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**RHODES**  **COLLEGE**  
**SCIENCE** **JOURNAL**

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Number 1

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The Rhodes College Science Journal

Volume III, Number I  
May 1985

Preface

In keeping with the renaming of the college, this year ushers in the first issue of the Rhodes College Science Journal, formerly the Southwestern Science Journal. Although our name has changed, our goals remain the same: to recognize the superior research and investigation of our fellow students and to encourage among them the continuing pursuit of scientific excellence.

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Our special thanks to alumni and friends whose generous contributions made this publication possible.

1

DNA-BINDING PROTEINS AND RNA TRANSCRIPTIVE FUNCTION  
IN DIFFERENT SUBCELLULAR FRACTIONS OF HeLa CELLS

by Beth Baxter

## INTRODUCTION

The organization of transcriptionally active chromatin (TAC) is important in gaining an understanding of the mechanism of cellular transcription. The spatial organization of TAC within the nucleus and the structure of TAC itself can reveal some variables affecting gene regulation. Research has indicated that the nuclear matrix (NM) may play a key role in the orientation of chromatin within the nucleus. On another level of organization, TAC can be shown to be composed of nucleoprotein complexes containing non-histone proteins. Studies in both of the areas may reveal the organization of TAC and thus further demonstrate the mechanism of transcription.

A number of studies have identified a subnuclear structure known as the NM, which consists of an extensive non-chromatin proteinaceous intranuclear network, residual components of the nucleolus, and a peripheral lamina (Bouieille, 1983). These proteinaceous fibrils are thought to retain the overall shape of the nucleus and confer structural stability upon the interphase nucleus (Agutter *et al.*, 1980). This structure is prepared from isolated cell nuclei after treatment with salt to remove loosely bound protein, treatment with nucleases to remove associated chromatin, and treatment with detergents to remove nuclear membranes. The composition of the structure isolated from this method is approximately 98% protein, 0.1% DNA, 1.2% RNA, and 1.1% phospholipid. The protein usually consists of three to five major polypeptides depending on cell type.

The NM has been implicated in the cellular processes of transcription and replication (Robinson *et al.*, 1983; Agutter *et al.*, 1980). Studies using radioactive precursors have demonstrated that newly replicative and transcriptive products can be found in association with the NM. These observations suggest that active chromatin may exist in association with the NM, and that transcription may occur on the eukaryotic NM in chromatin-NM complexes. Furthermore, studies have shown that initiation of DNA replication is localized in the matrix (Agutter *et al.*, 1980). This implies that the NM may serve as a scaffold on which DNA replication occurs.

The function of the NM in transcription can also be demonstrated by the presence of transcriptional cofactors associated with the NM. Studies (Abulafia *et al.*, 1984) have shown that the NM does have a relatively large amount of associated RNA polymerase. These findings, in addition to those previously stated, support the hypothesis that the NM plays a role in the spatial organization of chromatin during the cellular processes of replication and transcription.

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When seeking to elucidate the mechanism of transcription in eukaryotic systems, one should also examine the structure and composition of eukaryotic chromatin. Simian virus 40 (SV40) chromatin, composed of 70% viral chromatin and 30% eukaryotic chromatin, behaves in a manner which is empirically similar to eukaryotic chromatin. (Das *et al.*, 1981). SV40 chromatin can then be used to determine eukaryotic characteristics under the assumption of their analogous reactivities.

It is known that inactive chromatin consists of histone proteins bound to nucleic acid. Studies have recently revealed structures composed of nucleic acid bound with non-histone proteins, collectively known as nucleoprotein complexes (NPC) (Sinha *et al.*, 1982). These structures have been demonstrated to be transcriptionally active as viable templates. In addition, transcriptional reactions using non-histone NPC have been found to be less dependent on transcriptional factors, suggesting that NPCs are enriched in these factors. This evidence supports the hypothesis that non-histone NPCs may serve as an intermediate transcriptive template structure. It would follow from this that cellular regions containing relatively high levels of non-histone DNA binding proteins would be associated with cellular regions of relatively high transcriptive activity. The transcriptive function of the NM can then be demonstrated by determining the amounts of both transcriptive factors and non-histone DNA binding proteins associated with the NM.

Transcriptional cofactors such as magnesium ( $Mg^{++}$ ) are known to be important in affecting the conformation of chromatin. This affect can be indirect, through association with enzyme molecules, or direct, through electrostatic interactions with charged phosphate groups exposed on the nucleic acid structure (Ochiai, 1977). The conformational influence of magnesium can be demonstrated by varying its concentration in transcription reactions and noting resultant structural differences in the chromatin.

## MATERIALS AND METHODS

### Cells

HeLa cells (Strain S, CCL 2.2, mycoplasma-free) obtained from American Type Culture Collection were grown in suspension culture in 4 L flasks at 37°C in minimal essential medium containing  $NaHCO_3$  (0.22 g/l) and 5% fetal calf serum. The cells were grown to a density of  $5.5 \times 10^5$  cells per ml prior to harvesting, and the packed cell volume was 8 ml.

### Preparation Of HeLa Cell Extracts

Cells were harvested from cell culture medium by centrifugation for 10 minutes at 3500 rpm in a Sorvall RC2-B. Pelleted cells were washed well with phosphate-buffered saline at 0-4°C and collected by centrifugation. The HeLa cells were suspended in five packed cell volumes of buffer A (10 mM Tris-HCL, pH 7.9; 50 mM KCl; 0.1 mM EDTA) and allowed to stand at 0°Celsius for 10 minutes. Cell nuclei were isolated by homogenization according to Dignam *et al.* (1983). The

cytoplasmic protein supernatant was prepared according to Dignam et al. (1983); the nuclear extract was prepared from isolated nuclei as described by Dignam et al. (1983). The pellet obtained from the nuclear extract contained the NM and was treated with endonucleases and high salt to remove soluble proteins and membranes according to Staubenfiel et al. (1983), yielding the purified NM.

The NM was then divided into three sections, and each of the sections was treated with one of the following detergents: CHAPS (0.65%), CHAPSO (0.6%), and SDS (0.06%). This yielded three samples of NM proteins. The optical density (260 and 280 nm) of all five protein fractions was measured on a Zeiss spectrophotometer to determine protein concentration.

All five protein fractions (3 NM fractions, cytoplasmic supernatant, and the nuclear extract) were measured individually on a Zeiss spectrophotometer at 260 and 280 nm to determine protein concentration of each fraction.

### Transcription Assays

Assays were performed in a final volume of 50  $\mu$ l by a method described by Sinha et al. (1982). Reactions were carried out with 25  $\mu$ l of protein extract, 2.5  $\mu$ l of NTP with 1/6 less of UTP, 2.0  $\mu$ l of [ $P^{32}$ ] UTP (BRL), 0.5  $\mu$ M  $MgCl_2$ , 15  $\mu$ l EDTA/TE Buffer, and both with and without calf thymus DNA.

The reaction mixtures were incubated at 30 C; 5  $\mu$ l aliquots were withdrawn at timed intervals and precipitated with cold 5% trichloroacetic acid containing 10mM sodium pyrophosphate. After 10 minutes at 0°C, the solution was filtered through a Whatman glass paper (GF/C) disc. The disc was washed extensively with the same solvent, followed by cold ethanol, then dried under an infrared heat lamp, and placed in a scintillation vial. The radioactivity of the samples were determined with BBOT-Toluene scintillation fluid (4g of BBOT/liter of toluene) in a Packard Tri-Carb liquid scintillation spectrometer.

### Nucleoprotein Complex Formation and Isolation

The reaction mixtures for each extract contained 6 ng [ $^{3}H$ ] SV40 DNA, 12  $\mu$ l TE buffer, pH 7.9; 20  $\mu$ l of Dialysis Buffer 1 (20 mM HEPES, pH 7.9; 20% glycerol; 0.1 M KCl; 0.2 mM EDTA; 0.5 mM PMSF; and 0.5 mM DTT) and protein in varying amounts from one of the subcellular samples. The protein sample was added in several protein : DNA ratios, all within the range of protein:DNA saturation of SV40 DNA for each subcellular sample. Experiments were performed with nuclear extract and S100 samples at ratios of 75:1, 100:1, and 150:1. The NM proteins were added in 5:1, 10:1, and 25:1 ratios.

## Electron Microscopy

Peak fractions from Biogel A-15m columns were analyzed by electron microscopy following the method of Dubochet et al. (1971) as modified by de Murcia et al. (1978). Samples were placed on carbon-covered nitro cellulose film supported on a 200 mesh Copper grid performed in an atmosphere of amylamine, which yielded the grids hydrophilic. The sample on the grid was rinsed with H<sub>2</sub>O and Tris/EDTA buffer. The samples were stained 30 seconds with a 1% aqueous uranylacetate solution. The grids were then rotary shadowed with platinum at a 6 degree angle. Electron micrographs were prepared for each NPC sample.

All reactions were incubated at 30° Celsius for 30 minutes, diluted with 200 ml of buffer A and 10 ml of 0.1 N NaOH, mixed well, and chilled in ice for 10 minutes. NPC samples to be isolated were loaded onto 5 ml Biogel A-15m columns that had previously been packed using buffer A. Columns were eluted with buffer A and collected in 30-250 ml fractions. Aliquots were counted in aqueous counting scintillant on a Packard Tri-Carb liquid scintillation spectrometer.

## RESULTS AND DISCUSSION

### Transcription Assay

Results from this procedure indicated both the relative amount of RNA polymerase and its association with endogenous template in each subcellular sample. The assay with cytoplasmic (S100) proteins showed transcriptive activity, indicative of the presence of RNA polymerase. The level of transcriptional activity with exogenous DNA was approximately equal to that noted in the reaction without exogenous DNA. This suggests that the RNA polymerase within the S100 region was associated with endogenous template. The transcriptional activity demonstrated by the cytoplasmic fraction is evidence that nuclear contents leaked into the cytoplasm during isolation of the nuclei, as transcriptional factors are not found in the cytoplasm in vivo. Investigation is currently being done in developing methods which minimize this leakage. Reactions carried out with nuclear extract proteins showed transcriptive activity, implicating the presence of RNA polymerase. This activity appeared to be dependent upon exogenous template, as transcriptional activity increased with the addition of DNA. From this, it may be concluded that polymerase in the nuclear extract probably exists unbound and free from endogenous template.

The NM, however, demonstrated minimal and inconclusive transcriptional activity both with and without exogenous DNA. It is very possible that the detergent treatments used to extract the NM proteins from the NM interfered with the activity of the RNA polymerase by dissociating the constituent subunits. This experiment was of particular interest in reference to results of an investigation by Abulafia et al. (1984), which demonstrated a large degree of RNA polymerase activity associated with the NM. This information, if verified, would provide additional evidence implicating the function of the NM in transcription. The

investigation of Abulafia et al. (1984) was performed by isolating the NM through sucrose cushions without subsequent detergent treatments. These results indicate that additional research should be performed to develop a method which would extract RNA polymerase from the NM in an active form.

## Isolation and Characterization of NPCs

### Gel Filtration

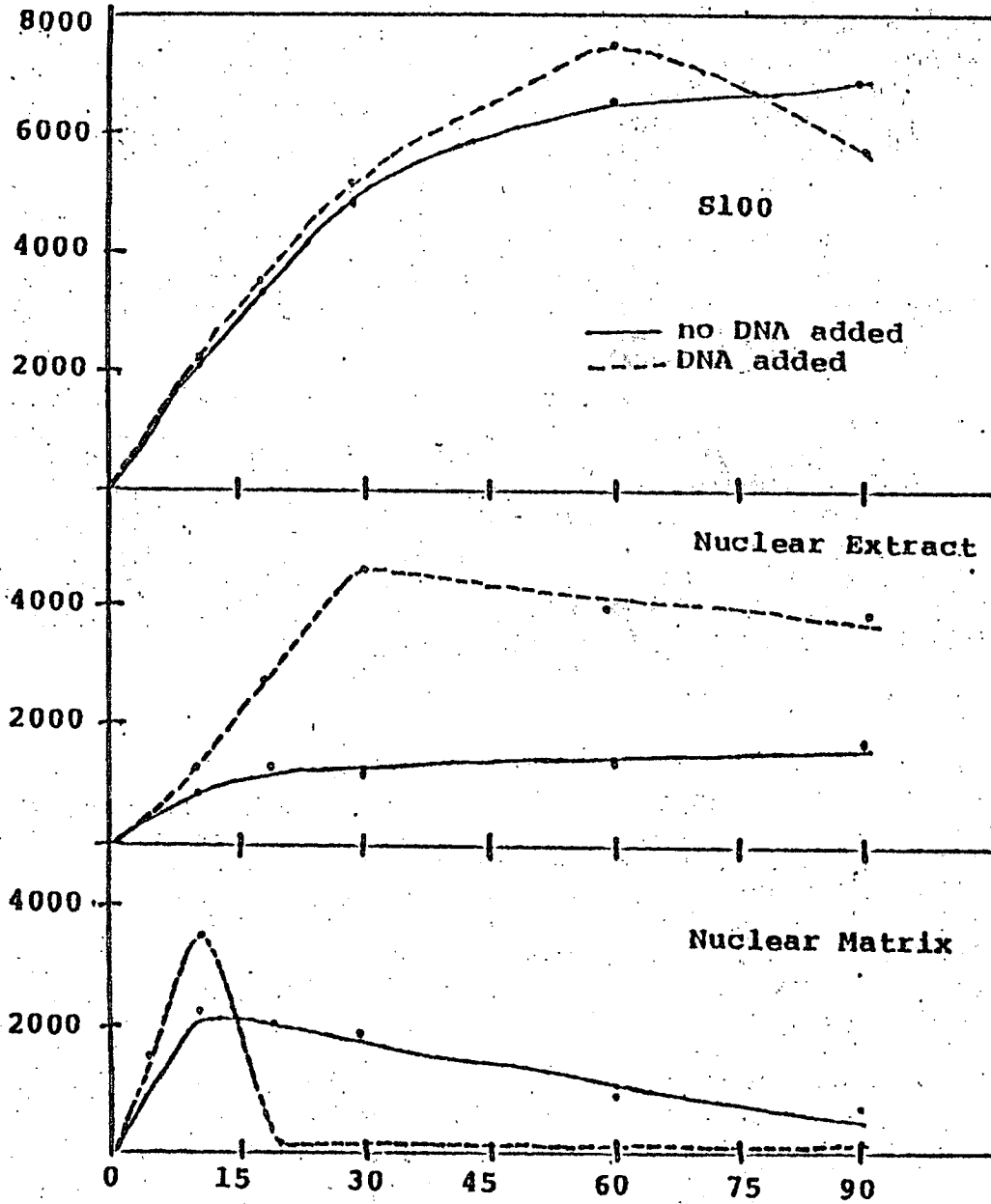
Several protein:DNA ratios were tested for each subcellular protein sample. The ratios of N.Ext and S100 tested were higher than those of the NM tested, due to the fact that the N.Ext and S100 contain many more proteins in addition to DNA binding proteins. Results from Figure 1 indicate that more DNA was bound by the NM protein sample than by the other two protein samples. Under the theory that NPCs are involved in active chromatin, this would demonstrate that there would be more transcriptionally active chromatin associated with the NM than with the other cellular regions tested. This conclusion would support evidence that the NM functions in gene regulation and control.

Reaction of the S100 proteins in the presence of  $MgCl_2$  yielded recovery of a large part of the product in a broad peak between the 15th and 20th fractions, rather than near the 10th fraction as was previously observed with other reaction products. This later peak, characteristic of degraded DNA, is evidence of a possible endonuclease contaminant present in the cytoplasmic fraction. It may be noted that the endonuclease is magnesium-dependent, demonstrated by the fact that the second peak is not observed when reaction with S100 protein is performed in the absence of  $MgCl_2$ .

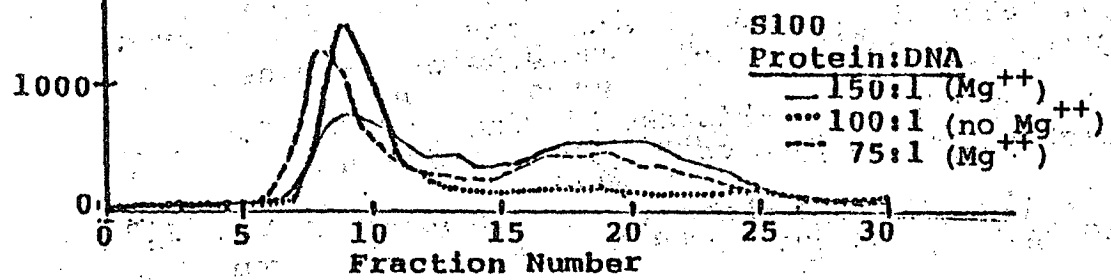
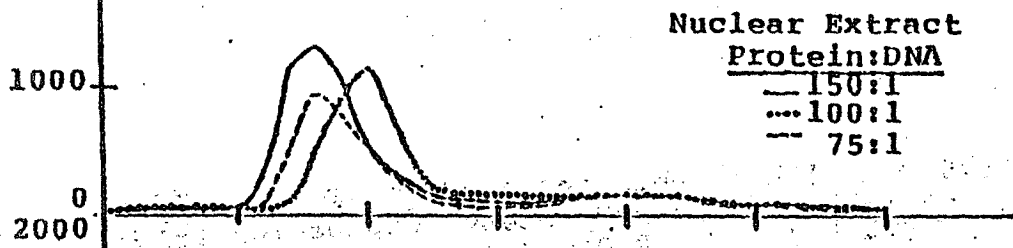
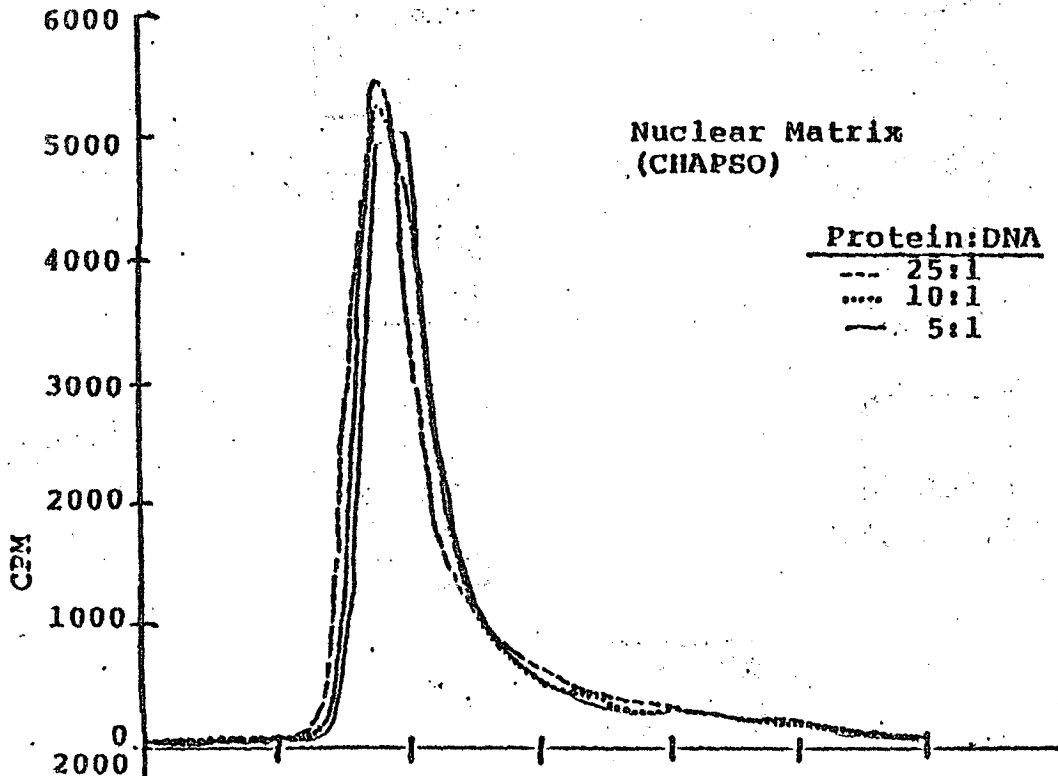
### Electron Micrographs

Electron micrographs of the isolated NPCs from each gel filtration peak revealed information of their structure and configuration. As the protein:DNA ratio was increased for each protein sample, the number of observed "beads" per DNA molecule increased. NPCs formed in the presence of  $MgCl_2$  appeared to be tight and compact, while those complexes formed in the absence of  $MgCl_2$  were open and circular. These observations held true for NPCs from peak fractions of each protein:DNA reaction with one exception. NPCs from the second peak of the S100 reaction with magnesium appeared to be degraded into linear structures. This supports the hypothesis that the S100 fraction contained an endonuclease contaminant. The concentration of magnesium used in the experiment (1.5 mM) is analogous to the external  $Mg^{++}$  concentration found in cells. The effects of  $Mg^{++}$ , as seen by comparison of NPC structures, suggest the role of  $Mg^{++}$  in gene regulation by influencing chromatin conformation.

TRANSCRIPTION ASSAY



### GEL FILTRATION



## KEY TO ELECTRON MICROGRAPHS:




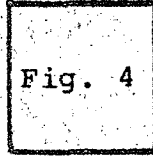
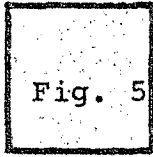
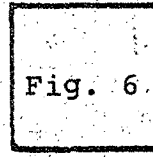

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Figure 1: SV40 DNA with non-histone binding proteins from nuclear extract fraction in presence of 1.5 mM  $MgCl_2$ .

Figure 2: SV40 DNA with non-histone binding proteins from nuclear extract fraction in absence of  $MgCl_2$ .

Figure 3: SV40 with non-histone binding proteins from S100 fraction in presence of 1.5 mM  $MgCl_2$ .

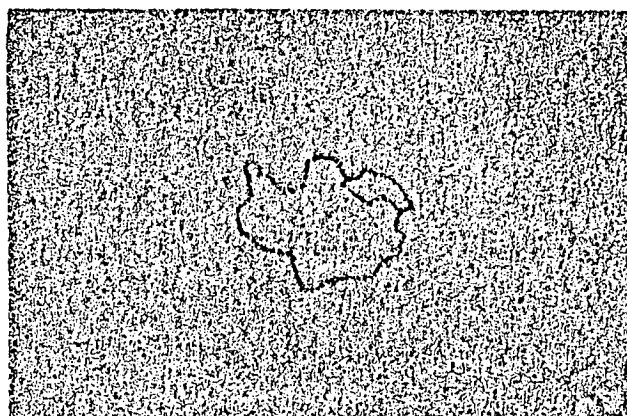
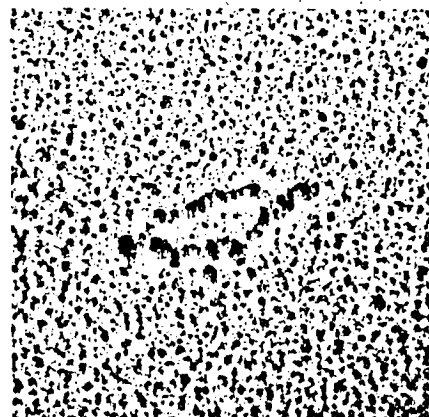
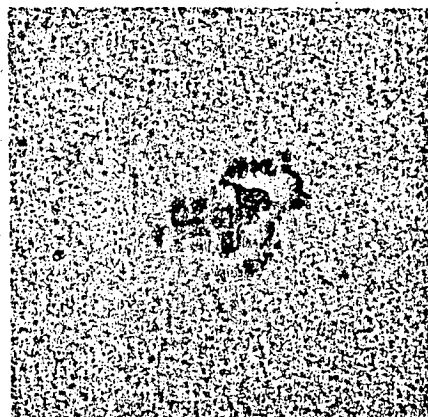
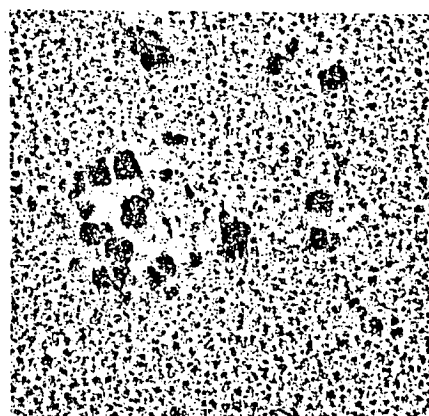
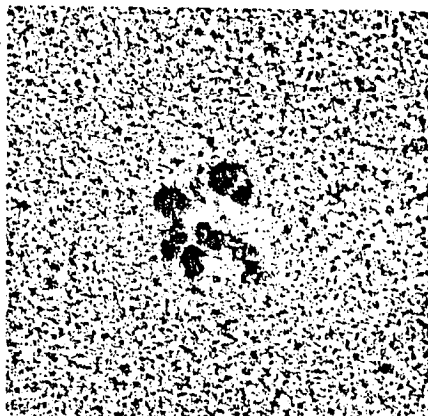
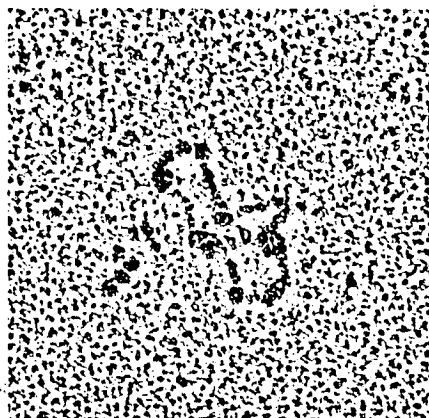
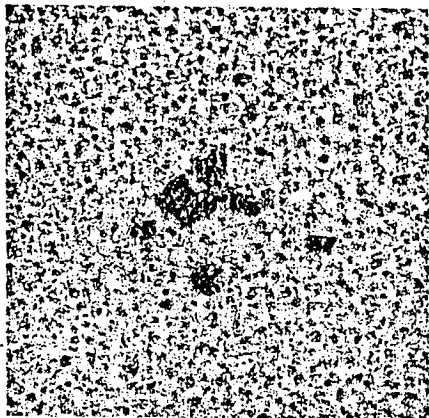
Figure 4: SV40 with non-histone binding proteins from S100 fraction in absence of  $MgCl_2$ .

