Some Effects of Cold Stress on the Morphology and Electrophysiological Function of the Retina of the Goldfish

By
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SOME EFFECTS OF COLD STRESS ON THE MORPHOLOGY AND ELECTROPHYSIOLOGICAL FUNCTION OF THE RETINA OF THE GOLDFISH

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The purpose of this research was to investigate parameters of and variables contributing to cone outer segment atrophy under extended low temperature. Specific problems attacked were to define the time course of structural degeneration and loss of electrophysiological function, to determine if morphological loss extended beyond the receptor cell and to investigate the influence of environmental lighting on the effects of cold stress on the cone receptor. Additionally, it was hoped that, through manipulation of stimulus parameters for electrophysiological recording, it would be possible to determine if there were differential effects on photopic (cone) and scotopic (rod) systems and on receptor and neural function for each.

In order to accomplish these ends, five groups of goldfish were established, with two receiving white light or no light and the other three receiving light from specific bands of wavelengths. These groups of animals were subjected to temperatures between 4° and 8° centigrade for 44 or 59

days respectively (cold stress), then were allowed to return to more temperate conditions for the remaining 62 or 47 days (recovery). Throughout this time each group was sampled periodically. Primary data were electrophysiological recordings taken from the cornea (ERGs) and counts of cellular constituents of each layer of the retina, with especial attention to inner and outer segments of cone receptors. Electrophysiological recording and histological processing of the eyes were by conventional techniques. Total ERGs were analyzed and A and B wave components, assumed to reflect receptor and neural activity from the inner nuclear layer respectively, were analyzed individually. Stimulation parameters were chosen in a manner such that photopic and scotopic function were differentiated.

It was found that both the ratio of residual outer segments to cone cells (actually ellipsoids) and gross electrophysiological function were reduced in an orderly manner with time at low temperature. Early rebound followed by stabilization at an intermediate level, indicating incomplete recovery, characterized electrophysiological function upon return to more normal temperatures whereas outer segment to ellipsoid ratios showed gradual total recovery. Animals recovering in the light absent condition failed to show electrophysiological recovery, as did one group recovering in blue light. Complex interactions involving receptor and neural function, light versus light absent conditions and photopic and scotopic adaptation were

seen. No reliable effects were seen as a result of spectral differences in environmental lighting except for the failure of one group, noted above, to show recovery. Analysis of food catching behavior suggested that functional visual deficits accompanied electrophysiological and structural losses incurred during cold stress, while partial restoration accompanied recovery of electrophysiological function and structure. No morphological loss was seen other than cone outer segments, however, due to difficulty in resolution of these very fine structures with available equipment, no attempt was made to evaluate loss of rod receptors or their outer segments.

It was concluded that cold stress effects were orderly over time with respect to both electrophysiological function and receptor structure, at least for cones. Recovery was generally less orderly and was essentially complete for outer segments but not for electrophysiological function. Photopic and scotopic systems were differentially affected, as were receptor and neural function. These relationships were modified with differential environmental lighting conditions. Results were discussed with emphasis on lines for future research related to elucidating anomalous results within the present experiment, disparate results with respect to previous research into the phenomenon and possible biochemical mechanisms underlying the effect of cold stress on the structure and function of the retinal cone receptors of the goldfish.

INTRODUCTION

One of the traditional techniques employed in the study of vision has been the utilization of abnormalities. Beginning with Dalton's careful study of his own color deficiency (Helmholtz, 1924, pp. 145-147), much of our knowledge of the function of the visual system in response to light of limited wavelengths has been deduced from the study of abnormal human subjects.

Even with the advent of the very powerful tools which are now available, allowing the vision researcher to work at the subcellular and molecular level, the use of these techniques has frequently been employed in conjunction with system abnormalities. Dowling's description of retinal atrophy resulting from Vitamin A deficiency and inherited retinal dystrophy (Dowling, 1964) has expanded our understanding of the photochemistry of vision. Similarly, research on retinal damage by visible light (Noell et al., 1966; Gorn and Kuwabara, 1967; Kuwabara and Gorn, 1968; Grignolo et al., 1969) has contributed to our concepts of retinal viability and limitations on its stimulation. Investigations of surgically induced retinal detachment and reattachment have contributed to the same end, have aided in understanding the etiology of this malfunction frequently seen in the clinic and have contributed to the understanding of resulting visual loss (Kroll and Machemer, 1968, 1969a, 1969b;

Machemer, 1968a, 1968b; Machemer and Norton, 1968; Hamasaki et al., 1969).

The research to be reported here takes as a point of departure a recently reported phenomenon (Dawson et al., 1969; Hope et al., 1970; Dawson et al., 1971) which results in degeneration of the outer segments of the retinal cone receptors of the goldfish (Carassius auratus). Since these reports are not readily available to all readers, this research will be treated in some detail, following a brief description of the structures involved. Reference to Figure 1 may aid this description.

The retinal receptors are composed of an outer segment, containing the photopigment upon which luminous energy acts to initiate the visual response, an inner segment, and a cell body. The cell bodies form the outer nuclear layer and the inner and outer segments the receptor layer of the retina. The pigment epithelium lies sclerally to the receptors. In some species, including the goldfish, processes of the pigment cells can migrate into and out of the receptor cell layer in response to light level, interdigitating with and screening receptors (John et al., 1967). The receptor cells also migrate in response to light, as well as demonstrating diurnal rhythms (John and Kaminester, 1969; John et al., 1967). In general, the cones and pigment epithelium move sclerally in dark adaptation with the rods moving vitreally. The reverse occurs in the light adapted animal. These movements have also been shown to occur at night and

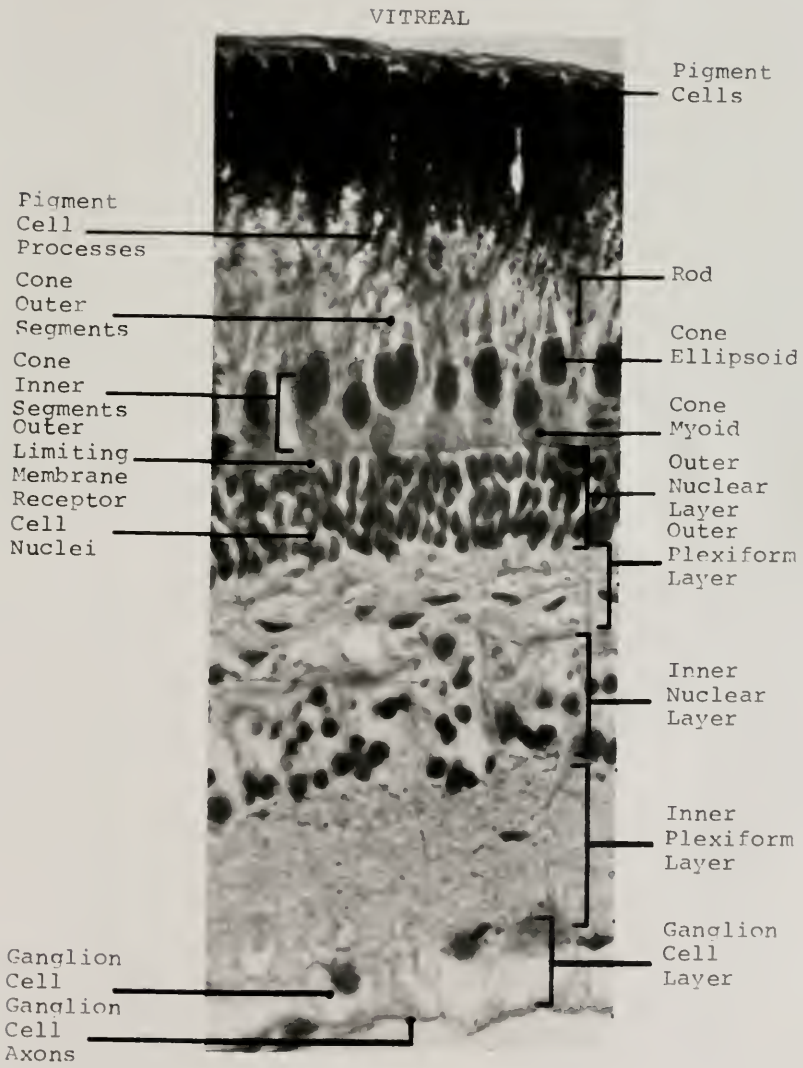

Figure 1.-- Anatomy of the Retina

daytime respectively, even in constant darkness (John et al., 1967; John and Kaminester, 1969).

The inner nuclear layer lies vitreally to the outer nuclear layer, separated from it by the outer plexiform layer (the region of synaptic contact between the receptors and cells of the inner nuclear layer). The cellular composition of the inner nuclear layer includes the horizontal, bipolar and amacrine cells in a scleral to vitreal arrangement. The inner nuclear layer is separated from a very sparsely populated ganglion cell layer by the inner plexiform layer. The synaptic relationships among the various cellular components have been studied and described in detail (e.g., Dowling, 1970) but will not be discussed here.

The research providing the incentive for the present study showed that cold stress resulted in atrophy of cone outer segments in the goldfish. The animals were placed in an aquarium and refrigerant was pumped through a coil submerged in the aquarium water. The temperature of the water was lowered to 5^{o} C and held at this level for up to 31 days. The water was then allowed to return to room temperature, 22^{o} C, and the animals were maintained at this level for the remainder of the experiment. During these periods, cold stress and recovery respectively, fish were periodically removed from the aquarium and electrophysiological signals in response to a light flash were recorded from the cornea and exposed contralateral tectum. These signals were referred to as the electroretinogram (ERG) and tectal evoked

response (TER) respectively. Following dark adaptation the eyes were removed and subjected to histological processing.

Electrical responses were not recordable at the cornea as early as four days into cold stress. The TER was recordable but grossly abnormal. Throughout the remainder of cold stress, no signals could be recorded in response to light stimulation. During recovery, all signals showed unimpressive recovery to, circa, 20% normal amplitude. Examination and counts of outer segments and inner segments indicated that there was significant loss of cone outer segments, abnormalities in remaining outer segments and loss of cone ellipsoids , implying loss of cone cells. During recovery these effects were reversed, but normal complements of complete outer segments and cone cells were not regained during 52 days of recovery.

