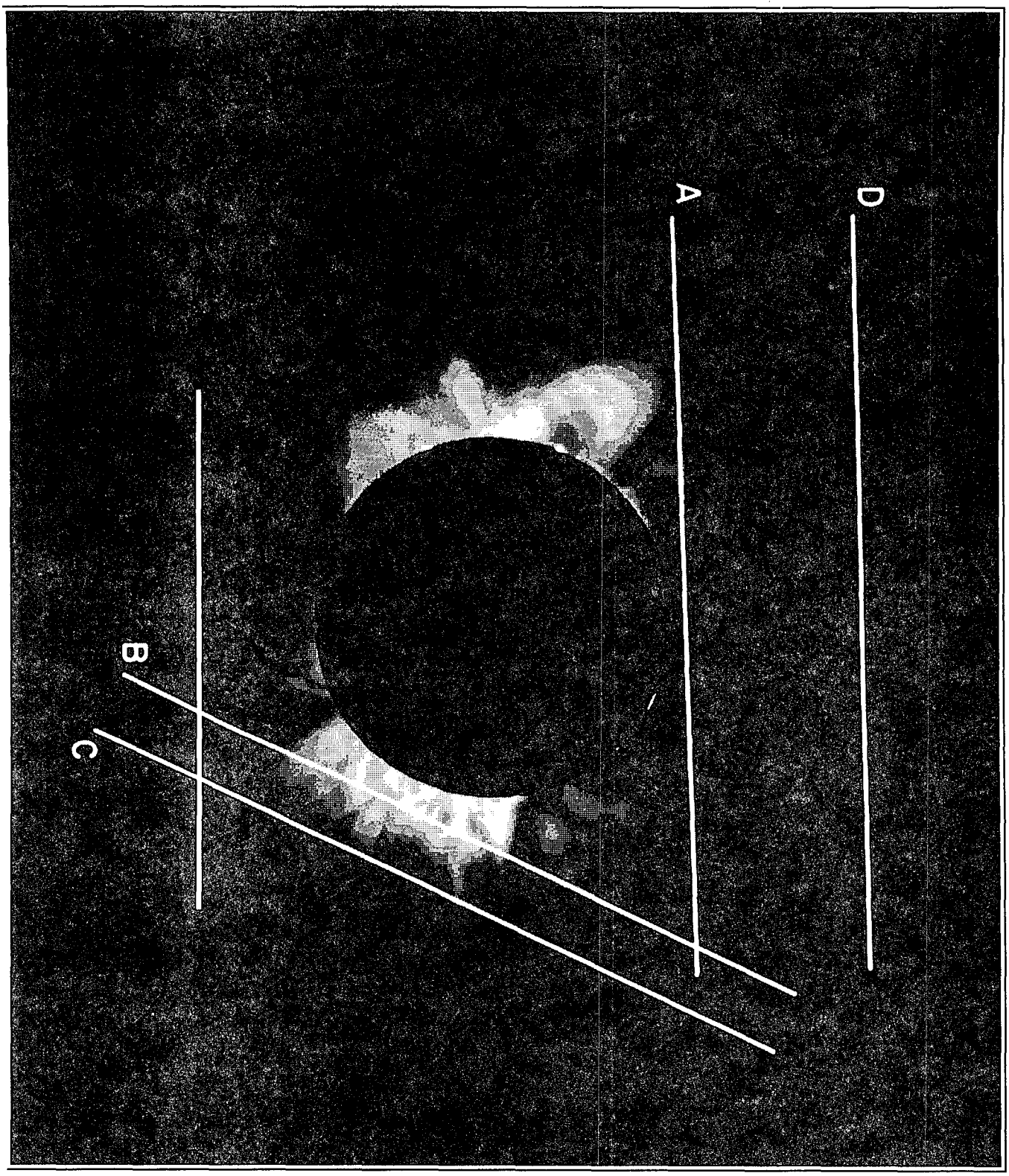

Figure 1: Eclipse 1980 **(Note ray at pole)**

to 15 arc second range.

CORONAL OBSERVATIONS

There are many sources of coronal data. The first task in the search for fine coronal structure was to decide what set of data to work with. First, we considered using coronagraph data. Such data is plentiful since with a coronagraph, images can be made hundreds of times every day. However, there are significant problems which render both space and earth-based coronagraph data inappropriate for this type of study.

Space coronagraphs are externally occulted, in order to block out all of the light in the solar disk and thus create the necessary artificial eclipse. Thus, the occulting disk actually includes a larger angular width than the sun's disk itself; it also covers most of the lower corona. Thus, the brightest and seemingly most structured coronal regions are occulted. We could have tried to examine the lowest region of the corona that is reproduced by the coronagraph, however, there was another problem there. Since light from the innermost coronal regions just clears the edge of the occulting disk, it does not illuminate the entire area of the objective lens of the coronagraph. Because the entire lens is not being illuminated, the full resolving power of the instrument is not being utilized. This effect is only overcome as you get farther out into the corona, and the coronal light illuminates the entire objective lens. Practically, therefore, externally-occulted coronagraphs are not optimum for examining small scale coronal structure.

Ground-based coronagraph data is also inappropriate for this type of study. Such coronagraphs are not externally occulted; it is inappropriate for a different reason. Since this coronagraph picks out the corona in terms of its polarization-brightness product, variations in the polarization (or brightness) of the earth's atmosphere set a limit on the signal:noise that can be observed with this instrument. Unfortunately, the spatial scales dictated by these considerations are somewhat above that which we wish to study.

Thus, eclipse photographs must provide the data set from which we will make our observations, and, obviously, such sets are not plentiful. Eclipses can only occur at most twice a year, an then, they have a nasty habit of occurring in the middle of the ocean or in a nearly inaccessible place such as Kenya (1973) or Siberia (1981). Even when one can mount an expedition to an eclipse, success is still dependent on good weather, reliable equipment, and instrument quality. Most coronal eclipse photographs, too, have the problem that a single exposure cannot pick up the huge dynamic range that the corona displays (over only a few solar radii the brightness decreases to a factor of one one-thousandth the brightness of the innermost corona). However, HAO has obtained a particularly unique set of photographs, made using a radially graded filter. This filter varies in transmission across its surface and is oriented so that its transmission is highest for the light of the outer corona. In this way the filter cancels the steep corona brightness falloff, so that the entire dynamic range can be collected in a single exposure.

A second motivation for this investigation involves a potential experiment at the 1991 eclipse. The path of totality will cross Baja, California around local noon; it will also cross Mauna Kea, Hawaii in the early morning, right over the 88-inch University of Hawaii telescope, equipped with CDD detectors, and the Canada-France-Hawaii telescope (CFHT). Data taken with either such instrument through a quiet, if extended, atmosphere could advance the study of fine coronal structure immensely. Clues concerning such fine coronal structure from the current study may help guide the definition of efforts at the 1991 eclipse.

DATA COLLECTION

The first step in the data collection was to make digital scans of the data set. We used 75mm negatives of eclipse photographs made at seven different eclipses, in 1966, 1970, 1973, 1980, 1981, 1983, and 1988. Linear (one-dimensional) scans were made of regions of interest on each eclipse image. The digital scans were made with a new HAO instrument, an Eikonix digitizer. The main element of the machine is a linear array of 4096 detectors (pixels). When used in conjunction with a 105mm lens, we were able to achieve a resolution of about 1 arc second per pixel, or about eight microns sample size on

the film. For the Kodak Linagraph Shellburst film used for these photographs, the film grain is on the order of 10-20 microns in size. Thus, with this resolution, we were confident that we were indeed detecting as much structure as might be present, given the limits of the film.

As noted above, on each of the seven negatives we made scans of "interesting" structures (streamers, polar plumes, rays, etc.) and a background scan away from coronal structure, in order to assist determining the background "noise" of the film, due to grain and grain clumping effects. (See figure 1 for an example of positioning of data scans.) Further, we made scans across the lunar disk to determine the exact spatial dimensions of our scan and scans across the lunar edge to assess the effective resolution of the telescope (to see how well the telescope was focused) and the atmospheric "seeing". To determine the spatial dimension for each eclipse, we scanned across the lunar disk at an equatorial region. Then after determining the number of pixels recording the lunar disk, and using the exact size of the moon (in arc seconds) at each of the eclipses we were able to calculate how many arc seconds were being scanned by one complete scan of the 4096 array (approximately one pixel per arc second, as mentioned above). To examine the effective image resolution we made scans of each image across the lunar disk vertically through the polar regions. We had to realize, however, that this test was limited by the real motion of the lunar disk with respect to that of the sun during the time the exposure was being made (the eclipse telescope compensated for solar motion with tracking).

DATA ANALYSIS

There are several ways in which we could have analyzed the data taken from the negatives. First, we could have simply taken the raw data, plotted it, and spread it out greatly. Such a plot could then be examined visually for small variations in intensity. This technique has been employed with some success by Eddy (1973) in his study of an apparent neutral sheet in the 9122 corona, and by Koutchmy (1988), who has discussed the importance of the study of coronal fine structure and image processing techniques which can emphasize small variations in intensity in coronal structure.

However, we decided to take a different course. By using the technique of Fourier transforms, a more qualitative examination of the amount of power present as a function of spatial frequency can be determined.

Perhaps a brief digression is appropriate to discuss Fourier transforms. A Fourier transform is essentially a Fourier decomposition of a function (see Bracewell, 1978, and Champeney, 1973). The result is a function whose value at a given spatial frequency is the coefficient of sine and cosine functions of that frequency that can be added together to make up the original function. Coefficients for a given frequency imply the amount of power present at that frequency in the original function. If an inverse transform were performed on this function of coefficients, the resultant would be the original function.

Before using this technique on coronal data, Fourier transforms were studied analytically and digitally. This was for pedagogic reasons and also to ensure that the computer program which performed the transforms deserved our confidence. The first step in the study involved performing an analytic transform on a simple object plane and plotting that function. Then the same image was created with a computer program, and its transform was taken with the computer routine and plotted. The results were exactly the same, proving the integrity of the program.

We found that the best way to study coronal transform data was in the following manner: after the transform of a scan was performed, it was normalized to one, and smoothed by performing a four-point running mean; then the difference was taken between that function and a four-point running mean of a normalized transform of a background scan; we then plotted all values of that difference greater than 10^{-5}. (All negative differences were set to 10^{-5}.)

CONCLUSIONS

A total of 25 different coronal scans have been examined, and this paper will summarize some preliminary conclusions from the examination.

Special attention has been given to each of these plots at the high spatial frequency range.

Consider, as a first example, the plot (as described above) for the scan of the 1980 ray (see unlabeled line in figure 1). Notice that this scan intercepted essentially no other coronal structure than the ray itself; thus we expect to see very little power in the high spatial frequency range. Our suspicions are verified (see figure 2). Furthermore, there is clear evidence of power at the spatial frequency corresponding to the half width of the coronal ray. (See figures 2 and 3).

In contrast to the lack of high spatial frequencies present in the scan of the isolated ray visible in the 1980 eclipse, many of the polar regions which we observed appear to have quite a bit of fine structure visible, even to the naked eye. Thus, we were not surprised when 5 out of the 7 plots with the most significant amount of power at high spatial frequencies were from polar scans. However, it was interesting to note the rather large amount of power above noise at the high spatial frequency range (3 to 4 arc seconds, between about 3.5 and 5 x $(104\ km)^{-1}$). (See figure 4.)

Therefore, as a preliminary result from this study we can report strong evidence for the existence of fine structure in the corona down to the several arc second spatial frequency range. Further refinement of the smoothing process will be done, additional scans will further investigate the background noise, and quantitative comparisons of the amount of power per spatial frequency intervals are anticipated.