Figure 5: SV40 DNA with non-histone binding proteins from nuclear matrix fraction in presence of 1.5 mM  $MgCl_2$ .

Figure 6: SV40 DNA with non-histone binding proteins from nuclear matrix fraction in absence of  $MgCl_2$ .

Figure 7: Naked SV40 DNA.

ELECTRON MICROGRAPHS:

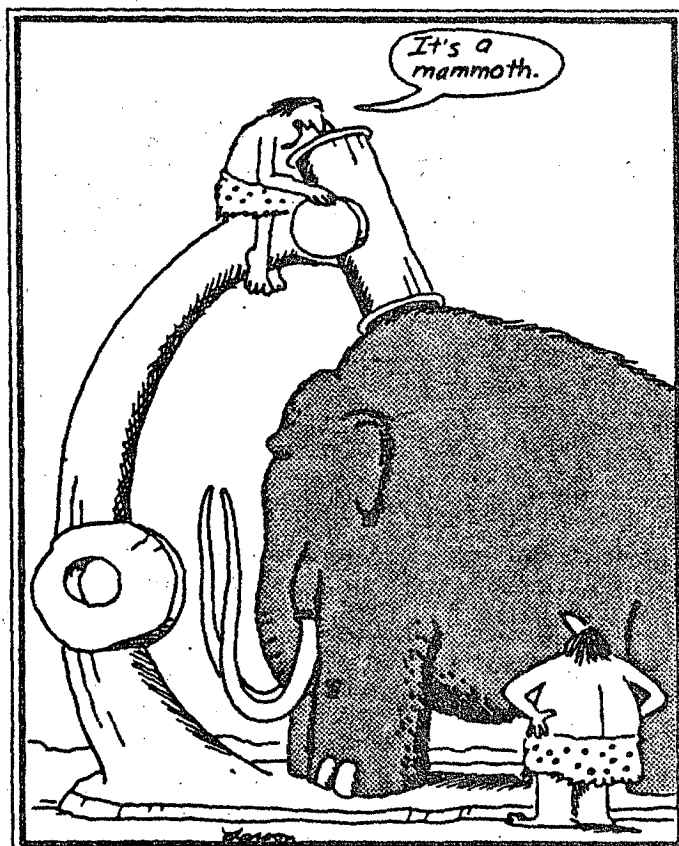


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Early microscope

The Far Side  
by Gary Larson

## HERBIVORY INTERACTIONS OF PINFISH

by John Marr

## ABSTRACT

Algal preferences and growth rates, with respect to different algal species, of pinfish (Lagodon rhomboides) were tested. Algal preference of spottail (Diplodus holbrooki) was also tested. Pinfish showed a significant preference for Hypnea and Neogardiella over Ulva and Sargassum, which were preferred over Padina and Codium. Gut analysis of pinfish demonstrated similar selectivity in the field. Red algae, including Hypnea and Neogardiella, constituted over 70% of total gut contents. Nutritional value and digestibility are believed to be higher in the Rhodophytes as compared to Chlorophytes and Phaeophytes.

## INTRODUCTION

Radio Island Jetty, a man-made reef, provides a rocky substrate offshore near Beaufort, N.C.. Habitats for many algal species, herbivores, omnivores, and carnivores are provided at this site. The interactions between these organisms and their physical environment provide a somewhat diverse community to be examined. Pinfish (Lagodon rhomboides) range in bays and estuaries from New York to Cuba (Smith, 1907) and reach a maximal length of 25.4 cm.. They forage on small fish, worms, crustaceans, mollusks, and algae (Smith, 1907). Foraging patterns and behavior of pinfish on different algae species was the topic of our study.

## MATERIALS AND METHODS

## Preference

Algal preferences were examined in the lab for two species of pinfish, Lagodon rhomboides and Diplodus holbrooki. Three large aquaria were each partitioned into three equal sized sections using screened frames. This allowed unfiltered, aerated seawater to circulate freely among partitioned sections of the tanks. For each experimental run there was one control per tank, consisting of the algal species without fish. This gave a total of three controls and six treatments per run. The sections without fish controlled for natural weight loss or gain for the individual algal species. Six algal species were tested for any pinfish grazing preferences. The algal species used in all the preference tests were Ulva, Codium, Sargassum, Padina, and Neogardiella. The algae were collected at Radio Island Jetty approximately two hours before each treatment. For each treatment and control, each alga was individually weighed (4.0-7.0g), strapped to a rock by rubberband, and located in the treatment and control sections of the tanks at specified points using a random number table. The six algal species remained in the tanks for 48 hours. After each run, the algae were removed, reweighed, and

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the mean change in control weights was used to adjust treatment weights to account for natural change in weights unrelated to fish grazing. There were three runs conducted for L. rhomboides and one run for D. holbrooki. The aquaria were emptied and cleaned after each run, and conditions were held constant for all replicates.

Pinfish used in the preference test (L. rhomboides) were collected in the field at Phillip's Island with a large seine prior to each run, and were allowed a 24 hour starvation and acclimation period. Pinfish with a standard length range from 4.2-7.0 cm were placed in each of the six grazing treatments; ten fish per treatment. The six previously weighed algae remained in the tanks for 48 hours. They were then reweighed to record any change due to feeding by the fish. For the pinfish, three different runs were done, giving eighteen replicates and nine controls.

Spottailed pinfish used in the preference test (D. holbrooki) were caught at Radio Island Jetty by hook and line. Six spottails were collected and brought into the lab to be starved for four days. The standard length ranged from 12-15 cm.. The feeding period was 48 hours and all other procedures were identical to those previously described for the pinfish. One run was done on the spottails, giving six replicates and three controls.

Using the results from the pinfish (L. rhomboides) preference test, three algal species were chosen for the growth rate experiment. We chose a highly preferred species, Hypnea; a moderately preferred species, Sargassum; and a non-preferred species, Padina. Thirty 3.8l jars with a continual flow of seawater and supply of air were set up; this gave ten replicate fish for each alga. Pinfish collected at Phillip's Island, with a mean standard length of 5.0 cm, were weighed and placed individually in each of the jars. Then each alga was separately strapped to a rock and randomly placed in a jar with a fish. The algae was replaced in excess every two days. The experiment ran for eight days. Then each of the fish were removed, remeasured and weighed to obtain any change due to the specified diet.

#### Field Observations

An algal abundance survey at Radio Island Jetty was taken in the following manner. A meter tape was stretched out in 5 m increments, in the sub-tidal zone, and 5 random points were located in each meter. At each point the understory, species beneath the canopy, and overstory, species forming the canopy were recorded. A total of 300 points were surveyed to give a general idea of the abundance of the different species.

A general fish survey at Radio Island Jetty was made during high tide in the mid-afternoon. With visibility less than 3 m, one sample was taken by swimming along a 100 m stretch next to the jetty. The number of individuals of the common species visible at this location was recorded.

## Gut Analysis

In order to assess what pinfish were consuming in their natural habitat, a gut analysis was taken for sixteen L. rhomboides. The pinfish, standard length range from 11-14 cm, were caught by hook and line. The guts were preserved in the freezer or immediately analyzed upon returning to the lab. A dissecting microscope aided in separating the contents into four categories: red, green, and brown algae, and animal tissue. The identified contents were placed in an incubator at 50°C until the dry weights were taken.

## RESULTS

Consumption of the various seaweeds by pinfish (L. rhomboides) is shown in Fig. 1. This graph illustrates that Hypnea and Neogardiella lost significantly more weight as compared to the other algae ( $p < .05$  ANOVA and Duncan's Multiple Range Test). Ulva and Sargassum lost significantly more weight than Codium and Padina. In general, the red algal species appeared to be preferred over either green or brown algal species.

Consumption by spottails (D. holbrooki) is shown in Fig. 2. This graph also illustrates that red algal species were consumed at a higher rate than green or brown algae. Again, the red algal species, Hypnea and Neogardiella, lost in excess as compared with the green or brown algae.

Pinfish fed three different types of algae exhibited no significant differences in growth rates. There is, however, a trend. Padina was the lowest preference alga (Fig. 1) and caused the greatest weight loss (Table A).

Table A

Alga	x wt.(g)	S.D.
<u>Hypnea</u>	-.32	.34
<u>Sargassum</u>	-.30	.42
<u>Padina</u>	-.59	.30

Pinfish gut content analysis is shown in Fig. 3. Red algae accounted for 70% of the total gut contents in the pinfish, thus, reds were significantly ( $.005 < p < .01$ , ANOVA tests) more than browns, greens, or animal material. The only readily identifiable algal species were as follows: Reds-Polysiphonia, Hypnea, and Neogardiella; Greens-Cladophora and Ulva; Browns-Sargassum.

The algal abundances at Radio Island Jetty are shown in Fig. 4. Sargassum, Codium, Ulva, and Gracilaria each comprise at least 10% of the total species according to the graph. This illustrates that the three main algal classes are represented in large proportions by at least one member of their class. According to the graph, the reds, as a group, are represented by five species, all at relatively low

abundances. The greens are mostly represented by Ulva and Codium; and Sargassum is by far the most abundant of the browns and possibly of all the algae. Also a large percentage of the reds are found in the understory along with a lot of greens.

Table B: Fish Survey

Species	% Abundance
<u>Diplodus holbrooki</u>	42
<u>Lagodon rhomboides</u>	40.19
<u>Micropterus sp.</u>	6.5
<u>Archosargus probatocephalus</u>	11.22

According to the fish survey taken at Radio Island Jetty, pinfish (L. rhomboides) and spottails (D. holbrooki) comprised more than 80% of all the fish encountered.

#### DISCUSSION

In order to study feeding patterns and behavior in Lagodon rhomboides and Diplodus holbrooki, the influences of their habitat complexity must be considered. Radio Island Jetty, the study site, may be one of the more diverse habitats in the immediate Beaufort, N.C. area. Much of the jetty's substrate is in the intertidal zone, being largely exposed during low tide and completely under water during high tide. Also strong currents and high wave action probably play a large role in physical disturbances. The physical structure of the habitat may be intricately related to the population and community dynamics of its inhabitants. "Variation in habitat structure over space and time may dramatically alter not only the behavior of the predator, but undoubtedly influences patterns of abundance and species richness in both prey and predator groups" (Stoner, 1979).

According to Eastbrook and Dunham (1976), the environment offers all animals a great variety of potential food items which vary greatly in the ease with which they can be found, captured, and eaten, and in their nutritive value. The pinfish and spottail are both known to be omnivores, feeding both on plant and animal material, which can potentially provide a large assortment in the diet, depending on the environment. "Schoener suggested that generalist feeding strategy is favored when: 1) food density is low and there is a premium on the ability of the animal to take a range of prey, 2) the predator has a relatively long period to gain energy, and 3) prey densities fluctuate widely." (Stoner, 1980). Perhaps the pinfish at Radio Island Jetty are forced, with a decrease in epifaunal species (i.e. amphipods) during certain seasons- (e.g. summer), to compensate by broadening their diet breadth. This prediction that foragers will generalize as food abundance declines is consistent with two temperate fishes' feeding strategies studies by Horn (1983). It can clearly be concluded from our data, obtained in mid-July, that red algae is consumed in much greater quantities

than animal matter in Lagodon rhomboides. Hansen (1963-65) also found that vegetation constituted a large majority of the diet for pinfish in the summer and fall. Seasonal variation in the diet of pinfish appears to be directly influenced by fluctuations of food abundance.

With the assumption that algae is an important part of pinfish diet, the preferences for certain algal species and any related importances should be examined. First, the differential digestive capabilities on different types of algae must be considered. The presence of cellulolytic activity in samples of pinfish alimentary tract may make cellulose digestion play a substantive nutritional role (Weinstein et al., 1982). If plant material does have nutritional significance to pinfish, then the nutritional value may vary amongst the different algae types. High selective preference for red algae shown in our results might be related to a high nutritional value as compared to other algae. Two temperate herbivorous fish (Cebidichthys violaceus and Xiphister mucosus) were shown to highly select for annual Chlorophytes and Rhodophytes as opposed to tough, low nutritional Phaeophytes (Horn et al., 1982). The fact that the Rhodophytes used in our experiment were both highly preferred and that over 70% of the gut contents analyzed were Rhodophytes is consistent with the high nutritional value of Rhodophytes over Chlorophytes and Phaeophytes. "Rhodophytes are known to have a greater concentration of the kinds of carbohydrates that are more susceptible to digestion by fishes..." (Horn et al., 1982). In a study conducted by Edwards and Horn (1982), on a temperate fish (Cebidichthys violaceus), protein, carbohydrate, and total organic material were absorbed more efficiently from Rhodophytes. Other related differences in digestible components between the different algae might be explained by differences in morphologies and herbivore defense mechanisms found in some algal species. Phaeophytes and other algae may be avoided due to tough thalli and because many contain polyphenolic compounds, which serve as herbivore defense mechanisms (Horn et al., 1982). Furthermore, there is a tendency for finely branched thalli of algae to be more palatable and of higher caloric content (Edwards and Horn, 1982). The morphologies of the two most preferred algal species, Hypnea and Neogardiella, are similar in that they are multi-branched and relatively tender, which supports the models for high nutritional value and increased susceptibility to digestion.

Results from the algal survey of Radio Island Jetty support the higher percentage of Rhodophyte consumption in that all species of red algae encountered were represented in low abundances. With pinfish comprising 80% of all fish encountered in the fish survey; the low percentage of red algae could be attributed to preferential foraging in pinfish. If pinfish are able to preferentially select the algal species based on high nutritional value and digestibility, then their foraging efficiency should be maximized, even at times of low food abundance.

Fig. 1 PREFERENCE: PINFISH  
Mean Consumption

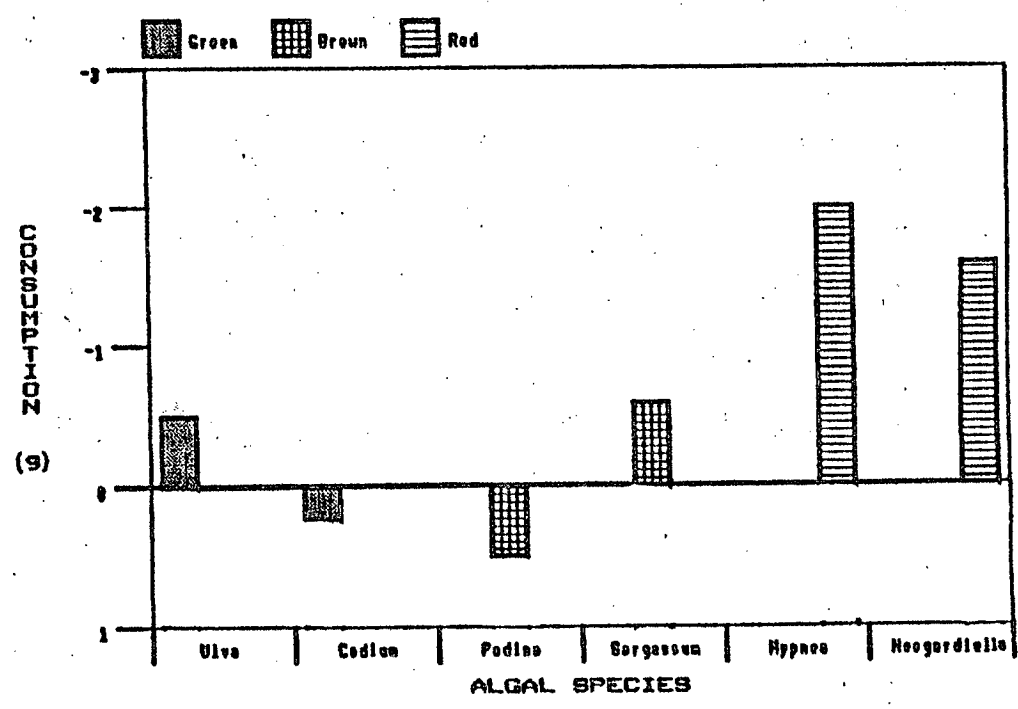
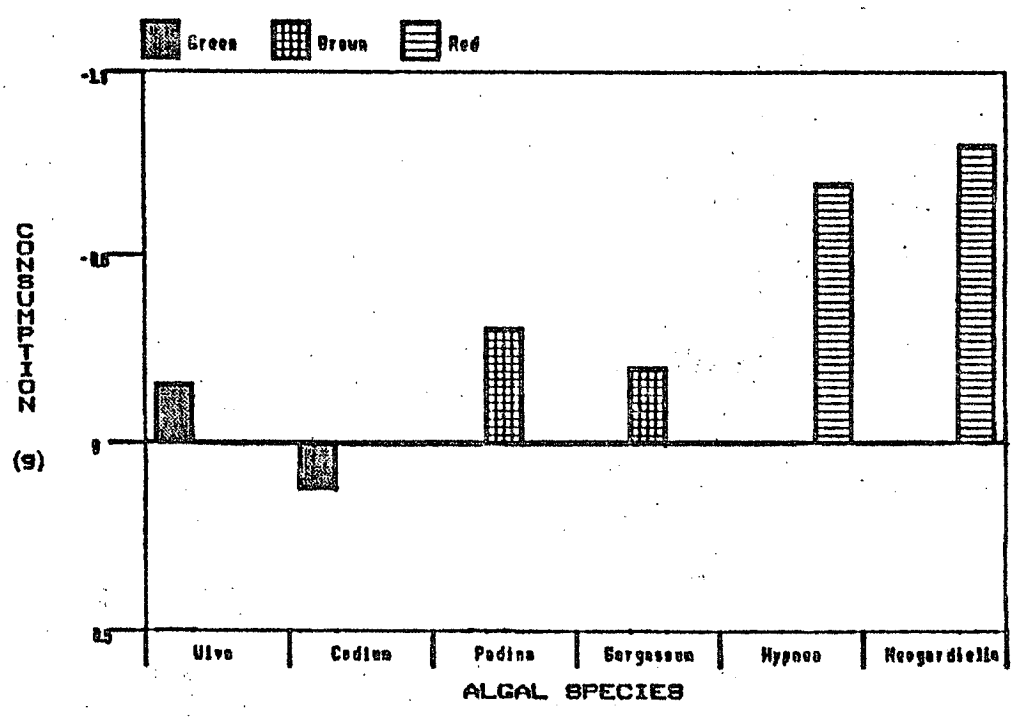
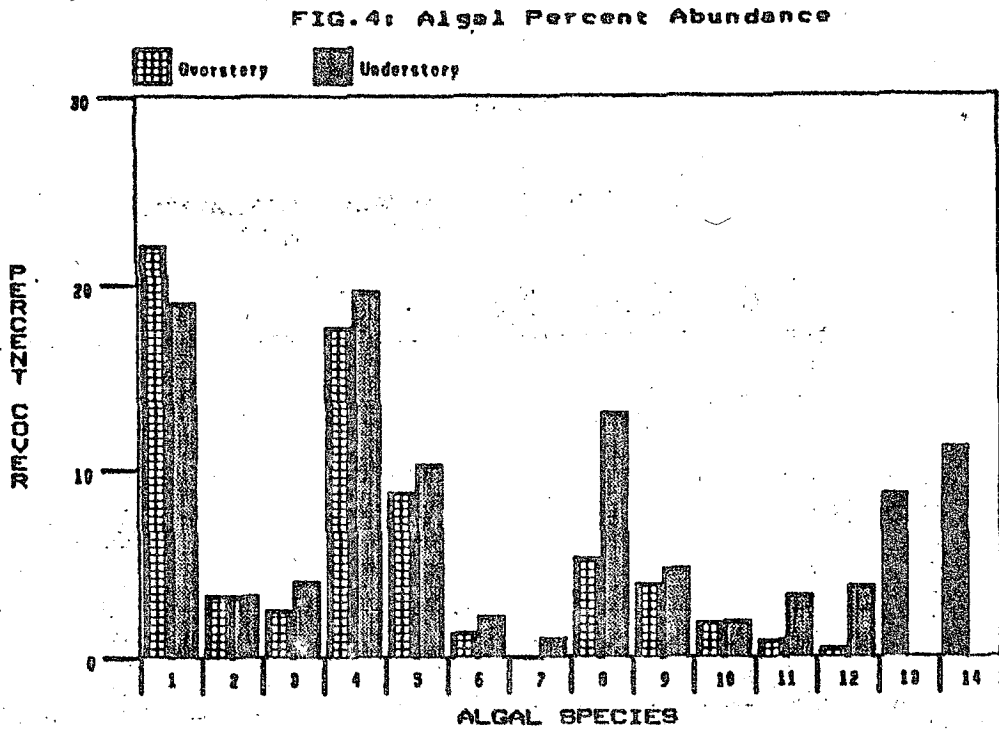
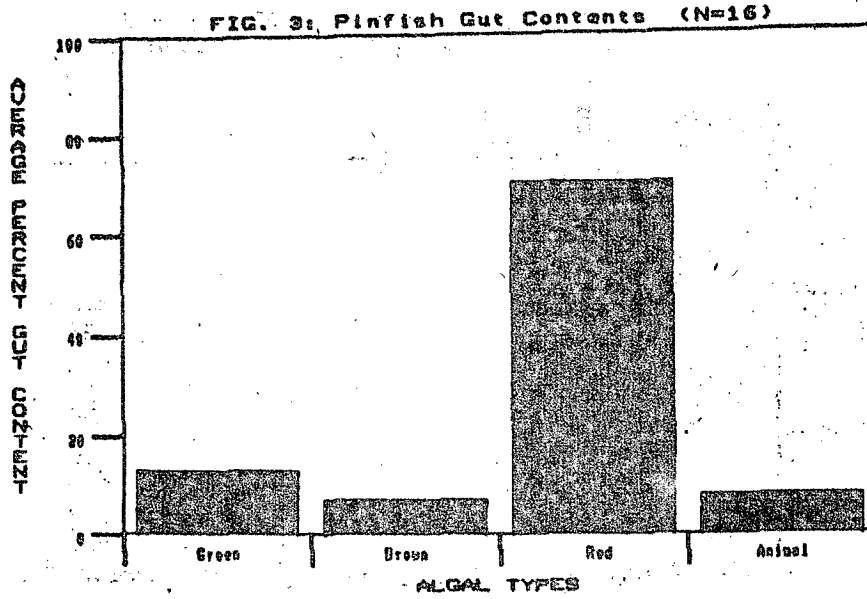


Fig. 2 SPOTTAIL PREFERENCE  
Mean Consumption





**Brown Algae:**

- 1-Sargassum
- 2-Dictyota
- 3-Padina

**Green Algae:**

- 4-Codium
- 5-Ulva
- 6-Cladophora
- 7-Siphonocladales

**Red Algae:**

- 8-Gracilaria
- 9-Hypnea
- 10-Chondria
- 11-Neogardiella
- 12-Gelidium

**Other:**

- 13-Sand
- 14-Bare Rock

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THE SYNTHESIS AND ELECTROPHILIC SUBSTITUTION  
OF 1-TRIFLYROMETHYLSULFONYLPYRROLE

by Paula Louise Millirons

STATEMENT OF RESEARCH PROBLEM

The synthesis and reaction of 1-trifluoromethylsulfonylpyrrole were investigated for their usefulness as a synthetic route to 3-substituted pyrroles and for what they might reveal about the mechanistic control of 3-substitution. Increases in the percent of 3-isomer formed during reactions of deactivated pyrroles with "hard electrophiles" have been postulated to result from increased charge control of the reaction, for example in the  $\text{AlCl}_3$ -catalyzed acylation of 1-phenylsulfonylpyrrole. While there does seem to be greater charge control in the reactions of the 1-sulfonylpyrroles, we believe that this increase does not alone account for the rise in 3-acylation. It is proposed that a previously uncharacterized complex between the 1-sulfonylpyrroles and  $\text{AlCl}_3$  may account for the increased stereospecificity beyond that expected by the hardness of the electrophile in these acylation reactions.