Similar results have been seen in several other studies using different species and treatments. One of these was the experimentally induced pathological state known clinically as the detached retina. Recently, a group composed of Machemer, Norton, Kroll and Hamasaki has reported a series of light microscopic, electron microscopic and electrophysiological experiments on retinae treated in this manner. The species employed were owl monkeys (Machemer and Norton, 1968; Machemer, 1968a, b; Kroll and Machemer, 1968, 1969a; Hamasaki et al., 1969) and rhesus monkeys (Kroll and Machemer, 1969b). Light microscopic studies of these retinae indicated that the outer segments of the

receptor cells disappeared and there were gross abnormalities of the retina in general (Machemer, 1968a). Electron microscopy revealed that a sequence of events followed detachment, culminating in the loss of the outer segments with relatively little change in the cellular components of the remainder of the retina. The sequence of events included loss of orientation of the outer segment lamellae, fragmentation of the outer segments, atrophy and phagocytosis of fragments of outer segments (Kroll and Machemer, 1969a). In general, surgical reattachment of the retina produced a reversal of these effects and the rod outer segments soon recovered their normal morphology (Machemer, 1968b) but the cones showed much slower recovery (Kroll and Machemer, 1969b). In this latter study Kroll and Machemer (1969b) report some alteration of the inner segments, most notable a paucity of mitrochondria.

Hamasaki et al. (1969) traced the electrical responsivity of these retinae following detachment and reattachment. The results were as one would expect, with the electroretinogram (ERG) being totally absent two weeks after detachment surgery if detachment was total. Small areas of attached retina were capable of supporting an ERG. With reattachment, progressive recovery of the ERG was seen, beginning at about 3 days after surgery with a minimal response as early as 17 hours. The eyes were tested for up to 12 weeks, with slight improvement being shown over the last 4 weeks. There is no indication as to whether or not the 12-week, or final, records were normal.

Another means of producing retinal degeneration which has been investigated in the last several years has been stimulation by low intensity light. This technique did or did not produce permanent damage and blindness, depending on the levels and durations of light used. A major factor contributing to the severity and permanency of the damage was body temperature, with slightly supranormal temperatures enhancing the damaging effect of light dramatically (Noell et al., 1966). The methodology typically involved subjecting a free ranging animal (rat) to constant illumination by small fluorescent tubes for varying durations while varying temperatures. The resulting damage was evaluated electrophysiologically or electron microscopically.

The results of the electron microscopic examinations (Kuwabara and Gorn, 1968; Grignolo et al., 1969) of the retinae of the subjects indicated that damage ranged continuously from simple loss of the distal one-third of the rod outer segments to total loss of the receptor cells. In the former case, regeneration occurred while in the latter the effect was irreversible. The fine structural description was very similar to that given by other authors following retinal detachment (e.g., Kroll and Machemer, 1968, 1969b). The electrophysiological (ERG) (Noell et al., 1966; Gorn and Kuwabara, 1967) measures followed the electron microscopic observations very well. The ERG was diminished or eradicated as one would expect from the morphology.

Interestingly, these studies (Noell et al., 1966; Gorn and Kuwabara, 1967) also employed monochromatic stimulation and found differential effects, depending on the bleaching power of the wave lengths employed.

Dowling (1964) has described the fine structural details of retinal rod degeneration under Vitamin A deficiency and inherited retinal dystrophy in rats. The latter is irreversible, whereas the former may or may not be, depending on the duration of the deficiency. The degeneration proceeded from slight disorientation of outer segment lamellae, to breaking up of lamellar material, to assumption of a spherical shape by the outer segment. With longer durations of the vitamin deficiency, the inner segments and cell bodies degenerated also. If the deficiency was corrected prior to degeneration of the inner segments, the outer segments regenerated. This process was characterized by the formation of a cilium on the inner segment terminating in a small bulb of vesicular material. This material expanded, taking on a lamellar appearance, and the lamellae assumed a normal orientation. The structure then elongated and increased to normal size (Dowling, 1964).

Another area of research applicable to receptor degeneration and regeneration is that dealing with normal renewal of rod outer segments. In general, this work has shown that labeled amino acids were incorporated into proteins which formed a disc at the base of the rod outer segment. This disc was displaced sclerally, by the formation of new

discs, until it was expelled at the peripheral end of the outer segment (Droz, 1963; Young, 1965, 1966, 1967, 1969; Herron et al., 1969, 1971) and was phagocytized by the pigment epithelium (Herron et al., 1969, 1971). The labeled protein has been identified as rhodopsin, the photopigment of the rod receptors (Hall et al., 1968; Bargoot et al., 1969). It has also been shown that rate of displacement was more rapid in light than dark adapted conditions and when ambient temperature was increased (Young, 1967). Young (1969) has recently shown that renewal as seen in the rods did not occur in cones - at least for the frog. While labeled protein was found in cone outer segments, it was diffusely located and did not progress along the outer segment as would be expected if renewal were occurring.

The general aim of the research reported here was to elaborate on the effects of cold stress on the retina of goldfish as well as to extend these results in several directions suggested by the research reviewed above. The initial exploratory work into this effect (Dawson et al., 1969; Hope et al., 1970; Dawson et al., 1971) suggested the need for revising the temporal patterning of sampling of experimental animals during the induction of cold stress. The first fish recorded in those efforts failed to give electrical signals at the cornea and presented a grossly abnormal TER. Similarly, the earliest animals recorded during the recovery phase of those experiments produced electrical signals of small amplitude. In general, during

the cold stress phase only one animal was sampled during the first two weeks. The first animal sampled during recovery was on day 11. Therefore, it seemed desirable to investigate the early portions of each of these phases more carefully, sampling animals at shorter intervals. It was hoped that this would enable evaluation of the time course of electrophysiological decay and morphological degeneration.

In the initial studies of this phenomenon, no effort was made to extend the morphological effects beyond the relationships and numbers of ellipsoids and outer segments. The loss of cone cells noted above was assumed on the basis of losses of ellipsoids. While none of the techniques described above resulted in degeneration beyond the receptor cell, it seemed desirable to investigate the possibility of cellular loss in the inner and outer nuclear layers and ganglion cell layer under cold stress. Since low temperature in a poikilotherm is known to lower the metabolism generally (Prosser, 1950), it was expected that the effects of cold stress may not be limited to the receptor cells.

Brown (1968) has presented compelling data from a number of experiments demonstrating that the A wave of the ERG has its origin in the receptor cell layer of the retina, whereas the B wave originates in the inner nuclear layer. The cells in the inner nuclear layer have been shown to be responsible for much of the retinal processing of visual information (Werblin and Dowling, 1969; Dowling, 1970). Thus, it seemed reasonable to assume that separate analyses

of these components of the ERG would differentiate between receptor function and neural processing. This defined a functional approach to the question of effects beyond the receptors.

Stimulation parameters for eliciting electrical activity of the retina in the preliminary experiments on the effects of cold stress were chosen such that they optimally stimulated cones. Additionally, because of their small size and relatively poor staining characteristics with the techniques employed, the rods were difficult to resolve in histological specimens. While it was possible to make general observations concerning the depletion of these structures, no concentrated effort was made to detect degeneration or loss of function of rods. Since some loss of the lightly stained rod material normally seen between the cone ellipsoids was observed in the preliminary experiments, stimulation parameters in the present experiment were manipulated in an effort to differentiate photopic and scotopic electrophysiological responses.

The manipulations consisted of two general types, variations in adaptation level during recordings and use of closely paired flashes of light as stimuli. The former is generally accepted as a means of separating function, with high (photopic) ambient light levels resulting in activity due primarily to cone function and low (scotopic) levels resulting in activity reflecting primarily rod function (cf. e.g., Bartlett, 1965; Riggs, 1965; LeGrand, 1968, p. 260).

In general, while these separations are probably not wholly dichotomous, the relative contributions of the two systems under these varied adaptation states can give useful information about their integrity.

The second type of manipulation, the use of paired flashes as stimuli, was incorporated as a result of a recent publication showing that, under scotopic conditions and with appropriate separation of the members of the pair, the response to the first flash represents activity of both rods and cones while that to the second flash stems only from cones (Elenius, 1969). The author demonstrated rationally, utilizing previously published data, that the effect was due to active inhibition of the rods by one of the photoproducts of the rod photopigment, rhodopsin, as it breaks down in response to light. Unpublished data taken informally by the present experimenter from the dark adapted cat supported Elenius' contentions regarding dissociation of photopic and scotopic function. It was felt that, in spite of the somewhat preliminary nature of the data purporting to establish the double flash technique, its inclusion in the present experiment would improve the probability of observing differences in loss or rate of loss of cone and rod function, with no a priori indication that inclusion would compromise other dependent variables.

Casual observations in previous work dealing with the cold stress phenomenon suggested that animals in cold stress and early recovery did not exhibit visually guided

feeding behavior of the sort seen in the normal fish, and that there may have been differences in cone outer segment degeneration between peripheral receptors and those in the central portion of these retinae. The present experiment attempted to quantify visually guided feeding behavior in the goldfish and to compare this behavior in the normal, cold stressed and recovering animal. Additionally, it was hoped that quantification of cellular structures in peripheral and central portions of the retina might allow verification of possible differences in the effect of cold stress on these two areas.

In the research (reviewed above) using other techniques which have resulted in retinal degeneration, all have one factor in common which stands in contrast to cold stress induced degeneration of outer segments. All of these techniques involved factors which can be thought of, at least in a general sense, as acting upon the outer segment from outside the cell. Vitamin A deficiency produced retinal degeneration by virtue of interrupting the rhodopsin regeneration cycle (by denying the outer segment this necessary constituent) (Dowling, 1964). Vitamin A is furnished to the receptors by the pigment epithelium. Retinal detachment, the physical separation of the retina from the pigment epithelium, may have produced degeneration in the same manner as did Vitamin A deficiency, since the receptors were removed from their source of Vitamin A. Inherited retinal dystrophy in the rat is now known to

result from the inability of the pigment epithelium to phagocytize rod outer segment material which is extruded by the receptors (Herron et al., 1969, 1971). Finally, light induced retinal degeneration obviously resulted from the impinging of energy on the receptor. Thus, the precipitating factor in all these cases appeared to be imposed on the receptor from outside its encompassing membrane. On the other hand, low temperatures are known to inhibit general metabolism in the poikilotherm (Prosser, 1950). Therefore it might be argued that outer segment degeneration induced by cold stress may be the result of the inability of the receptor cell to furnish the outer segment with metabolic products necessary for its maintainence. Under this assumption, one might expect that light stimulation would perhaps interact with cold stress to produce more severe degeneration, since light stimulation would be expected to force the receptor to function, exhausting stores of available nutrients. In this respect, inherited retinal dystrophy in the rat has been shown to progress more slowly in the dark (Dowling, 1964) and protein renewal proceeded more rapidly at constant high light levels in Young's (1967) rats. In order to test the hypothesis that light stimulation would interact with cold stress, thus increasing loss or rate of loss of electrophysiological function, varying conditions of light stimulation were imposed on the animals during cold stress and recovery.