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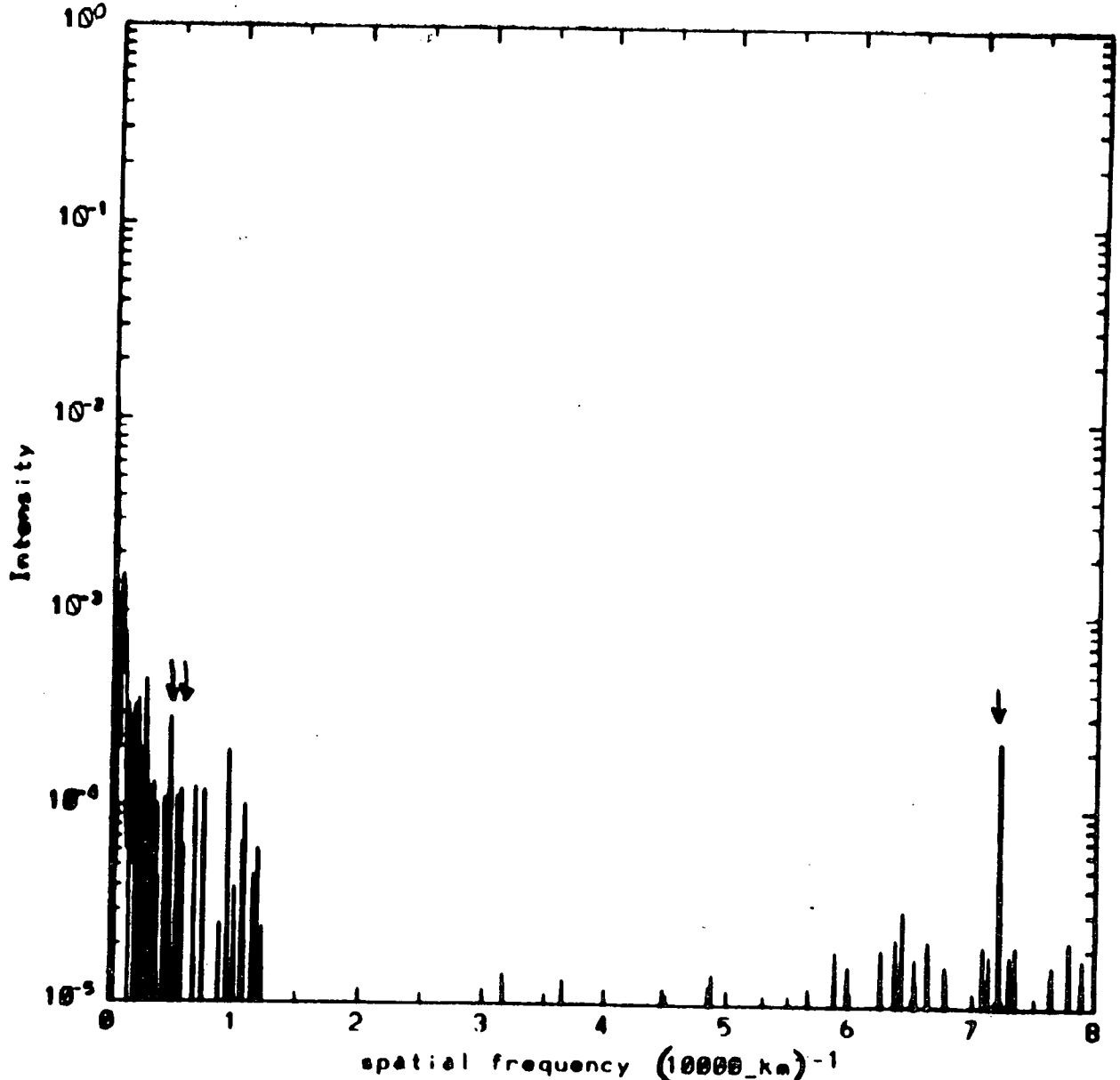
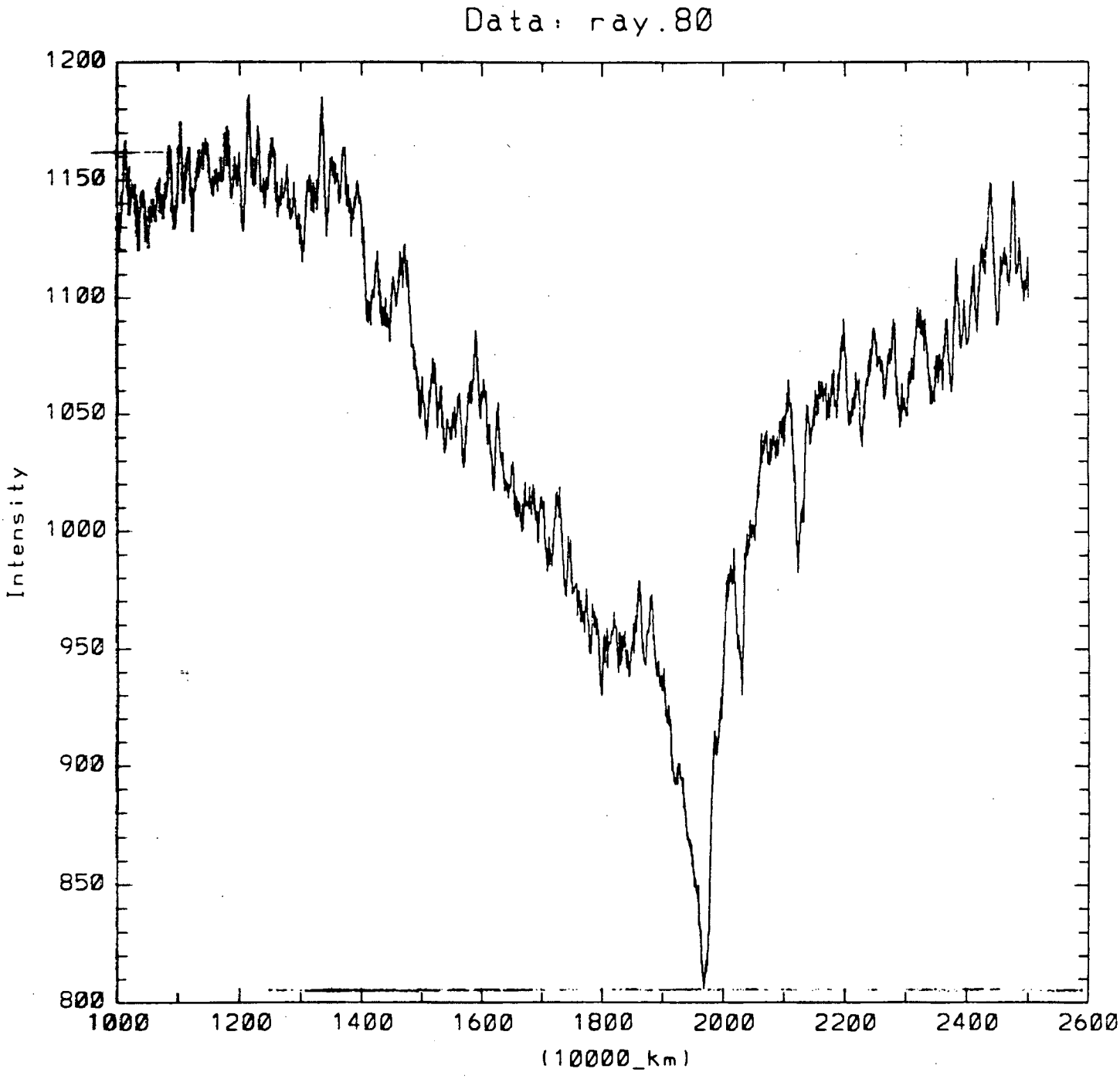


↓ = max spatial frequency

↓↓ = Spatial frequency = 1/2 width of ray

Figure 2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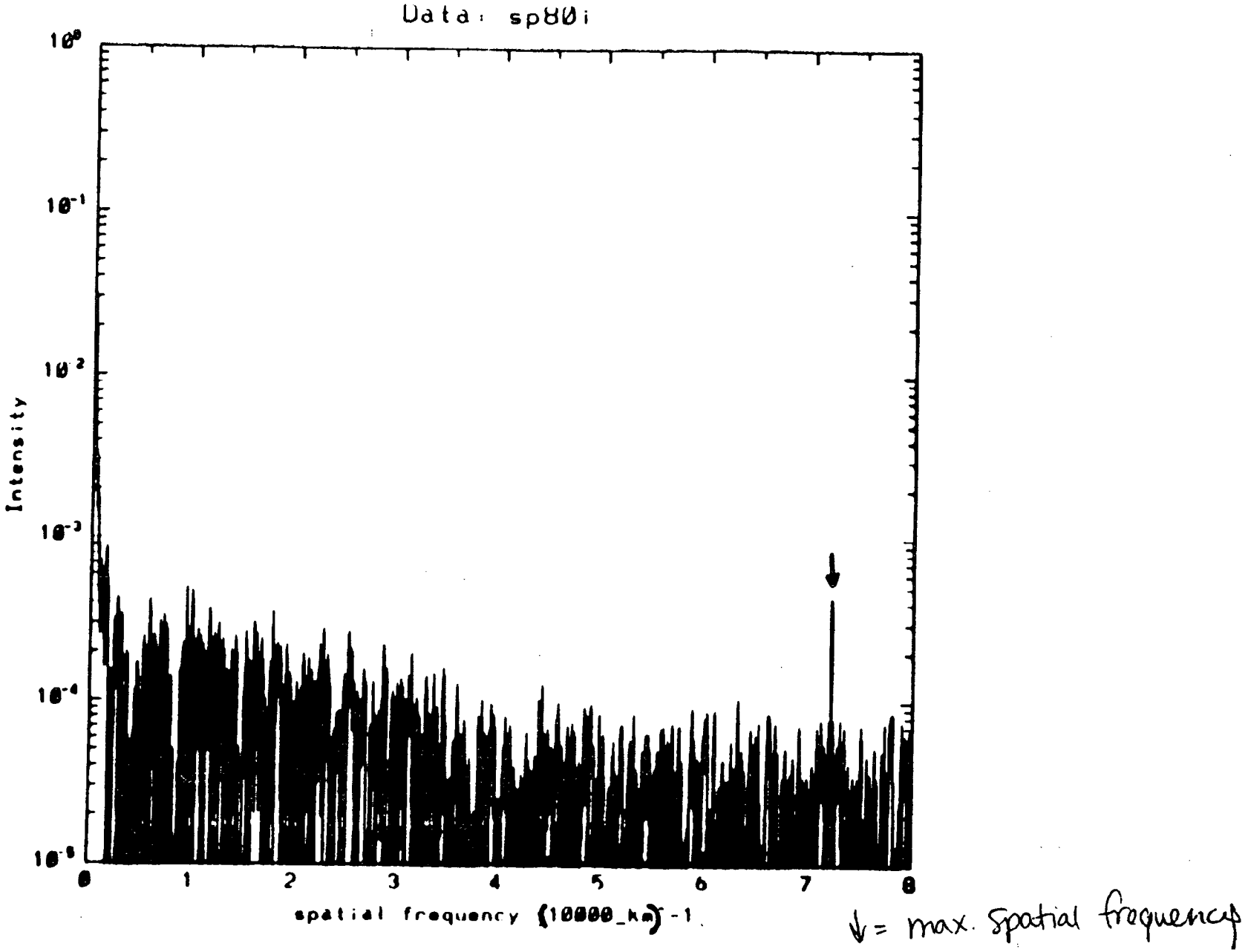
1200
1150
1100
1050
1000
950
900
850
800
Intensity
1000
1200
1400
1600
1800
2000
2200
2400
2600
(10000_km)

Figure 3

Figure 4


Data: sp80i
Intensity
spatial frequency (10000_km)-1
0
1
2
3
4
5
6
7
8
↓ = max. spatial frequency

DEVELOPMENTAL VARIABILITY IN THE CHICK EMBRYO *GALLUS DOMESTICUS*

ANN E. HAIGHT
Department of Biology, Rhodes College, Memphis TN 38112

ABSTRACT

Through this study, I attempted to determine whether the embryological development of the chick Gallus Domesticus is synchronous, and, if not, where the variations lie. Development was quantified by measuring structures in populations of chick whole mounts at five stages in earlydevelopment. No comprehensive trend of variations was determined, though isolated evaluations and trends were explored within functional groupings of the structures studied.