The ring nucleus of pyrrole exhibits resonance stabilization, and it is extremely susceptible to electrophilic attack. Electrophilic attack occurs predominantly at the 2-position of pyrrole, which can be readily explained by the increased stability of the sigma-complex during 2-substitution over that during 3-substitution. However, in some instances predominantly the 3-isomer is formed, especially with 1-substituted pyrroles. Such 3-substitution can be rationalized using any of three explanations or some combination of them. 1) Steric hindrance at the 2-position from the neighboring 1-substituent could promote substitution at the sterically less-hindered 3-position (Katritzky and Rees, 1984). 2) Acid-catalyzed rearrangement of the 2-isomer could lead to the more thermodynamically stable 3-isomer (Katritzky and Rees, 1984). 3) Some reactions with "hard electrophiles" appear to be "charge controlled" and hence substitution would occur at the more electron dense 3-position. It has been shown that in the gas phase, with no solvent or catalyst effects, pyrrole undergoes predominantly 3-substitution with a variety of ionic electrophiles (Speranza, 1981).

The factors promoting 3-substitution in pyrrole are important because of their relevance in the synthesis of biological molecules such as the prodigiosins, a family of antibiotics (Jackson, 1979). Also, from a theoretical point of view, the electrophilic substitution of pyrrole is an interesting system to examine using approaches such as the polyelectronic perturbation theory. This theory tries to predict whether a reaction will be charge or frontier controlled by looking at the relative "hardness" or "softness" of the attacking electrophile. The effect which the solvent or environment

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may have on the electrophile and acceptor molecules is another factor influencing the site of substitution which even further complicates the control of reactions in solution since solvation can alter the relative hardness of the electrophile and the basicity of the pyrrole ring carbons (Klopman, 1968).

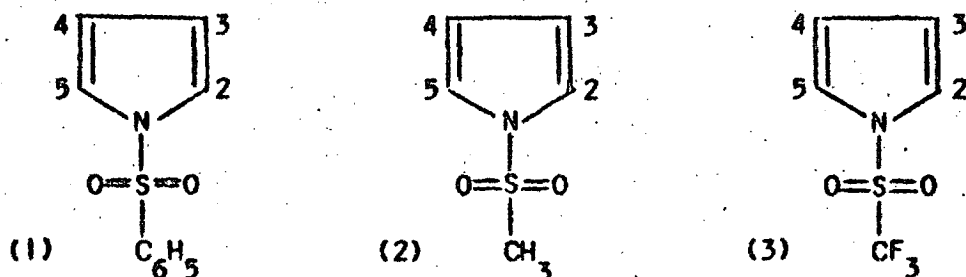
Certain 1-substituted pyrroles such as 1-phenylsulfonylpyrrole (1) have lower ground state ring energies than does pyrrole itself. Hence, 1 is more likely to exhibit charge-controlled behavior in its reactions. It has been reported that  $\text{AlCl}_3$ -catalyzed Friedel-Crafts acylations of 1 typically give the 3-substituted product.

Attempts to introduce one-carbon acyl groups is reported to uniformly result in the 2-isomer however (Xu *et al.*, 1981; Rokach *et al.*, 1981; Kakushima *et al.*, 1983).

The factors promoting this selective 3-substitution have not been fully explained. Although the phenylsulfonyl group will cause some steric hindrance at the 2-position, it is felt that the extreme stereospecificity of the  $\text{AlCl}_3$ -acylations cannot be fully justified by such steric effects arising from the phenylsulfonyl group alone (Rokach *et al.*, 1981; Kakushima *et al.*, 1983). However, steric effects could also arise if the  $\text{AlCl}_3$  were mixed in 1,2-dichloromethane (Kakushima *et al.*, 1983). The remaining alternative is that the  $\text{AlCl}_3$  produces harder acylium ion electrophiles which interact with 1 in a charge-controlled reaction. However, this theory does not fully explain the results of the one-carbon acylations.

The synthesis of 1-trifluoromethylsulfonylpyrrole (3) had not been reported, and it was hoped that this even more strongly electron-withdrawing substituent could lead to new synthetic pathways as well as help to further elucidate the control of these reactions. Its reactivity could also be related to the sterically-similar 1-methylsulfonylpyrrole (2) whose synthesis had been reported (Prinzbach *et al.*, 1973). Differences in the reactivity and stereospecificity of these pyrroles should reflect the relative differences in the electron densities of their ring carbons. In this paper, a successful synthesis of 3 is reported and a comparative look at the reactivity of 2 and 3 is set forth, and the likely controlling mechanisms further elucidated.

Figure 1



## EXPERIMENTAL INVESTIGATIONS AND CONCLUSIONS

Both 1 and 2 can be synthesized by reacting the appropriate sulfonyl chloride with potassium pyrrole. The analogous procedure was not applicable for introducing the trifluoromethylsulfonyl group. However, the trifluoromethanesulfonic imidazolide can be used to transfer the trifluoromethylsulfonyl group to potassium pyrrole.

This reaction was found to give an 81% yield of 3 as a sweet-smelling liquid. Due to its volatility and the length of the purification procedure, the first attempts at isolation only afforded a 16% yield but this has been markedly improved with practice to about 50%. 3 was characterized by its various spectra and elemental analysis.

Molecular orbital comparisons of pyrrole, 1, 2, and 3 were made using the MNDO program on the Rhodes' computer beginning with the pyrrole ring geometry established by Bak et al. (1956). Table I shows that, for 1, 2, and 3, the total electron density is greater at the 3-carbons than at the 2-, with all densities being lower than those for pyrrole itself. Of these derivatives, 3 is the most strongly deactivated. Although its greater deactivation will make 3 less reactive in general than 1 or 2, 3 should show more charge-controlled behavior than do either 1 or 2.

Table I. Electron Densities

Atom:	Pyrrole	1	2	3
C <sub>2</sub>	4.029	3.993	3.996	3.987
C <sub>3</sub>	4.129	4.117	4.114	4.096

Various acylations were investigated and these results are summarized in Table II. Mass spectra were obtained to confirm the identity of the products, and often the -SO<sub>2</sub>CF<sub>3</sub> group would be hydrolysed to give the free pyrrole derivative for further isomeric confirmation. The acylations require a Lewis acid catalyst, AlCl<sub>3</sub>, to proceed; typically, 2 mmol of catalyst and 1 mmol of acylating agent would be stirred for 10 minutes in dichloromethane and then a solution of 1 mmol of 2 or 3 would be added. From the results, it can be seen that AlCl<sub>3</sub>-catalyzed substitution on 3 with both acetyl and benzoyl chloride appeared to occur almost exclusively at the 3-position, while 2 showed less stereospecificity.

One-carbon acylations were performed on 3 only. It had been reported that 1 gave only the 2-isomer in these reactions (Albert, 1968; Kosower and Bauer, 1960). However, when 3 was reacted with dichloromethyl methyl ether, it gave a good yield of what we characterized to be the 3-aldehyde. This is the first direct synthesis of the 3-aldehyde of which we know.

The reaction with oxalyl chloride was hoped to give the dipyrrolyl ketones (Kakushima *et al.*, 1983; Triebs and Kreuzer, 1969) (3,2- or the unreported 3,3-) after initial formation of the acid chlorides of 3. Although this reaction does produce the acid chlorides, it is not highly stereospecific and no dipyrrolyl ketone could be characterized.

Table II. Summary of Acylation Reactions

3	Reagent	Catalyst	-R	Time	Overall Yield	% of Isomers	
						2-	3-
a)	CH <sub>3</sub> COCl	AlCl <sub>3</sub>	$\begin{array}{c} \text{O} \\ \parallel \\ -\text{CCH}_3 \end{array}$	2 hrs	96%		99
b)	C <sub>6</sub> H <sub>5</sub> COCl	AlCl <sub>3</sub>	$\begin{array}{c} \text{O} \\ \parallel \\ -\text{CC}_6\text{H}_5 \end{array}$	2 hrs	87%		100
c)	Cl <sub>2</sub> CHOCH <sub>3</sub>	AlCl <sub>3</sub>	$\begin{array}{c} \text{O} \\ \parallel \\ -\text{CH} \end{array}$	1 hr	98%		100
d)	$\begin{array}{c} \text{O} \\ \parallel \\ \text{ClCCCl} \end{array}$	AlCl <sub>3</sub>	$\begin{array}{c} \text{O} \\ \parallel \\ -\text{CCl} \end{array}$	2 days	99%	40	60

2	Reagent	Catalyst	-R	Time	Overall Yield	% of Isomers	
						2-	3-
a)	CH <sub>3</sub> COCl	AlCl <sub>3</sub>	$\begin{array}{c} \text{O} \\ \parallel \\ -\text{CCH}_3 \end{array}$	2 hrs	99%	58	42
b)	C <sub>6</sub> H <sub>5</sub> COCl	AlCl <sub>3</sub>	$\begin{array}{c} \text{O} \\ \parallel \\ -\text{CC}_6\text{H}_5 \end{array}$	1 hr	50%	8	92

The nitration and bromination reactions were investigated to observe the behavior of 2 and 3 in the absence of a Lewis acid catalyst and with electrophiles of differing softness. The results, summarized in Table III, show definite acid-catalyzed rearrangement is occurring. When equimolar amounts of pyrrole and 90% HNO<sub>3</sub> are mixed, it appears that the electrophile attacks at the 2-position with a much greater rate than is witnessed by the final equilibrium ratios of the isomers. The brominations were carried out using an equimolar addition of N-bromosuccinimide, a very soft electrophile; for both 2 and 3 a predominance of the 2-isomer was seen. This does seem to indicate that such a soft electrophile does promote more frontier control of the reaction and hence greater 2-substitution is seen.

Table III. Summary of Nitration and Bromination Reactions

3	Reagent	Solvent	-R	Time	Overall Yield	% of Isomers	
						2-	3-
e)	HNO <sub>3</sub>	$(\text{CH}_3\overset{\text{O}}{\text{C}})_2\text{O}$	-NO <sub>2</sub>	2 hrs	63%	30	70
				1 day	99%	10	90
f)	NBS	DMF	-Br	50 min.	27%	75	25

2	Reagent	Solvent	-R	Time	Overall Yield	% of Isomers	
						2-	3-
e)	HNO <sub>3</sub>	$(\text{CH}_3\overset{\text{O}}{\text{C}})_2\text{O}$	-NO <sub>2</sub>	10 min	99%	80	20
				1 day	100%	37	63
f)	NBS	DMF	-Br	50 min	41%	100	

Having looked at the electrophiles of various strengths, we wanted to examine a reaction which involved a very hard electrophile and so we chose to study the rates of deuterium exchange on 2 and 3. In acidic solutions, pyrroles will behave as carbon bases, undergoing protonation on one of the ring carbons, and we expected to see a predominance of 3-protonation. The rates of exchange of 2 and 3 are given in Table IV and were obtained by adding the deuterated solvent to 0.03 g of pyrrole in an NMR tube.

This was quickly shaken and integrations of the hydrogen peaks made at time intervals from 30 seconds to 24 hours. The exchange should be pseudo-first order, so a plot of the natural log of the integrations versus time should give a straight line whose slope is the relative rate. Partial rate factors were derived from the relative exchange rate of durene under these conditions related to its known partial rate factor (Lauer and Stedman, 1958).

For both 2 and 3, it can be seen that the 2-position is more reactive than the 3-position. Although solvent effects may be important here, we were still surprised to see that exchange with the harder deuterium electrophile did not seem to exhibit the same charge-controlled behavior as had the acylation reactions with relatively softer electrophiles.

Table IV. Deuterium Exchange Rates

	Solvent	2-Hydrogens		3-Hydrogens	
		Rate	Partial Rate <sub>5</sub> Factor x 10 <sup>5</sup>	Rate	Partial Rate <sub>5</sub> Factor x 10 <sup>5</sup>
<u>2</u>	CF <sub>3</sub> COOD	0.86	33	0.021	0.80
<u>3</u>	CF <sub>3</sub> COOD	0.00011	0.0042	---	---
<u>3</u>	10% D <sub>2</sub> SO <sub>4</sub> in CF <sub>3</sub> COOD	0.85	---	0.028	---

Although no spectral evidence of an AlCl<sub>3</sub> complex with 1 had been reported, we felt that there must be an additional interaction at work in these acylations besides charge control. Complex formation between "pi-excessive" centers such as these pyrroles and "pi-deficient" centers such as AlCl<sub>3</sub> has been well documented (Albert, 1968; Kosower and Bauer, 1960). We decided to examine the spectral data ourselves for definite signs of complex formation. The UV spectral data which we obtained is shown in Table V. Although these absorptivities seem unusually low across the board, the significant factor is the definite increase in absorptivity upon the addition of AlCl<sub>3</sub> for all three pyrroles (which have a maximum absorbance around 250 nm).

Table V. UV Data on Complex Formation

Molar Absorptivity of:	1	2	3
a) Without AlCl <sub>3</sub>	1480	46.1	68.7
b) With AlCl <sub>3</sub>	2290	82.5	146
b/a	1.55	1.85	2.13

The NMR spectral data, given in Table VI, also shows evidence of complex formation. For each pyrrole, there is a downfield shift for both the 2- and 3-hydrogens with that at the 3-H being more pronounced. When identical studies were done with SnCl<sub>4</sub>, no changes in the chemical shifts of 1, 2, or 3 were observed. This indicates that the SnCl<sub>4</sub> is not interacting with these pyrroles in the same manner as AlCl<sub>3</sub>.

Table VI. NMR Data on Complex Formation

Chemical Shift of:	1		2		3	
	2-H	3-H	2-H	3-H	2-H	3-H
Without AlCl <sub>3</sub>	6.91	6.08	6.89	6.17	6.90	6.29
With AlCl <sub>3</sub>	7.03	6.38	7.02	6.43	7.03	6.53
Change in Shift	0.12	0.32	0.13	0.26	0.13	0.24

## CONCLUSIONS

Although we conclude that a complex is forming between the AlCl<sub>3</sub> and the pyrroles, we cannot be sure of the site or sites at which it occurs. It might involve any of the electron-rich centers in the molecule: the pi ring system, or the nitrogen or oxygen molecules. At any of these sites, the complex could increase the steric hindrance of the 2-position and thus further promote 3-acylation. This is supported by the fact that the bulkier benzoyl chloride shows a greater percentage of 3-substitution than does acetyl chloride. Also, the decreased regioselectivity of one-carbon acylations on 1 and 3 can be explained since the smaller electrophiles in these reactions (especially that with oxalyl chloride) will be less sterically hindered by such a complex and will have greater access to the 2-position. We do believe that this AlCl<sub>3</sub>-pyrrole complex is an integral factor in the regioselective 3-acylations of 1, 2, and 3 and in the absence of this catalyst, more frontier-controlled behavior is exhibited by these pyrroles. Decreased 3-substitution is seen in the acylation catalyzed with SnCl<sub>4</sub>, in the initial nitration mixtures, in all of the brominations with NBS, and even in the deuterium exchange reactions.

## PLANS FOR FUTURE INVESTIGATIONS

The findings from these studies open the door to many new investigations. 1) The exact site or sites at which complex formation is occurring and the extent of the electronic interaction between the pyrrole and the AlCl<sub>3</sub> could be studied and compared to other such complexes (Albert, 1968). 2) Since 3 is quite volatile, it might be feasible to attempt gas phase substitution experiments on it, which could be related to the work done on pyrrole itself (Speranza, 1981). Such data could also be used to see how well the MO calculations predict the degree of charge-controlled behavior. 3) Finally, the effects of the solvent on these reactions could be more systematically studied. Although various unreported deuterium-exchange solvents were tried in this research, no comprehensive study of solvent effect was made in terms of such parameters as dielectric constants.

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## COLOR DISCRIMINATION IN FISH ANALYZED USING CLASSICAL CONDITIONING

by Claire de Saussure

### ABSTRACT

In this project, I conducted experiments using classical conditioning to investigate color discrimination in fish. Colored light and electrical shock were used as the conditioned stimulus and unconditioned stimulus, respectively. The response was change in ventilation rate. The results of conditioning of four Swordtails (Xiphophorus helleri) are presented. No conclusions were reached concerning ability to discriminate color, because learning was not achieved due to habituation of the fish to the unconditioned stimulus (UCS). It was determined that the fish perceived the colored light.

### INTRODUCTION

Classical conditioning can be a useful tool in determining sensory capabilities in certain animals. The goal of this experiment was to determine if certain types of fish have color vision using classical conditioning. Color vision may be more ecologically important in some kinds of fish than others. Perhaps for colorful, territorial fish, it would be more adaptive to be able to distinguish color than for schooling fish, because color patterns on some fish are stimuli for mating or territorial responses.

Another type of conditioning, instrumental, has been used successfully in previous experiments to indicate color discrimination in goldfish (Muntz, 1966). Classical conditioning has also been used successfully in mullet, with visual and auditory stimuli and an increase in respiration rate as the observed response (Denny, 1970).

If classical conditioning could be used successfully to determine color discrimination in fish, it would be a relatively simple and reliable quantitative method. This project was an attempt to accomplish this.

### MATERIALS AND METHODS

The method of classical conditioning involves an unconditioned stimulus (UCS), which elicits a consistent and detectable unconditioned response (UCR). The conditioned stimulus (CS) does not originally elicit a significant response. When the CS is presented consistently in association with the UCS, the association is learned and a response is elicited by the CS only. The conditioned response (CR) should be similar to the UCR.

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I used mild electrical shock as the UCS. This was a single pulse across two wire electrodes in the water with the fish. The pulse was produced by a Grass Stimulator and ranged from 8 to 95 volts, depending on what elicited a response (change in ventilation rate). Because the electrodes were not placed directly on the fish, the actual amount of electricity affecting the fish was less. I assumed it to be related to the value given by the stimulator.

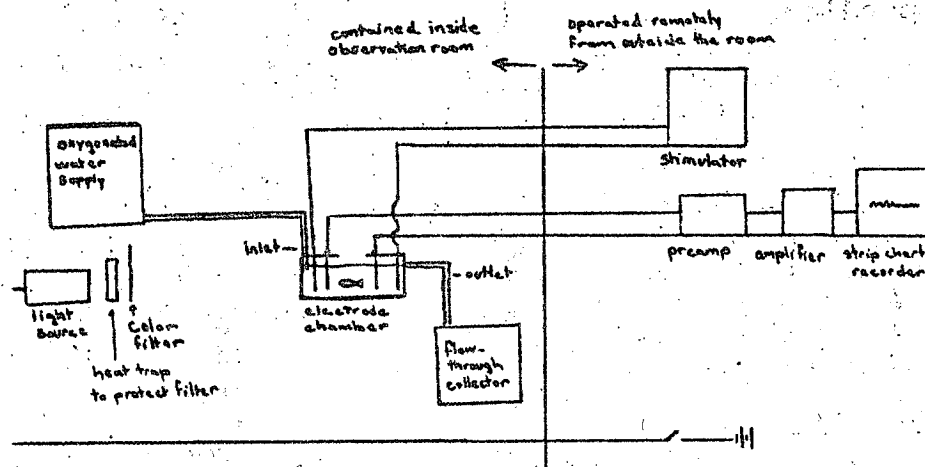
The conditioned stimulus was colored light. A long pass or band pass filter which transmitted only light of certain wavelengths was placed in front of a 150 watt flood lamp. Because the light source used emitted light mostly in the red end of the visible spectrum, the intensity of light transmitted through the green and blue filters was lower than for the red filter. The minimum distance used was 150 cm. If the light was closer to the chamber, the electrodes picked up static from it, which interfered with the recording of ventilation rate. Hence filters which transmitted light of lower intensities (green and blue) could not be placed close enough to the electrode chamber to be of equal intensity to a red filter placed 150 cm from the electrode chamber.

In all of the experiments, red light, of principal wave-length 635 nm, was used. The light was presented for 10 seconds directly before the electric shock and turned off immediately following each trial.

The response of the fish was measured as the change in ventilation rate. The ventilation rate was measured as the rate of movements of the opercular flap covering the gills (Kesler, 1972). Each fish was contained in an electrode chamber which allowed the fish to move freely, but was sensitive to opercular movements. The electrodes were stainless steel screens which covered the cross-sectional area of the chamber and were attached, by shielded cable, to a preamplifier, amplifier, and a strip chart recorder. The fish's opercular movements caused a potential difference between the two electrodes which was amplified and recorded on the strip chart recorder (Kesler, 1972). The potential difference caused by the electrical stimulus was also detected and recorded as an obvious peak. A constant flow of oxygenated water was maintained in the chamber to prevent an effect of oxygen concentration change on ventilation rate. Changes in ventilation rate were determined by counting the number of peaks per 10 seconds before the CS (red light), during the CS, and after the electrical shock.

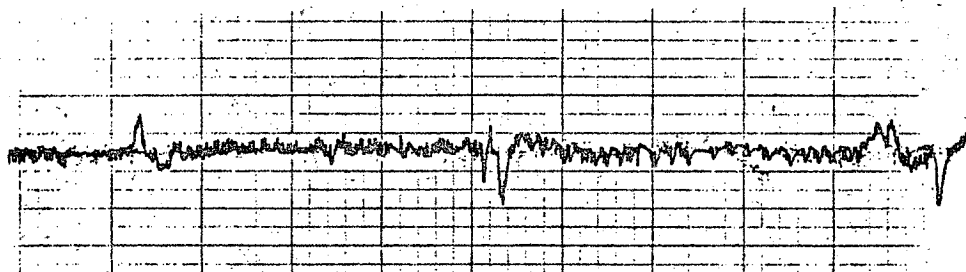
The electrode chamber, containing the fish, was in a separate room, with an overhead light on. Stimuli were presented and responses recorded from outside the room (see Figure 1). This was required because direct observation causes variation in ventilation rate (Kesler, 1972; de Saussure, personal observation).

Figure 1  
Schematic diagram of experimental apparatus



Originally, goldfish (Carrasius aurtus) were used because previous studies indicated color vision in these fish (Muntz, 1966). However, the irregularity of their ventilation rate and increased movement made it impossible to distinguish any effect of the stimulus from random fluctuations in breathing patterns (see Figure 2). Swordtails (Xiaboabarus belleri) were more acceptable for monitoring of ventilation rate because of their consistent base rate and relatively little movement.

Figure 2  
Physiograph tracing of base ventilation rate of goldfish



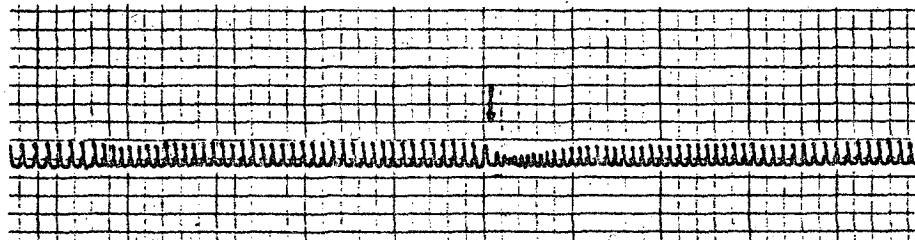
To see if conditioning could be successfully accomplished with this method, I initially attempted to condition a response to the red light only, with the intention of later introducing other colors. The Swordtails were conditioned and tested three times a week between 6 and 9 p.m. Inter-trial periods were varied with all Swordtails to avoid time conditioning, i.e., learning to respond at certain time intervals regardless of stimulus. S1f (female) was conditioned in three successive sessions of 1 hour each, with trials an average of every 10 minutes. Shock pulses ranged from 6 to 11 volts as read on the stimulator. S1m (male) was conditioned in four successive sessions, two half hour sessions per night with an hour rest period in between. Inter-trial periods averaged 5 minutes and UCS was 35-40 volts. After two sessions, S1m died. S2m was conditioned seven sessions in the same pattern as S1m, except that inter-trial periods were lengthened to an average of 10 minutes on the last three

sessions, and voltage used was 40-60 volts. Conditioning of S2f was attempted for one half hour session with no detectable UCR with up to 95 volts. Variations in average inter-trial periods and lengths of sessions were done in attempt to achieve successful conditioning. Other Swordtails were also tested for response to red light alone and shock alone at different voltages.

## RESULTS

The test for unconditioned response elicited by the CS (red light) showed interesting results (see Figure 3). There appeared to be no change in ventilation rate in response to the light coming on, but an increase in ventilation rate and decrease in amplitude in response to the light turning off. This also occurred during conditioning. At times this made it difficult to determine the cause of the response, i.e., light going off or electrical shock. However, an increase in ventilation rate was also observed when the UCS (electrical shock) was presented alone.

Figure 3  
Physiograph tracing of ventilation rate of swordtail  
(Arrow indicates where red light is turned off)



Non-parametric one-sample Wilcoxon signed-rank tests of the null hypothesis,  $H_0: \text{center}=0$ , were performed on the data to determine if there were significant differences in ventilation rates in response to stimuli.

TABLE 1

P-values from Wilcoxon signed-rank tests

Note: Direction of variation from base rate indicated in parenthesis  
(+=greater than base rate, -=less than base rate).

Fish	After light	After shock	Number of trials
S1m	(-) .013 *	(+) .000 **	21
S2m	(-) .047 *	.114	39
S1f	.889	(+) .078 ?	25
S2f	(+) .061 ?	.138	8

\* =  $p < .05$

\*\* =  $p < .01$

? =  $.1 > p > .05$

## DISCUSSION

Various types of conditioning have been carried out successfully with different kinds of fish and using electric shock as an UCS (Denny, 1970). I did not find any previous work involving conditioning of Swordtails. However, I did observe the swordtails to exhibit a behavior described by Denny (1970). In his study using mullet, a light was used as the CS and electric shock as the UCS, and the UCR was a frenetic dash toward the positive electrode. The CR he observed was a slow withdrawal from the positive electrode. He was able to obtain a 98 percent level of conditioning within 70 trials, and he also observed a progressive increase in respiration rate (opercular opening) following the UCS.