Light stimulation was varied along two dimensions.

Variations in intensity of white light were expected to evoke quantitative differences in the dependent variables observed, while differences in wavelength composition of stimulating light were expected to result in qualitative effects on the morphology and/or electrical function of the retina. This latter point rested on the ability to differentiate, at least to some degree, between (relatively) stimulated and unstimulated cones. A possible means of accomplishing this was provided by the observation (Glicstein et al., 1969) that selective migration of cones follows adaptation to long wavelength light. Thus, it was hoped that long wavelength stimulation during cold stress might result in relatively greater degeneration of migrated cones versus unmigrated cones and stimulation by short wavelength light during cold stress might produce just the reverse effect. It was also hoped that differences in sensitivity to stimulation by monochromatic light might be detected electrophysiologically.

In order to attack the questions posed above it was necessary to subject goldfish to low temperature for an extended period of time then allow them to recover under more temperate conditions while subjecting different groups to various forms and intensities of ambient light. During these phases of the experiment, animals were sampled at specified intervals in order to obtain electrophysiological and histological data. The following section describes the equipment used and the procedures followed in accomplishing this end.

METHOD

Procedure

The general procedure was to adapt goldfish to low temperature by progressive lowering to a nominal value of 5° C. Reaching 5° C identified day 1 of the experiment. Initial temperature of the water in the tanks was 22° C. The temperature was reduced from this level to 12-15° over a span of three days. The temperature was then held between 12 and 15° for a period of three weeks. At this point the temperature was further reduced to 5° C. The temperature was then held at this level for different periods of time, depending upon the general category of environmental light stimulation to which the animals were assigned. The temperature of the water in the tanks was then allowed to return to normal room level (20-22° C) overnight and was held constant for the remainder of the experiment. These two conditions represent the cold stress and recovery phases of the experiment respectively. During each of these two phases animals were selected randomly according to a predetermined temporal schedule for electrophysiological recording and histological processing of retinae. The remainder of this section will describe in detail the equipment, techniques and procedures involved in each aspect of this general procedure.

Subjects

Subjects were common goldfish, Carassius auratus. A total of 159 fish was used in this research. Of these, ten served as normal controls for the various treatments, seven as controls receiving treatments other than those in the basic study and the remainder were distributed among the various groups defined below.

Environmental Control

All fish were housed in 60-quart styrofoam picnic coolers. Each cooler (a total of six) was equipped with a water filter and aerator. These tanks and related equipment were placed in a "walk-in" cooler which was capable of maintaining the air temperature at approximately 8 ($\pm$1)^{o} C indefinitely.

Temperature was controlled by simply adjusting the thermostat regulating the compressor of the cold room in which the aquaria were housed. This was adequate for temperatures above 8^{o} C. Below this level, the water in the tanks was chilled by the addition of frozen, hermetically sealed, cans of an "anti-freeze" solution. These were placed in the tanks once or twice daily, as needed, to maintain water temperature between 4 and 8^{o} C.

Four of the six tanks were rendered opaque by painting all outer surfaces with several coats of flat black paint. All air and filter tubing entered these tanks through heavy opaque plastic elbows. All transparent tubing and elbows

were covered with aluminum foil to further insure that extraneous light was excluded. Three of these masked tanks were fitted with sandwiched filters to admit only light of specifiable wavelength bands in equal amounts. The tops of these four tanks were then covered with an additional layer of foil and fitted with a foil skirt extending well past the junction of the cover and walls. In general, this procedure insured that one of these four tanks was light-tight and that only light passing through appropriate color filters was admitted to the other three. The remaining two tanks were untreated, with the exception of removing their covers and draping them with transparent polyethelene film to retard evaporation and contain the fish.

Light filters, fitted to the three tanks as described above, admitted light in either the red or blue portions of the spectrum. The red filter (Kodak Wratten #92), and associated glassware, passed energy in the band from 635 nanometers (nm) to 780 nm. Maximum transmission was 34% at 660 nm and the vast majority of the total transmitted light was of wavelengths longer than 640 nm since the transmission curve (Figure 2) showed a pronounced skew toward the longer wavelengths. The blue filter (Kodak Wratten #49B) passed light having wavelengths between 380 and 500 nm (Figure 2). Peak transmission was 28% at 438 nm and the bulk of the light was of wavelengths below 460 nm. The transmission curves presented in Figure 2 are those for the filters together with the glass between which they were sandwiched

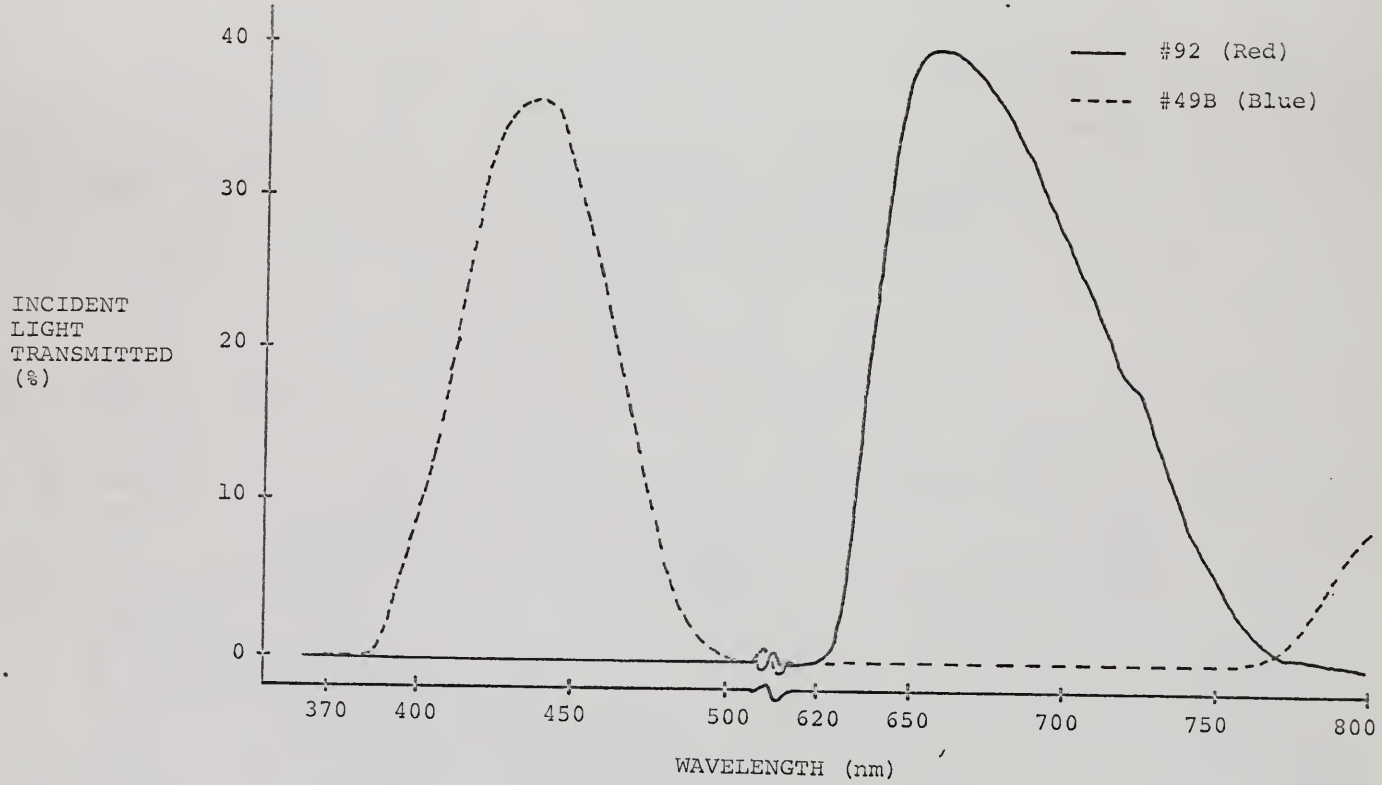

Figure 2.-- Spectral Transmission Characteristics of Wratten Filters.

and a heat absorbing filter (Kodak) interposed between the light source and the sandwich. As such, they indicate the wavelengths actually admitted to the tanks as measured spectrophotometrically.

Fish in one tank received light through the blue filter, those in the second through the red and those in the third through one of each. As will be described in more detail below these subjects were compared in the analysis of the data, thus it was necessary to equate the light in the three tanks. In order to accomplish this, the transmission characteristics of the filters, and associated glassware, were measured spectrophotometrically at wavelengths between 300 and 800 nm. The percentage of energy transmitted at 10 nm intervals over this range was multiplied by the relative energy output of a 3000° K source (Wyszecki and Stiles, 1967, p. 21) at each of these steps and this product summed over the visible spectrum. Since the blue filter passed less energy than the red and the energy output of the lamp was lower at the blue end of the spectrum, a 0.70 log unit neutral density filter (Kodak #96) was added to the red filter sandwich to equate the two. In addition, 0.3 log unit neutral density filters (50% reduction in transmission) were added to the two sandwiches placed in the tank receiving light of both wavelength bands in order to equalize the energy in this tank to the others.

Illumination for the filters was provided by a simple optical system consisting of a 500 watt tungsten -

Halogen lamp (Sylvania 500 QCL), an aspheric condensing lens and a heat absorbing filter. These lamps have an energy distribution approximated by a blackbody radiator at 3000° K when operated at 120 volts. The system provided a diverging image (defocused) of the lamp filament which was confined to the open area of the filter sandwich and filled it. The lighting system was powered by a voltage regulator set at 120 volts, the output of which was passed through a timing circuit then to the lamps. The system was operated on a 30 min. on, 60 min. off cycle to avoid the temperature rise which resulted from more prolonged operation of the 2000 watts lighting system. The lamps and optical system were enclosed in a metal hood, the open bottom of which was masked except for exit ports for the light, and which was exhausted to the outside.

Illumination levels in the aquaria were determined from direct measures of the luminance of a surface placed in the water in each tank. The surface was chosen for high diffuse reflectance and impregnability to water. Nondirectionality and high reflectance were required in order to make the necessary conversion from luminance to illuminance (LeGrand, 1968, pp. 202-203). Luminance was measured with an SEI Photometer at the approximate center of the open tanks at the surface and floor in the water. The cover was removed from one of the three tanks receiving light through the colored filters and measures were made at the surface and floor directly below the source and at the most remote

point in the aquarium.