INTRODUCTION

Morphological differences among taxa may be attributed to the alteration of developmental timing of a character in phylogeny (e.g. heterochrony) as well as to direct selection on the adult morphology (Emerson, 1988; Gould, 1977; Hafner and Hafner 1984). Heterochrony involves either acceleration or retardation in growth rate or appearance time of a feature in a descendant relative to its ancestor ; the result is an altered developmental sequence on which selection may act. Thus, relatively small differences in size or appearance time of morphological structures in embryos may lead to large scale changes in adult structure. For example, heterochrony is considered a contributing factor to the development of such features as high encephalization in geomyoid rodents (Hafner and Hafner 1984) and hind limb morphology in the frog, *Hyla Crucifer* (Emerson 1988). Alberch and Alberch determined that morphological features of a salamander (*Bolitoglossa occidentalis)* such as small body size, short tail, carpal and tarsal fusion, fully webbed hands and feet, and others were not adapted features, but instead were the result of the truncated development of *B. occidentalis* as compared with other species of *Bolitoglossa* (Alberch and Alberch, 1981).

Because heterochrony acts through changes in timing of developmental events, it is of interest to study the variation in these developmental events in a population of organisms. Such variability may be common in nature if heterochrony is an important part of evolution. The goals of this study were to measure and evaluate the degree of developmental variability in a population of chick embryos (*Gallus Domesticus*) and to test whether degree of variability differs over time (age).

Hypothesized correlations between function and time of appearance in development have heen made for quite some time (Menhert 1891, Keibel 1898), including J. Massart's conclusions that organs functioning first develop first, and those functioning at the same time develop according to final size (1894, cited in Gould, 1977). With such correlations in mind, I will examine changes in variability over time for groups of characters with differing functions: musculo-skeletal system, circulatory system, central nervous system, and sensory nervous system. For each groups of characters, I hypothesize that variability will increase with time, so that structures developing earlier will be less variable than those developing late. This is based on an idea that as more and more cells and structures develop, there are more chances for a "mistake" or deviance from the normal growth rate. Alternatively, there may be some stabilizing selection force keeping growth rate constant and undeviating among a population; or variability may decrease with time if growth rates and structures have a tendency to "catch up" to be ready for functioning at the same time as all other members of the population.

MATERIALS AND METHODS

I used the chick as a developmental model because it is readily available, its development is well studied and differentiation easy to follow, and its embryological structures are relatively easy to quantify by measuring them in whole mounts up to the age of 72 hours. In addition, the chick is representative of all vertebrate groups in its basic organ systems and body formation processes (Patten, 1971). The chick's heavily yolked, telolecital egg undergoes a partial cleavage in which cell division occurs only in the uppermost layer of cells (blastodisk) and does not include the yolk. By 13 hours, invagination (gastrulation) has begun, producing the three germ layers and the "primitive streak," which represents the future anteroposterior axis of the chick. By 20 hours, the neural fold develops anterior to the primitive streak, and folding progresses posteriorly. The primitive streak regresses and the first somites appear by 24 hours, along with the development of a head fold. By 33 hours a true neural tube has formed and the brain begins to differentiate into regions. A simple heart forms and blood supplied with nutrients begins to circulate through the vitelline veins. As the embryo grows larger, by 48 hours it undergoes flexion of the head and torsion to the right due to crowding by the large yolk. The brain differentiates from three regions (prosencephalon, mesencephalon, rhombencephalon) into five (telencephalon, diencephalon, mesencephalon, metencephalon, myelencephalon), the optic vesicles of the 33 hour chick invaginate into optic cups, and auditory vesicles (precursors to the ears) are visible by 48 hours. By 72 to 96 hours, the chick is developed further; wing and leg buds are apparent, ventricle walls have visibly thickened, and somites, which have been increasing in number since about 20 hours, are now too numerous to count. This brief summary outlines most of the early developmental processes characteristic of birds and other vertebrates, and describes all of the morphological structures that were analyzed in this investigation.

The populations of embryos studied were collections of whole mounts of chicks ranging in age from 13 hours (just after gastrulation) to 72 hours (just after appearance of wing and leg buds. Twenty-four structures were measured on four age groups (Table 1). Although I originally planned to use chicks of ages 13 to 72 hours, the measurements of chicks under 20 hours were of little comparative value, since the only measureable structure in these was the primitive groove. Not all features measured in each age group (see Table 1) could be measured in every other age group, because features were either not yet present, had developed into something else, or had dissappeared in one group or another. For example, brain regions measured in 33 to 72 hour embryos could not be measured in 20 to 24 hour embryos because they had not yet developed.

The features were classified into categories based on organ system (e.g. function) for later comparison. For example, somite number and size are of the musculo-skeletal system; other structures were classified as central nervous system, sensory nervous system, or circulatory system. Structures were measured primarily using an ocular micrometer installed in a light microscope (unless otherwise noted), and measurements were later converted to millimeters. Commonly used "ages" (in hours) of chicks are actually slightly variable because hens will not lay eggs during the night; instead they retain the eggs until daylight, so the actual "age" of a chick is uncertain to an extent (Patten, 1971). To adjust for age variability within the four age classes, a length measurement was taken of each chick by which the measurements of the structures were standardized . These length measurements in the early embryos (20 to 24 hour) were the primitive groove measurements. However, due to the torsion and flexion of the later embryos (33 to 72 hour), length measurements were performed on these chicks by tracing their image from a projecting scope with a string (following the notochord from tail until torsion, then around the dorsal outline of the chick to the developing eye) , then measuring the string. Figures 1 through 5 illustrate measuring techiques for each structure at each stage.

RESULTS

Table 2 summarizes the measurements taken, organized by functional classes for comparison. Each measurement was converted to millimeters and standardized by its measurement of the length of the embryo. For each age group the variance (standard deviation squared) and 95% confidence intervals of that variance were calculated. Variances ranged from 0.0025 to 7.198.

Table 1. Summary of chick embryo Age Classes, Sample Size, Functional Class for each structure (ms=musculo-skeletal system, cns=central nervous system, sns=sensory nervous system, circ=circulatory system), Structures Measured, and Type of Length Measurement used to standardize the structure measurements.

Age (hours)	Number of Individuals	Type of Length Measurement	Functional Class	Structures Measured
20-22	17	Primitive Groove	ms	Somite Number
			ms	Somite Size
24	20	Primitive Groove	ms	Somite Number
			ms	Somite Size
33	19	Traced Length	ms	Somite Number
			ms	Somite Size
			cns	Mesencephalon Width
			cns	Prosencephalon Length
			cns	Prosencephalon Width
			cns	Rhombencephalon Width
			circ	Vitelline Vein Width
48	31	Traced Length	ms	Somite Number
			ms	Somite Size
			ms	Notochord Width
			ms	Tail Bud Width
			cns	Mesencephalon Width
			cns	Telencephalon Width
			sns	Optic Cup
			sns	Auditory Vessicle
			circ	Truncus Arteriosus
72	8	Traced Length	ms	Limb Bud Width
			cns	Mesencephalon
			cns	Metencephalon
			sns	Auditory Vessicle
			sns	Eye
			circ	Vitelline Artery

DISCUSSION

Although there are no apparent trends of variability over time across the entire set of data, the four groups of structures can be analyzed to detect patterns within functional groups. In the musculoskeletal group, variance of somite size (SoS) remains remarkably consistent over four age groups (20-48 hours), increasing only a small amount from .0006 at 20-22 hours to .0016 at 48 hours. Because the confidence intervals for these variances do overlap, none of them are significantly different. Appearance of somites occurs very early in the chick, so perhaps these structures are on a strict developmental timeline. Similiarly, the variances for Tail Bud Width (TBW), Notochord Width (NoW), and Limb Bud Width (LBW) are similar, having overlapping confidence intervals. These structures develop in the later ages studied yet still are only slightly more variable than the somites.

Within the Circulatory group there is a decrease in variance over time, from the substantially variable Vitelline Vein Width (VVW) at 33 hours (variance of .2285), down to the Truncus Arteriosus Width (TAW) at 48 hours (variance of .0064) and the Vitelline Artery Width (VAW) at 72 hours (variance of .0142). The confidence intervals of the last two structures overlap and can be considered similar in variance. Perhaps this decrease in variance is due to the fact that at 33 hours, circulatory structures are just beginning to form, but by 48 hours they are becoming functional. They may be more room for variability in growth rate while a circulatory structure is forming, but by the time it becomes functional it may be under stricter control due to the critical nature of nutrient distribution.

None of the structures of the Central Nervous System are extremely variable, and all of these developing brain regions have overlap in the confidence intervals of the variances and are therefore very similar in developmental variability. Although it seems that CNS structures might, in fact, be relatively variable in embryonic development since communication can wait until oxygenation, perhaps the central nervous system structures are needed as an axis for the chick's growth to proceed.

Within the Sensory Nervous System group, there is a slight increase in variability of the Auditory Vessicles (AuV), from a variance of .0036 at 48 hours to .0185 at 72 hours. This increase may support my hypothesis that developmental variability increases with age, since as more structures form, there is more chance for genetic differences to come into play. The variances of eye structures (OpC and Eye) at 48 and 72 hours are small (.0077 and .0156, respectively) and not significantly different because their confidence intervals overlap. It would seem that SNS structures would be the most variable of all functional groups, since they are nowhere near being used during embryonic life but begin functioning only after the chick hatches. On the other hand, from a purely day to day observational viewpoint, these sensory structures in most vertebrates (primarily humans) appear to be the least variable of all body parts as finished products.

In conclusion, there is no comprehensive trend of increasing variability with time in this population of *Gallus Domesticus*. However, the development of this chick is not completely synchronous, as illustrated by the presence of variation shown in Table 2. Structures of the Musculoskeletal and Central Nervous Systems had variances that were very similar over time within their functional groups, and Circulatory structures were found to decrease in variability over time, while Sensory Nervous structures increased in variability over time. Using this research as a base, further interesting studies related to this subject would include a comparison of the variability in one chick species with another, or perhaps a comparison of variability in precocial versus altricial species.

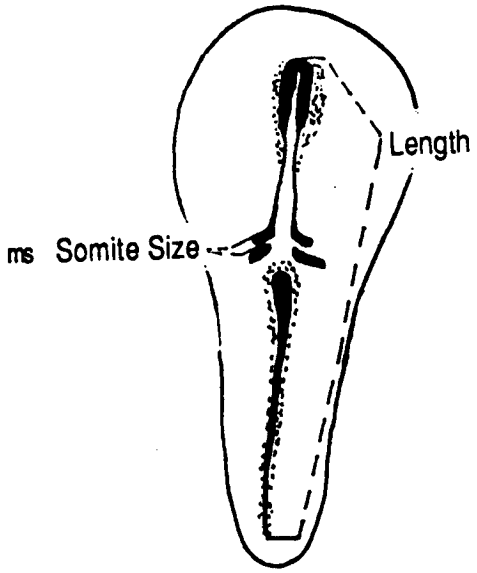


Figure 1. Measurements on 20-22 hour chick. Length: measured using ocular micrometer as shown. Somite Number: counted whole somite pairs present. Somite Size: measured largest somite as shown. (Modified from fig. 2-6, Wischnitzer, 1975, p 49.)

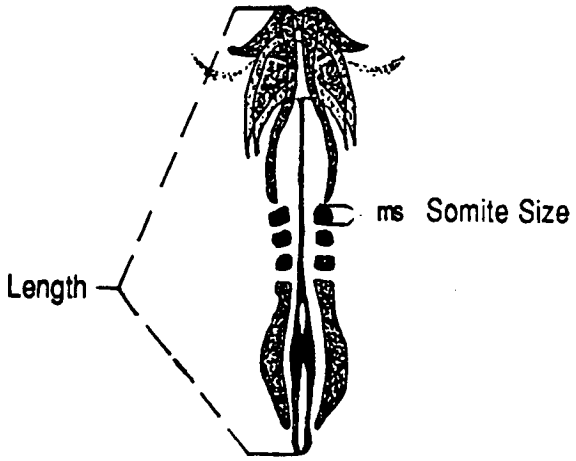


Figure 2. Measurements on 24 hour chick. Length: measured using ocular micrometer as shown. Somite Number: counted whole somite pairs present. Somite Size: measured larges somite as shown. (Modified from fig. 3-1, Wischnitzer, 1975, p 53.