It is now well established that most species of fish, especially teleost fish, have trichromatic color vision (Denny, 1970; Reynolds, 1977). One of the main problems, however, in testing for color discrimination is accounting for changes in intensity at different wavelengths. This must be controlled in order to be sure that it is the differences in wavelength and not intensity to which the fish is responding. One experiment using operant conditioning of goldfish successfully controlled for this variable (Muntz, 1966). In Muntz's experiment, the fish were trained to go toward one colored panel over another to get food and continued to go to the correct panel even when no food was given. Varying degrees of intensity of the colored panels were used in varying combinations. His results suggest that goldfish have a predisposition to respond to a visual stimulus on the basis of its color rather than on the basis of brightness (Muntz, 1966).

It is necessary in classical conditioning that the CS be perceived by the fish, but that it is not aversive enough to elicit an UCR. It was evident from Figure 3 that the Swordtails perceived the red light. The response to the light being turned off is also an interesting phenomenon. It could possibly be a defense response to the dimming of surrounding light caused by a predatory fish overhead. This response could cause difficulty in the experiment if the red light going off was acting as a UCS to the fish rather than the electric shock. However, tests performed with shock alone showed a response to the shock, especially in the males. The greater sensitivity to the shock in males could be due to their generally smaller size and larger surface area to volume ratio.

The results, especially from S2m, would seem to indicate that instead of learning to respond to the CS, the fish were habituating to the UCS. This can be seen by the extinction of the response and an increase in ventilation rate following electric shock. As the response began to diminish, an attempt was made to avert this problem by increasing the voltage, but even at a very high voltage the response continued to decline.

The results for S1m seemed to show more potential for learning because the UCR was maintained, although unfortunately this fish died. I cannot conclude whether or not learning occurred, based on these observations, because of the habituation to the UCS, which may

actually be considered learning in itself. However, because of this shortcoming in the experimental method, no testing for discrimination was attempted.

Further attempts at investigating color vision in fish using classical conditioning may be more successful if changes are made in my procedure. Perhaps mullet could be used, since they have previously been conditioned using electrical stimulation (Denny, 1970). Some response other than change in ventilation rate may be more salient and could be measured using other methods. One possible method of observation would be a remotely-monitored video camera. Also, since variations between individuals was so evident in this experiment, a greater number would be necessary to obtain significant results. However, even though the initial goal of this experiment was not achieved, I did demonstrate habituation to shock and perception of the colored light. These observations may help further the exploration of fish physiology and behavior and the methodology of classical conditioning.

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# TURBULENT EDDY FLUX MEASUREMENT USING A PRESSURE MODULATED RADIOMETER: AN OVERVIEW

by Scott Alan Budzien

## ABSTRACT

This paper is based upon a Directed Inquiry performed during the summer of 1984 under the direction of Dr. W.G. Mankin at the National Center for Atmospheric Research. The use of a pressure modulated gas cell as a selective modulator in a radiometer will be discussed in relation to turbulent eddy gas flux measurement. The feasibility of measuring methane fluxes with such a filter is evaluated based upon simulated spectra calculated on a Cray computer from McClatchey spectral line data. This paper is an excerpt from the original feasibility study, and it serves as an overview of the qualitative aspects of flux measurement.

## FLUX MEASUREMENT

Until recently it had been thought that much of the variability of atmospheric components was due to geosphere-atmosphere interactions. However, it has become apparent that biosphere-atmosphere interactions play a much larger role than previously anticipated, at least for certain gases. Both types of interactions can be thought in terms of sources and sinks of gas in the ground-atmosphere interface and gas fluxes across surfaces parallel to the ground. The intensities, locations, influences, and causes of these sources and sinks is of interest to atmospheric chemistry. Turbulent eddy flux measurement can yield information concerning their intensities and locations.

Measurement of gas fluxes is key to understanding biosphere-atmosphere interactions. Several methods have been employed in the study of such fluxes. One of the simplest is the box method, which involves covering a patch of ground with a box, measuring the initial gas concentration, letting it sit for a period of time, and measuring the final gas concentration. Although this method has the advantage of simplicity, it perturbs the system it is measuring by isolating the air in the box from the rest of the atmosphere. This suggests that there might be a more accurate alternate method available.

## TURBULENT EDDY FLUX MEASUREMENT

Turbulent eddy flux measurement is somewhat more complex than the box method, but it perturbs the atmosphere very little. The measurements involve determining the velocity and gas content of parcels of air. The gas content can be found radiometrically, and the velocities can be measured with a sonic anemometer. From these data, instantaneous fluxes can be calculated and averaged to yield the flux.

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## ATMOSPHERIC TURBULENCE

It is well known from everyday experience that the atmosphere is turbulent: the wind blows, thermal currents rise, weather systems pass, etc. Obviously, this indicates several scales of atmospheric turbulence from the highly-localized to the global. In essence, there is a spectrum of turbulence sizes; in general, the higher the turbulent eddies occur in the atmosphere, the larger they tend to be (fig. 1a and 1b).

In the case of turbulent eddy flux measurement, the turbulence of interest occurs in the surface layer, a portion of the boundary layer of the troposphere. The boundary layer is characterized by large diurnal variations and eddy exchange. The vertical extent of the boundary layer varies, depending on atmosphere-surface interactions. Depending upon surface roughness, wind velocity, and temperature, this may extend from a height of from a few hundred meters to over a kilometer above the ground. In this layer the effects of pressure gradient, the Coriolis force, and eddy viscosity forces play an important role in circulation, compared to the atmosphere above the tropopause (Mateev, 1967).

Within the boundary layer lies the surface layer. The surface layer is defined as the region of the atmosphere where the coefficient of turbulence increases with height under any condition. This region typically extends to a height of 50 to 100 meters. Turbulent eddy flux measurement is concerned only with the vertical velocities of turbulence in the surface layer of the atmosphere.

The vertical wind velocity in the boundary layer is given by

$$W = -\frac{H}{L}U \left( \frac{\frac{\partial U_1}{\partial x_1} + \frac{\partial v_1}{\partial y_1}}{\frac{\partial w_1}{\partial z_1}} \right)$$

where	$W$ = vertical velocity	$v_1 = v / U$
	$U$ = horizontal velocity	$x_1 = x / L$
	$L$ = horizontal scale	$y_1 = y / L$
	$H$ = vertical scale	$w_1 = w / W$
	$u_1 = u / U$	$z_1 = z / H$

$u$ ,  $v$ , and  $w$  are the  $x$ ,  $y$ , and  $z$  components of the mean velocity of air volume.

The quantitative details of this relationship are really of no concern in getting a general feel for vertical wind velocity.  $H$  corresponds roughly to the height of the troposphere, the quantities  $u_1$ ,  $v_1$ ,  $x_1$ ,  $y_1$ ,  $w_1$ ,  $z_1$ , and the bracketed terms are on the order of unity, and  $U$  is generally about 10 m/s, so  $W$  depends mainly upon the horizontal extent of the phenomenon. The characteristic horizontal scale of turbulent eddies can vary from several centimeters to tens of hundreds of meters. These lengths correspond to the size of an

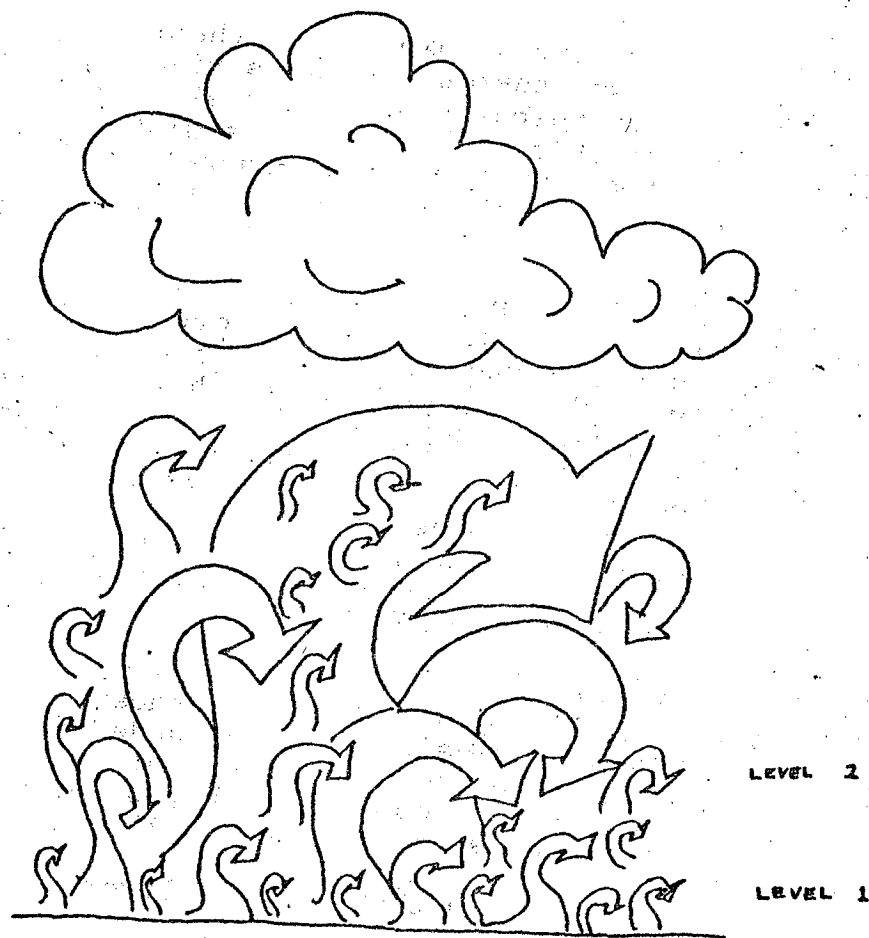


FIGURE 1a.

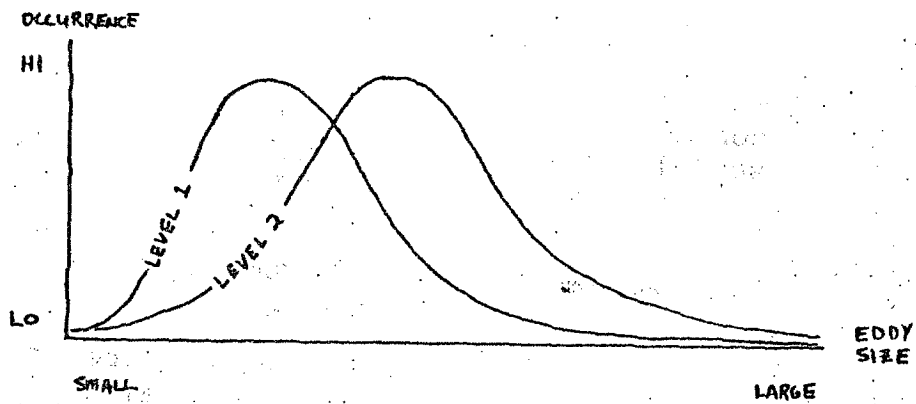


FIGURE 1b.

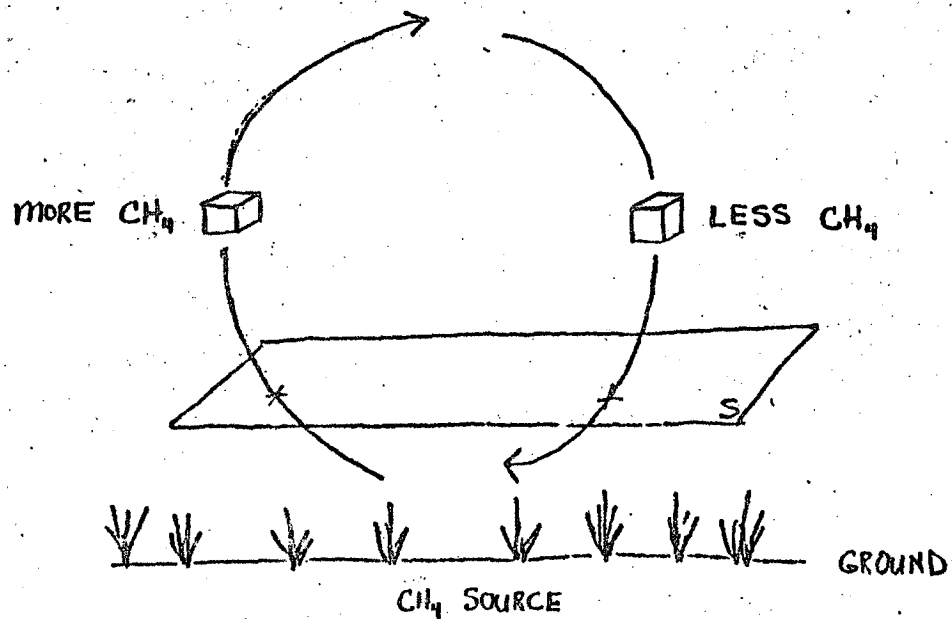


FIGURE 2a.

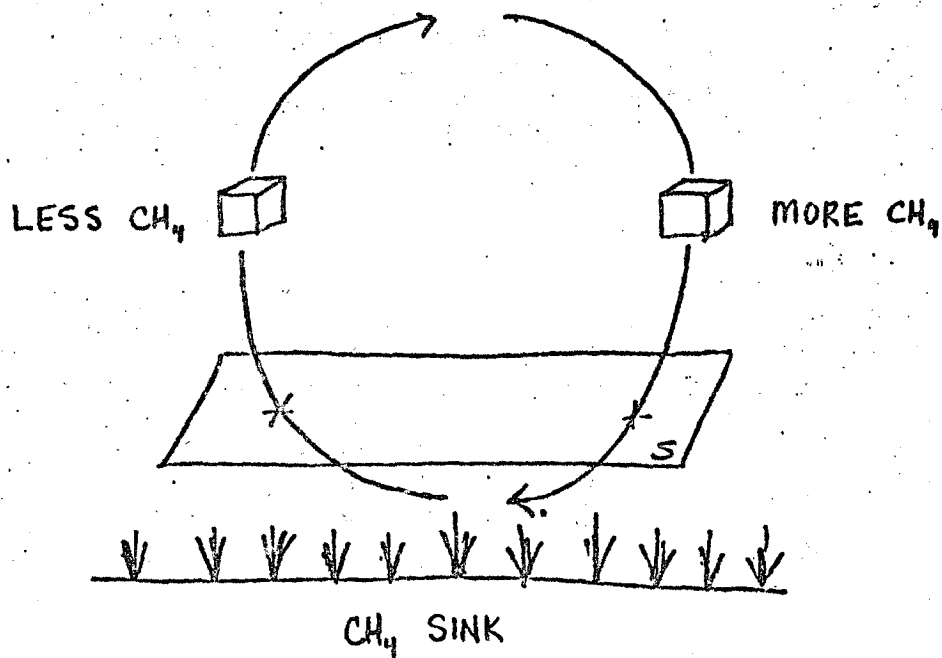


FIGURE 2b.

area in which the velocity of air has the same direction, up or down (Mateev, 1967). This characteristic length is very important to turbulent eddy gas flux measurement: its goal is to measure the gas concentration and the vertical velocity of an individual parcel of air and the characteristic length indicates the size of the parcel.

Since both size and velocity can be associated with eddies, they can be thought of in terms of frequencies as well. High frequency eddies of around 10 Hz occur near the ground, and lower frequencies, say 1 Hz, occur a few meters up. The lowest frequency eddies have periods of about 20 minutes, and are of the size associated with a cloud.

Generally, turbulence comes in groups of eddies containing a full range of characteristic frequencies. When making flux measurements, the highest frequency eddy must be sampled at the Nyquist frequency, twice the characteristic frequency of the eddy. In order to make measurements over a sufficient portion of the frequency spectrum, the sampling time should correspond to the period of the largest eddy of interest. Thus to make measurements on one group of turbulent eddies, the measurement time needs to be about 20 minutes.

#### ATMOSPHERIC METHANE

Methane is a minor constituent of the atmosphere with a mixing ratio of approximately 1.6 parts per million. It is a fairly stable gas, produced in biological processes. Its major source is decay in wet environments, such as swamps, marshes, and paddy fields. The sinks of methane are for the most part unknown; it may be destroyed biologically or oxidized (Iribarne, 1980).

Infrared spectroscopy confirms chemists' classical tetrahedral model of the molecule. The molecule is a spherical top and is therefore highly degenerate. Only two of the fundamental vibration modes are allowed due to symmetry considerations: the  $\nu_3$  and  $\nu_4$  bands, centered at 3019 and 1306  $\text{cm}^{-1}$  respectively (Goody, 1964). For the purposes of this study, the  $\nu_3$  band is of primary interest.

#### MEASURING FLUX

Consider a localized region of the ground-atmosphere interface (fig. 2a and 2b). Naturally, there is a considerable amount of circulation and turbulence over the interface. Note an individual parcel of air as it circulates with respect to the flux surface S. In this feasibility study the gas of interest is methane. If S is a methane source, then the downward traveling parcels will contain less methane than the upward traveling ones. On the other hand, if a sink lies beneath S, then the rising parcels will contain less methane than the descending parcels.

Nearly every quantity can be expressed as the sum of a mean and a deviation:  $x = \bar{x} + x'$ . In flux measurement it is convenient to use this scheme to express vertical wind velocity and concentrations. Hence

$$w = \bar{w} + w' \quad \text{and} \quad c = \bar{c} + c'$$

where  $w$  is vertical wind velocity and  $c$  is the methane concentration. Note that  $\bar{w}$ , the mean vertical wind, is zero (i.e. no wind coming out of the ground), and that  $\bar{c}$ , the mean concentration, has a definite value greater than zero, equivalent to approximately 1.6 ppm. In measuring flux,  $w'$  and  $c'$  are determined. The product  $w'c'$  is the amount of material moving up or down at any given time, and the time average of these values  $\langle w'c' \rangle$  is the flux.

For investigations of surface fluxes the time resolution of the instrument must be on the order of a few Hertz and the period of integration should be about 20 minutes. This sampling procedure is sufficient to sample the full range of boundary layer turbulence (Delaney et. al., undated).

Another consideration in making flux determinations is the area from which the flux information is actually coming. This will depend upon the average horizontal wind velocity and the vertical position of the instrument (fig. 3a, 3b, and 3c). When the horizontal wind speed is high or the instrument is well above the ground, the sampled area is far from the instrument and large. The reason for this is that the concentration information is being blown downwind to the instrument. Depending on the speed of any given gust, the data corresponds to slightly different sampling points. In this way the wind distribution can be directly correlated with the sampling location distribution (fig. 4).

#### INSTRUMENT DESIGN AND FUNCTION

To perform flux measurements conveniently, an instrument positioned a couple of meters off of the ground is desirable. Being close to the ground means that the turbulence measured will be generally small and fast. This means that the instrument must have a small characteristic length, and be able to make fast measurements. This also implies that the sampled area will be fairly close and small, assuming a moderate wind speed.

The length of the sampling cell must be on the order of the size of the smallest turbulent eddies. Since  $w'$  and  $c'$  need to be measured for each turbulent eddy passing through the cell, if the cell is too long, two or more eddies might be measured at once. In this case rather than finding  $w'c'$  for each eddy, a space average of  $c'$  would be measured, while  $w'$  would be found for only one of the eddies. Obviously,  $w'c'$  in this case is simply not the flux of a single eddy, or even an acceptable approximation.

The concentrations are found by computing the molecule number density based upon the transmission of the gas in the cell. The absorption cell could be a Pfund cell or a White cell, depending on

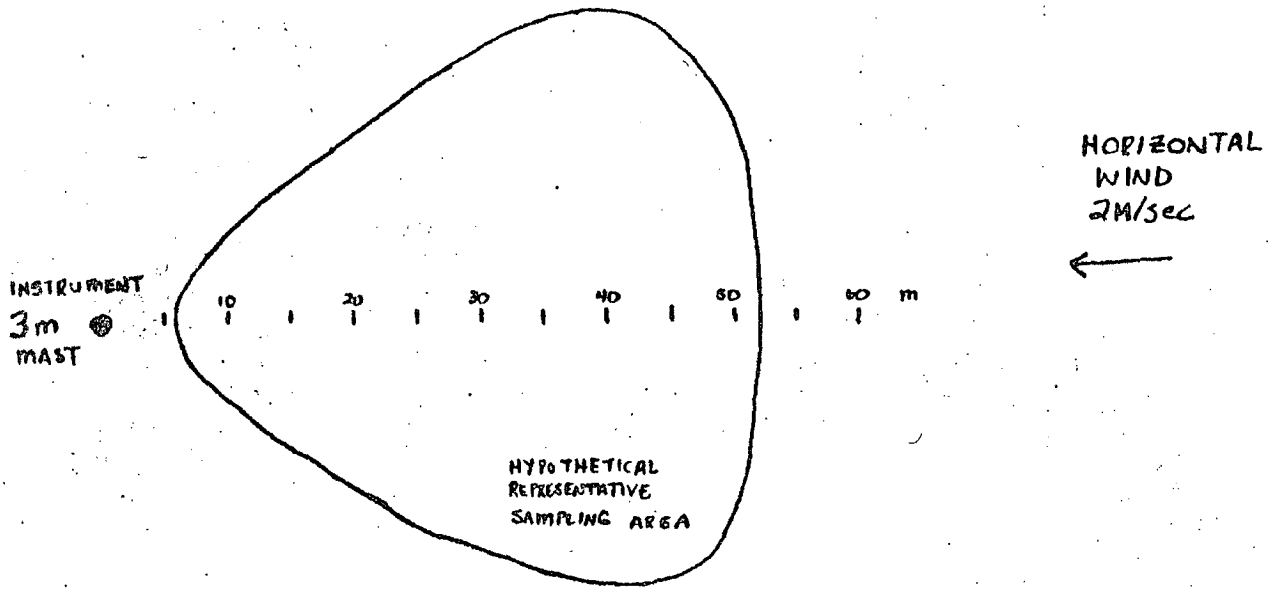


FIGURE 3a. [7]

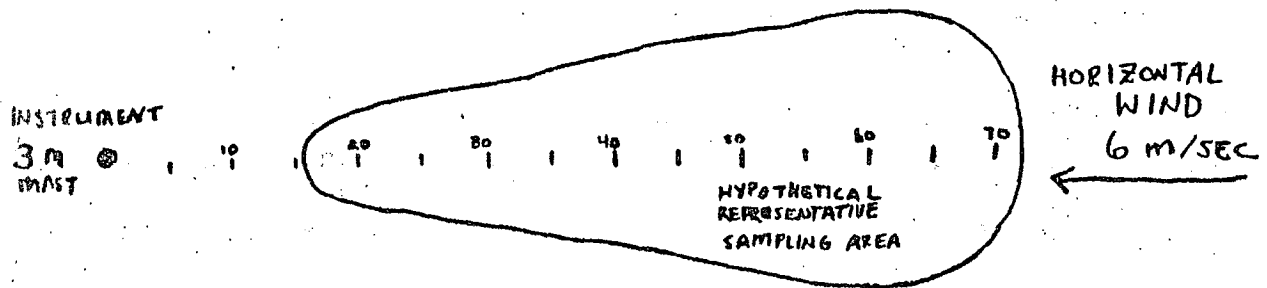


FIGURE 3b. [7]

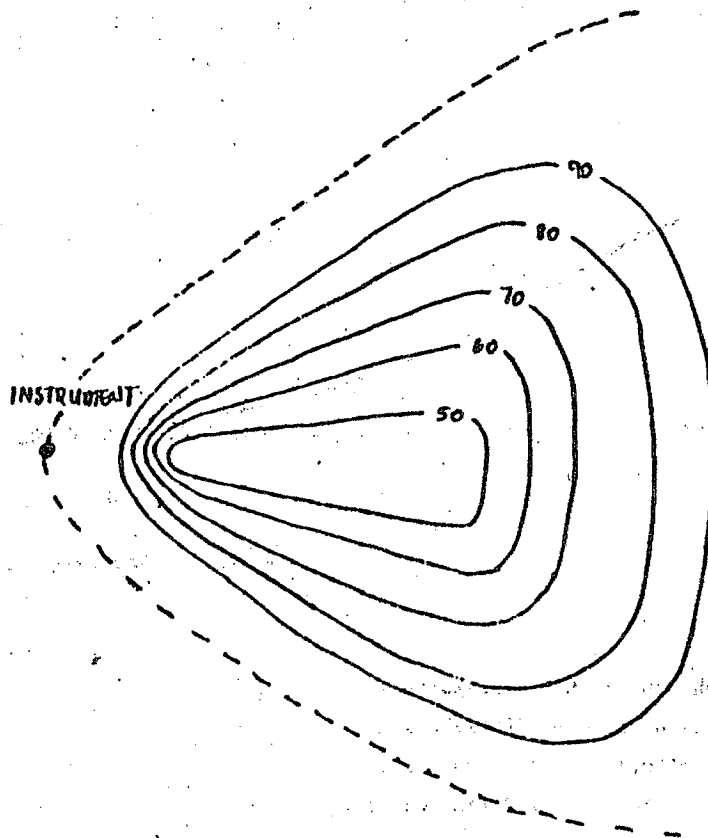
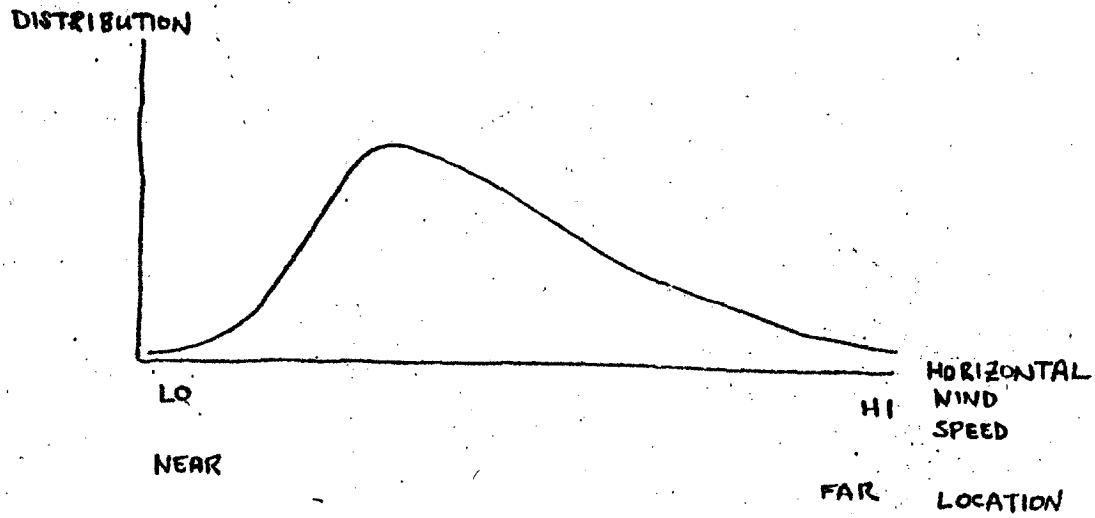


FIGURE 3c. This figure depicts a typical sampling area. The numbers on the curves indicate the percentages of sampling information originating from the area inside the curve. Thus, due to the wind speed distribution, 50% of the data originated in the smallest region, while 90% of the data originated in the largest region. Note that 100% of the data originated in the dashed curve, which extends from the instrument to infinity, in order to take into account wind speeds from 0 to an infinite number of meters per second. [7]



**FIGURE 4.** This graph depicts the relationship between a hypothetical wind speed distribution and sampled data distribution. Since velocity and distance are proportional by a factor of time, each datum carried by an eddy moving with a certain horizontal velocity corresponds to a particular location in the sampling area.

the line intensities and the concentration of the absorbing gas of interest. (Since the cell length must be fixed to the characteristic size of the turbulence, the only way to get increased absorption is to increase the number of reflections.)