Light received at the photometer from the reflecting surface placed at the floor of the aquaria necessarily passed through the water twice. Turbidity was unavoidable, therefore the water acted as a filter and the measured luminance was not the true value. An approximation to the true value was calculated to be the luminance measured at the water's surface less ½ the difference between this measure and that at the floor. The mean of the surface and actual floor values was then computed and this value converted to illumination incident to an object placed in the center of the tank.

This value was valid as described for the open aquaria but it was necessary to correct in accordance with the limitations imposed by the filters in the various spectral tanks. This correction required that the luminance measure be distributed across the spectrum in accordance with the relative spectral sensitivity of the observer. Since the light levels, without the interposed filters, were quite high (2.05 - 3.80 log foot lamberts), tables for relative photopic sensitivity (Wyszecki and Stiles, 1967, p. 378) allowed an evaluation of the percentage of the measured luminance contributed by light from portions of the spectrum at ten millimicron intervals. Multiplying these values by the percentage transmitted at that interval on the spectrum and summing the products gave the percent of the measured

luminance passing the filter. This value was calculated for the mean of surface and floor measures in the remote position and directly under the source and converted to illumination.

Average illumination in the open tanks was 1.9 lumens / sq. ft.; in the spectral tanks receiving red and blue light without filters interposed it was 2136.8 lumens / sq. ft. and ½ that for the other two spectral tanks. Calculated values for illumination in the spectral tanks with the filters in place were 1.9 lumens / sq. ft. for the tank receiving red light, 0.9 for the one receiving blue light and 1.4 for the one receiving both. The illumination values were valid for relative white light levels but the filtered light levels were valid only for the human observer and represent only gross estimates of the relative stimulating efficiency of these illuminations on the goldfish.

Sampling

Animals were randomly assigned to the aquaria receiving the various environmental lighting conditions. Twenty-two animals were assigned to each of the tanks which received colored light, 20 to the tank which received no light and 56 to the two tanks receiving ambient white light. Animals which received white light were designated group L, those maintained in darkness group D and those receiving light through the red, blue, and red and blue filters group R, group B and group RB, respectively.

Electrophysiological recordings were taken from the

cornea of at least one group L animal each day through day 16 of cold stress. Thereafter, during cold stress, animals from this group were sampled at four-day intervals. Histological specimens were collected from group L at four-day intervals throughout cold stress. Group D was sampled at each alternate sampling time for group L, that is on alternate days through day 16 and at eight-day intervals thereafter. Each of the three spectral groups was sampled at seven-day intervals.

During recovery, groups D,R, B and RB were sampled (electrophysiology and histology) at seven-day intervals. Group L was sampled on days 4, 8, 12, 16, 32 and 62 of recovery. The duration of the cold stress stage was 44 days for groups L and D. The recovery phase lasted for 62 days. An additional 14 days of cold stress were given to four animals from group L, with two of these being sampled at the end of this period, day 59, and the remaining two were allowed to recover for the remainder of the experiment and were sampled on day 106. These four animals comprised group E.

On day 45 the cover was removed from the tank housing group RB, exposing them to the full 2136.8 lumens / sq. ft. from the unattenuated sources, in order to establish a zero baseline for observation of recovery under spectral lighting. This group and groups R and B were then given an additional 14 days of cold stress. Electroretinograms were recorded from four group RB animals on day 45 and again on day 59.

At this point the animals from group RB were marked with suture clips clamped on the dorsal fin, distributed evenly between the tanks receiving red and blue light and allowed to recover under these conditions with groups R and B. Thereafter, these animals were referred to as groups RBR and RBB respectively. Fish from groups RBR and RBB were sampled at seven-day intervals at the same time as groups R and B during recovery, except for days 92 and 99 when fish in groups RBR and RBB were almost exhausted and one fish from each group was saved until day 106. Figure 3 presents a graphic presentation of the sequence of events described above.

Electrophysiological Measures

The electrophysiological responses recorded from the goldfish were the electroretinogram (ERG) and the response of the contralateral optic tectum (TER). The ERG was recorded from the cornea by a circular stainless steel electrode, fitted to the cornea and masked and insulated except where direct contact with tissue occurred. The reference electrode was clipped to the external naris and the signals were fed directly into the inputs of two sequential Tektronix Model 122 differential AC amplifiers having a pass band set at 0.2 to 10,000 Hz. The TER was recorded and amplified in a similar manner except that the electrode was a tungsten shaft insulated except at the 15 to 20 micron tip.

These signals were led directly, and through Krohn-

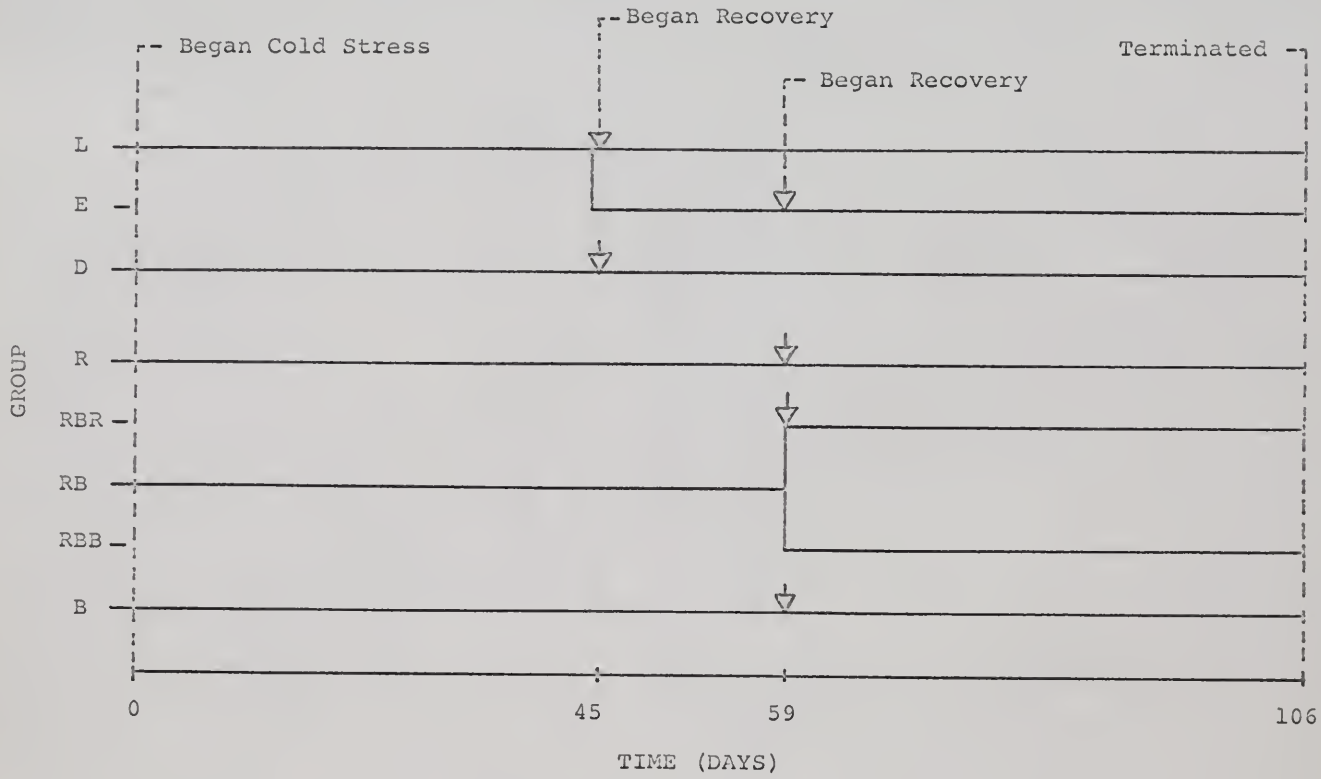

Figure 3.-- Order of Experimental Events.

Hite Model 330A and 330 NR electronic filters set to exclude frequencies below 50 or 30 Hz and above 500 Hz, to the four channels of a Fabritek 1052 signal averaging computer. The unfiltered ERG and TER were converted to frequency modulated representation then recorded, with appropriate triggering signals (unconverted), on a Roberts 770 tape recorder for further analysis off line. In addition, the unfiltered ERG and TER were displayed on a Tektronix dual beam oscilloscope for monitoring. Total gains at the inputs of the averager and oscilloscope were 10,800 for the ERG and 13,200 for the TER.

The raw ERG was passed through one of the filters (50 - 500 Hz pass band) and the raw TER through the other (30 - 500 Hz pass band). The signals resulting were the fast retinal potential (FRP) and fast tectal potential (FTP). Gains for these signals were as for the ERG and TER respectively.

During recording, goldfish were held in a holder which allowed perfusion of water through the mouth then out the gills and along the ventral body. Anesthetics were not generally necessary for recording but small amounts of a neuromuscular blocking agent (Flaxedil) were administered by injection if required. All recordings were taken with subjects at controlled room temperature (20 - 22^{o} C). The fish were kept moist at all times. Fish have been maintained in this apparatus for as long as 10 hours with no detectable ill effects.

Stimuli eliciting electrical activity were provided

by an especially constructed photostimulator utilizing a xenon arc source and optics such that the stimulus was presented in Maxwellian view (Westhiemer, 1966). Intensity was variable over a wide range through internal filters. A variable intensity adapting light was available as part of the system. Wavelength of both the stimulus and adaptation field could be varied by addition of monochromatic filters (Baird-Atomic).

Stimulating conditions for eliciting electrical activity were tailored to each individual group. For animals in Group L the stimuli and electrophysiological recordings were intended to enable the investigator to look at electrical responses at photopic or high light levels, scotopic or low light levels, and mesopic or intermediate light levels. It is generally accepted that at high photopic light levels the majority of the electrical activity is a result of activity in cone receptors, whereas at scotopic levels the activity is primarily the result of activation of rods. The mesopic level is believed to represent the intermediate case where the activity is mixed, that is, results from stimulation of both rods and cones. The photopic adaptation level was achieved by raising the ambient light level to 1.2 log foot lamberts measured on a tangent screen placed 10 cm in front of the animal's eye and, in some cases, adding the secondary adaptation light. Sixty-four stimuli were delivered from the stimulating device described above with each discrete flash delivering 0.7 ergs, retinal irradiance uncorrected

for absorption of media of the eye (LeGrand, 1968, pp 87-93) or the Stiles-Crawford effect (Stiles and Crawford, 1933). Each stimulus was composed of two flashes separated by 250 milliseconds. Stimuli were delivered at a rate of one pair per second at intensity setting W4 on the photostimulator. The mesopic condition was achieved by lowering ambient light level to 0.1 log foot lamberts measured on a tangent screen 10 cm from the eye. Thirty-two stimuli were delivered, with each consisting of two flashes as described above, delivered at a rate of one pair per three seconds. Scotopic conditions were obtained by turning off all light and allowing the animal to dark adapt for 20 minutes. During the process of dark adaptation the stimulator was operating, delivering one stimulus, a pair of flashes, every 15 seconds. Following dark adaptation, sixteen stimuli (flash pairs) were delivered at one per 15 seconds. Electrical activity evoked by the pairs of flashes were averaged, as described above, for each condition. Beginning on day 10 the process of dark adaptation was also monitored. This was accomplished by averaging responses to the four stimuli delivered during alternate, odd-numbered, minutes. That is, responses to the four stimuli delivered during minute one were recorded and printed out and so on for alternate minutes through minute 19. On the 20th minute the standard dark adapted series described above was initiated. Thus, for animals in group L, primary data consisted of an averaged ERG tracing representing 64 signals under low photopic conditions

(beginning on day 14), one representing thirty-two signals under mesopic conditions, one representing sixteen signals under scotopic conditions, and a series of averages of four responses, each representing odd numbered minutes during dark adaptation (from day 10).