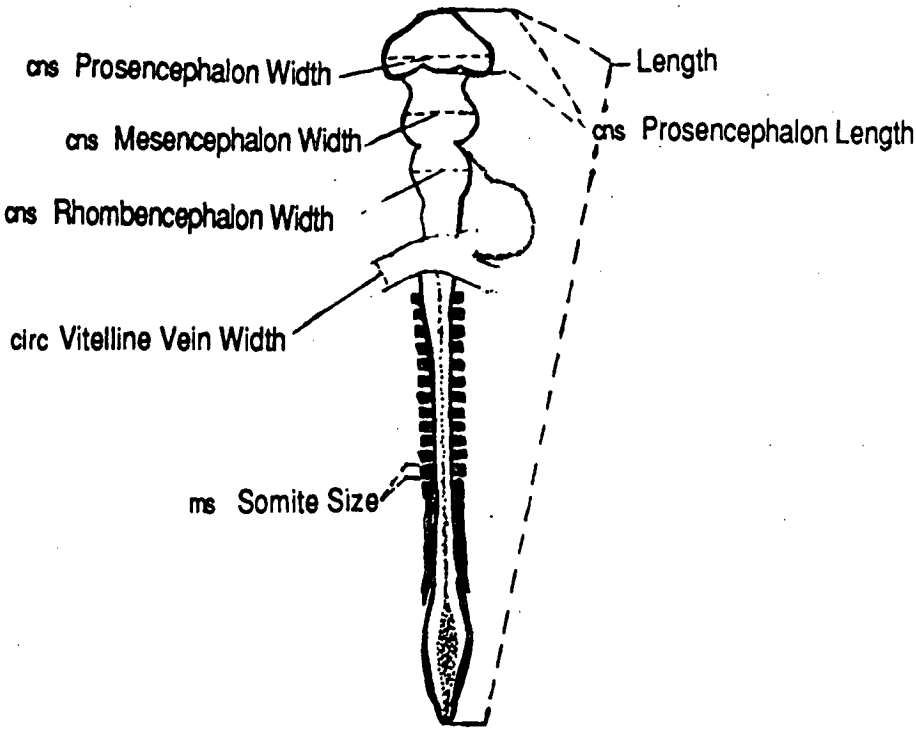


Figure 3. Measurements on 33 hour chick. Length: traced with string using projecting scope; end points as shown. Somite Number: counted whole pairs present. Somite Size: measured largest somite as shown. Mesencephalon Width: measured across widest part of mesencephalon. Prosencephalon Length: measured down center of prosencephalon as shown. Prosencepahlon Width: measured across widest part as shown. Rhombencephalon Width: measured across widest part of rhombencephalon. Vitelline Vein Width: measured across widest clearly formed part of vein before branching. (Modified from fig. 4-2, Wischnitzer, 1975, p 61.)

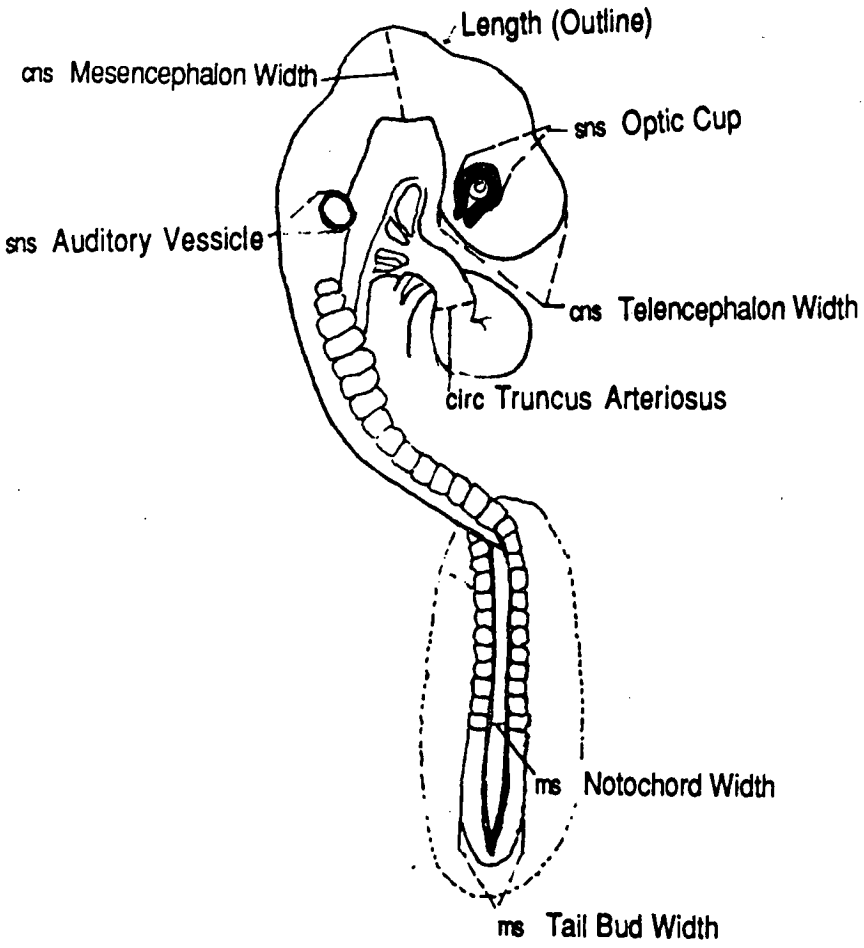


Figure 4. Measurements on 48 hour chick. Length: Projected image traced with string along colored outline shown; began at tip of tail, followed notochord then head outline to choroid fissure of eye as shown. Somite Number: counted whole pairs. Somite Size: measured largest somite as in fig. 3. Notochord Width: measured width at last posterior somite pair as shown. Tail Bud Width: measured width at widest point as shown. Mesencephalon Width: measured widest part as shown. Telencephalon Width: measured across widest part as shown. Optic Cup: measured width as shown. Auditory Vessicle: widest measurement taken as shown. Truncus Arteriosus: measured as shown. (Modified from fig. 5-1, Wischnitzer, 1975, p 67.)

Figure 5. Measurements on 72 hour chick. Length: projected image traced with string along colored outline shown; began at tip of tail, followed notochord then head outline around to choroid fissure of eye as shown. Limb Bud Width: measured across widest part of posterior limb buds as shown. Mesencephalon Width: measured across widest part as shown. Metencephalon Width: measured across widest part as shown. Auditory Vessicle: widest measurement taken as shown. Eye: measured width as shown. Vitelline Artery: measured width of largest artery before branching. (Modified from fig. 6-2, Wischnitzer, 1975, p 79.)

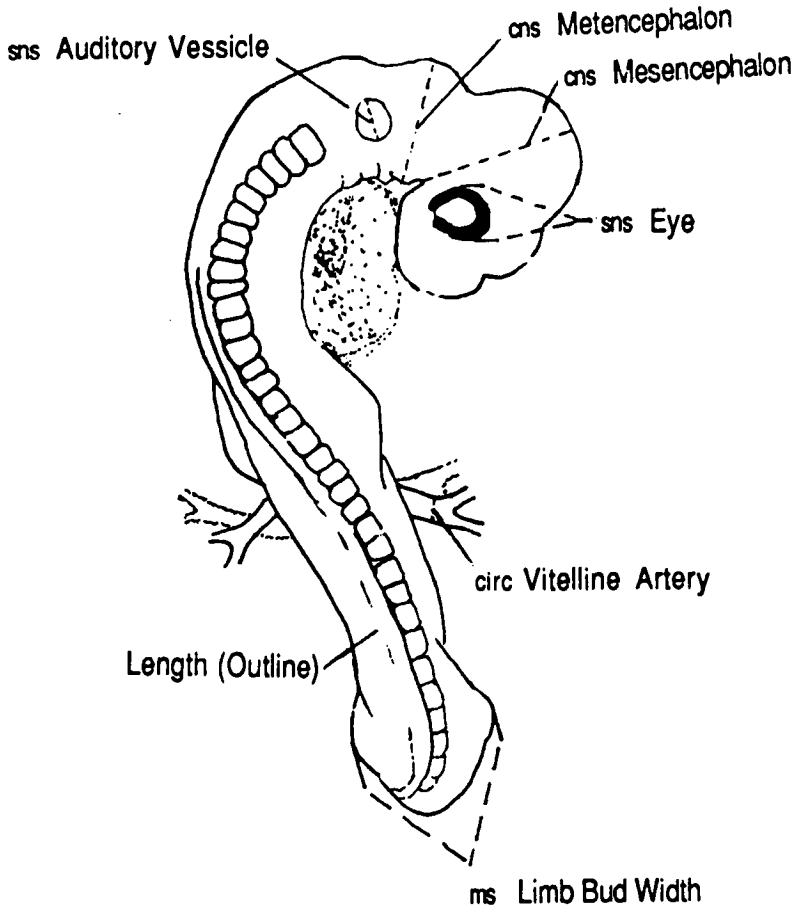


Table 2. Variance (abbreviated as Var), individuals per group (n), and 95% confidence intervals for each structure in each age group. Abbreviations for structures are: So#=Somite Number; SoS=Somite Size; TBW=Tail Bud Width; NoW=Notochord Width; LBW=Limb Bud Width; VVW=Vitelline Vein Width; TAW=Truncus Arteriosus Width; VAW=Vitelline Artery Width; HFL=Head Fold Length; PrW=Prosencephalon Width; PrL=Prosencephalon Length; MsW=Mesencephalon Width; RhW=Rhombencephalon Width; TeW=Telencephalon Width; MtW=Metencephalon Width; OpC=Optic Cup; AuV=Auditory Vessicle; Eye=Eye Width.

Age	Length			MS				Circ				CNS				SNS			
(Hrs)	Var	n	CI	Struc	n	Var.	CI	Struc	n	Var	CI	Struc	n	Var	CI	Struc	n	Var	CI
20-22	.1621	17	.0866 .3579	**So#**	17	.3080	.1645 .6801												
				SoS	16	.0006	.0003 .0013												
24	.1492	20	.0835 .3058	**So#**	20	1.250	.7001 2.621												
				SoS	20	.0006	.0004 .0013												
33	.0025	19	.0014 .0052	**So#**	19	.0288	.0189 .0604	**VVW**	17	.2285	.1220 .5045	**PrW**	19	.0700	.0386 .1467				
				SoS	19	.0007	.0004 .0014					**PrL**	19	.0122	.0067 .0255				
												MsW	19	.0138	.0076 .0289				
												RhW	18	.0068	.0037 .0147				
48	.0029	31	.0017 .0047	**So#**	29	7.198	4.434 12.83	**TAW**	27	.0064	.0039 .0117	**MsW**	31	.0209	.0131 .0365	**OpC**	28	.0077	.0047 .0139
				SoS	30	.0016	.0010 .0029					**TeW**	30	.0425	.0264 .0742	**AuV**	29	.0036	.0022 .0065
				TBW	28	.0547	.0334 .0985												
				NoW	30	.0019	.0012 .0034												
72	.0118	8	.0437 .3995	**LBW**	8	.1289	.0519 .4742	**VAW**	8	.0142	.0057 .0522	**MsW**	8	.0050	.0020 .0212	**Eye**	8	.0156	.0062 .0573
												MtW	18	.0137	.0055 .0504	**AuV**	8	.0185	.0074 .0679

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THE DEPENDENCE OF MAXIMUM BRDICKA CURRENT ON PROTEIN SULFHYDRYL CONTENT AND MOLECULAR WEIGHT

S. STEVEN HIPP

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ABSTRACT

Previous studies have shown that Brdicka current is caused by catalytic reduction of H^+ at cobalt-sulfhydryl complexes in proteins or peptides. Wave I and wave II increase with increasing protein concentration until the mercury drop is saturated. Further increases in concentration cause a drop in current, possibly due to inhibition of hydrogen ion diffusion caused by the crowding of still more proteins near the saturated electrode. This study has shown that the maximum current attained for a given protein under fixed ionic and temperature conditions is a linear function of % cysteine content. Furthermore, an analysis of the charged amino acids adjacent to cysteine residues demonstrated that positively charged environments cause the sulfhydryls of a protein to be more polarographically active, giving a higher maximum current than found for proteins with negatively charged cysteine environments.