The instrument consists of a global source, the atmospheric absorption cell, the pressure modulated cell, a filter, a condensing lens, and the detector (fig. 5). Radiation is produced by the global, partially absorbed by the atmospheric cell, further absorbed by the pressure modulated cell, passed through the filter to isolate the spectral region of interest, and focused onto the detector by a collecting lens. Then the signal is amplified, and the power reaching the detector can be found. Knowing the source function of the global and the detected power, the absorption within the atmospheric cell can be computed, and from that the methane concentration in the sampling cell. This is then correlated with velocity measurements to yield the flux.

#### THE PRESSURE MODULATED RADIOMETER

The pressure modulated radiometer is an instrument that uses a gas cell whose pressure is being varied in time. The cell is filled with the atmospheric gas being measured. As the pressure is varied, the transmittance of the cell changes, so that the radiation passing through the cell onto the detector is modulated. Hence, the signal may be amplified by an a.c. system. But more importantly than that, the only portions of the spectrum being modulated are the places at which absorption lines occur for the gas being studied. So, ideally, the a.c. system only "sees" those absorption lines, and the output reflects only the concentration of the absorbing gas.

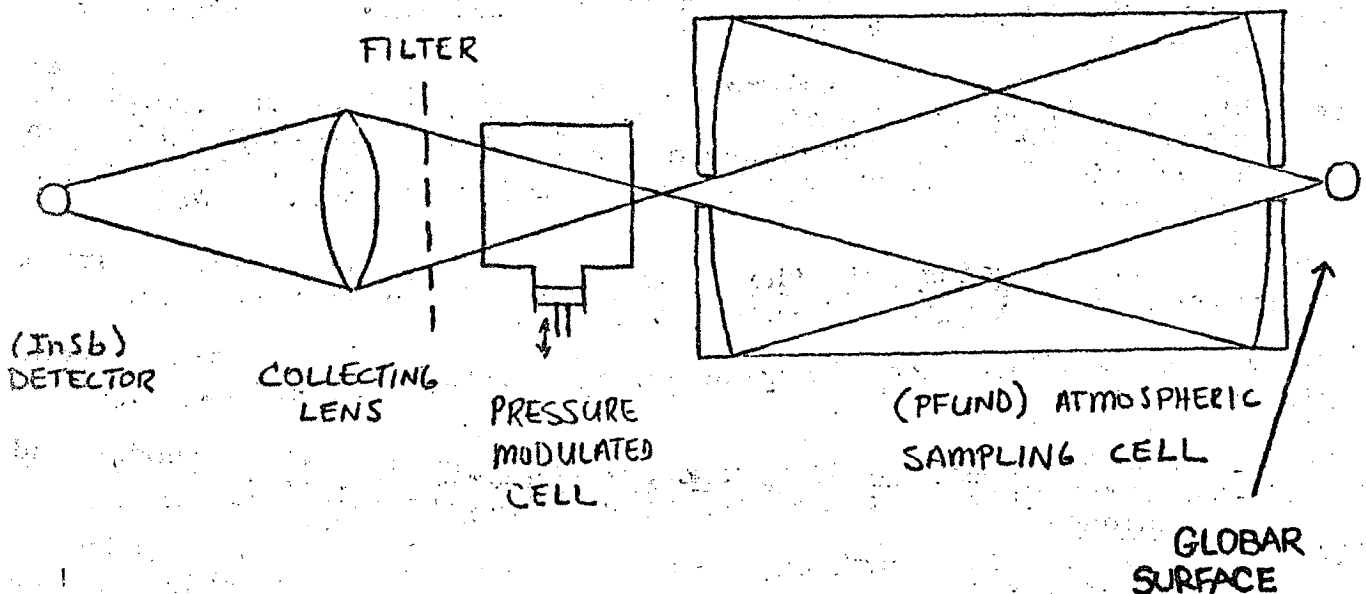


FIGURE 5. THE TURBULENT EDDY FLUX MEASUREMENT SYSTEM USING A PRESSURE MODULATED RADIOMETER.

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## A DIURNAL AND SEASONAL INSECT SURVEY

by Richard Samuels

## ABSTRACT

A diurnal and seasonal sweep net survey of the insect population of a cow-grazed pasture in southern Mississippi was performed. Three diurnal sweep net trials were made. Each trial consisted of five sweep net collections (with three samples per collection) per 24 hours. The seasonal survey consisted of collections on five dates from June 9 to August 10. Ninety-nine percent of the insect community (by number of individuals) consisted of six orders: Orthoptera, Hemiptera, Homoptera, Coleoptera, Diptera, and Hymenoptera. Many spiders (Class Arachnida, Order Araneida) were also captured. The Orthoptera, Coleoptera, Diptera, Hymenoptera, and Araneida showed circadian behavior. Generally, activity was low at 06:00, moderate at 11:00, low at 15:20, and highest at 20:30. The seasonal survey revealed changing population size of the Orthoptera, Coleoptera, Hemiptera, Homoptera, and Diptera. The changing population size was attributed to changing grass height and possible seasonal reproductive cycles.

## INTRODUCTION

This experiment was a sweep net survey with two objectives. The first objective was to record which orders of insects inhabited a section of pasture land near Wiggins, a town in the southern tip of Mississippi. The second objective was to record changes in the numbers of insects in each order over three 24-hour periods and over the summer months from early June to middle August.

There are many methods for capturing insects: the sweep net, suction, pitfall trap, dry funnel, and photo-electric methods. Auchennorrhyncha (1982) found that the pitfall trap complements the sweep net by capturing individuals of certain sexes and ages that the sweep net often misses. Tormala (1982) found that the suction method was the most effective method (74-100%) for capturing all orders of insects. However, because of the ease of handling and minor expense, the sweep net alone was used, and inaccuracies with its use were expected.

Basically, the sweep net works on the premise that, since most insects are phytophagous (Borror, 1976), the activity of individuals in an order is directly proportional to the numbers of insects feeding on the available vegetation. Inactive, nonfeeding insects may be on the ground or on low portions of stems in order to evade predators or heat, or both; these insects would be out of the range of the net. Active, feeding insects might seek the leaves and tender stems. Because most of this vegetation is high enough above the ground to be in the range of the sweep net, more individuals would be

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captured when the insects are active (feeding) than when inactive. Thus, by recording the numbers of insects captured at regular intervals over a 24-hour (diurnal) period, circadian cycles in insect abundance may be noticed.

Obviously, both population size and activity will affect the number of insects captured. One does not expect population size to change greatly over 24 hours; any changes in the numbers of individuals captured should reflect changes in the location of the insects on the vegetation and not changes in the actual number of insects in the field. However, over three months, population sizes could change greatly, and thus changes recorded over this length of time may not result from a circadian cycle. When only comparing data collected on different days at the same time of day, any changes in the numbers of insects collected per order should be due to changing population size over time or changing activity due to varying temperature and not to changing activity due to a circadian cycle.

## MATERIALS AND METHODS

### A. Study Area

A 45x25 meter rectangular section of pasture land near Wiggins, Mississippi, was sampled. The land was moderately grazed by cows but otherwise undisturbed. Three parallel, 45 meter paths were marked at each end with stakes. Each path was separated by 8 meters so that a collection on one path would not disturb the others. Native centipede grass, bahea grass, and various weeds covered the plot.

### B. General Outline

In the diurnal study, the insect population changes over a 24-hour period were recorded in three trials: on July 3, July 13, and July 20. On each of these dates, a sweep net collection (consisting of three separate samples per collection) was performed every 4 hours 50 minutes for 24 hours. Because sunset varies slightly (approximately 45 minutes) from June through August and insects react to the position of the sun (Borror et al., 1976), these times can be translated into circadian time (based on sunset) only to + 45 minutes. The total number of insects in each order from each collection was plotted against time of day; the number of insects was plotted both absolutely and relatively by the percent maximum. One-way variance tests were performed to compare the data of each order for each day; the probability (p) values were recorded. The coefficients of variation [(standard deviation of the samples/the mean of the samples)x100] were calculated for each collection to compare the collection mean to the individual samples in order to determine the consistency of the samples.

In the seasonal study, changes in the numbers of individuals per order collected at three times of the day (05:45, 15:15, and 20:10) on five dates from June 9 to August 10 were recorded. The results from the three 24-hour trials, as well as data from two other dates (June 9 and August 10) were used. Only data collected at the same time of day were compared. The total number of insects in each order

and of all insects grouped together was plotted against the date of collection. The total number of insects in each order was also plotted against the temperature at collection; the total number was plotted both absolutely and relatively by the percent maximum. One-way variance tests were performed to compare the data of each order of one time of day over the five dates; the p values of these tests are recorded in Table 3. The coefficients of variance were calculated for each collection (Table 4).

### C. Method of Collection

Each collection of insects was composed of three separate samples, one sample collected per path. Thus, three killjars were required. Ethyl acetate killjars were prepared according to a procedure described by Borror (1976).

Immediately before each collection, one killjar was placed at the north end of each path. The first sample was started at the south end of the path and performed by slowly walking northward while making 80 consecutive sweeps with the net. Approximately two sweeps were made per meter. A sweep consisted of one short rapid stroke per step; the rim of the net travelled as low to the ground as possible without being grossly impeded by the ground or thick vegetation. After the 80th sweep, the insects were transferred into the appropriate killjar. To do this, the net was swished three more times above the vegetation to force the insects farther back into the net. The killjar was then opened, the insects were emptied into it, and the lid was quickly replaced. The next two samples were collected in the same manner.

The killjars were allowed to remain closed for 20 minutes. If there had not been enough ethyl acetate in the jars to kill the insects, then the jar was placed in a freezer for a few minutes.

After the insects were dead, the vegetation accidentally collected in the net was removed, the insects were divided into orders, and the numbers in each order were recorded. In addition, all spiders caught were classified together, counted, and recorded. If the order to which a type of insect belonged could not be identified, a few of the individuals were preserved in 70% ethanol to be identified later.

## RESULTS

(Note: In Figures 1, 2, and 3, dark segments above the x-axis indicate hours of darkness.)

## ORDER KEY

* . . . *	Orthoptera
* — — — *	Hemiptera
* — — — *	Homoptera
* — — — *	Coleoptera
* — — — *	Diptera
* — — — *	Hymenoptera
* — — — *	Araneida

FIG. 1: Total Insect Abundance x Time

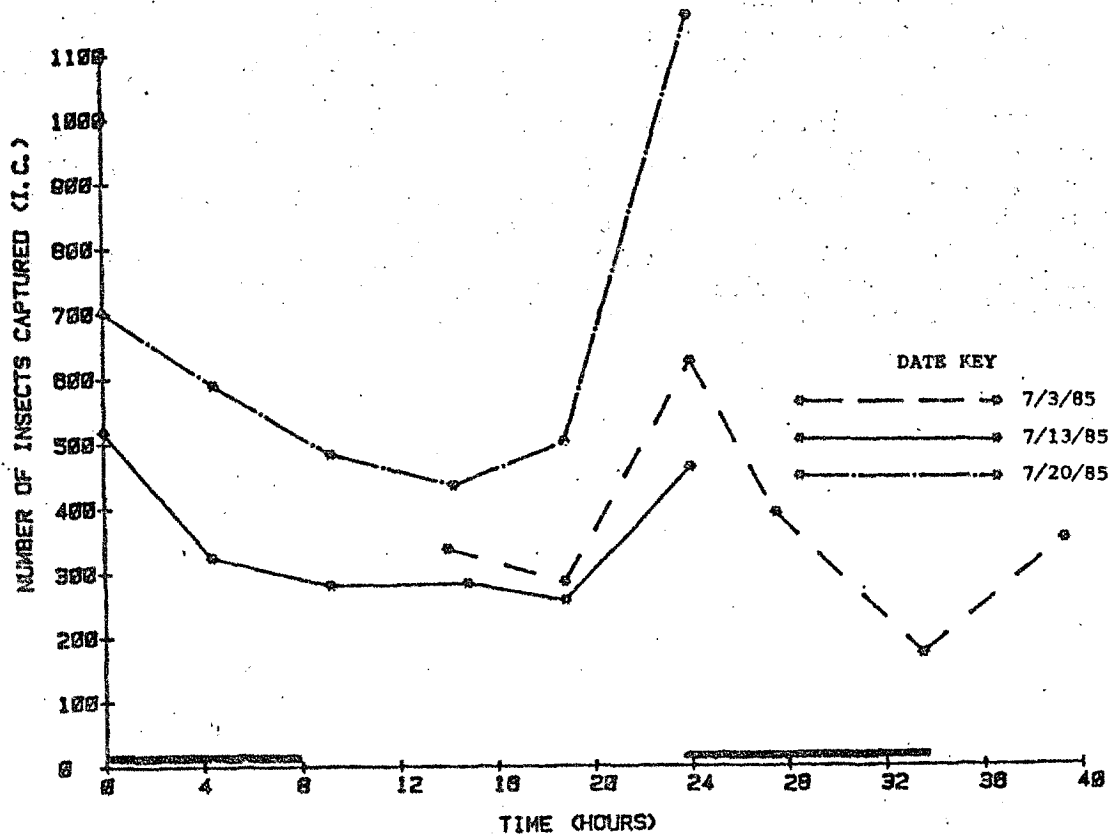


FIG. 2: Insect Abundance (by Order) x Time

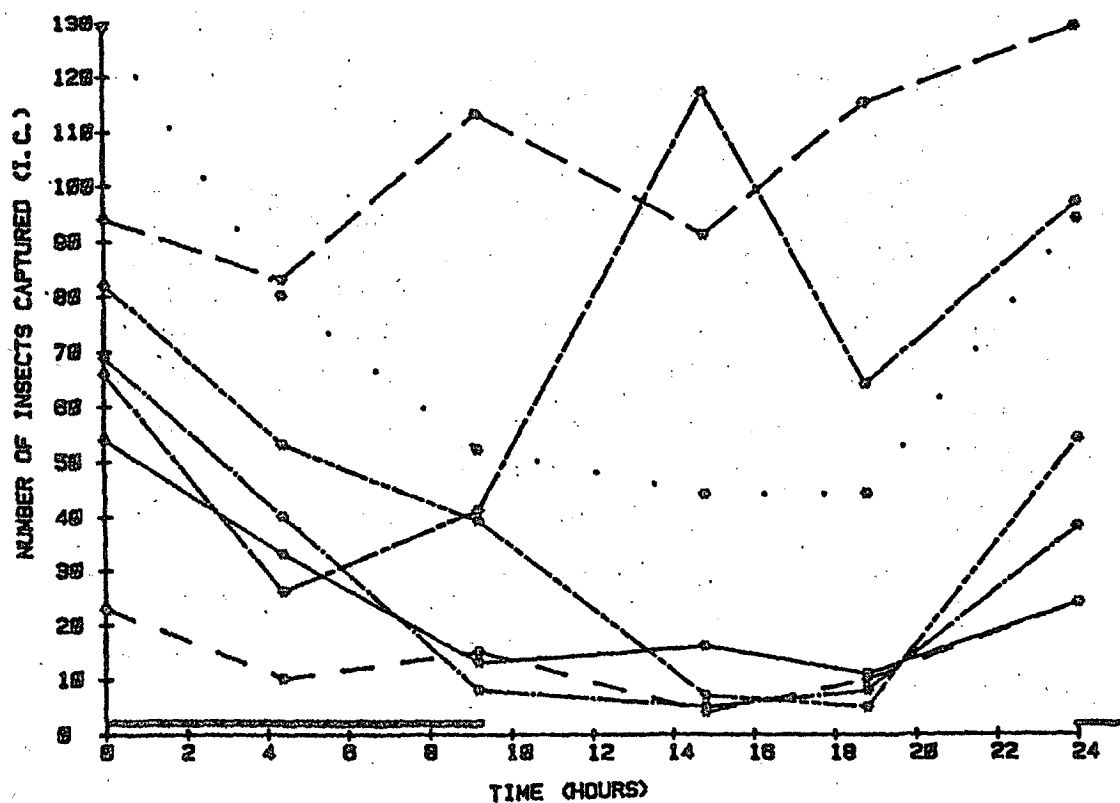


FIG. 3: Relative Insect Abundance (by Order) x Time

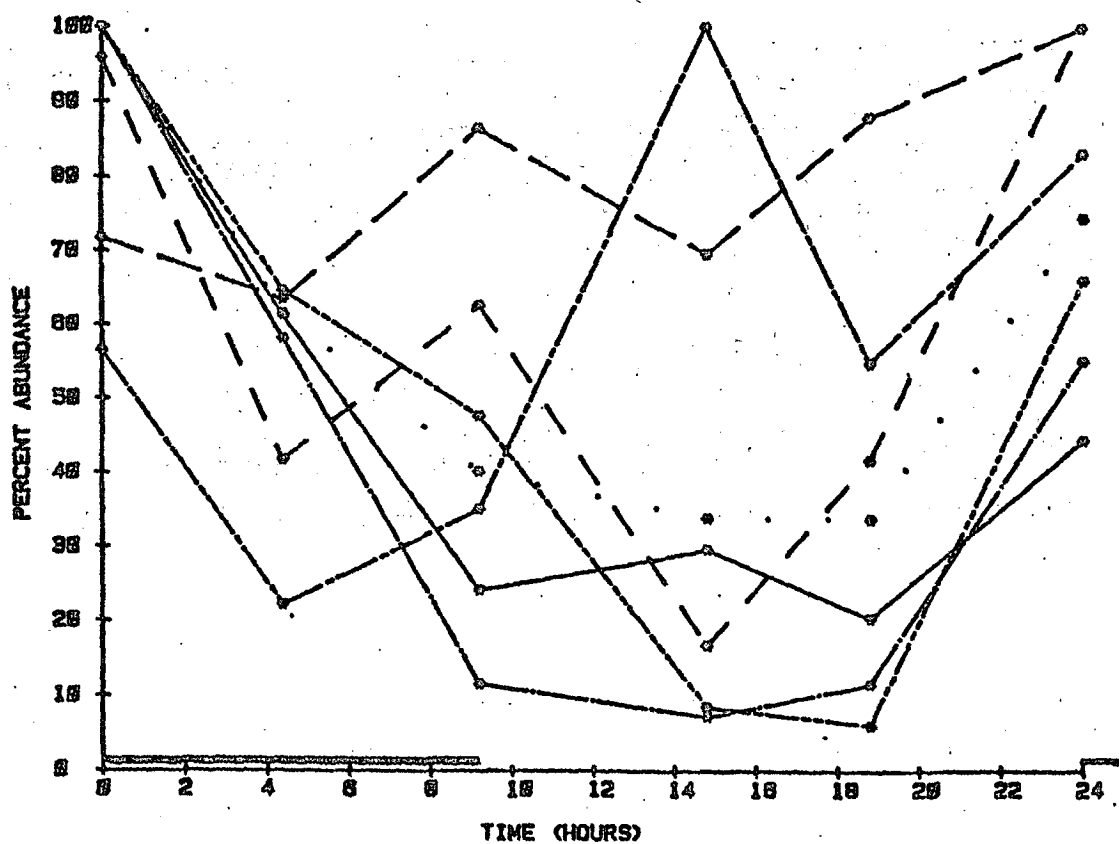


TABLE 1: P Values from One-Way Variance Tests of Diurnal Trials

ORDER \ DATE	7/3/84	7/13/84	7/20/84	AVERAGE
Orthoptera	0.002	0.008	0.002	0.004
Hemiptera	0.219	0.447	0.126	0.264
Homoptera	0.051	0.782	0.171	0.335
Coleoptera	0.002	0.006	0.000	0.003
Diptera	0.006	0.032	0.000	0.013
Hymenoptera	0.020	0.028	0.310	0.119
Araneida	0.000	0.191	0.001	0.064
AVERAGE	0.043	0.213	0.087	

TABLE 2: Coefficients of Variation [ $x$  ( $\pm$ range)] of Diurnal Trials

ORDER \ DATE	7/3/84	7/13/84	7/20/84	AVERAGE
Orthoptera	35.0 (14.7)	43.0 (37.5)	21.8 (10.1)	33.3
Hemiptera	66.9 (32.4)	82.6 (5.38)	64.9 (32.7)	71.5
Homoptera	55.7 (32.2)	39.9 (24.9)	34.5 (15.4)	43.4
Coleoptera	49.4 (43.1)	63.3 (45.7)	50.3 (42.5)	54.3
Diptera	40.1 (29.1)	47.2 (45.6)	35.9 (29.9)	41.1
Hymenoptera	68.5 (51.5)	69.7 (54.8)	83.0 (36.2)	73.7
Araneida	18.0 (16.5)	57.4 (30.1)	26.8 (19.1)	34.1
AVERAGE	47.7	57.6	45.3	