Stimuli delivered to animals in the spectral groups differed from those for group L. Stimuli for these animals were from the same stimulus source but at a higher intensity (setting W16) and passed through monochromatic filters (Baird-Atomic) which had nominal peaks at 430 nm, 550 nm, and 630 nm. These filters were selected in order to optimally stimulate discrete cone populations. That is, the filter having a peak transmission at 430 nm stimulated cones having maximum sensitivities in the short wavelength end of the spectrum, the 550 nm filter stimulated cones having peak sensitivity in the middle of the spectrum as well as rods, and the 630 filter stimulated those cones having peak sensitivity in the long wavelength end of the spectrum. These stimulating wavelengths were chosen on the basis of published absorption spectra for goldfish single cones (Marks, 1965; Liebman and Entine, 1964), and while the filters chosen quite probably did not selectively stimulate a single population, they very probably did result in a relative imbalance which tended to favor one population over another. To a human observer, the 430 nm filter appeared blue-violet, the 550 green and the 630 filter orange-red. The blue filter had a peak transmission at 430 nm of 73% with an 8 nm band width at 50% transmission,

the 630 or red filter 80% at 630, 4 nm at 50% transmission and the green filter 70.5% at 747 nm with a 6 nm band width at 50% transmission. For stimulation through these filters the stimulating device was operated at setting W16, representing maximum intensity for this source, and at this level delivered 6.9 ergs, retinal irradiance (uncorrected, as noted above) under these conditions without the filters interposed. Since this energy is spread across the visible spectrum, and since the filters employed pass only narrow portions of the spectrum it was necessary to determine the percentage of energy within those bands passed by each of the filters and then, through the use of neutral density filters, to equate each of the spectral filters in a manner such that they passed the same amounts of energy. The absolute energy levels delivered to the eye with the filters interposed were not determined. However, using ERG amplitude as a criterion, the efficiency of this system with only the monochromatic filters interposed was approximately equivalent to the stimulus setting W4 without these filters. Since this was the condition under which the stimulator was operated for group L animals, the data for spectral group animals were comparable to that for animals in group L when the highest intensity settings on the neutral density filters were used for the former. The major factor, however, was that the stimuli could be corrected so as to deliver equal energy at the three wavelengths being studied.

All stimuli for animals from the spectral groups

were delivered while the animal was in the dark adapted state. Four responses were averaged at each intensity setting for each of the monochromatic filters. Initial intensity settings were the maximum obtainable from the stimulating system and were reduced in 0.3 log unit steps. Amplitude-by-intensity curves so generated take the general form of an ogive and, in some cases, slightly larger intensity decrements were used during the rapidly falling portion of these curves. Stimulus intensity was reduced until the ERG reached a lower asymptote or became indiscernible.

Goldfish which were deprived of light during cold stress (group D) were run under dark adapted conditions. Stimuli were paired flashes of white light (setting W4) with each pair delivered at a rate of one per 15 seconds. Responses to 16 of the stimuli were averaged.

All animals were transported to the recording chamber and prepared for recording in light appropriate to their environmental stimulation condition, with two exceptions. Animals in the dark group were prepared in dim red light. Animals from the group receiving blue light in cold stress were prepared in dim blue light except for a brief period during which surgery required for tectal exposure was conducted. It was extremely difficult to perform the tectal exposure in dim blue light, since very little light at the shorter wavelengths was reflected from the orange fish.

Tectal exposure was accomplished by cutting through

the cartilaginous tissue overlying the tectum. A triangular section of this tissue was removed exposing the heavy fat deposit lying below. This deposit was removed by aspiration, exposing the very prominent tectum. The tectal electrode was lowered into the approximate center of the dorsal surface of the tectum while stimuli appropriate to the recording conditions for animals from each group were being delivered. Responses were monitored on an oscilloscope. The tectal electrode was advanced past the point at which the maximum signal amplitude was obtained. Then by a process of titration the electrode was placed at the point of maximum signal. Upon completion of the collection of electrophysiological data the animals were enucleated and the eyes processed histologically.

Histology

Eyes were fixed in 4% gluteraldehyde carried in a physiological solution (Ringers). All eyes were fixed for a minimum of 12 hours. During the first 3 to 6 hours of fixation the tissue was shielded from light. The tissue was then washed, dehydrated in alcohol, and imbedded in paraffin (Paraplast). Eyes were sectioned at 4 to 6 micra on a rotary microtome (Americal Optical). Eyes were carefully oriented in the block during imbedding and the block carefully oriented on the microtome prior to cutting such that sections passing through the optic papilla also passed through the central retina. Thus, sections extended from

the inferior nasal to the superior temporal poles, at a slight angle from the horizonal with reference to the normal position of the eye in the intact animal. Sections were cleared in xylene and stained with hematoxylin and eosin. Additionally, in one set of sections, the pigment epithelium was bleached with hydrogen peroxide prior to staining. All sections were cover slipped with permount.

Histological materials were viewed through a Zeiss phase contrast binocular microscope. Primary data consisted of counts of ellipsoids and outer segments and for animals in the light and dark groups, counts of cell bodies, actually nuclei, in the three nuclear layers of the retina. For animals in the spectral groups ellipsoid and outer segment counts were classified into migrated and unmigrated. Red light adaptation of the retina of the goldfish has been reported to result in migration of approximately one-third of the cone cells (Glicstein et al., 1969). Red light adaptation was provided during enucleation in the present experiment. Thus, it was presumed that migrated cones were those maximally responsive to light from the longer wavelength, i.e., red, portion of the section.

Nuclei in the central retinal layers were not counted for animals in the spectral groups, and ellipsoid and outer segment counts were limited to the central portion of the retina. For groups L and D, all counts were performed for one central and one peripheral retinal locus. The central position was defined as the area just temporal to

the optic papilla, and the peripheral locus as an area approximately 30° temporal or nasal to the central locus. Counts were made over a retinal extent of 0.18 or 0.45 millimeters at nominal optical gains of 1250 or 500. Counts were corrected for eye size, since this extent in smaller eyes represented a larger portion of the retina.

Correction for eye size consisted of calculating the geometric angle subtended by the measured portion with reference to the length of the eye from the plane of the iris to the retina, then correcting and expressing data in terms of 10° angle. Simple proportions of associated outer segments to migrated and unmigrated ellipsoids were calculated for groups R, B and RB and outer segments to ellipsoids for groups L and D. In the latter case, since data were proportions, no correction was necessary for eye size.

Behavioral Testing

Evaluation of feeding behavior was carried out on animals in group L. Tests were conducted prior to cold stress, on days 14, 15 and 16 of cold stress and days 14, 15 and 16 of recovery. Testing was carried out in conjunction with normal feeding and consisted of simply counting the number of food bits which were caught while sinking to the bottom of the aquaria. Food for conducting the tests was standard laboratory rabbit chow (Purina) broken into irregular bits with no dimension greater than approximately 2 mm. The animals were fed a subnormal amount of their

regular diet (Bio-Rel goldfish flakes, Longlife) to stimulate feeding behavior. When the animals were vigorously feeding, or a reasonable time had elapsed, two trials, each consisting of dropping 20 test bits, were conducted. The number of food bits caught, or toward which a clear movement was directed, was tabulated for each trial. There was seldom any doubt as to whether to count a bit. The bits drifted to the floors of the aquaria with a sliding oscillatory motion, requiring two or three seconds to reach the bottom. Typically one or more fish would suddenly wheel and attack the food, usually catching it as it floated down. Few fish were seen to respond to the food bits as they hit the water. Bits were always dropped into the area of the aquarium having the highest density of fish at that moment. After the tests were conducted, the remainder of the normal daily ration was delivered.

Statistical Analyses

Most of the data from the experimental procedures described above were analyzed statistically utilizing multivariate designs presented by Winer (1962) in chapters five through seven. In some cases designs from this source were adapted slightly to fit the experimental procedures utilized in collecting the data. These modifications involved simple adaptations to allow incorporation of corrections for unequal cell frequencies when these corrections were not provided by the author. In general, the bulk of the data were analyzed using one-, two-or three-way analyses of

variance (ANOVs) with repeated measures on one or more factors in the latter two cases. Modifications employed to allow use of these designs with unequal cell frequencies were the least square type, justified in the present case since the unequal cell frequencies were dictated by the temporal sampling schedule rather than the result of missing data.

The design of the experiment was such that these analyses were appropriate for observing effects pertinent to the questions under investigation. In many cases relevant effects were interactions between variables rather than main effects due to the variables per se. Multifactor analyses of variance are uniquely effective in cases of this type. However, in some cases it was clear that one of the basic assumptions underlying these designs was violated.

Several animals in groups L and D contributed electrophysiological data at more than one point in the experiment. That is, an animal was sampled then returned to the tank and sampled again at a later date. Since no attempt was made to identify these animals it was not possible to remove data contributed by these animals prior to statistical analysis. The effect of this multiple sampling was to insure that error or within cells variances were not independent as assumed in the theoretical design. The situation was somewhat analogous to that obtaining when one incorporates a control group into such an analysis so that the control group appears at all levels of some main factor (see Winer,

1962, p 264). A partially compensatory procedure in this case has been to reduce the degrees of freedom for calculating the error term by an amount equal to the number of dependent cells.