The possibility that conformation could play a large role in determining the activity of cysteines in a protein was explored by repeating the experiment with the proteins denatured in 4 and 8 molar urea with 2-mercaptoethanol. The results of this denaturation experiment did not alter the relative positions of the data points; therefore, conformation is not important in determining polarographic activity. Other than % cysteine and charge environment, which have been demonstrated by this research effort to be important factors in maximum polarographic activity, one or more unknown factors other than conformation probably also play a role in fixing the maximum Brdicka current for a protein.

INTRODUCTION

The phenomenon of Brdicka current was discovered in the 1920's when R. Brdicka accidentally found two polarographic peaks at about -1.4V and -1.6V vs. SCE when cobalt salts were added to NH_3-buffered protein solutions in which a dropping mercury electrode (DME) was placed. A necessary condition for observing these waves is the presence of sulfhydryl or disulfidic groups in the protein.

Understanding of the exact mechanism of Brdicka waves is incomplete, but a clear general picture of the reaction has emerged. Cobalt atoms of uncertain valency complex with sulfhydryl groups. The valency of the cobalt atoms in the complex is a matter of dispute, with some authors claiming 0 valency and some claiming a valency of II (Maironovskii 1987). After electrodeposition of the proteins on the DME, the cobalt-sulfhydryl complexes take up protons from the acid component of the buffer and set them up for reduction at the electrode. (Brdicka 1965).

A characteristic feature of all Brdicka catalytic waves is the dependence of their height on both the concentration of proteins as well as their cysteine content. The waves can be observed at very low protein concentration and increase in height as the protein concentration increases. The wave height increase follows a pattern roughly like a Langmuir adsorption isotherm, since a leveling off and decrease of the current occurs at high protein concentration.

The reason for the decrease in current at high protein concentration has not been determined with certainty, but a good guess is that once the mercury drop is saturated with a layer of proteins, further increases in concentration cause the crowding of a second layer, and this inhibits the diffusion of hydrogen ions and possibly cobalt into the reactive layer.

Hence, for each protein a characteristic maximum Brdicka current is obtained under fixed ionic and temperature conditions. The maximum current is obtained at different concentrations for different proteins, and in this study was found anywhere from 0.2 to 0.8 mg/mL.

Many authors have noted that at a given concentration, a protein with more cysteine residues will give a larger Brdicka current than one with fewer cysteines at the same concentration. Kalous and Pavlicek

(1962) correlated Brdicka current with % cysteine with some success. Millar (1953) studied the proteins insulin, pepsin, bovine serum albumin, chymotrypsin, and trypsin in a revealing fashion. He plotted the maximum Brdicka current obtained for a protein versus its number of cysteine residues divided by the volume of the protein as determined by x-ray crystallography. For these five data points he obtained a good linear fit to a line. This fit implies that all of the sulfhydryls in a protein are available for polarographic activity (but not necessarily equally).

The goal of this study was to plot the sum of the maximum Brdicka currents ($h_{1,max} + h_{2,max}$) versus the number of cysteine residues divided by the molecular weight of the protein. Division by molecular weight takes into account inhibition of hydrogen ion diffusion due to non-cysteine residues more precisely than division by protein volume, since the protein could be loosely or tightly coiled.

METHODS AND MATERIALS

All proteins were purchased from Sigma Chemical Company in a 90% or greater pure form. The proteins were dissolved in 0.85% NaCl, and only the two caseins had to be heated in order to dissolve.

The polarograph used (Princeton Applied Research Model 174A) was set to the differential pulse mode. The working electrode was a dropping mercury electrode, and the reference electrode was a saturated calomel electrode from Corning Glass works. The counter electrode was a platinum wire. Scans were carried out from -0.5 to -2.0 V at a scan rate of 2 mV/s with a pulse modulation amplitude of 50 mV and a drop time of 1 second.

For each run 7.0 ml of electrolyte solution (0.2M NH_4OH, 0.2M NH_4Cl, 0.002M $[Co(NH_3)_6]Cl_3$) was added to a jacketed polarograph cell at 25.0°C. Distilled water and protein solution were added until the total volume was 20 mL. (The volume of protein solution varied in order to vary the protein concentration from about 0.025 mg/mL to several tenths of a mg/mL.) 0.05 mL of 0.1% Triton X-100 was added to the cell to reduce the occurrence of maxima, and the pH was adjusted to 9.3 with a Corning Model 7 pH meter. 3 drops of octyl alcohol were added to the cell before N_2 was bubbled through the solution for 5 minutes. After nitrogen bubbling, the scan was taken.

Runs for several concentrations of each protein were made so that a current vs. concentration plot could be constructed. The maximum current was the highest point on this curve. Figure 1 is an example of this kind of plot for one of the proteins studied. Both wave I and wave II have their maxima, and these two currents are added together.

The procedure described above was repeated twice, in order to establish reproducibility for the data. Furthermore, so that the role of conformation in Brdicka current could be explored, the experimental procedure was repeated under two sets of denaturing conditions: once with 4 M urea and 2mM 2-mercaptoethanol in the cell, and once with 8 M urea and 4 mM 2-mercaptoethanol in the cell.

In order to plot $h_{1,max} + h_{2,max}$ vs. # cysteines / mol. wt., the amino acid sequence of each protein and its molecular weight were found in the chemical literature (Dixon and Webb 1979, Hartley 1964, Mitchel 1970, Dayhoff 1972, Smyth *et al.* 1963).

RESULTS AND DISCUSSION

Figure 2 demonstrates the linear dependence of maximum Brdicka current on % cysteine. The data points are the average for three runs, with standard deviation error bars. In cases where the point has no error bar, the standard deviation is smaller than the symbol.

The data in its current state of completeness is an affirmation of the conclusion Millar made from his similar plot: that all of the sulfhydryl groups in a protein are available for polarographic activity. Within the accuracy allowed by a 0.97 correlation coefficient, the internal, hydrophobic cysteines contribute just as much height to the Brdicka wave total as do the external, hydrophilic cysteines. This conclusion holds for intraprotein cysteines only.

The work of Kuznetsov (1988) also supports the same conclusion. This scientist correlated the height of wave I with the number of hydrophobic cysteine residues in a protein, and obtained a correlation coefficient of 0.97. Correlation of the height of wave II with the hydrophilic sulfhydryls yielded an analogous coefficient. What Kuznetsov's work is demonstrating is that wave I arises from cysteines found in hydrophobic environments, and wave II arises from cysteines found in hydrophilic, exterior protein

environments.

An interesting experiment relating to Kuznetsov's theory of the origin of waves I and II was carried out as part of this project. Experimental runs were made with two peptides, bacitracin and glutathione, one of which contains a cysteine in a hydrophilic environment, and one of which contains a cysteine in a hydrophobic environment. Kadlecek and Kalous (1979) have pointed out the importance of small peptide studies for revealing information about the Brdicka current mechanism. They note that very little data has been published on peptide Brdicka current.

Glutathione (amino acid composition: Glu-cys-gly [Stryer 1988]) contains cysteine in a very hydrophilic environment, and gave only wave II, with no hint of wave I. Bacitracin, a peptide antibiotic of 11 amino acids, contains one cysteine in the mainly hydrophobic sequence Ileu-cys-leu-glu-ileu (Abraham 1953). Polarograms of this peptide gave a large wave I and only a very small wave II (about 10% of wave I). These facts are consistent with Kuznetsov's theory, and deserve to be followed up with a systematic study of peptides with cysteines in environments of varying hydrophobicity/hydrophilicity. A great effort was made to locate inexpensive natural peptides which could supply more data for the peptide aspects of this research effort. Unfortunately, no other cheap peptides could be located. The capacity of systematic peptide studies to reveal information about the mechanism of Brdicka currents is so great, this researcher feels, that a series of peptides should be synthesized on a peptide synthesizer and studied at some time in the future when the Rhodes chemistry department acquires a peptide synthesizer or has sufficient funds to buy these custom made peptides.

The data in figure 2 suggests several possible ways to use this function to explore the mechanism of Brdicka current. One question which could be asked is if the fact that a point on the graph is above or below the line has any theoretical meaning. One possible explanation could be that a protein below the line has more hydrophobic sulfhydryls, and hydrophobic sulfhydryls are polarographically somewhat less active than hydrophilic sulfhydryls. An experiment to test this possibility was run. Proteins were denatured with urea and 2-mercaptoethanol, because these denaturing agents were not polarographically active themselves (in any way that would have affected the Brdicka waves). Denaturing the protein increases the ratio of hydrophilic to hydrophobic sulfhydryls. If the sum of the two wave heights were still the same after denaturation, then this hypothesis would have been disproved. As it turned out, urea had the effect of weakening the current somewhat, so the urea denaturation experiments could not reveal any information on the relative polarographic reactivity of hydrophobic and hydrophilic cysteines.

The urea/2-mercaptoethanol denaturation experiments revealed that conformation is not an important factor in determining the position of one of the data points in figure 4 with respect to the line. Denatured proteins did not, generally speaking, change their positions relative to the line (See figures 3 and 4). This fact supports Kuznetsov's claim that previous to the catalytic reaction the protein is unfolded and flattened onto the surface of the Hg drop to such an extent that only a single layer of amino acids is present (Kuznetsov and Shumakovich 1975).

Perhaps a better hypothesis to explain the position of a point below or above the line is to be found in considering the positive or negative charges of the domains in the protein which contain the cysteine residues. If a domain were negatively charged, it would tend to move away from the negatively charged electrode, so its sulfhydryl group would not be as available for activity at the mercury drop surface. Likewise, if a domain were positively charged, it would be further sucked into the reactive layer. Each protein was analyzed to see if the amino acids immediately adjacent to its cysteines would be positively or negatively charged at pH 9.3. The ratings, positive, negative, or neutral, were assigned to each cysteine, and then added up for all the cysteines in a protein to give the protein an overall charge rating. Figure 5 repeats the data found in figure 2, except this time the points have their "charge rating" printed next to them. Finally, some light is being shed on the question of a point's relative position with respect to the line. The following rule is supported by the data: All points with a positive rating are on or above the line (more polarographically active), and all points with a negative charge rating are on or below the line (less polarographically active). This fact makes good intuitive sense, because a positively charged environment would be "sucked" into the negatively charged Hg electrode due to electrostatic forces. The cysteine would be closer to the source of electrons, and hence more reduction current could flow. On the other hand, if a cysteine's environment were negatively charged, it would be repelled from the Hg electrode by electrostatic forces, and less current would flow because the cysteine would be farther from the source of electrons. This charge analysis is only a partial explanation for the relative reactivities of the proteins, however, because

there are, after all, both positively and negatively rated proteins on the line.

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Figure 1. Brdicka plot for chymotrypsin

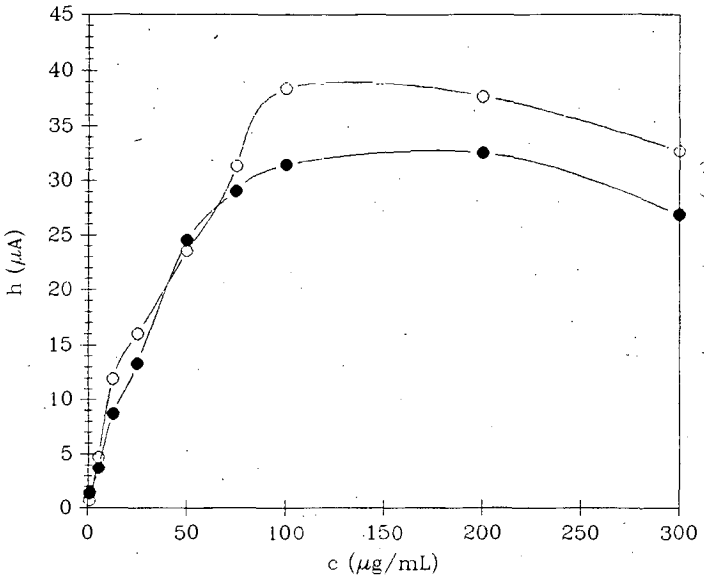


Figure 2. The linear dependence of Brdicka current on % cysteine.