FIG. 4: Total Insect Abundance x Date

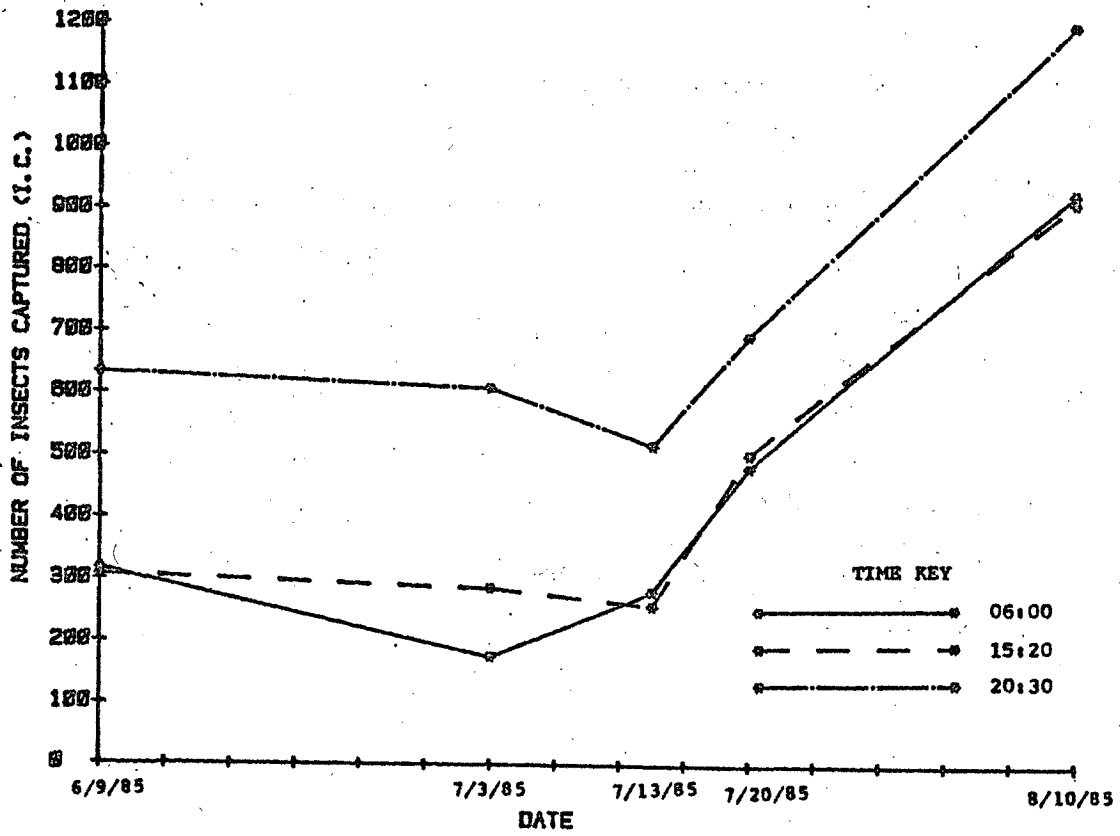


FIG. 5: Insect Abundance (by Order) x Date

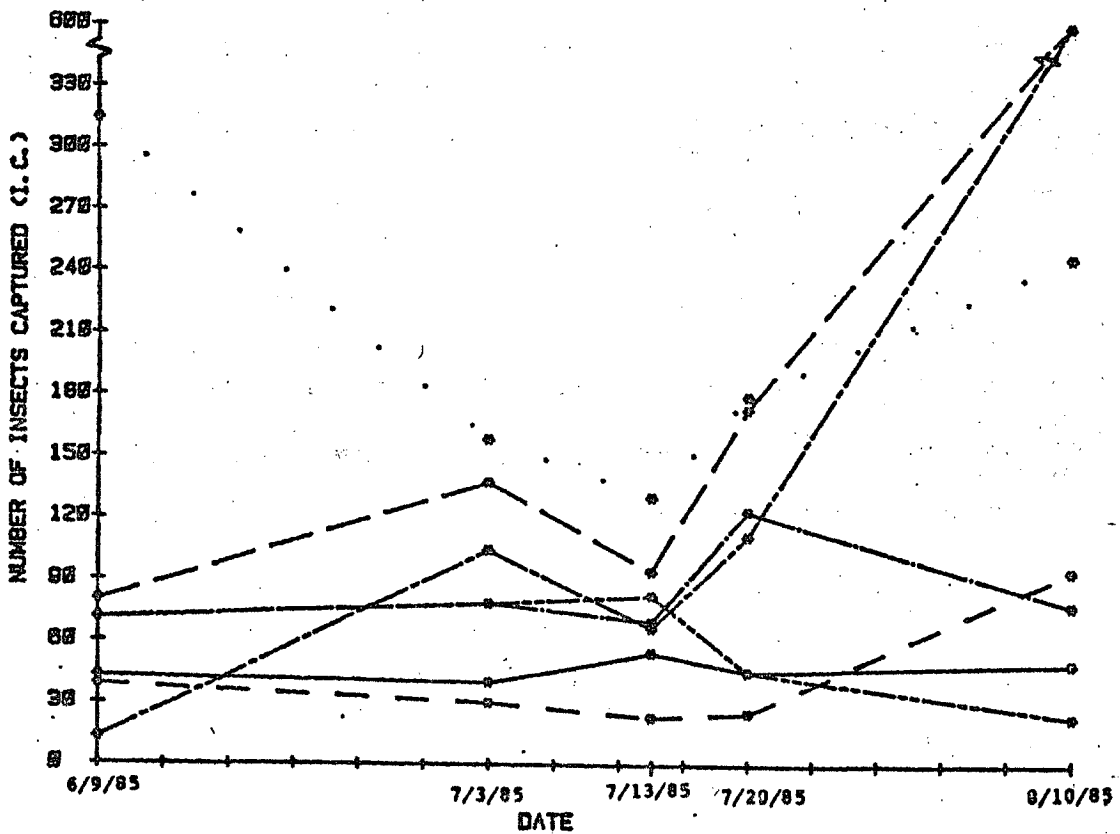


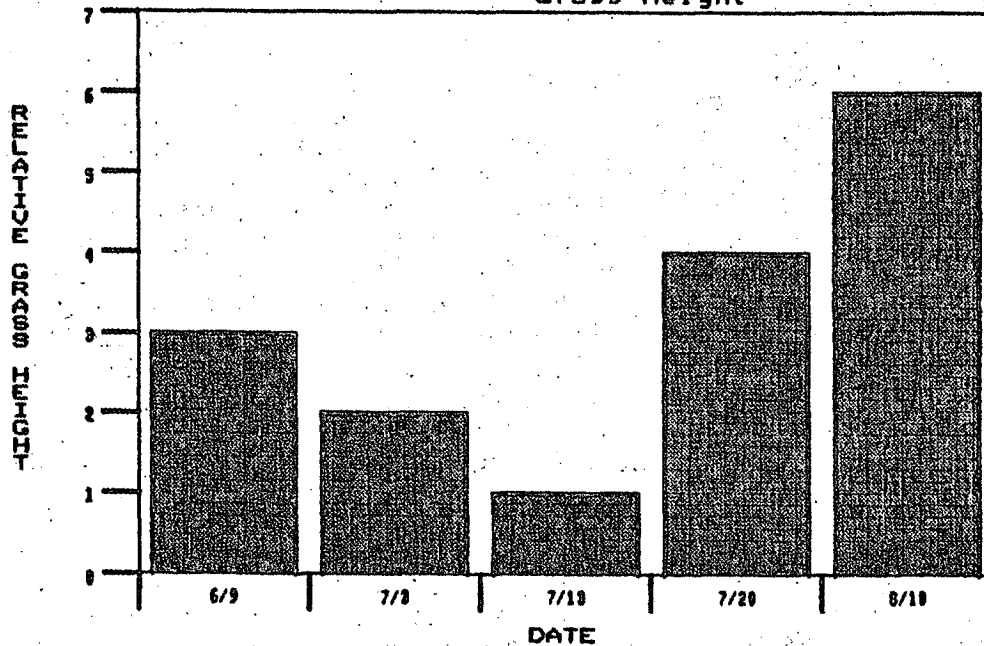
TABLE 3: P Values from One-Way Variance Tests of Seasonal Trials

ORDER \ TIME	06:00	15:20	20:30	AVERAGE
Orthoptera	0.036	0.013	0.004	0.018
Hemiptera	0.086	0.011	0.010	0.036
Homoptera	0.000	0.008	0.000	0.003
Coleoptera	0.002	0.002	0.316	0.107
Diptera	0.000	0.000	0.000	0.000
Hymenoptera	0.403	0.518	0.099	0.340
Araneida	0.444	0.109	0.958	0.504
AVERAGE	0.139	0.094	0.198	

Table 4: Coefficients of Variation [x (+range)] of Seasonal Trials

ORDER \ TIME	06:00	15:20	20:30	AVERAGE
Orthoptera	35.5 (11.8)	31.9 (30.7)	20.0 (12.4)	29.1
Hemiptera	45.7 (15.9)	70.8 (36.6)	68.9 (51.9)	61.8
Homoptera	36.0 (43.5)	34.5 (18.0)	39.7 (33.3)	36.7
Coleoptera	41.8 (41.7)	53.3 (34.9)	27.1 (25.4)	40.7
Diptera	39.0 (37.9)	51.0 (82.1)	31.6 (26.7)	40.5
Hymenoptera	58.0 (31.2)	102. (31.2)	48.1 (43.6)	69.4
Araneida	43.7 (18.1)	40.9 (26.4)	41.9 (35.1)	42.2
AVERAGE	42.8	54.9	39.6	

TABLE 5: Approximate Relative  
Grass Height



## DISCUSSION

### A. General Observations

Although a casual walk over the pasture revealed little insect activity, a few sweeps of the net showed that the field was teeming with insect life. Ninety-nine percent of all insects collected were in one of the following orders: Orthoptera (grasshoppers and crickets), Hemiptera (true bugs), Homoptera (hoppers), Coleoptera (beetles), Diptera (true flies), and Hymenoptera (ants, wasps, and bees). Many spiders (class Arachnida, order Araneida) were also collected. In all 27 collections, less than ten insects were captured in each of two other orders, Odonata (dragonflies and damselflies) and Lepidoptera (butterflies and moths); thus data for these orders were too sparse to be included in the graphs. Almost all hymenopterans captured were ants. All data collected on insect abundance were recorded in terms of numbers of insects per order rather than biomass per order; thus 600 small gnats (dipterans) would seem more significant than 50 orthopterans. By rough estimation, orthopterans consistently occupied the most biomass in a collection while coleopterans usually occupied the second highest biomass.

### B. Diurnal Surveys

The activity of many insects follows a diurnal or circadian cycle. For instance, many insects are active during the day and inactive during the night. This rhythmic behavior is mostly controlled by the sun. However, because certain insects continue the circadian cycle for a while when placed in continuous light or darkness, some physiological factors must be responsible for the

behavior as well (Borror et al., 1976).

Insect activity is also affected by temperature. Because the insect is poikilothermic or cold-blooded, its internal temperature drops as the environmental temperature drops. Consequently, the insect's physiological processes are slowed (Borror, 1976). High temperatures can also reduce the activity of an insect. Because the insect has a large surface area to volume ratio, there is a relatively large area for water loss through the exoskeleton. In addition, the waterproof nature of the lipid (wax) covering of the exoskeleton can be reduced in high temperatures. Insects threatened with water loss might seek refuge from the sun by staying in the shade of vegetation close to the ground and thus be out of range of the sweep net. Because the changing temperature and light cycle are closely related, it is difficult to determine whether diurnal activity cycles are due to light or temperature or both. The temperature changes over the 24-hour periods followed this pattern: beginning at dawn, the temperature increased until approximately 15:20; then it decreased through dusk and night until dawn, when it began to rise again.

Finally, fluctuations in insect activity reflect the need to elude predators. Insects may remain low to the ground during daylight to avoid capture by predators, such as birds, who locate prey by sight. During dusk and night, periods of low visibility, the insects may be able to safely feed higher up on the vegetation.

Upon examining the changes in the "number of insects captured" (I.C.) of all orders grouped together, it is obvious that activity varies according to some circadian pattern (Fig. 1). The general pattern is 1) a decrease in I.C. from dusk (20:30) until dawn (06:00), 2) little change in I.C. from dawn to 15:20, and 3) an increase in I.C. at dusk (20:30).

Orthopterans, coleopterans, hymenopterans, and araneidans exhibited similar activity patterns (Fig. 2 and Fig. 3). The general pattern, beginning at 20:30, was 1) a large decrease in I.C. until a minimum was reached at dawn; 2) a smaller decrease or slight increase in I.C. until 11:00; 3) a larger decrease in I.C. until 15:20; and 4) a large increase in I.C. to a maximum at dusk. The sharp decrease in I.C. during the night could be explained by a decrease in activity due to the drop in temperature. (From 01:00 to 06:00 in Trial 3, the I.C. of Orthoptera remained fairly constant and the I.C. of Hymenoptera actually increased; possible reasons for these irregularities are not known.) The smaller decrease or slight increase from 06:00 to 11:00 may have indicated that the insects were somewhat more able to feed at the temperatures (75-85 deg. F) of this period. From 11:20 to 15:20, the sharp decrease in I.C. may have indicated that the insects need shade at the hottest part of the day. Then, as the temperature fell to approximately 80 deg. F at sunset, the I.C. increased to its maximum; at this time the insects were most active at their presumed optimal temperature.

The dipterans, homopterans, and hemipterans exhibited different activity patterns. The I.C. of Diptera reached a minimum at 01:00 (instead of 06:00), after which the I.C. increased slightly at 06:00. The I.C. then increased to a maximum at 11:00, decreased at 15:20, and increased greatly at 20:30. Perhaps the 06:00 increase in the I.C. was due to some advantage that sucking insects (like dipterans) have in dew-covered grass. The afternoon increases in I.C. at 11:00 may have been due to the fact that the temperature of flight muscles must be maintained above a certain point in order to function optimally; an increase to an optimum temperature may have brought more insects into the range of the net.

A circadian pattern was not discernable for Homoptera and Hemiptera. The I.C.s of Homoptera alternately rose and fell at each collection, with an overall tendency to increase from dawn to dusk. The I.C.s of Hemiptera remained fairly constant throughout the trial.

The one-way analysis of variance of the diurnal surveys (Table 1) measures by a standard method the probability (to a 95% level of confidence) that the I.C.s of different collections of the same order are "not equal"; thus the one-way tests can tell if change in I.C. occurred between collections. The probability (p) value must be less than 0.05 to indicate a significant change. Homoptera and Hemiptera were the only orders that had p values greater than 0.05 in all three trials, indicating that the I.C.s of this order remained fairly constant, as mentioned above.

The coefficients of variation (c.v.) of the collections were approximately the same, except for those of the hemipteran and hymenopteran collections. The c.v.s of these orders were the highest, showing that variability among the samples of these orders was greatest. The population of ants (hymenopterans) tend to be highest around their nests. Thus, a net sweep over a nest would pick up an unusually large number of ants. Hemipterans may also concentrate in certain areas of the pasture. The orthopterans and araneidans exhibited the smallest coefficients and thus the greatest consistency between samples. The orthopterans and araneidans probably spread out evenly over the pasture.

### C. Seasonal Trials

Because only collections taken at the same time of day were compared, changes in I.C. of each order should not be the result of differences in activity due to a circadian cycle. Changes could be caused by temperature contingent activity or by changes in population size due to seasonal reproductive cycle or changing grass height (a variable not accurately measured).

In Fig. 4, the I.C.s of all orders captured in a collection were grouped together; thus the fluctuation in total insect abundance was plotted against the date of collection. The I.C.s varied consistently for all three collection times (06:00, 15:20, and 20:30). There was little or no change in I.C. from June 6 to July 13. However, there was a marked increase in I.C. for July 20 and August 10. Although this marked increase could be due to some

seasonal reproductive cycle, it seems more reasonable to attribute it to changing grass height, as discussed below.

The activity patterns of Orthoptera, Homoptera, Hemiptera, Coleoptera, and Diptera can be attributed both to a reproductive cycle and changing grass height. An estimation of relative grass heights over the seasonal trial is provided in Table 5. Logically, higher grass should provide food and protection for a greater population of insects. The changing abundance of Hemiptera seems to approximately follow the changes in grass height. The changing abundance of Orthoptera seems also to correspond to grass height changes until the collection on August 10. On this date, when the grass was highest, the I.C. of Orthoptera increased, but it did not exceed the I.C. on June 9 when the grass was shorter. Perhaps on August 10, the population of Orthoptera is beginning to decline because of some reproductive cycle. The I.C.s of Coleoptera underwent changes similar to Orthoptera. The changes in the I.C.s of Diptera and Homoptera generally also corresponded to changes in grass height except on the first collection, June 9. The I.C.s of both orders were greater on July 3 than on June 9, yet grass was higher on June 9. Perhaps a seasonal population increase had occurred between June 10 and July 3.

One-way variance tests performed on the seasonal data showed that the I.C.s of Hymenoptera and Araneida underwent no apparent significant changes (Table 3). Also, no observable connections could be discerned between the changes of the I.C. of any order and the temperature of collection.

As in the diurnal samples, the c.v.s of all of the collections were approximately the same, except that the c.v.s of the hemipteran collections were again somewhat greater than most. Again, the c.v.s of the orthopteran collections were the smallest.

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THE ROLE OF MICROTUBULES IN THE CELLULASE SECRETION  
OF Trichoderma reesei

by Marion Herndon

ABSTRACT

The filamentous fungi Trichoderma reesei was tested for its sensitivity to anti-microtubular drugs in hopes of further understanding the role of microtubules in the secretion of cellulase. The compounds nocodazole, IPC and griseofulvin were used to test for microtubular sensitivity; a 2 M solution of nocodazole and a 200 M solution of IPC were found to inhibit secretion by at least 50%. Griseofulvin was found to have no effect.

INTRODUCTION

In 1882, Sigmund Freud described longitudinal elements in the living crayfish nerve fiber (Soifer, 1975). As the biological study of the cell continued, it became clear that microtubules could be found in many areas of an organism. In the early 1930's, regulation of cell growth and function was a quickly growing field. Colchicine, a toxic alkaloid from Colchicum autumnale, seemed to stimulate cell mitosis in germative tissue, therefore aiding the study of cell division. It was hypothesized though, in 1939, that colchicine arrested division by destroying the fibrillar apparatus. In 1955, it was stated that colchicine was a very specific inhibitor of cell division and that the alkaloid attached to a particular component of the spindle fiber. This was confirmed again in 1965 using labeled colchicine. In 1967, a colchicine-binding protein was isolated, finding it to be abundant in nerve cells of the brain as well as in dividing cells. On the basis of identical structure in higher and lower plants and animals, this colchicine-binding protein was determined as the structure of microtubules, which had been named in 1963. It was later suggested that the colchicine-binding site was a subunit of the microtubule (Dustin, 1980). This subunit is now known to be tubulin.

Tubulin is often mistaken to be one protein but is rather made up of two subunits, alpha-( $\alpha$ ) and beta-( $\beta$ ) tubulin, which are globular molecules. The tubulins form dimers, and if present in high enough concentration, they associate to form various intermediate structures with a tube as the final product. The formation of microtubules from these subunits occurs in a region of both plant and animal cells known as the microtubule organizing center (MTOC). An MTOC appears to function as a focus for microtubule growth and, in fungi, it has been noted that they possess discrete structures at the poles of the spindle apparatus known as the spindle pole structures, and, in Ascomycetes, appear to be an integral part of the nuclear envelop (Hepler and Palevitz, 1974). The control of an MTOC is due to an equilibrium process whereupon the subunits polymerize given the

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proper conditions. This may infer proper pH, an optimal level of ions (most notably  $Mg^{++}$  and  $Ca^{++}$ ) or temperature (Soifer, 1975).

Microtubules are a common cytoplasmic feature of all eukaryotic cells. They are involved in a variety of cellular processes including chromosome movement, ciliary and flagellar movements and the maintenance and development of cell form. Of recent interest is the role of microtubules in the release and/or movement of stored secretory products. Many theories have been presented in recent years, but the relationship between secretory granules and microtubules has yet to be solved. A very general hypothesis was made by Poisner and Bernstein in 1971 which stated that microtubules assist in the transport of secretory granules to the cell surface involving mechanical and chemical initiated movement and that the final discharge of the secretory product was controlled by microtubules as well. Microtubules have been shown to regulate membrane processes (Garraway and Evans, 1984). In support of Poisner and Bernstein's theory, Richard Allen proved that vesicle-to-microtubule binding exists and that the binding appears to be of a filamentous material rather than discrete bridges (Allen, 1975). (Discrete bridge binding was the general theory in the early 1970's.) Additional evidence for the role of microtubules in the movement of membrane-bounded vesicles comes from the use of microtubule-active agents such as colchicine. Another example is the drug oncodazole or nocodazole, a benzimidazole 2-yl carbamate fungicide, which binds to the colchicine binding site of mammalian tubulin, but has varying effects in fungi (Heath, 1978). Another group of drugs which effects microtubule systems are the phenyl carbamates of which IPC is a member. IPC releases  $Ca^{++}$  from plant mitochondria (Gunning and Hardham, 1982), mentioned above as essential to tubulin polymerization. Griseofulvin, a Penicillim product, may also have antimitotic colchicine-like effects but also works in other ways which are not antimicrotubule in nature (Heath, 1978; Turner, 1971).

The use of microtubule inhibitors is important because many organisms secrete substances which can be monitored, and if they are sensitive to substances such as colchicine, we can then theorize the mechanism of the organism's secretion. The filamentous fungus Trichoderma reesei is one such organism, and its secretion of cellulase is of great industrial importance. However, the mechanism by which this happens still remains unclear. Therefore, the primary goal of this directed inquiry was to expose the fungus to antimicrotubule agents and note any effect the compound had upon the secretion of cellulase.

#### MATERIALS AND METHODS

Spore Culture: A culture of Trichoderma reesei QM6a (ATCC 13631) was maintained on slants of potato dextrose agar (PDA). Cultures were grown at 25 C under constant fluorescent illumination.

Medium: The medium used was a modification of that used by Mandels and Reese (1957) and included the following: 1.4 g  $(NH_4)_2SO_4$ , 0.3 g urea, 14.7 ml of 0.2%  $KH_2PO_4$ , 2.4 ml of 0.03%  $MgSO_4 \cdot H_2O$ , 5.5

ml of 0.3%  $\text{CaCl}_2$ , 0.005 g of  $\text{FeSO}_4$ , and 1 ml of 0.012 M  $\text{ZnCl}_2$ . This was mixed with one liter of distilled water. One gram of glucose was added if the medium was to be used for growth (MRG) or 0.34 g of lactose was added if the medium was to induce secretion of mycelium (MRL). The pH of the medium in either case was adjusted to 5.3 using 0.1 M NaOH. This was autoclaved (except for a white precipitate) and stored for later use.

Growth of mycelium: Prior to inoculating growth medium (NRG), a spore suspension was prepared by pouring 5 ml of sterile 0.1% Tween 80 over a seven day old slant of *T. reesei* and agitating the surface of the slant with a sterile glass rod. The suspension was transferred to 25 ml of sterile 0.1% Tween 80, and a one ml sample was aseptically transferred to a hemacytometer for spore counting. The standard aseptic inoculation of spores in MRS was  $7.5 \times 10^6$  spores per 500 ml of growth medium, each 500 ml volume of MRG being in a one liter Erlenmeyer flask. These cultures were incubated in a New Brunswick Environmental Shaker (reciprocal) at 29°C, 100 rpm, under constant fluorescent illumination.

Induction: Mycelia from forty-eight hour old MRG cultures were harvested by filtration over miracloth, washed with an equal volume of distilled water and weighed. The standard inoculation for induction was 1 g of mycelium (wet weight) per 100 ml of MRL contained in 250 ml flasks. These were incubated in the New Brunswick Environmental Shaker at 29 C, under constant fluorescent illumination.

Sampling: Three ml samples were removed from cultures at intervals, usually about seven hours after induction. These were assayed for cellulase activity after being centrifuged at 2400 rpm in an IEC clinical centrifuge for 30 seconds, and the mycelium-free medium was decanted.

Cellulase Assay: A viscometric assay specific for endocellulase activity was used. The substrate consisted of 1.2% carboxymethyl cellulose (CMC) in 0.018 M citrate-NaOH buffer, pH 5.0, made in 0.05K% in merthiolate. Five ml CMC in a size "300" Ostwald-Fenske viscometer was equilibrated at 30 C in water bath for 15 minutes. Once activity was noted, 1 ml of decanted sample was added, the solution was mixed for 10 seconds, and an initial flow rate ( $t=0$ ) was measured. The flow rate was measured again at 30 minutes, and fresh samples taken once more after the initial reading ( $t=0$ ).

Enzyme units were expressed as an increase in relative fluidity x 1000.

$$\frac{\Delta \frac{1}{(np)}}{\Delta t} \times 1000$$

where  $np = \text{specific viscosity} = \frac{t - t_0}{t_0}$

$t_0 = \text{flow time of water as a reference to liquid}$

## EXPERIMENTAL PROTOCOL

In Experiment A, 100, 200, and 300  $\mu\text{M}$  solutions of IPC were made using 10  $\mu\text{M}$  stock. The appropriate weight of IPC was mixed with one ml of DMSO and diluted slowly with distilled water to achieve a stock solution. Each group of mycelia had a final concentration of 1% DMSO.

In Experiment B, 0.5, 2, and 5  $\mu\text{M}$  solutions of nocodazole were made using 500  $\mu\text{M}$  stock. The compound was weighed and mixed with ten ml DMSO. Each group of mycelia had a final concentration of 1% DMSO.

In Experiment C, 20, 50, and 100  $\mu\text{M}$  solutions of griseofulvin were made; the substance was dissolved in acetone and the final concentration in all trials was 1%.