An analogous procedure utilized in the present case was to reduce the degrees of freedom for error terms by an amount equal to the number of multi-sampled animals. This was possible since, under the least squares correction for unequal cell frequencies, the total number of subjects (N) was utilized in calculating degrees of freedom rather than cell frequencies (n) in the usual manner. Under these conditions, the reduction in total degrees of freedom was especially significant since the reduction was, in some cases, multiplied by the number of levels of the various factors; thus, an additional degree of conservatism was involved.

It might justifiably have been argued that this compensating procedure was overly conservative since, in addition to the multiplicative factor noted above, it involved the implicit assumption that the multi-sampled animals contributed nothing to the error scores, an extremely improbable condition. It appeared likely that the valid condition lay somewhere between the standard and conservative analyses. For this reason, summary tables for analyses in which the compensation was necessary were prepared with two sets of F ratios, those calculated in the usual manner, ignoring the multiple samplings, and a second set of

conservative values. In general, effects seen to be significant under both procedures were considered to be real and probably very strong. Effects found to be significant under the former but not the latter procedure were assumed to be, at best, questionable or borderline.

While it was felt that the compensating procedure was adequately conservative to allow use of the analyses in interpreting the data, the procedure ultimately reduces to an attempt to correct for an effect, the magnitude of which cannot be estimated. Prudence therefore dictated that one not accept the statistical levels per se with the trust normally accruing to powerful statistical techniques. Thus, while the significance level was probably unreliable, significance, in conjunction with visual evidence provided by accompanying figures, was considered sufficient to establish an effect.

Additional Considerations

During the course of the experiment the data departed from results seen previously in research on the effects of cold stress on the goldfish retina. In order to evaluate the effect of several procedural differences between the previous and present work two partial replications of Dawson et al.'s (1971) original experiments were performed.

These replications utilized the same equipment and procedures employed in the original studies but recording of electrophysiological data was as described above for

animals from group L in the present experiment. There were three primary differences in environmental control between the replications and the treatment of group L animals. First the temperature was dropped more quickly from 22° C to 5° C. Second, the ambient illumination, provided by standard room lighting, at the center of the tank was higher, 8.8 lumens / ft^2 vs. 2.0 lumens / ft^2. Finally, the temperature was less variable. While the temperature range was approximately the same, the excursions within the range were less frequent. The differences in temperature control were possible because of the more sophisticated means of manipulating this variable in the replications, as the equipment allowed precise thermostatic control.

Electrophysiological data were recorded from the cornea (FRGs) prior to cold stress, for one replication, and after two and three weeks of cold stress in both. Since the procedures were essentially the same in both replications, the animals were treated as one group and were referred to as group RC (replication controls).

This section of this paper has attempted to describe the methods, procedures and equipment employed in collecting the data presented in the following section. Collection of all data has been treated above even though some did not contribute to the results of the experiment and was not considered pertinent to the questions under attack in this research. The complete description was given in order that possible effects on the experimental results due to the

addition of procedures necessary to collect this ancillary data might be assessed.

The ERG's composed the primary electrophysiological data. The TER was recorded mainly to provide added sensitivity, with the electrode inserted among the cells producing the electrical activity, rather than distal as is the case when recording the ERG from the cornea. Additionally, the tectum amplifies effects of stimulation due to areal summation occurring through the system. In spite of the advantage in sensitivity, however, the TER, as recorded in this experiment, was not a good variable for quantification, since small movements of the animal, relative to the fixed tectal electrode, produced variability in signal amplitude. Movement was not a significant factor for the corneal recordings as the electrode was free to move with the eye. The TER was useful as a casual on-line qualitative check when corneal signals were not recordable but did not provide quantitative data.

Similarly, the FRP was included in the recorded data for informal qualitative evaluation only. Under the recording conditions this signal was rather small and fragile. Being small, it was free to vary over only a limited range and therefore was not precise enough for quantification. Being fragile, it disappeared too quickly in cold stress to allow evaluation of excursions over even the limited range available. It was included primarily in order to assess the early effect of cold stress in a qualitative manner and in hopes that it

might aid in evaluating possible photopic losses, as it has been described as being primarily related to photopic function (Adams and Dawson, 1970).

The fast tectal potential (FTP) was recorded in a relatively small percentage of the animals. This signal has not been described in the literature and this task seemed beyond the scope of the present experiment. When the FTP was recorded, it was done primarily out of curiosity and for the purpose of evaluating the feasibility of possible future research in that direction. Consequently, this signal did not contribute significantly to the present experiment.

RESULTS

Behavior

In general, the fish were extremely lethargic during cold stress, tending to lie on the floors of the aquaria in groups or schools. Orientations of fish relative to their neighbors suggested schools rather than groups. Movement could sometimes be aroused by prodding and spontaneous movements did occur. Casual observations suggested a relationship between the temperature of the water and the amount of spontaneous movement seen, with higher temperatures within the range specified for cold stress being associated with greater frequencies of movement.

Figure 4 presents histograms showing the results of quantifications of feeding behavior. It can readily be seen that visually guided feeding behavior, as measured by the test described earlier, was inhibited in cold stress and was reduced in recovery relative to normal. A one-way analysis of variance was performed on these data (Table 1). The results indicated that the main effect was highly significant, consistent with the clear differences shown in Figure 4. General observations indicated that the fish were eating but that they were feeding from the bottom of the aquaria. The fish were not observed to feed during the lower temperature ranges during cold stress, but did eat from the floor when the temperature rose to 6° or 7° C.

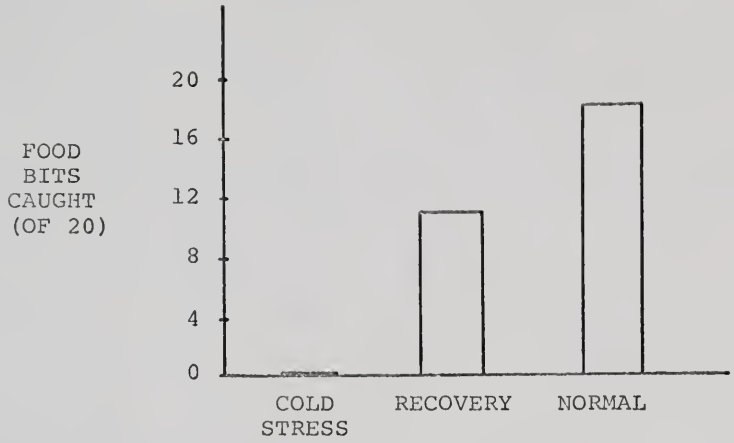

Figure 4.-- Food Catching Behavior of Goldfish in Cold Stress and Recovery.

TABLE 1

SUMMARY OF ANALYSIS OF VARIANCE:
FOOD CATCHIN G BEHAVIOR

Source	SS	df	MS	F
Experimental Condition	1007.44	2	503.72	294.57**
Error	25.67	15	1.71	
Total	1033.11	17		

**p < .01

There were two factors which may have contributed to these differences other than possible visual effects. During cold stress the fish were very inactive during the testing periods and it was impossible to assess the degree to which the failure to catch food might have been attributable to this factor. This was not a problem during recovery, however, there were 50% fewer fish in the aquaria in recovery than in the pre-stress period. The reduced population density may have reduced the probability that a food bit would be seen by a given fish. Since bits were dropped into the area of highest fish density in all cases, the reduction in probability of seeing was not directly related to numbers of fish in the aquaria and could not be assessed. The fish has a field of view of about 195°, monocularly, and binocularly the field is continuous except for about 25° obscured by the body (Trevarthen, 1968). Under these conditions it seemed unlikely that the reduced numbers of fish could have had a significant influence, but the possibility must be considered.

Electrophysiology

Figure 5 presents mean ERG amplitudes versus time in cold stress and recovery for animals from group L. Data presented in this figure were recorded under mesopic and photopic adaptation conditions and include responses to both the first (AB_1) and second (AB_2) flash. Amplitudes, on the ordinate, are in microvolts (μv) at the cornea. For the

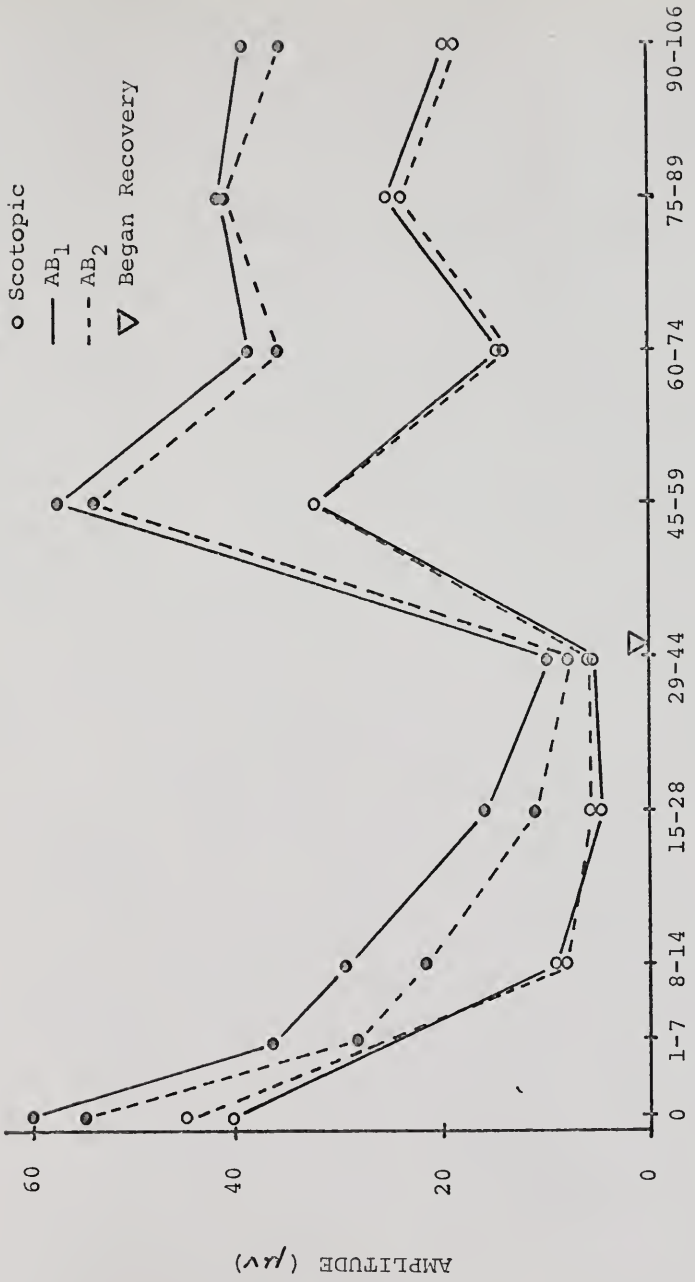

Figure 5.-- ERG Amplitudes from Group L Under Photopic and Mesopic Adaptation.

purpose of analysis and presentation, data for two-week periods (±2 days) were averaged, with one exception. The first two weeks in cold stress, under mesopic conditions, were treated separately in order to more closely observe the time course of signal loss. These periods constituted the abscissa of Figure 5.