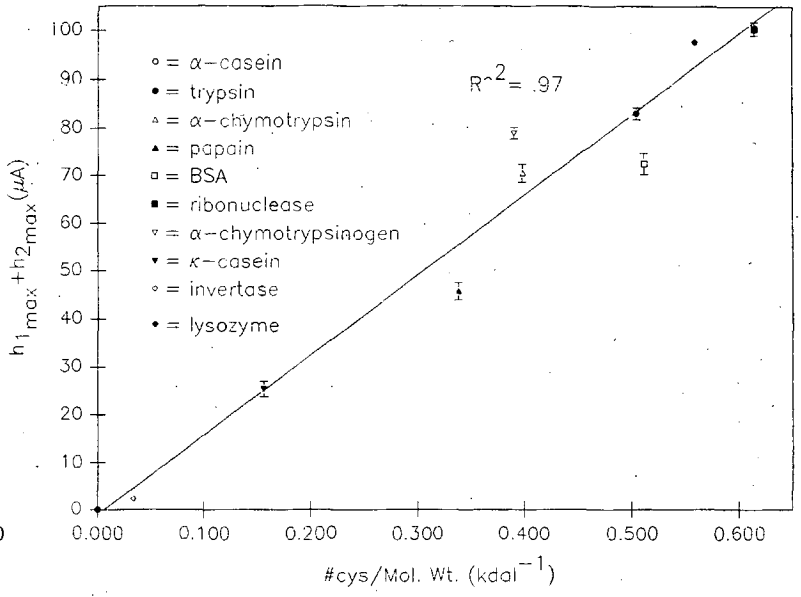


Figure 3. The data taken with proteins denatured in 4 M urea.

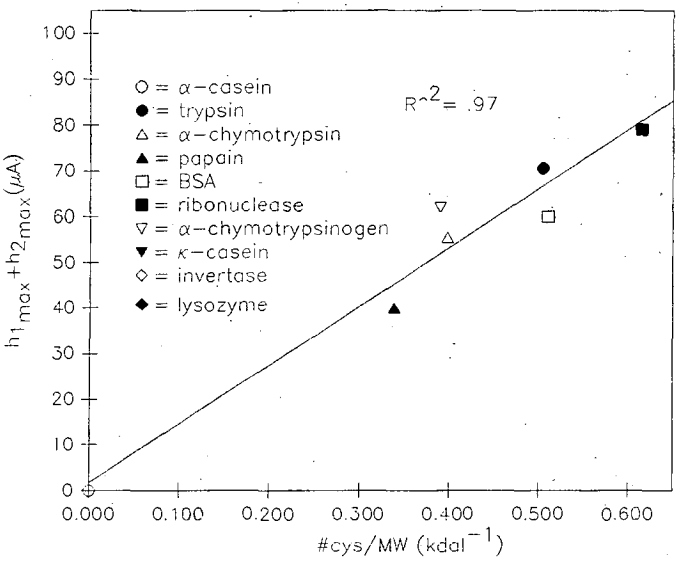


Figure 4. The data taken with proteins denatured in 8 M urea.

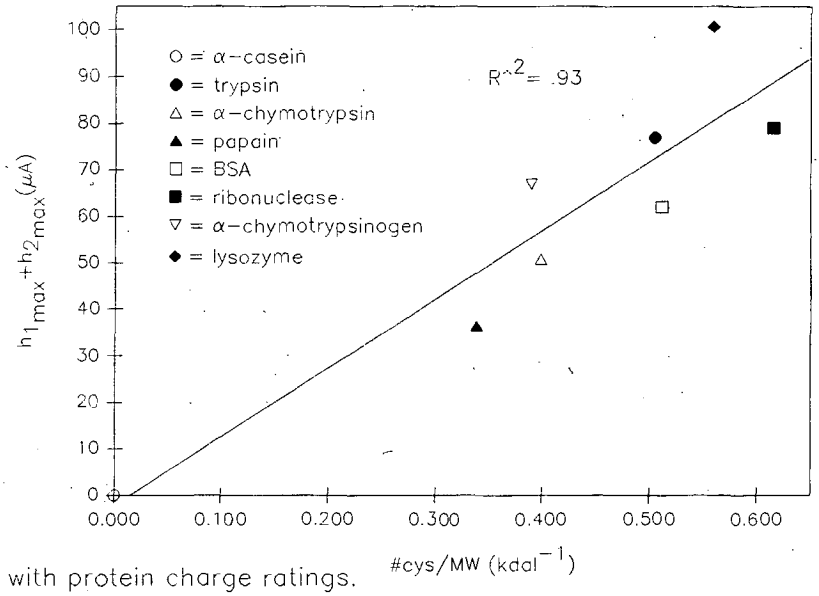
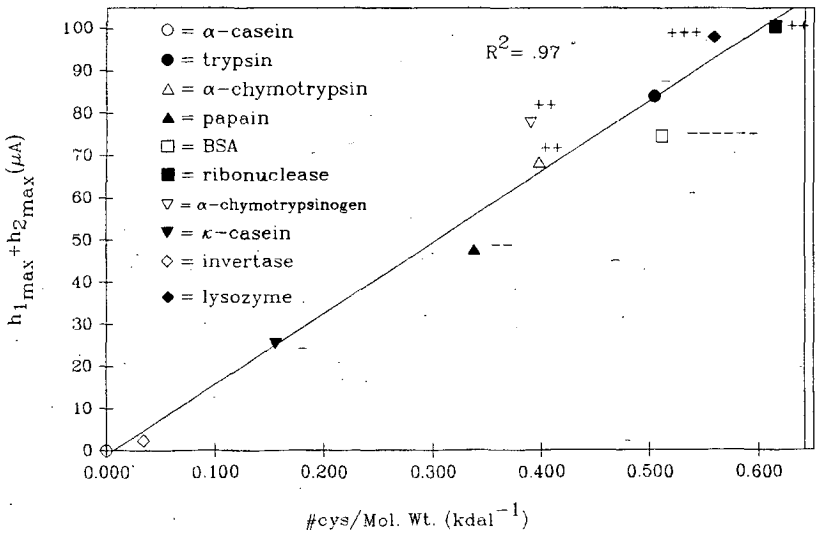


Figure 5. The data with protein charge ratings.



URINARY CHEMOSIGNALS AND THEIR EFFECT ON THE SEXUAL MATURATION OF FEMALE MICE

JANINE LISSARD

ABSTRACT

The influence of urinary chemosignals on the onset of puberty was observed in four groups of the three female mice each. Prepubertal female mice were treated with three types of urine and monitored by vaginal smears for first estrus. First estrus or puberty was recognized by the abundance of cornified epithelial cells. In addition there were four control groups in which water was applied to calculate the average age of puberty without treatment. The experiment consisted of successfully sexing the litters by day 21, then beginning treatment on day 22. Vaginal smears were taken to monitor the vaginal cellular growth, leading to the onset of puberty. Male urine causes the acceleration of puberty whereas, grouped female urine and a mixture of both male and female urine results in the delay of puberty.

INRODUCTION

The sexual maturation of female mice is influenced by a hierarchy of cues such as the light cycle, nutrition and chemosignals. Several of these chemosignals are found in the urine of other mice. Odorous substances produced by mammals contain volatile hydrocarbons, or pheromones which can affect the estrus cycle and therefore the rate of sexual maturation of mice. (Parkes and Bruce, 1961)

A considerable amount of research has been done concerning various reproductive aspects of mice in relation to phermones. It is known that odor can constitute an extroceptive factor affecting the estrus cycle and pregnancy through neurohumoral mechanisms. For example, as a result of olfactory discrimination the pregnancy of a recently inseminated female mouse can be blocked due to presence of an "alien" male, especially if of a different strain than the stud male. This is called the Bruce effect. Another interaction, the Lee-Boot effect is the suppression of the estrous cycle due to the crowding of female mice. In contrast, the presence of a male with a large group of females will induce and accelerate the estrous cycle of the females, the Whitten effect. (Parkes and Bruce, 1961)

The endocrinological basis for these changes in the onset of the estrous cycle is the control of FSH. Pheromones from females suppress pituitary release of the follicle-stimulation hormone (FSH), whereas male phermones stimulate the release of FSH which is followed in normal sequence by LH release and ovulation. The sexual pheromones involved in these effects are modified steroids (steroid metabolites) which are transmitted via the urine of grouped females or a male mouse to the olfactory apparatus of a female. (Norris, 1980)

The primary goal of my experiment was to test urinary chemosignals and their effect of acceleration and delay on the timing of puberty. In contrast to the Whitten and Lee-Boot effects, I tested juvenile female mice, prepubertal, rather than adult female mice. The experimental procedure was based on accurate vaginal smears. This entailed being able to sex the mice early enough to know that the estrus observed was their first estrus. It was necessary to be very familiar with the vaginal stages in order to immediately recognize the estrus stage. This stage characterizes the onset of puberty.

From the first practice trial, I observed a pattern leading to first estrus. Initially there are essentially no cells or only parts of cells. Then gradually the smears consist of a mucus-like substance which eventually becomes an accumulation of cornified epithelial cells. This is the estrus stage, which indicates that active growth is in progress in various parts of the genital tract.

METHOD

Sixteen juvenile female mice of the strain NSD:ICR were divided into four groups and tested. Each mouse was housed separately. In addition, there was one group of eight adult female mice housed together and two groups of adult male mice with three in each. The juvenile female mice were held in a different room from the others but under identical conditions. The lighting system of the rooms were on a twelve hour cycle, 12 dark:12 light. The mice were fed ad libidum and the cages were cleaned regularly.

To obtain experimental juvenile females, several matings were completed. During this period, the cages were carefully observed in order to record the date of birth of the offspring. It was crucial to know the age of the mice throughout the experiment since the onset of puberty was distinguished by age. On day 21, after their birth, the mice were weaned and the females were placed in separate cages. On day 22, the treatment began.

There were four different treatments with four mice in each. On day 22 each mouse received treatment daily, between approximately 3pm to 5pm. The four treatments were male urine, grouped female urine, a mixture of the two and water only. Each day .5ml of urine was applied to the nares of the mice with a syringe. The mixture of the two urines consisted of .25ml male and .25ml grouped female urine. In addition, vaginal smears were taken daily. These vaginal smears were obtained by pippette or lavage method according to the techniques suggested in Experimental Endocrinology (Zarrow, 1964).

The urine used for treatment was collected every two days. This was necessary to ensure the effectiveness of the urine (Drickamer,1986). I collected urine from both the adult male mice and female mice in the same way. The mice were placed in a cage with a wire lid and this cage was turned upside down on top of another open cage. The bottom cage served as a collecting device which worked sufficiently.

The urine collected was applied daily until first estrus occurred and was verified. Even when clumps of cornified epithelial cells were viewed, I continued the treatment to monitor the estrous cycle. The vaginal smears following first estrus were taken to validate my conclusions of the onset of puberty. If correct, the last two stages of the cycle occurred which are metestrus and diestrus. The metestrus is characterized by a variety of cells, cornified epithelial cells, nucleated epithelial cells and leukocytes, and diestrus is mainly leukocytes with a few nucleated epethelial cells. If the last two stages did occur then it was clear that puberty had begun. If metestrus did not occur, then further clumping of cornified epithelial cells was observed extending the date of first estrus.

RESULTS AND DISCUSSION

The acceleration and delay of puberty in prepubertal female mice was observed due to the effect of urinary chemosignals. According to the data collected, male urine results in acceleration whereas grouped female urine and a mixture of both types causes a delay in the onset of puberty.

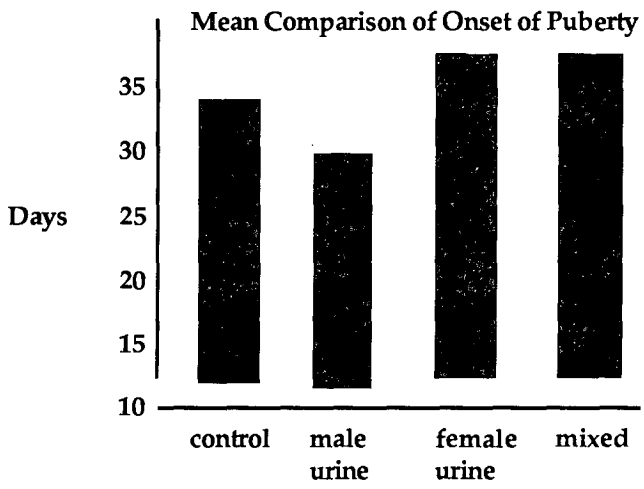
Data:

Table 1
Day of First Estrus

	Control	Male Urine	Female Urine	Both-(M&F)
	29	27	33	34
	30	28	33	34
	31	28	34	35
	31	26	36	33
mean	30.25	27.25	34.00	34.00
S.E.	0.48	0.48	0.41	0.41

* The averages are presented in Graph

Graph 1



From these data, three different statistical test were run. They were analysis of variance, a Scheffe test and the Mann-Whitney Confidence Interval test.