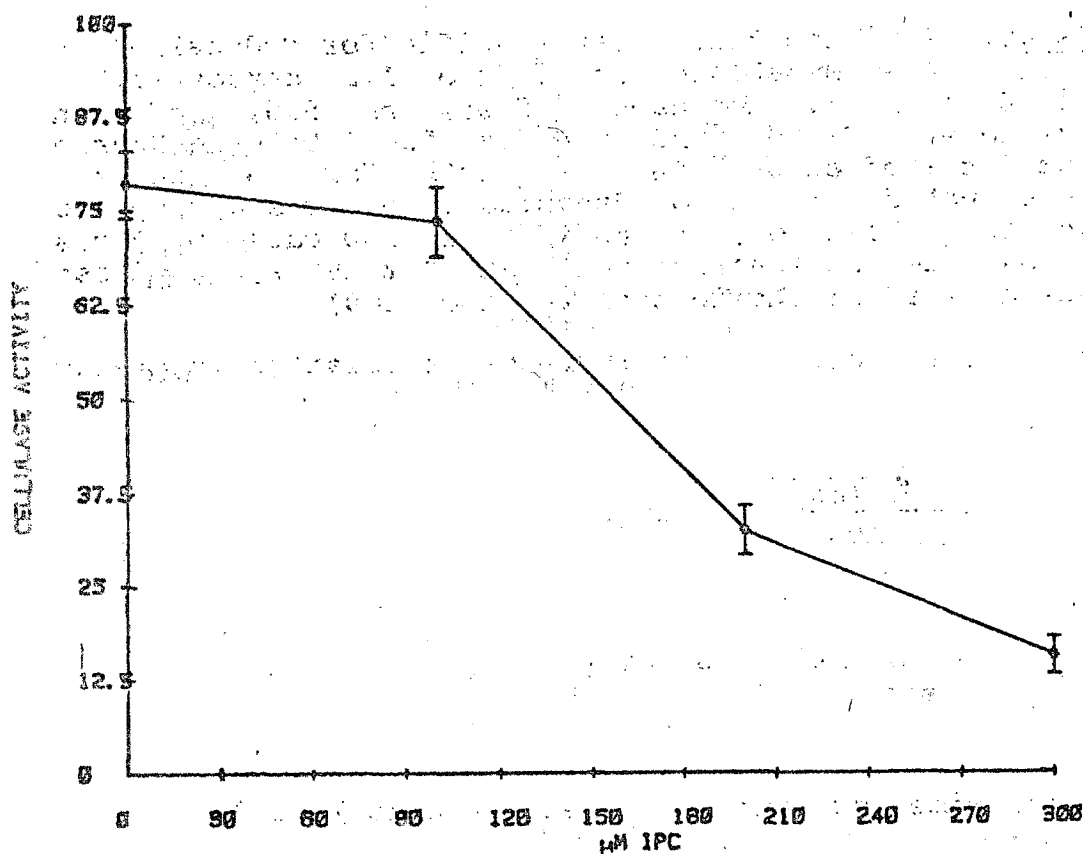
## RESULTS

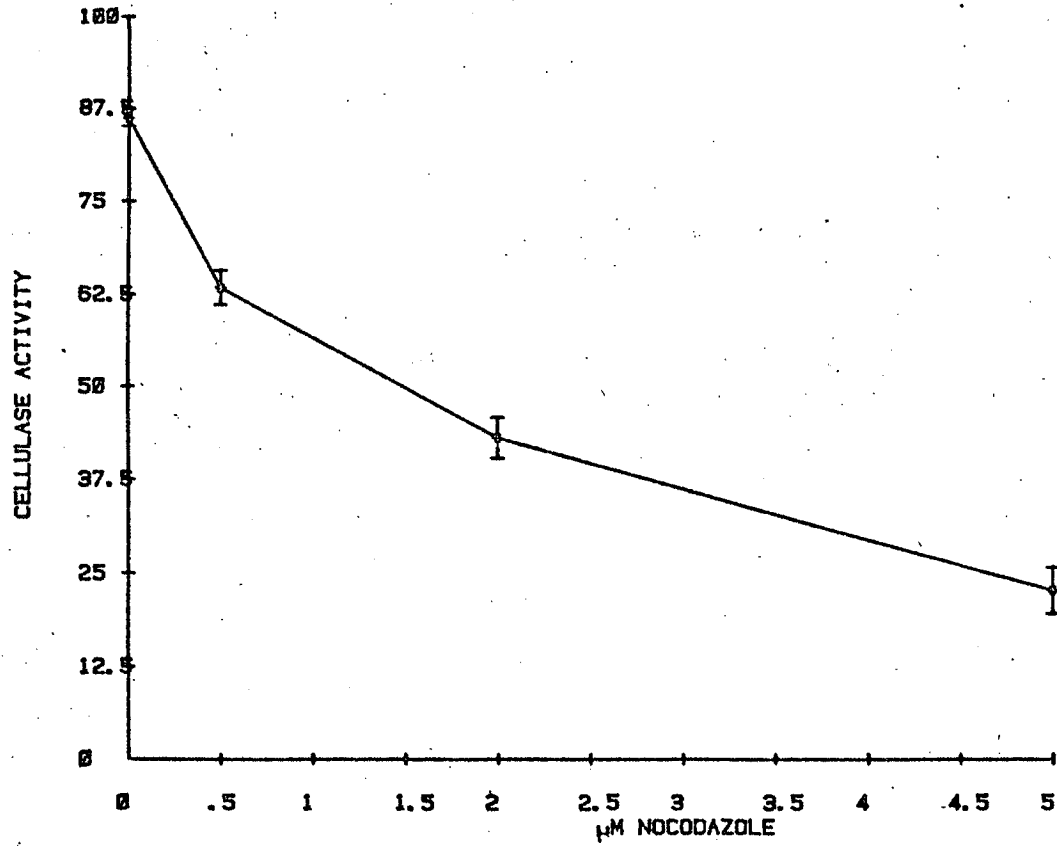
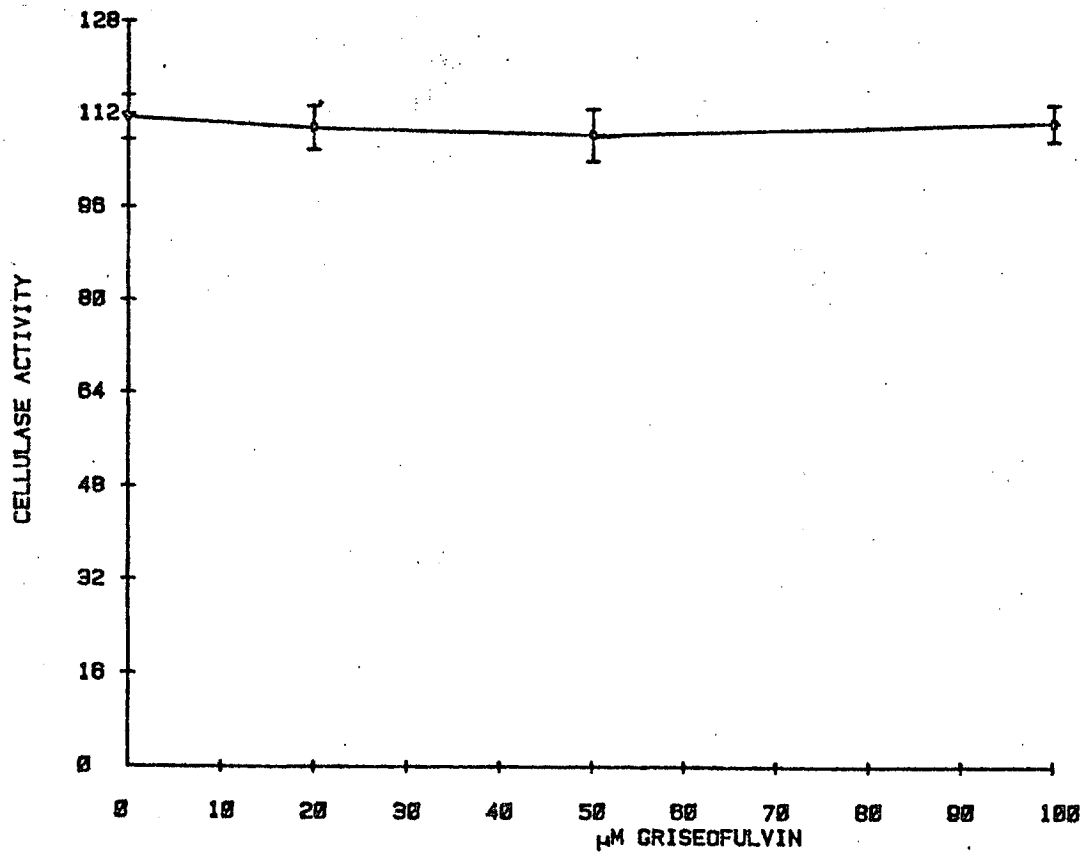
Graphical results of the experiments are at the end of the discussion. Graph A shows IPC clearly has an inhibitory effect upon secretion; however, it is noted that a 100  $\mu\text{M}$  concentration is not strong enough to give desired inhibition.

Graph B of nocodazole expresses the suppressing effect of the drug in seemingly small concentrations. The greater the concentration, the smaller the activity for cellulase secretion.

Graph C without a doubt states griseofulvin had no effect. As well, it seems apparent that using acetone as a solvent had little, if any, effect.

GRAPH A: EFFECT OF IPC ON THE CELLULASE SECRETION OF *T. reesei*



GRAPH B: EFFECT OF NOCODAZOLE ON THE CELLULASE SECRETION OF T. reeseiGRAPH C: EFFECT OF GRISEOFULVIN ON THE CELLULASE SECRETION OF T. reesei

## DISCUSSION

The graph of IPC shows relatively little inhibition of  $100\mu\text{M}$  IPC while increased concentration certainly curtailed secretion. IPC has been shown to be antimicrotubular in algae and induces abnormalities of cell division in higher plant and animal cells. However, larger concentrations have been used in the past. A ten millimolar concentration was used by Gull and Trinci in 1973. They found that IPC produced an array of cells arrested at metaphase but that a number of multinucleate cells were produced. This infers that IPC caused disorganization of spindle microtubules in the dividing cell Basidiobolus ranavum and therefore decreased the growth rate of the colony. However, since the growth rate of Trichoderma was not observed, such an assumption cannot be made. It has been noted that IPC causes the release of calcium from mitochondrial stores. If this is in fact true, then it seems that secretion would have been repressed linearly. At this point in time, I am unaware as to the mechanism of IPC. Since the fungus was able to build up calcium stores during growth and had extracellular calcium available at the time of induction, it might be that the fungus was able to withstand a slight depletion of its stored calcium in the presence of  $100\mu\text{M}$  concentration of IPC. It would have been interesting to note the effects of concentrations of IPC in the presence of added calcium to see if stores of the ion could be replenished or at least equilibrated. It might be added that a prior experiment was performed using 2.5, 5, and  $10\mu\text{M}$  concentrations and no effect was seen.

An analysis of variance was performed in order to determine if there were two or more data sets in which results were synonymous. This test showed that the absence of IPC and the  $100\mu\text{M}$  concentration portrayed no metabolic effect, which is what the graph shows as well.

The graph of nocodazole displays inhibition of secretion of cellulase, correlating to some degree with concentration. Nocodazole (oncodazole) has been shown to induce mitotic arrest in Dicotyostelium, and inhibit growth in Aspergillus (Walker, 1981). There is now increasing evidence that nocodazole has a mechanism of action similar to MBC as it is a competitive inhibitor of colchicine for the binding site on tubulin (Hoebeke, J et.al. 1976; Havercroft, J., et.al., 1981). One micromolar concentrations gave almost complete inhibition of microtubule formation, and while we cannot weigh this experiment heavily because the variable was mammalian tubulin, it can be noted.

Assembly of amoebal microtubule protein was almost completely inhibited using  $2\mu\text{M}$  solution of nocodazole (Quinlan et. al., 1981), and it was interesting that in this particular study, similar micromolar concentrations of nocodazole were just as effective during in vitro growth as in in vivo growth. Its inhibition does vary, however, from organism to organism. Nocodazole affects ascomycetes (of which Trichoderma is a member) but no oomycetes or zygomycetes (Gunning and Hardham, 1982).

If in fact nocodazole binds to tubulin, then this would prevent alpha and beta tubulin from polymerizing. Thus, microtubules would not form in order to aid the movement and/or secretion of cellulase vesicles.

An analysis of variance was performed as well on the given data and merely reaffirmed graphical results that each concentration of nocodazole had a unique and visible effect.

The results of an experiment using griseofulvin as a microtubule inhibitor clearly shows that there was no inhibition. This certainly agrees with some experimentation but is puzzling in light of other results. In 1960, it was noted that low doses of griseofulvin produced a characteristic hyphal distortion. It seemed that the effect was due to the fact that the cell wall consisted of chitin (Ainsworth and Sussman, 1965). However, interesting results were obtained using the fungus Aspergillus nidulans. Griseofulvin was shown to affect spindle functioning and selectivity interfere with microtubule assembly but did not affect colchicine binding at 1000 $\mu$ M concentrations (Davids and Flach, 1977). This might imply that griseofulvin and colchicine do not compete for the same binding site, and once colchicine has bound to tubulin, a conformational change takes place covering up griseofulvin binding sites. Perhaps though, that part of griseofulvin's activity is due to the proteins associated with microtubules which control polymerization. If griseofulvin's action is upon a microtubule component and not tubulin, then it would explain the variability of effectiveness upon different organisms; this may imply that organisms have microtubule associated proteins unique to their genera or class. For the most part, griseofulvin has been shown to be ineffective (Heath, 1978). Another criteria to consider is that it has been reported that enzyme degradation of griseofulvin has occurred in plants and mammals (Heath, 1975).

The data also shows that acetone had no real effect upon the fungus, although a control against acetone should have been done in order to rule out all possibilities.

An analysis of variance only reaffirmed by theory that griseofulvin had no effect upon the cellulase secretion of Trichoderma reesei.

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THE TEMPERATURE DEPENDENCE OF THE ABSTRACTION OF  
BENZYLIC HYDROGEN BY BROMINE

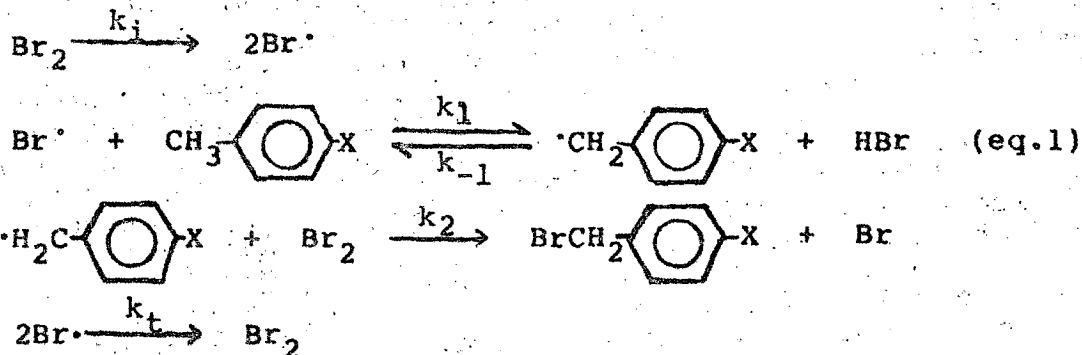
by Jeff Wright

ABSTRACT

The abstraction of benzylic hydrogen from toluene and 4-chlorotoluene by molecular bromine has been studied at several temperatures. A plot of  $\ln(k_x/k)$  vs.  $1/T$  yields a "two point" isokinetic temperature of  $-31.0$  C. The dependence of the relative rates on temperature as expressed by the Arrhenius equation exhibits excellent correlation ( $r^2 = 0.998$ ) for the 5 points above the isokinetic temperature, but exhibits a dramatic departure from linearity below the isokinetic temperature. The results also indicate that our understanding of the transition state in the usual terms of "polar effects" is inadequate; rather the results are discussed in terms of entropic contributions to the free energy of activation. The failure of the isokinetic relationship is discussed in terms of enthalpic vs. entropic control of the reaction.

INTRODUCTION

Substituent effects in the homolytic abstraction of hydrogen from substituted toluenes have been widely studied (1,2). Brominations using molecular bromine, as well as N-bromosuccinimide (NBS), have also been extensively studied (3,4,5,6). The results of these studies, in conjunction with their correlation in Hammett-type linear free energy relationships (7), have been given considerable weight in interpreting the nature of the transition state for these reactions, as well as for hydrogen abstractions in other analogous systems (1). The generally accepted reaction mechanism (40) is



The commonly held model for the transition state, as first applied to benzylic bromination by Russell (8), and summarized by Pryor (9), involves charge separation in the abstraction step (eq.1), involving three resonance structures (eq.2).

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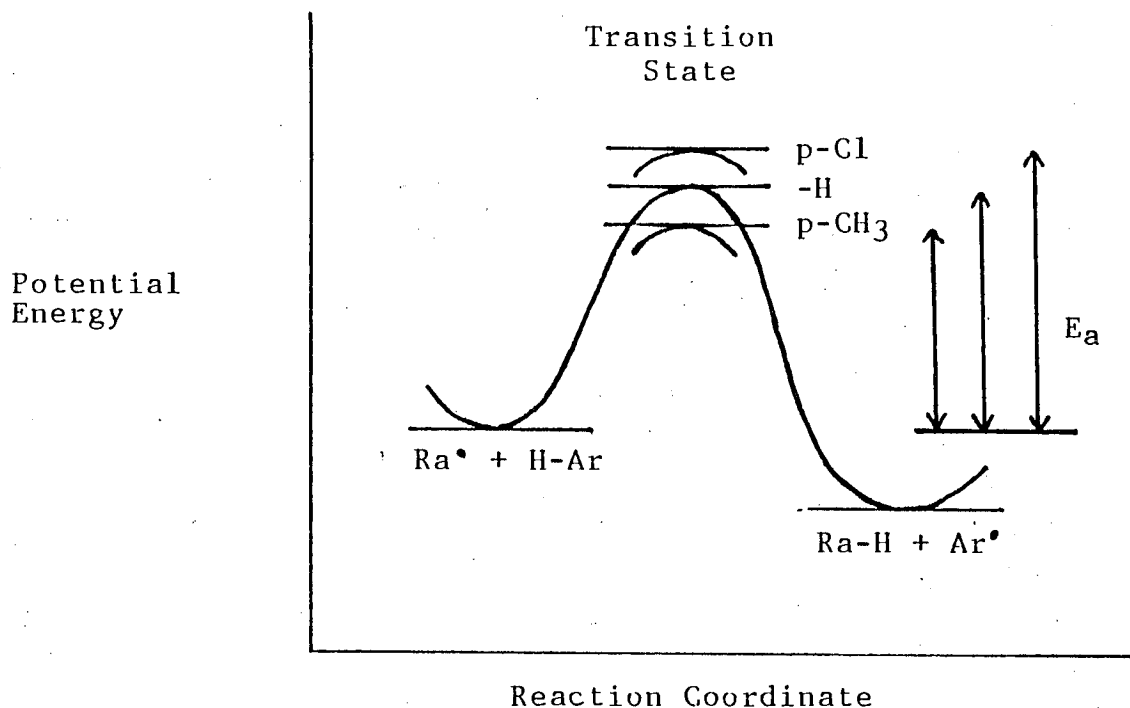
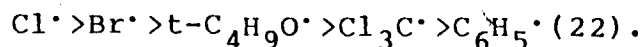
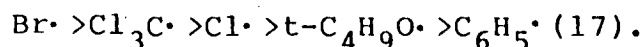


Figure 1. Evans-Polanyi diagram illustrating "polar effects."

As late as 1972, no positive  $\rho$  values had been observed for abstractions of hydrogen from substituted toluenes (21). Furthermore, of the five radicals then extensively studied in benzylic hydrogen abstractions (chlorine, bromine, trichloromethyl, t-butoxy, and phenyl), the electron affinities closely paralleled the absolute magnitude of  $\rho$  (17). The electron affinities of these five radicals are in the order



Taking the absolute magnitude of  $\rho$  as a measure of the polar effect, *vide supra*, the order of sensitivity to the polar effect for these radicals decreases in the order



The inconsistencies of these values have been explained in terms of the extent of bond breaking in the transition state (8,17).

A novel viewpoint of this reaction was suggested in 1972 by Zavitsas and Pinto (21). The observation that there was a linear correlation between  $\rho$  and  $H$  of reaction for toluenes with the five radicals mentioned above led Zavitsas and Pinto to conclude that  $\rho$  is a function "only of the extent of bond breaking" (since  $H$  reflects the extent of bond breaking by approximating the bond dissociation energy (BDE) of the  $\alpha$ -hydrogen) and that "charge separation in the transition state either does not occur or is an

unnecessary assumption" (21). Concurrent with this hypothesis is that all values of  $\rho$  must be negative, i.e., electron withdrawing substituents will reduce the reactivity of toluene, whereas electron donating substituents will enhance the reactivity, regardless of the nature of the abstracting radical. Thus, the value of  $\rho$  is independent of the electrophilic nature of the abstracting radical. Figure 2 illustrates the BDE explanation and its effect on activation energy. (19)

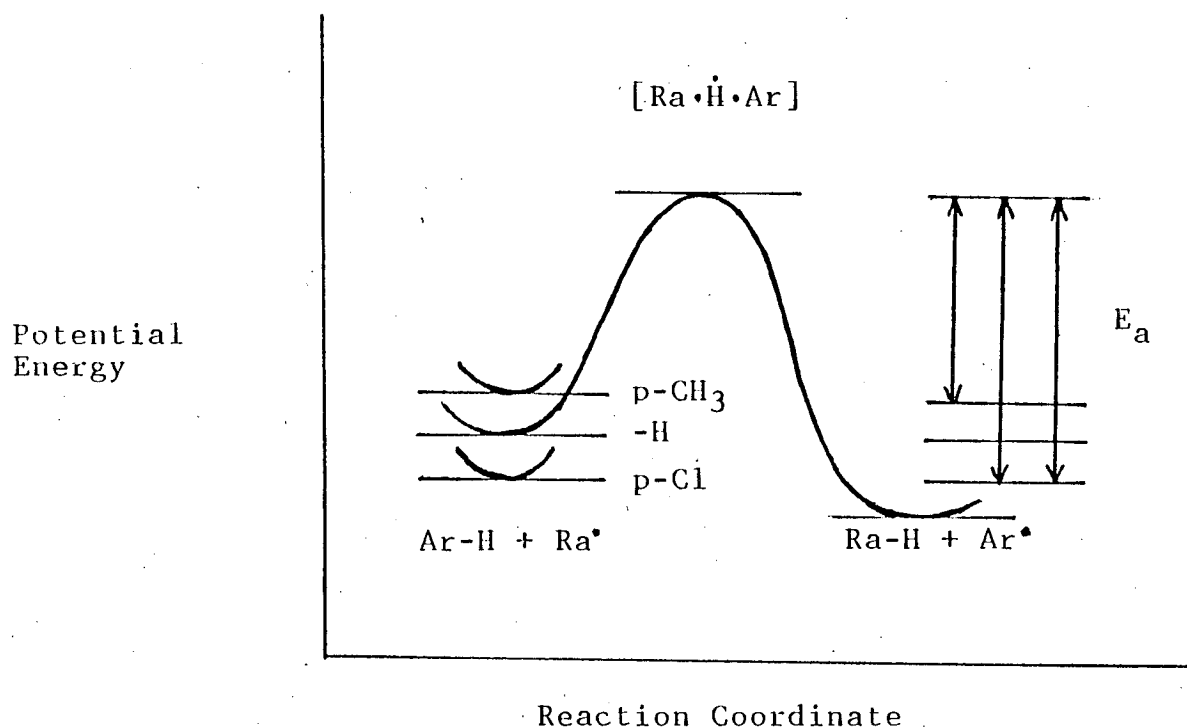


Figure 2. Evans-Polanyi diagram illustrating the BDE hypothesis of Zavitsas and Pinto. (21)

The BDE hypothesis put forward by Zavitsas and Pinto has generated a wealth of chemical literature in response to their hypothesis. At this time it is evident that this reaction requires further study. Empirical data for abstraction reactions gave ambiguous mechanistic indications, e.g., relative rate data give equally good correlations with  $\sigma^-$  as with  $\sigma^+$  substituent constants (21). Additionally, there is still a great deal of uncertainty in the bond dissociation energies of  $\alpha$ -hydrogens for substituted toluenes, as well as for toluene (24). There has been some difficulty in reproducing  $\rho$  values for different groups of workers (21,23), and early work (6) demonstrated the dependence of Hammett reaction constants ( $\rho$ ) on experimental method.

Although once disputed (25), Pryor, *et al*, have observed a positive  $\rho$  value of the *tert*-butyl radical (26); this value has since been reconfirmed (27). These findings have generally been interpreted as evidence for polar effects (28,29), with contribution

to the transition state by the dipolar canonical structure III. Positive  $\rho$  values have since been reported for several other alkyl radicals, including isopropyl (30) and undecyl (26,28). The occurrence of positive  $\rho$  values would seem to seriously question the validity of the BDE hypothesis; alternatively, Pryor has shown that in at least one system, the hydrogen abstraction from iodobenzene by 4-nitrophenyl radicals, BDE effects must be considered to explain the observed results (29,30). Davis and Pryor have postulated that both the effects of substituents on BDEs and on the free energy of activation must be considered in explaining the results of Hammett equation correlations (29,30). Recent work by Gilliom *et al.*, (10) on the temperature dependence of the abstraction of hydrogen by bromine from toluene have indicated that entropic contributions to the free energy of activation must be considered over both BDEs and dipolar stabilization of the transition state, since both of these interpretations are based on the potential energy surfaces of the reaction (10), as illustrated by Figures 1 and 2.

In light of this observation by Gilliom (10), it is clear that the temperature dependence of these reactions must be studied. The available literature seems to indicate that the temperature variable has been largely neglected in previous studies (31). We present a study of the temperature dependence for the abstraction of benzylic hydrogen from toluene and 4-chlorotoluene.