Several features of these data were noteworthy. During cold stress, ERG amplitudes in response to both the first and second flash decreased to a very low level, approximately 15% of normal (time 0 on the abscissa). With return to more normal temperatures (recovery) signals increased initially to normal or near normal amplitude then stabilized at approximately 60-65% normal. There was no difference between responses to the two flashes, AB_1 and AB_2, under photopic conditions but there was a tendency for AB_2 to be somewhat reduced relative to AB_1 under mesopic adaptation. There appeared to be a tendency for the rate of loss under the photopic condition to be somewhat more rapid than under the mesopic condition, but this was difficult to evaluate without data for the first week under the photopic condition.

Analyses of variance performed on these data are summarized in Tables 2 and 3. Effects due to period, or time in cold stress and recovery, were significant in both sets of data ($p<.01$). Additionally, there was a significant ($p<.01$) effect due to response (AB_1 vs AB_2) (Table 3) under mesopic conditions. The insignificant period x response interaction suggested that there was no differential cold stress effect on the responses to the first and second flash.

TABLE 2

SUMMARY OF ANALYSIS OF VARIANCE:
ERGS FROM GROUP L UNDER PHOTOPIC ADAPTATION

Source	SS	df	MS	F	Fc
Between Subjects	153.76	39			
Experimental Period	106.06	6	17.68	12.99**	10.379**
Subjects within Groups	47.70	33	1.45		
Within Subjects	7.10	40			
Response (Flash)	0.14	1	.14	<1	
Period X Response	0.97	6	.16	<1	
Response X Subjects within Groups	5.99	33	.18		

**$p < .01$

TABLE 3

SUMMARY OF ANALYSIS OF VARIANCE:
ERGS FROM GROUP L UNDER MESOPIC ADAPTATION

Source	SS	df	MS	F	Fc
Between Subjects	424.6750	64			
Experimental Period	216.4565	8	27.0570	7.277**	6.108**
Subjects within Groups	208.2185	56	3.7181		
Within Subjects	19.5040	65			
Response (Flash)	6.2480	1	6.2480	29.320**	24.608**
Period X Response	1.3204	8	.1650	<1	<1
Response X Subjects within Groups	11.9356	56	.2131		

**p < .01

The data recorded under mesopic conditions were further analyzed in order to investigate the possibility of differential cold stress and recovery effects on A and B wave components of the total ERG's evoked by the two flashes. That this effect obtained can be seen in the significant ($p<.01$) period x component interaction in the summary of this ANOV in Table 4. Inspection of the graphic presentation of these data (Figure 6) suggested that the A waves were relatively less affected by cold stress than the B waves and that recovery was also not so great as for the B waves. There also appeared to be a tendency during early recovery for the amplitude of the B wave following the second flash (B_2) to more closely approximate that of B_1 or, to put it another way, for the relationship between these components to more nearly approximate that seen under photopic adaptation for AB_1 and AB_2. Normalizing these data and plotting them as percentage of normal (Figure 7) allowed direct comparison of relative changes in A and B wave amplitudes during cold stress and recovery. The normalized data supported the suggestion that the A waves were spared somewhat during cold stress relative to the B waves and that A_1 showed this tendency to a greater extent than A_2. In recovery, the A waves appeared to remain depressed to a greater extent than did the B waves. There was also a tendency for A_2 to show relatively less and B_2 relatively more recovery initially than their first flash counterparts. These differential

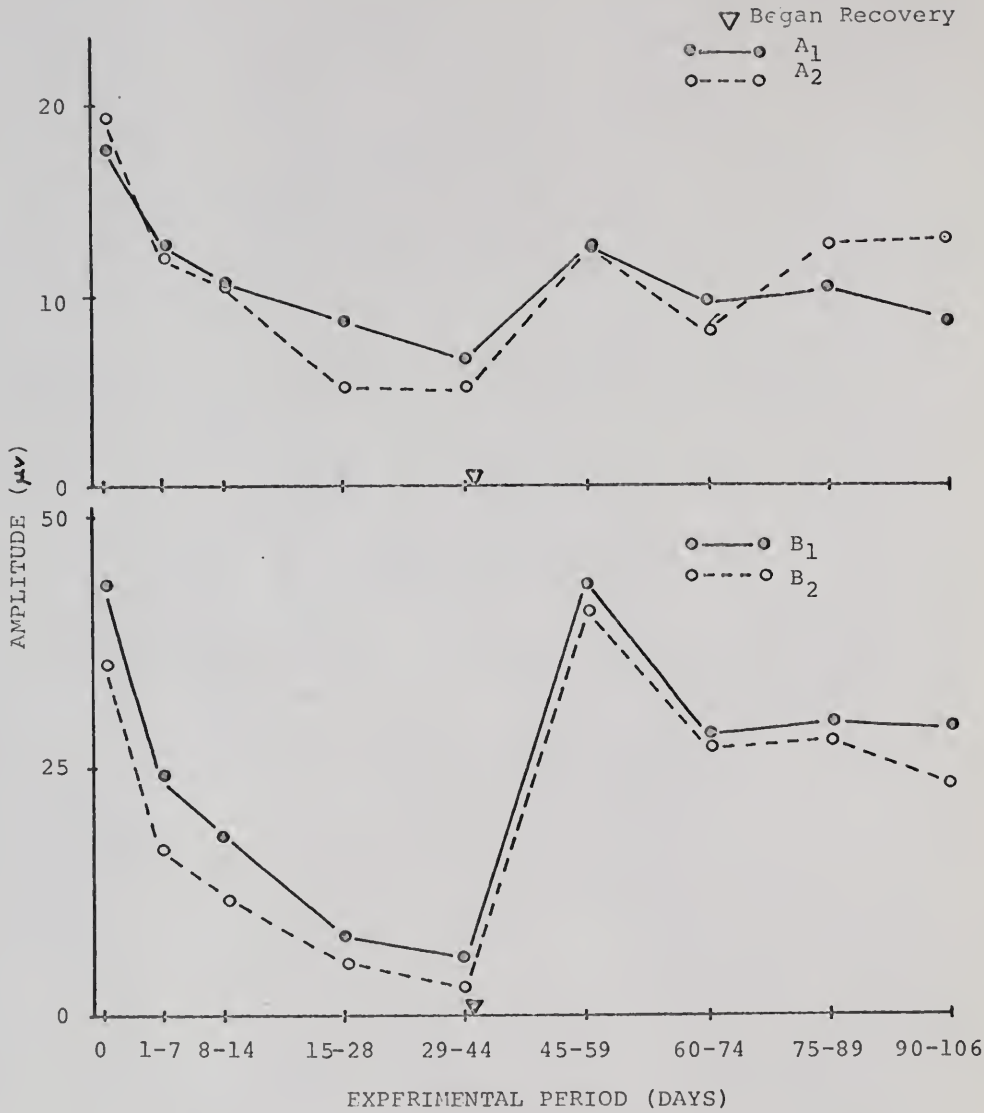

Figure 6.-- A and B Wave Amplitudes from Group L under Mesopic Adaption.

TABLE 4

SUMMARY OF ANALYSIS OF VARIANCE: A AND B WAVES FROM GROUP L UNDER MESOPIC ADAPTATION

Source	SS	df	MS	F	Fc
Between Subjects	200.7945	64			
Experimental Period	102.4802	8	12.8100	7.297**	6.124**
Subjects within Groups	98.3143	56	1.7556		
Within Subjects	182.5658	195			
Component (Wave)	67.5200	3	22.5066	55.503**	46.588**
Period X Component	46.9198	24	1.9549	4.821**	4.047**
Component X Subjects	68.1260	168	.4055		

**p <.01

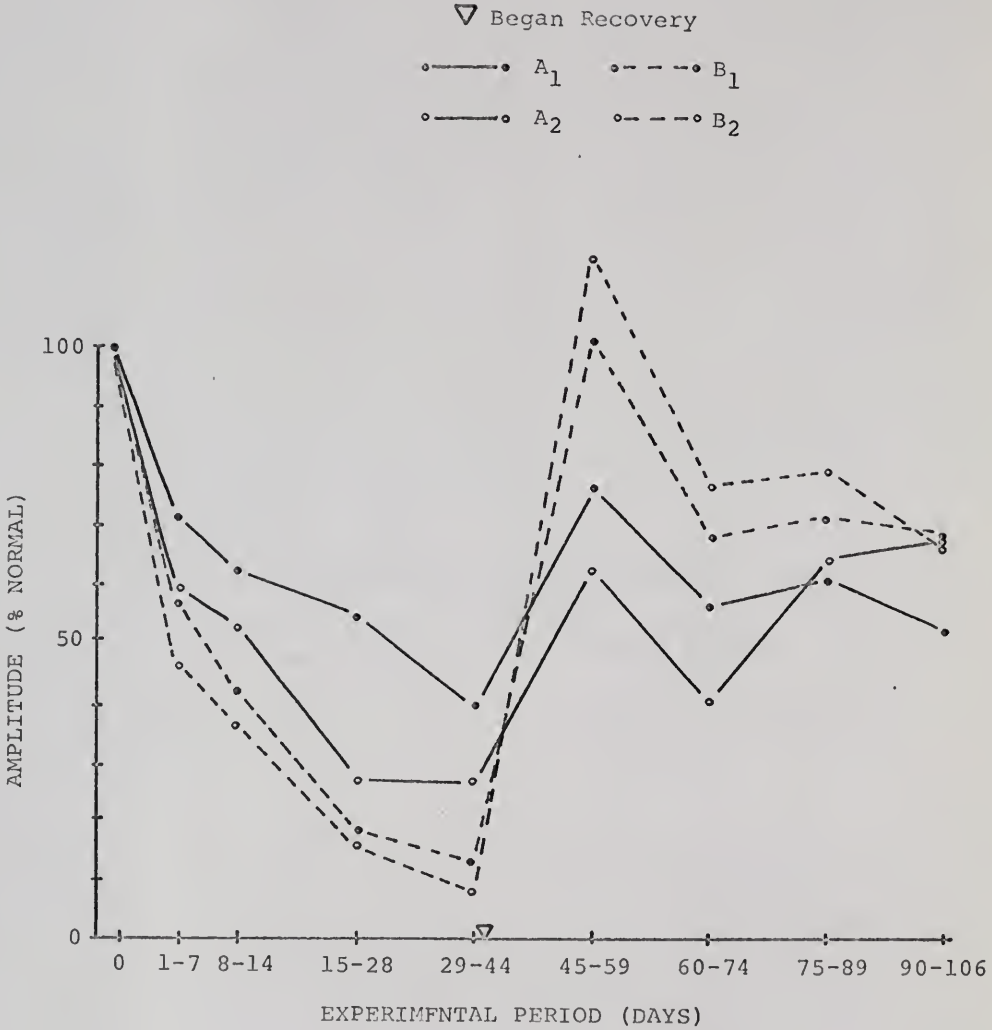

Figure 7.-- Normalized A and B Wave Amplitudes from Group L under Mesopic Adaptation.

changes in cold stress and recovery appeared to adequately account for the interaction effect seen in the statistical analysis.