The one-way analysis of variance showed a treatment effect ($F=38$; $df=3,12$; $p<.001$). The Scheffe's test ($a=.05$) showed the following unconnected means to be significantly different.

male-27.25 control-30.25 female-34.00 mixture-34.00

Because of the small sample sizes I also performed a nonparametric Mann-Whitney test. The results of this test agreed with those of the analysis of variance. It showed that there was a significant difference ($p<.05$) in the onset of puberty between each group except for between the female and mixed urine treatments.

These results give strong evidence that male urine will accelerate and grouped female urine or mixed urine will delay the onset of puberty. If I had more time with this experiment, I would continued treatment and monitored its effect by vaginal smears. I hypothesize that the male urine would continue to accelerate the estrus cycle so that the period between each cycle is shortened. In addition, it seems that the grouped female urine and mixed urine would increase the period between each cycle causing the females to rarely be receptive to a male.

The fact that female mice are more sensitive to the delay chemosignal proven by the mixed urine, may be of evolutionary significance. A young female mouse may be more successful in reproducing if she is better attuned to a signal that indicates social or other environmental conditions are not adequate, the delay signal, rather than to urinary chemosignals accelerating sexual development. Female mice do not release this signal unless they are experiencing unfit conditions such as over-crowding, food deprivation, or temperature extremes. The ability to produce this signal may have evolved as a safegaurd against wasting reproductive energies. For males however, the ability to produce an acceleration signal is to their reproductive advantage. Males are capable of acquiring potential additional or new mates. In addition, although puberty acceleration may be a somewhat risky reproductive strategy for females, it does play an important role in sustaining reproduction. (Drickamer, 1986 &1988)

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A CORRELATIONAL STUDY OF THE ESTERASE CONTENT FOR NYMPHAL VS. ADULT WHOLE BODY COCKROACH HOMOGENATE FOR PURPOSES OF DETERMINING A NON-LETHAL PROCESS OF SELECTION FOR CHLORPYRIPHOS RESISTANCE

PAMELA M. McQUILLEN

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ABSTRACT

Various methods of hemolymph extraction were attempted in order to obtain an assay for resistance that allowed the organism to survive to reproductive maturity. It was found that the hemolymph of the insect is not a good indicator of esterase activity or resistance due to its variable nature. Further testing procedures are suggested and are currently being examined.

INTRODUCTION

Blattella germanica, the German cockroach is one of the most loathed insect pests. Cockroaches are economically and medically menacing. They devour human foods in homes, ships, and restaurants (Mallis, 1964). Researchers have established the presence of "phage-typable Staphlococcus aureaus" in the feces of strains of B. germanica that infest homes and businesses(Mallis, 1964). Despite repeated attempts by pest control programs to develop an insecticide that is capable of eradicating total populations, control failures and subsequent cockroach resistance continue to be the norm (Milio, Koehler, and Patterson, 1987). Two possible methods of evolved cockroach resistance are amplification of an "esterase gene" (Mouches et al., 1986) and/or genetic mutation (Oopenworth and Van Asperen, 1960).

It is known that a directly proportional correlation exists between resistance to organophosphorous insecticide and esterase activity in Blattella germanica (Jones and Bancroft 1983,unpublished). However, attempts to further establish the relevance of this link have been hampered by the nature of LD50 and esterase content assays (see Domon and McCanless, 1989 for explanation of techniques). Insects killed by exposure to the insecticide (in LD50) or homogenized (for esterase activity assay) cannot be used for further studies. Therefore, in the absence of a non-lethal protocol, it has been impossible to directly establish the mechanism by which resistant animals survived (because it is impossible to run the same test before and after treatment with insecticide).

The purpose of this experiment was to develop a new selection procedure that allowed the insect to remain alive for subsequent testing. The nymphal hemolymph presented itself as an easily obtainable, non-lethal animal product that could be tested for esterase content without killing the roach. Hemolymph is defined as a clear, colorless fluid, "the medium through which all chemical exchange between organs are affected." It is through the hemolymph that hormones are conveyed, food and oxygen are brought to somatic cells, and waste products are carried to excretory organs (Wigglesworth,1982). Extraction of small volumes of hemolymph causes little stress to the animal (Wirtz and Hopkins, 1987). An attempt was made to correlate the esterase content of a known volume of hemolymph to that of the whole body of the adult in order to establish improved selection and esterase measuring protocols.

The author wishes to thank Dr. Bobby Jones for his patient guidance.

MATERIALS AND METHODS

A list of the materials used can be found in Appendix 1. The Hazard-WARF strain of Blattella germanica were used. They are a susceptible strain originating from Wisconsin Alumni Research Foundation and obtained from cultures kept at Texas A&M in 1986. The roaches were kept in five-gallon plastic buckets with fine-mesh lids and supplied with water (with a small amount of bleach to

prevent infection) and Dog Chow.

A sample of forty, mixed stage, last instar nymphs from the strain were isolated. Individual roaches were separated in individual collection vials with sponge stoppers. Each roach was anesthetized with CO2 and punctured with a 27.5 gauge needle between the second and third shield on the dorsal abdomen lateral to the medial line of the insect. The drop of hemolymph forming on the back was collected with a 1ul innoculation loop and placed into a test tube with 1ml of buffer in it. The test tubes were vortexed and the innoculating loops removed.

To each test tube 5ml of B-napthyl acetate substrate was added and the tubes were allowed to incubate for thirty minutes at 37oC in the water bath. After this amount of time 1ml of 3.4% Sodium lauryl sulfate with Fast garnet (made up 25 minutes prior to addition) was added to each tube. Tubes were again vortexed and allowed to develop for 5 minutes. After 5 minutes the absorbance of the solutions in the tubes was read on a spectrophotometer set at 560nm.

The roaches were allowed to recover in a 90-110oF incubator. They were checked every twenty-four hours for molting. Five days after molting, adults were sacrificed by freezing and homogenized according to body weight in 0.04M sodium phosphate buffer 1/10 ml buffer/mg of roach. 10ul of the homogenate solution was added to tubes containing 1ml of buffer and the tubes were processed as outlined above. Data were collected over a period of seven days.

RESULTS

Various correlations were attempted of the whole body homogenate absorbance values and the hemolymph absorbance values. Raw data and statistical manipulations may be found in Appendix 2. The data were processed as a single group although they were collected over a period of seven days. A correlation was attempted of the whole body homogenate of the adults and the nymphal hemolymph absorbance values. Only 9.7% of the variation may be explained by this regression (R-sq=9.7%). A better correlation was obtained when the animals were grouped according to sex (R-sq=14.7% for females, and R-sq=12.0% for males). These percentages are not high enough to support a link between hemolymph and whole body homogenate absorbance values.

When the absorbance values are restricted to those nymphs that became adults within a twenty-four hour period of one another, the R-sq value increases dramatically (R-sq=26.4%). Unfortunately, the sample size for this facet of the experiment is small (n=5) and replications were not performed.

DISCUSSION AND SUGGESTIONS FOR FURTHER RESEARCH

The purpose of this experiment was to develop a non-lethal selection procedure for resistance and lack of resistance to chlorpyriphos insecticide in Blattella germanica. The experiment focused on a new selection procedure based on examination of the nymphal hemolymph. The idea was to use the higher and lower hemolymph absorption values as indicators of resistance and/or lack of resistance. It was hoped that a correlation of nymphal hemolymph absorbance values and adult whole body absorbance values would be obtained. However, the correlations seen were not high enough to justify the employment of nymphal hemolymph as an indicator of esterase activity levels in the roach.

Possible reasons for the unreliability of hemolymph were revealed by further experimentation and literature searches. Hemolymph has an extremely complex nature. These complexities induce variables that must be controlled in future research. Dessication of the animals and its effect on the concentration of various components of the hemolymph (Wigglesworth, 1982 and Hyatt and Marchal, 1985 Tucker, 1977)), variations in hemolymph due to bodily preparations to undergo ecdysis, and increase of the osmotic pressure of the hemolymph due to deprivation of O2(Wigglesworth, 1982) are three of the variables discovered which may have skewed the data obtained and must be controlled in further research.

Appendix 1: List of Solutions and Equipment Used

Solutions:
0.04 Sodium phosphate buffer
3.4% Sodium lauryl sulfate with Fast Garnet
Beta-napthyl acetate substrate

Equipment:
dissecting microscope
glass insect dissecting dish with styrofoam insert
CO2
27.5 gauge hypodermic needles
disposable 1ul innoculating loops
test tubes
waterbath at 37oC
SMI topsider dispenser
featherlight forceps

Appendix 2: Raw data values and statistical tests

Female hemolymph absorbances:	Adult (F) wholebody absorbances:
0.031	0.470
0.019	0.528
0.055	0.502
0.005	0.460
0.013	0.553
0.023	0.561
0.021*	0.428*
0.008*	0.444*
0.007*	0.447*
0.068 *	0.378*
0.065 *	0.487*
0.128	0.425
0.063	0.512
0.011	0.405
0.061	0.138
0.091	0.206
0.066	0.446

Male hemolymph absorbances:	Adult(M) wholebody absorbances:
0.042	0.512
0.023	0.653
0.019	0.385
0.055	0.490
0.010	0.351
0.024	0.478

*Molted in the same 24 hour period.

Correlation of all hemolymph absorbances to all whole body absorbances:

regression equation-- wholebody=0.489-1.08 hemolymph

Predictor	Coef	Stdev	t-ratio	p
Wholebody	0.48933	0.03620	13.52	0.000
Hemolymph	-1.0844	0.7230	-1.50	0.149

R-sq=9.7%

Correlation of male hemolymph absorbances to male whole body absorbances:

regression equation-- wholebody=0.414+ 2.23 hemolymph

Predictor	Coef	Stdev	t-ratio	p	
Wholebody	0.41389	0.09833	4.21	0.14	
Hemolymph	2.229		3.021	0.74	0.502

R-sq=12.0%

Correlation of female hemolymph absorbances to female whole body absorbances:

regression equation-- wholebody=0.488-1.23 hemolymph

Predictor	Coef	Stdev	t-ratio	p
Wholebody	0.48832	0.04191	11.65	0.000
Hemolymph	-1.2264	0.7629	-1.61	0.129

R-sq=14.7%

Correlation of hemolymph absorbances and whole body absorbances that were obtained from roaches that molted within a 24 hour period*:

Predictor	Coef	Stdev	t-ratio	p
Wholebody	0.4403	0.1302	3.38	0.43
Hemolymph	-2.311	2.231	-1.04	0.376

R-sq=26.4%

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THE EFFECTS OF ATRAZINE ON SCHOOLING BEHAVIOR IN THE FISH BRACHYDANIO RERIO

Lee Robin

ABSTRACT

Schooling behavior in the fish Brachydanio rerio was observed and separated into subunits of schooling (horizontal, vertical, stationary, and non-orienting). Five fish were observed in an unpolluted aquatic environment to determine time spent schooling and non-schooling and time spent in each of the subsets of schooling within a thirty-minute period. These same five fish then were then observed in water containing Atrazine, a herbicide, at increasing concentrations of 0.04, 0.10, 1.00, and 4.00 ppm. The varying concentrations were used to determine if there is a correlation between schooling behavior and Atrazine concentration. The use of Atrazine in this study was intended to stimulate the effect of a stream polluted by agricultural runoff.

Overall schooling behavior decreased as a linear function of increasing Atrazine concentration. Also, increased proportion of time spent in the less active subsets of schooling was observed as Atrazine concentration was increased.