## EXPERIMENTAL

### Procedure for competitive brominations.

The relative reactivities were determined via competitive bromination with molecular bromine at various temperatures in  $\text{CFCl}_3$  and  $\text{CCl}_4$ . Solutions approximately 1:1 for each of the toluenes were prepared by accurately weighing 25 millimoles of each toluene into a 50 ml volumetric flask which upon dilution would give solutions of about 0.5M (1.0M in  $\text{CCl}_4$ ). Prior to dilution, the inert internal standard, chlorobenzene, was added to give concentrations near 0.375M in the fluorotrichloromethane determinations. Epoxides have been shown to be particularly effective in decreasing or eliminating hydrogen bromide reversal in hydrogen abstractions which generate HBr (20) by acting as an HBr trap. 1,2-Epoxybutane was added such that it would be in at least 3:1 molar excess over the total amount of bromine to be added. Bromine solutions for addition were prepared by weighing liquid bromine into a 25 ml volumetric flask followed by dilution with carbon tetrachloride or fluorotrichloromethane to give solutions of ca. 0.67M. The ratio of total molar quantities of toluenes to total bromine was at least 3:1.

10 ml aliquots of the toluene solution were volumetrically pipetted to a 50 ml round-bottomed or pear-shaped three neck flask. The flask was equipped with a gas-delivery tube, water-cooled condenser, and pressure-equalizing addition funnel. The flask was placed in a constant-temperature bath where it was allowed to thermally equilibrate for 20 minutes while dry nitrogen was slowly bubbled through the solution. No precipitation of reactants was

observed at the end of this time. Three to five ml of 0.67M bromine solution was then added drop-wise via the pressure-equalizing addition funnel. Nitrogen bubbling slowly through the reaction vessel provided agitation throughout the course of the addition. The solution was continuously irradiated with a 275W Sylvania sunlamp placed externally at a distance of 30 cm. The rate of bromine addition was maintained so that only a faint yellow color was observed in the reaction vessel. Reaction products accounted for 75% to 90% consumption of the bromine added.

Gas chromatographic analysis was performed on a Perkin-Elmer Sigma 3B gas chromatograph with flame ionization detector using a 6 ft. by 1/8 in. Teklab column packed with 10% OV-101 on Chromosorb W. Chromatograms were recorded and measured with a Hewlett-Packard 3392A integrating recorder. Temperature programming was used with a ramp rate of 5/min for temperatures from 60 to 120 C. Relative weights for the peak areas for both of the toluenes to the peak area of chlorobenzene were determined from a sample of the unreacted toluene solution taken when an unreacted aliquot was transferred to the reaction vessel. The relative weighting factor,  $X_i$  was determined as

$$X_i = A_i C_{is} / A_{is} C_i$$

where C is the concentration (or mass) of the compound and A is the peak area of the compound. The concentration (or mass) of component i remaining was determined from

$$C'_i = A'_i C_{is} / X_i A_{is}$$

## RESULTS

The relative reactivity of 4-chlorotoluene to toluene ( $k_x/k$ ) was determined by direct competition in carbon tetrachloride and in fluorotrichloromethane, through a range of temperatures from 45 C to -50 C. The relative reactivities were determined from the initial and final concentrations of the reactants according to the equation (32,33)

$$k_x/k = \ln ([CH_3C_6H_4X] / [CH_3C_6H_5]_0) / \ln ([CH_3C_6H_5] / [CH_3C_6H_5]_0)$$

Table I summarizes these calculations. The limits of error represent the sample standard deviation.

Table I. Summary of relative reactivities.

C	solvent	no. expts.	$k_{chl}/k_{tol}$	"point"
45	CCl <sub>4</sub>	3	0.7362 ± 0.0069	1
25	CCl <sub>4</sub>	2	0.7801 ± 0.0148	2
10	CFCI <sub>3</sub>	2	0.8232 ± 0.0173	4
0	CCl <sub>4</sub>	2	0.8652 ± 0.0030	3
0	CFCI <sub>3</sub>	4	0.8655 ± 0.0537	5
-19	CFCI <sub>3</sub>	3	0.9387 ± 0.0008	6
-32	CFCI <sub>3</sub>	3	0.9846 ± 0.0151	7
-42	CFCI <sub>3</sub>	3	0.7929 ± 0.0272	8
-50	CFCI <sub>3</sub>	2	0.7589 ± 0.0197	9

The temperature dependence of reactions often follow the Arrhenius equation:

$$k = A \exp(-E_a/RT)$$

which can be written

$$\ln k = \ln A - E_a/RT$$

For the case of competitive reactions, this becomes

$$\ln k_x/k = \ln A_x/A + (E^* - E_x^*)/RT$$

Thus, with a plot of the natural logarithms of the relative reactivities vs.  $1/T$ , we obtain a straight line with slope  $(E_{tol} - E_{chl})/R$  and intercept of  $\ln(A_{chl}/A_{tol})$ . We can thus obtain the differential energy of activation for temperature dependence. We have performed these plots and present the activation parameters in Table II.

Table II. Activation parameters for abstraction.

"Entry"	"points"	$E_{tol} - E_{chl}$	$\ln A_{chl}/A_{tol}$	$r^2$
a	1,2,3	0.6232	-1.295	0.9970
b	4,5,6,7,8,9	----	----	0.1639
c	1-9	----	----	0.0102
d	4,5,6	0.6618	-1.368	0.9969
e	4,5,6,7	0.5781	-1.214	0.9874
f	1,2,4,5,6	0.6272	-1.303	0.9975

\*Point 3 was excluded since this value and 5 are the same in both solvents. \*\*Subscripts "chl" and "tol" correspond to 4-chlorotoluene and toluene, respectively.

As seen from the accompanying graph of  $\ln k_x/k$  vs.  $1/T$ , the Arrhenius relationship exhibits a sharp deviation from linearity below -32.0 C. This shall be discussed later. Table "entries" (a), (d), (e), and (f) all exhibit good correlations. Entry (e) contains the point for the relative rate at -32.0 C, where the curve is beginning to show deviation from the Arrhenius relationship. Omission of this point, as well as for those at -42.0 C and -50.0 C, gives the line with the best correlation ( $r^2 = 0.998$ ) and includes a larger range of temperatures. The excellent agreement between the plots for carbon tetrachloride (a) and fluorotrichloromethane (d), lead us to report the Arrhenius

relationship for this reaction, in carbon tetrachloride and fluorotrichloromethane, for the temperature range 45 C to -19 C, to be best expressed by the equation (f):

$$\ln k_{\text{chl}}/k_{\text{tol}} = (315.6 \pm 17.8)/T - (1.3031 \pm 0.063)$$

As can be seen from Table II, this corresponds to  $E_{\text{tol}}^{\ddagger} - E_{\text{chl}}^{\ddagger} = 0.627$  kcal/mol (+ 0.035 kcal/mole), which is in agreement with the value of 1.38 kcal/mol obtained by Gilliom, et al. (10).

## DISCUSSION

As can be seen from Table I, 4-chlorotoluene is less reactive than toluene; this is consistent with the negative  $\rho$  values reported for this reaction (6,11). However, the value for the activation parameters,  $E_{\text{tol}}^{\ddagger} - E_{\text{chl}}^{\ddagger} = 0.627$  kcal/mol questions the validity of both the BDE explanation and the traditional "polar effects" argument. The observation that the Arrhenius energy of activation is larger for more reactive species is inconsistent with both of these explanations; we would expect the species with the lowest  $E_a$  to more reactive. It is evident that entropic contributions should be considered in this reaction, as has been suggested (10,11). To determine the magnitude of these contributions, we must turn to the transition state theory. The transition state theory predicts that the rate constant of a reaction will be expressed by

$$k = \frac{k_B T}{h} e^{-\Delta G^{\ddagger}/RT} = \frac{k_B T}{h} e^{-\Delta H^{\ddagger}/RT} e^{\Delta S^{\ddagger}/R}$$

For a pair of competitive rate constants, this can be rearranged to give

$$\ln k_2/k_1 = (\Delta S_2^{\ddagger} - \Delta S_1^{\ddagger})/R - (\Delta H_2^{\ddagger} - \Delta H_1^{\ddagger})/RT$$

So by plotting  $\ln k_{\text{chl}}/k_{\text{tol}}$  vs.  $1/T$ , as we did for the Arrhenius relationship, we obtain a line with slope  $-(\Delta H_{\text{chl}}^{\ddagger} - \Delta H_{\text{tol}}^{\ddagger})/R$  and intercept  $(\Delta S_{\text{chl}}^{\ddagger} - \Delta S_{\text{tol}}^{\ddagger})/R$ . Table III summarizes these calculations as obtained from the plot "f" of Table III. We obtain  $\Delta H_{\text{chl}}^{\ddagger} - \Delta H_{\text{tol}}^{\ddagger} = -0.627$  kcal/mol and  $\Delta S_{\text{chl}}^{\ddagger} - \Delta S_{\text{tol}}^{\ddagger} = -2.589 \times 10^{-3}$  kcal/mol K.

Table III. Contributions by entropy of activation to free energy of activation.

Kelvins	$\Delta H_{\text{chl}}^{\ddagger} - \Delta H_{\text{tol}}^{\ddagger}$	$-T(\Delta S_{\text{chl}}^{\ddagger} - \Delta S_{\text{tol}}^{\ddagger})$	$\Delta \Delta G^{\ddagger}$
318.15	-0.6272	+0.824	0.197
298.15	-0.6272	+0.772	0.145
283.15	-0.6272	+0.733	0.106
273.15	-0.6272	+0.707	0.080
254.65	-0.6272	+0.659	0.032
242.20 <sup>a</sup>	-0.6272	+0.627	0.000

<sup>a</sup>calculated two point "isokinetic point"

A reaction series observing the proportionality

$$\Delta H^\ddagger = \beta \Delta S^\ddagger$$

is said to exhibit an isokinetic relationship (34). A large number of reactions fitting this relationship have been observed (35). A pair of rate constants may exhibit a two-point "isokinetic relationship" (36). Substitution of the expression for the isokinetic relationship into the expression for the Gibbs free energy gives

$$\Delta G^\ddagger = (1 - T/\beta) \Delta H^\ddagger$$

Examination of these equations should make it obvious that when  $T$  is greater than  $\beta$ , entropic factors dominate the activation energy, whereas at temperatures below  $\beta$ , enthalpic effects will dominate the activation energy (36).

Although we do not have sufficient data to create a Hammett type plot, we do observe a decrease in selectivity as temperature is reduced; the data of Kim, *et al.*, seem to suggest the same results in their work. This corresponds to a decrease in the absolute magnitude of  $\rho$ .

If a reaction exhibits an isokinetic relationship, it will undergo an inversion of sign in  $\rho$  as it passes through the isokinetic temperature, going to zero as the rates become equal and  $\rho$  changing sign as the dominance of the reaction reverses. The temperature dependence is expressed (37) by

$$\rho_i = \rho_\infty (1 - \beta/T)$$

The isokinetic temperature from our Arrhenius relationship does not correspond to a change in sign of  $\rho$ , since the reactivities do not reverse dominance, i.e., toluene appears to be the more reactive compound above and below the isokinetic temperature.

This failure of the isokinetic relationship may be due to the perturbation caused by an additional interaction mechanism (38). Since this perturbation first appears significantly in the neighborhood of the isokinetic temperature, we can speculate that our results may be an observation of the switch from entropic to enthalpic control of the reaction. Data from other substituents is obviously needed. At temperatures below  $\beta$ , we might then argue for one of the traditional enthalpic arguments, e.g. BDE effects or polar effects.

Kim *et al.*, have suggested that the toluene behavior in brominations by NBS is controlled by entropic contributions. An abstract of their work states that the entropic control is due to a transition state with "considerable translational degrees of freedom" (11). Since we usually do not expect translational entropic factors to arise internally (39), we interpret the nature of the entropic factors differently. As the incipient radical begins to form, the odd electron becomes increasingly conjugated with the aromatic system; the entropy decreases as rotational and torsional vibrations of the ring become increasingly hindered. The observation of a slightly smaller  $\Delta S^\ddagger$  of  $-0.00259 \text{ kcal mol}^{-1} \text{ K}^{-1}$  is consistent with this interpretation, since we would expect the inductive effects of an electron withdrawing substituent to increase the degree of conjugation of the odd electron with the aromatic system.

## CONCLUSIONS

Our results, as well as recent work of others (10,11), suggest caution should be maintained in considering the mechanistic significance of  $\rho$  in free radical reactions. Davis and Pryor have postulated that substituent effects on both the BDE and stabilization of a dipolar transition state (29,30) must be considered in interpreting the results of Hammett-type correlations; these generally tend to be enthalpic arguments (10). Our results suggest that at least one more interaction mechanism should be considered, and further, that this must be an entropic effect. We have postulated that this is due to increased constraints on rotation of the ring as the odd electron formed in the free radical is conjugated with the aromatic system; this effect is heightened by the inductive effects of an electron withdrawing substituent such as 4-chloro. Our empirical data agree with this hypothesis.

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## A WORD WITH DR. ALAN JASLOW

by Claire de Saussure

The latest addition to the Biology department Dr. Alan Jaslow, joined the faculty this past fall. Dr. Jaslow, primarily an evolutionary biologist, has always loved nature and animals, with a special fascination for snakes and frogs. With a Bachelor's degree from the University of Wisconsin, Dr. Jaslow did his graduate work at the University of Michigan on the population biology of tadpoles.

Since then he has explored the evolution of hearing on land, focusing on hearing in frogs. The morphology of the frog's ear varies among different species, with some completely lacking the middle ear. These "earless" frogs have been a main interest in Dr. Jaslow's research. It had been thought that the middle ear was necessary for hearing high airborne frequencies. However, Dr. Jaslow found that these "earless" frogs could hear, in some cases even as well as frogs having a middle ear. This raises questions concerning the actual function of the middle ear structures.

Dr. Jaslow's research took him to Panama, where he spent two and a half years studying behavioral, ecological, and evolutionary aspects of the population biology of frogs. Dr. Jaslow has also been involved in research at the University of Chicago, where he will be continuing to work during the summers, concentrating on three projects related to ear morphology and hearing. One involves studying acoustical impedance in frogs, by looking at what the middle ear does in terms of absorbing or reflecting sound. In another project, he will be investigating underwater hearing in tadpoles. Finally, Dr. Jaslow will be studying the evolution of mammalian middle ears, in conjunction with two paleontologists. They will study the intermediate stages of the evolutionary sequence from a reptile condition to a mammalian one, and will try to identify the hearing capabilities for that lineage.

There are a variety of research projects that Dr. Jaslow will be able to pursue here. He is mainly interested in continuing his work on hearing in frogs. One such project would use a sound pressure level meter, which he hopes to acquire soon, to measure sound levels in the field.

Another project he will be working on is studying the muscle of the arms of sexually dimorphic frogs. In these frogs, the males have relatively larger arms which are used in fighting and holding onto the females. He will be doing a histological survey of the muscle to see if there are muscle differences, such as fast and slow fiber types.

Dr. Jaslow's research is not limited to frogs. Another project he is working on at Rhodes is a histological study of the tongue integuments of snakes. Apparently the pattern of shedding of the epidermis of the tongue is unrelated to the shedding of the outer skin layer, which is regulated by hormones.

In the near future, Dr. Jaslow hopes to go to Australia to continue work on the "earless" frogs. The frogs in Panama were uncooperative in breeding in the laboratory, and eggs of these frogs are hard to find. Dr. Jaslow is interested in investigating the different families of frogs in Australia. One "earless" group there will be easier to collect as eggs and tadpoles, which will be used to study the development of the ear in these frogs. He is interested in knowing how the pattern of development changes in frogs that have secondarily lost middle ears. Do these missing structures arise then disappear during development or do they fail to arise at all?

With all of these fascinating involvements in research, Dr. Jaslow still devotes considerable time and energy to his teaching. So far, he has taught Biology 100, Comparative Vertebrate Morphology, and is currently teaching Evolution. Dr. Jaslow finds the Comparative Morphology class very exciting and enjoyable, and he has worked hard to develop this new course. The course has more of an emphasis on the evolution, function, and development of structures in organisms than the traditional anatomy approach. He has also worked to improve the specimen collection for the course by improving access to them and by acquiring new ones which will be valuable for teaching.

In the short time that Dr. Jaslow has been here he has already made significant contributions to the Rhodes College community in terms of interesting research, as well as excellence and enthusiasm in teaching.

## BIOLOGICAL ILLUSTRATIONS

by Janet Freytes

The following illustrations are part of a Directed Inquiry on Biological Illustration. These drawings are the result of a study of pen and ink as one of the techniques used in Biological Illustration. The next pages show different views of both the skull and the jaw of a cat skelton.

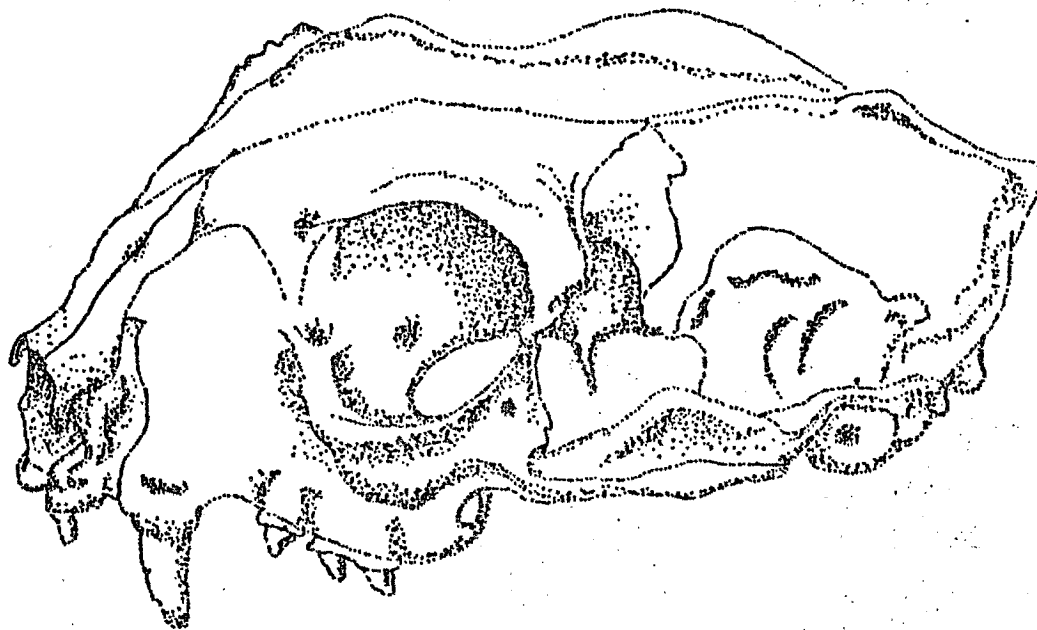


Fig. 1: Left side view of a cat skull

Janet Freytes is a senior Biology major from Rio Piedras, Puerto Rico.

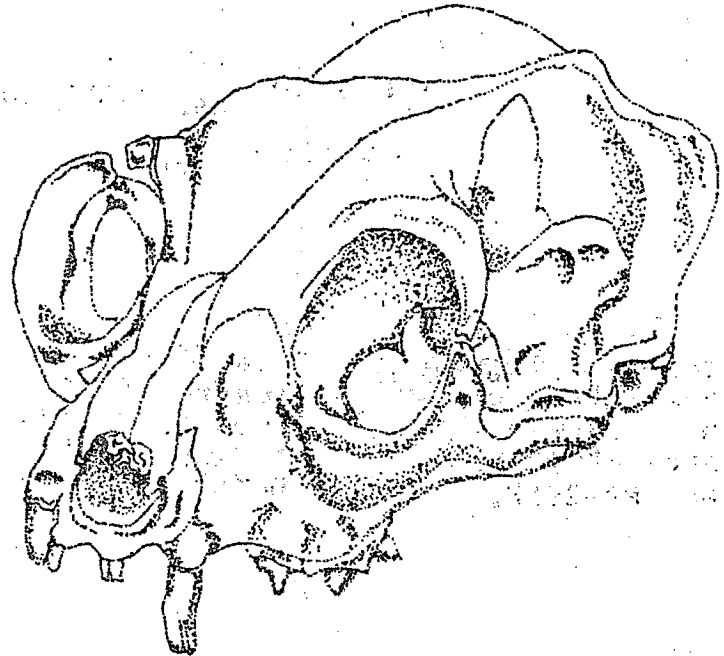


Fig. 2A: Three-quarters view of a cat skull

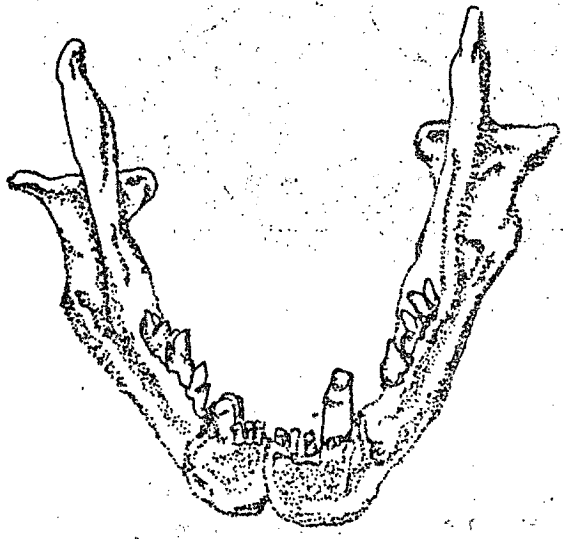


Fig. 2B: Front side view of a cat skull

Fig. 3: Right side view of a cat jaw

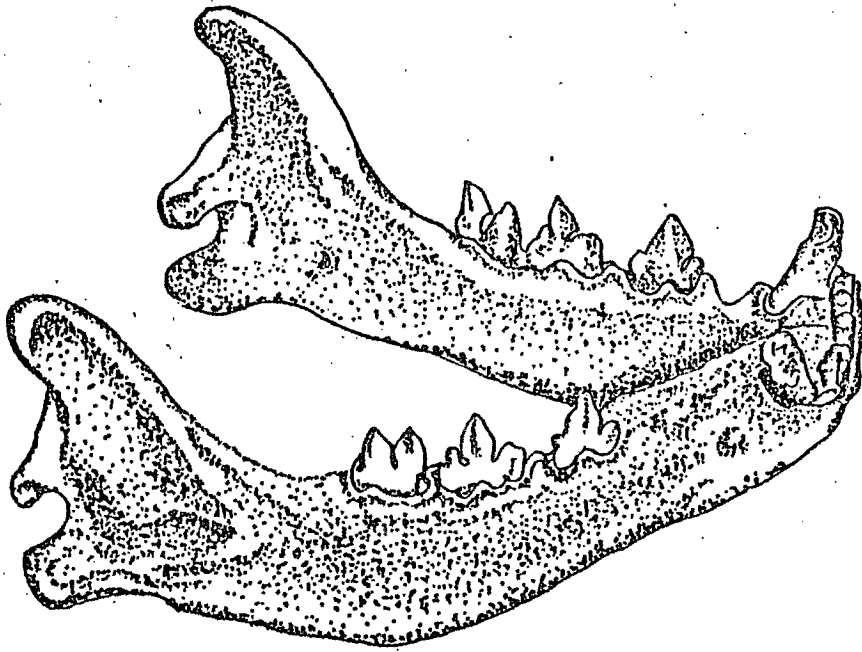
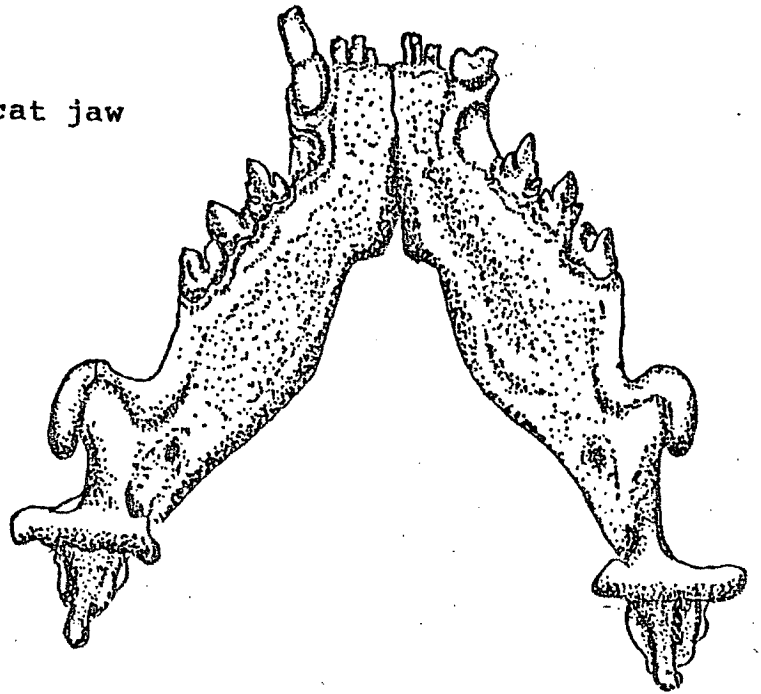


Fig. 4: Back side view of a cat jaw



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