INTRODUCTION

One of the most widely published areas of study on fish behavior has been that of fish schools. Morrow (1948) defines a school as "a closely-knit cohesive group in which there appears to be a definite centripetal influence existing between fish and fish." Permanent schools are characterized by a "high degree of stability from external factors" (Parr, 1927), which indicates the presence of internal factors (within the school or within the individuals) that elicit the schooling behavior. Proof of this theory comes by way of Parr's (1927) experiments with newly hatched fish that immediately schooled before there was any time for them to learn that this behavior is beneficial, and also by the fact that two fish were enough to form a school. This school of two indicates an individual rather than a group attraction (Parr, 1927). Breder and Halpern (1946) studied the fish Brachydanio rerio and found that their schooling behavior was also unlearned. Fish of this species do not school until they are several months old. To determine if schooling behavior was unlearned, fertilized eggs were isolated and the young were raised singly until they reached an age of several months. These fish were then placed in an aquarium with a school of young of comparable age and were observed to immediately form a school with these other fish (Breder and Halpern, 1946).

The phenomenon of schooling in fish, although widely documented, has raised a number of questions. For example, what is the mechanism for this behavior? In addition, what external factors may affect this behavior and in what manner? In the following paragraphs, I will cite the more strongly documented theories on mechanisms for schooling behavior and also some factors that have been determined to affect this behavior.

One of the most commonly published factors in affecting schooling behavior has been that of vision. Parr (1927) removed or cover the eyes of the fish in his experiment, and schooling immediately ceased. Also, Parr's experiment showed that schooling stops during darkness or when light falls below a certain threshold (Morrow, 1948). Formation of a school seems to depend upon the attraction that one fish exerts upon another. Fish usually remain "parallel to and at a given distance from "the other fish in the school, and it is believed that this distance is maintained by a "balancing of visual stimuli from each side" (Morrow, 1948). Spooner (1931) supported the vision theory by evidence that a fish will

school with its own reflection in a mirror.

Breder and Nigrelli (1935) suggested that fishes school because each of the fish provides a point f reference to which the other fish react. They wrote that schooling is influenced by the form and size of the other fish, by color vision, and by motion detection. Most researchers, therefore, agree that vision is of primary importance in schooling, yet there is evidence of other contributing factors. A possible avenue to determining the mechanism is to study the external factors that can influence schooling behavior. The problem with this approach is that many factors influence schooling, and these factors act in different ways in different species.

Changes in the physical properties of the water and exposure to certain toxic chemicals have been shown to produce marked alterations in schooling behavior. Breder (1967) wrote that schools are permanent "excepting only when physical conditions in the environment suppress the functioning of some essential system, usually the optical." In general, "an aquatic community exposed to a toxic chemical will be altered By the combined responses of its members" (DeNoyelles, 1982). As organisms are initially disturbed by the chemical, interactions such as predation and competition can be changed throughout the community. The herbicide Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) has been shown to cause disturbances in aquatic communities (DeNoyelles, 1982), although specific references to its effect on schooling behavior were not found.

In 1978, Atrazine was the most heavily used pestacide in the United States. Forty-one million kilograms (active ingredient) of the chemical were applied on United States farms, mainly for weed control (DeNoyelle,1982). DeNoyelle wrote that Atrazine usually degrades in nature in 1.5 years, yet this says nothing of the immediate effects on organisms exposed to the chemical. One source linked Atrazine pollution to decreased sodium uptake in fish (McKim et al, 1976), yet made no mention on behavioral effects. Other sources merely published LD-50 values. This study examines the effect of Atrazine from a behavioral standpoint, specifically that of schooling behavior.

METHODS

The Zebra Danio, Brachydanio rerio, was chosen for this experiment because of its availability and widely documented schooling behavior. A school of thirty Zebra Danios was obtained from a local pet store, and these fish were housed in a 30-gallon aquarium filled with aged tap water (unpolluted by Atrazine). An experimental aquarium also was set up, and a diagram of this tank appears on the following page. This 30-gallon aquarium was divided in half with tape as a marker (no actual barrier existed within the aquarium), and a clear, one gallon jar was placed on either side (equidistant from the tape). Into one of these jars, a school of 15 Zebra Danios was introduced, and the other jar was filled only with water as a control. To determine the subsets of schooling, the other 15 fish were introduced individually into the open area of the aquarium and their behavior was observed. From this, the five subsets of schooling behavior were determined.

Three of the subsets occur only when the fish is on the "schooling side" of the aquarium, ie., the half of the aqurium containing the jar with fish. Horizontal schooling is exhibited when a fish passesback and forth in a horizontal orientation (that is, swimming close to the jar with its body situated in a similar manner to the other fish) with the fish in the jar or circles around the jar in a horizontal manner. Vertical schooling occurs when a fish swims up and down in orientation with the other fish. Stationary schooling occurs when a fish is in orientation with the fish in the jar yet remained virtually motionless.

Non-schooling, in contrast to the previous three subsets, occurs when a fish is on the side of the aquarium with the empty jar and therefore is not interacting with the school. Non-orienting is the behavior exhibited when a fish is on the "schooling side" yet is not in any way orienting towards them. Non-schooling is also considered a part of non-orienting.

After these subsets had been determined, five of the fish were chosen at random as the experimental fish and were housed singly in one-gallon jars (resting in a water bath to maintain constant temperature) for ease in identification. The other fish were returned to the original holding tank. The temperature in the jars, the holding tank, and the experimental tank was a constant 24 degrees Celsius.

Before each run, a school of 15 fish was placed in the jar on the "schooling side" of the aquarium

and an experimental fish was introduced directly in the center of the open part of the aquarium and allowed to acclimate for thirty minutes. After this time, the activity in the aquarium was videotaped with a video camera on a tripod. To control for outside influences, the room was empty during filming. Following this time period, I viewed the video tape and time spent on the "schooling side", the "non-schooling side", and in each of the subsets of schooling was recorded and analyzed using paired t-tests, one-way analysis of variance, Fisher's Least Significant Difference test, and regression analysis.

This technique was performed for each of the fish in a control environment where the water was uncontaminated by Atrazine, and then for the same five fish in environments of increasing Atrazine concentrations. These concentrations were 0.04, 0.10, 0.40, 1.00, and 4.00 ppm (the 4.00 ppm trial was actually performed several months prior to the other trials, but the control data for both occasions were similar so that the data for 4 ppm were included in the overall analysis). The Atrazine conditions were created by dissolving the appropriate amount of 80% active Atrazine wettable powder in a small volume of methanol (for solubility) and then introducing this solution into the aquarium (not into the jars) with good mixing. The same amount of methanol was introduced into the control environment to correct for any effect that the methanol may have had on the behavior of the fish.

RESULTS AND DISCUSSION

The data obtained from control conditions were compared with the data from each fish subjected to the various Atrazine concentrations. The same fish used as control #1 was used as Atrazine #1 to control for any variation among fish.

In Figure 1., the total schooling time of each of the five fish at concentrations of Atrazine was plotted and a regression analysis was run to determine the "closeness of fit" of the line through these points. There was significant negative relationship between schooling time and the Atrazine concentration. Although the sample size in each Atrazine concentration was small, the close fit of the data ($r = 78.4\%$) suggests that Atrazine does indeed cause suppression of schooling behavior in the Zebra Danio.

Following the regression analysis, a one-way analysis of variance was run on the mean schooling time at each concentration of Atrazine, and the difference was found to be significant ($F=25.30$: $df=5, 24$; $p<0.0001$). Since the difference between means was significant, a Fisher's Least Significance Difference test was used to determine which concentrations were significantly different from each of the others. The results showed that the mean schooling time at the four lowest concentrations (0, 0.04, 0.10, and 0.40 ppm) were not significantly different from the others. That is, there was a significant effect of the Atrazine on the schooling time starting at 1 ppm.

The next group of data that illustrates the effects of Atrazine on schooling behavior is contained in Table 1. In this table, the mean time (minutes) spent in each of the subsets of schooling (HS=horizontal schooling, VS=vertical schooling, SS=stationary schooling, NS=non-schooling, and NO=non-orienting) is listed for each concentration of Atrazine. This table shows the general trend as a function of concentration. Separate paired t-tests were run on the data from each subset, and those sets that are significantly different from the control are indicated by the asterisk.

As this table illustrates, horizontal and vertical schooling, which are the more active subsets of schooling (ie., they entail more movement), decrease in total time with increasing concentrations of Atrazine. That is, the fish engage less frequently in these subsets as their environment becomes more concentrated in Atrazine.

Conversely, the table shows that stationary schooling and non-orienting increase as Atrazine concentration increase. That is, the less active subsets of schooling are exhibited more frequently when the concentration of Atrazine is greater. This finding is supported by many studies that list decreased activity as a general effect of change in water quality. The fact that non-schooling increases as a function of increasing concentration merely emphasizes the data from Figure 1 where schooling time decreases as a function of increasing Atrazine concentrations.

What, then, is the mechanism for this decrease in overall schooling activity with increasing Atrazine exposure? Since there were no studies found on this specific topic, a synthesis of studies was used to form a conjecture. Langlois (1936) showed that adverse physical conditions of water caused a fish's "slime coat" to disappear, and that either because of the loss of this protective covering or

because there was a disturbance of other membranes, the fish was "uncomfortable". Langlois' study was specifically with chlorine as the contaminent but can possibly be applied to Atrazine contamination as well. The fish in Langlois' study were also described as "sluggish", which describes perfectly the movements of the fish in my experiment. If, therefore, there has been degredation of the protective membranes of these fish, there could also have been some effect on the eyes of the organism, and vision has been widely demonstrated as a major portion of schooling mechanism. This could possibly cause a lower rate of schooling activity because the fish simply are not able to see the other fish well.

An experiment that could be designed to test the conjecture would have three parts. First, it would be necessary to determine if fish do have a depression in schooling behavior with increased turbidity of the water. The experiment by Parr (1927) where lowered visability caused termination of schooling follows along these lines and was already explained in the introduction of this report. Second, it would be necessary to demonstrate a relationship between the mucous coat and vision. That is, does destruction of the mucous membrane of the eye have an effect on vision? The final part of this experiment should be to introduce some type of know mucous-dissolving agent into the water to determine if it had the same affects on schooling behavior as did the Atrazine. This experimental design would help to determine if vision plays a major role in schooling behavior, and if it does, would also determine if dissolving mucous coating causes a decrease in schooling and therefore probably a deterioration of vision.

Whatever the mechanism for schooling behavior, however, this experiment illustrates that increasing concentrations of Atrazine do affect schooling behavior in a negative linear relationship in the Zebra Danio. If this were to occur in nature, the selective advantage of schooling would be lost, which would confer upon the individual a great selective disadvantage.

First, schooling is a mechanism for predator avoidance (Partridge, 1982). When a predator attacks a school of fish, they usually react by one of two mechanisms. Either they engage in the "flash expansion" where each fish darts outward away from the others to give an explosion-like appearance or they engage in the "fountain effect" where the school parts in the middle and circles around behind the predator (Partridge, 1982). Both of these mechanisms create sensory confusion so that the predator has a difficult time homing in on one individual to attack. If a fish swims alone, it stands a much less chance to escape because there is nothing to distract the predator from that fish (Partridge, 1982)

Another important advantage that schooling holds is that it aids in mating success. Phillip (1957) demonstrated on a mathematical basis that a social population has a distinct advantage over an asocial one in relation to reproductive potential. If an individual strays from the school, it is virtually lost from the "reproductive pool" (Breder, 1959). Along these lines, loss of the schooling response would not only decrease efficacy in mate competition but also in competition for other resources such as food.

Therefore, the fact that Atrazine does have a sublethal effect on Zebra Danios by depression of schooling behavior and that this effect increases with increasing Atrazine concentrations is quite significant. Though these concentrations do not kill the fish , in a natural setting they would probably not survive due to their increase vulnerability. Also, even if the fish were not consumed by predators, they would have little chance of successful reproduction.

There were several possible problems in this experimental design. First, methanol is not present in natural environments to said in dissolving the Atrazine, so larger amounts of the chemical would be necessary to create reasonable solubilities. Also there is a chance that methanol in conjunction with Atrazine actually increases the effects of the Atrazine on the fish in an additive manner. And finally, there could have been an additive effect on the individual organisms since the same five fish were used at each concentration (although the fact that the fish used in the 4 ppm trials were different and still conform to the same linear relationship partially negates this arguement). These problems could be overcome by merely changing the experimental conditions to a more natural setting with greater resources and more time.

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