Several conclusions could be reached on the basis of this evidence. It appeared clear that signal loss under cold stress followed an orderly time course, the general function appearing to be negatively accelerated and decreasing. Recovery was characterized by a rebound with some overshoot then stabilization at a level suggesting permanent or long term partial loss of function . Differential effects on the A and B waves under mesopic adaptation suggested that there were effects on both receptor function and neural processing, reflected by A and B waves respectively (Brown, 1968; Witkovsky, 1971), with neural processing showing greater loss and recovery. Additionally, there were two suggestions that cone function was affected to a greater extent than rod function. The tendency for photopic, primarily cone function, data to show a somewhat higher rate of loss than mesopic, receiving rod and cone contributions, data implied this effect. Differences in A_1 and A_2 in the normalized mesopic data added support.

Data recorded under scotopic conditions from groups L and D, Figures 8 and 9 for AB_1 and AB_2 respectively, pointed up additional cold stress effects. In Figure 8 it can be seen that both group L and D showed loss in amplitude of AB_1 during cold stress and that the loss followed a course very similar to that seen under mesopic and photopic conditions. There

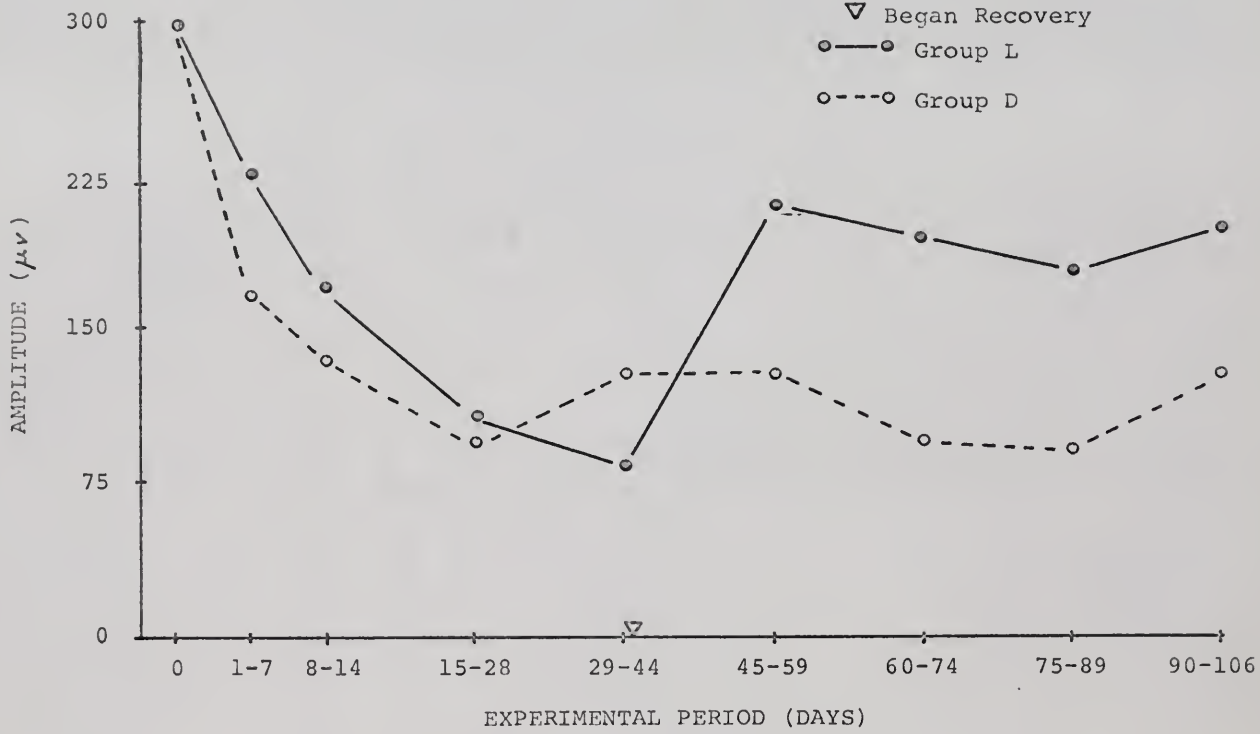

Figure 8.-- ERG Amplitudes: Response to the First Flash under Scotopic Adaptation.

appeared to be little or no difference between group L and D during cold stress but the curves diverge during recovery. Clearly, group D showed little or no recovery. Group L showed partial recovery but without the initial overshoot seen under mesopic and photopic conditions.

Figure 9 presents similar records for AB_2. It was found, in the process of conducting the present experiment, that double flash stimulation of the dark adapted normal goldfish produced results which departed significantly from expectation based on Elenius' (1969) reports on humans and from unreported data collected by this author from cat. Rather than maintaining an amplitude level equivalent to that at which the rod-cone break occurs in dark adaptation as expected, the second flash response decreased in the normal goldfish to an almost indiscernable ripple in the records (see below, p 100, for additional discussion). For this reason, the ordinate of Figure 9 had to be expanded by a factor of about six in order to observe changes in the AB_2s. The major points in these records were that the general form of the group L curve was very similar to those seen under light adapted conditions and that the curve for group D animals appeared to be supranormal during most of both cold stress and recovery.

As seen in Table 5, most of these observations received statistical support. Significant ($p<.01$) main effects due to time in cold stress and recovery and response, AB_1 vs AB_2, were clear in Figures 8 and 9. Interaction

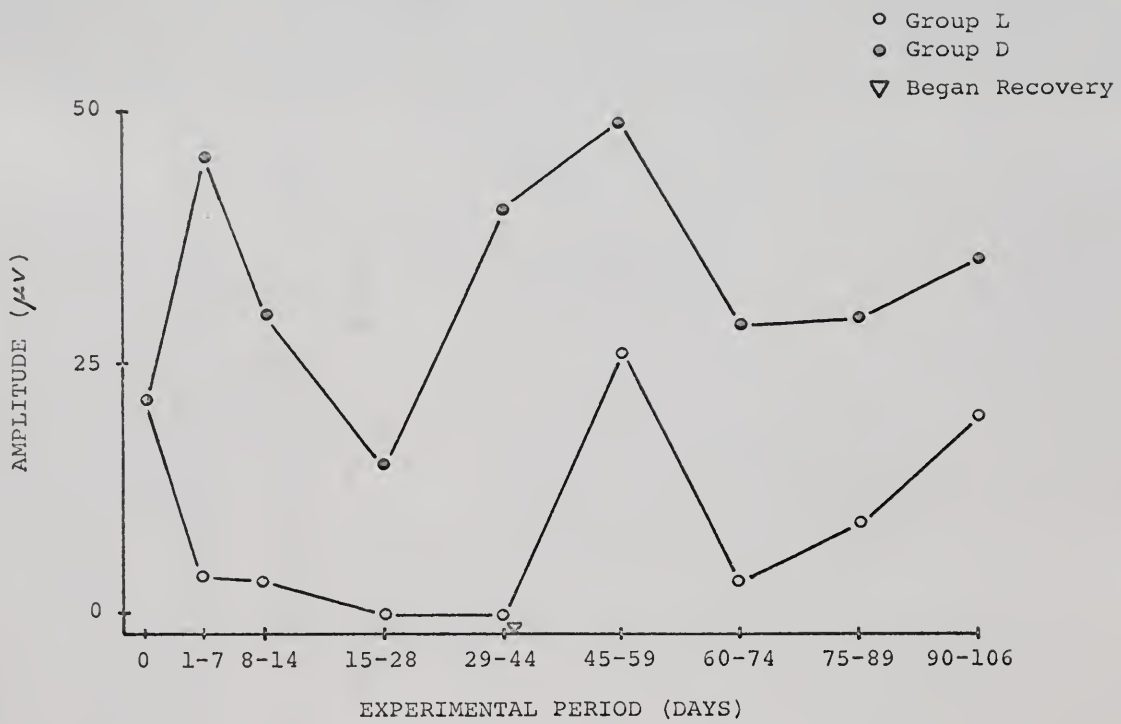

Figure 9.-- ERG Amplitudes from Groups L and D in Response to the Second Flash Under Scotopic Adaptation.

TABLE 5

SUMMARY OF ANALYSIS OF VARIANCE:
ERGS FROM GROUPS L AND D UNDER SCOTOPIC ADAPTATION

Source	SS	df	MS	F	Fc
Between Subjects	3,122.3580	102			
Group	22.3012	1	22.3012	1.054	
Experimental Period	1,178.4946	8	147.3118	6.961**	4.475**
Group X Period	143.8793	8	17.9849	<1	
Subjects within Groups	1,777.682	84[a]	21.1629		
Within Subjects	11,285.1288	103			
Response (Flash)	8,213.3522	1	8,213.3522	451.663**	290.356**
Group X Response	356.8717	1	356.8717	19.625**	12.160**
Period X Response	933.9399	8	116.7424	6.420**	4.127**
Group X Period X Response	253.4532	8	31.6816	1.742	
Response X Subjects within Groups	1,527.5118	84[a]	18.1847		

**p < .01

[a]correction for control group (Winer, 1962, 263-267)

effects, response with group and response with time in cold stress and recovery ($p<.01$) supported the observation that AB_1 was of smaller amplitude generally, and especially during recovery, in group D animals than group L, while AB_2 was just the reverse. The significant ($p<.01$) time x response interaction was seen as probably resulting from the relatively large difference in amplitude between AB_1 and AB_2. Due to this difference, if plotted on the same ordinate and abscissa AB_2 would have appeared to be extremely stable while AB_1 showed a large change with time in the experiment. The expanded ordinate in Figure 9 enhanced the change over time of the AB_2s whereas the analysis saw the actual small numerical differences relative to the large differences for the AB_1s. The proper interpretation of this interaction would have been that the AB_1s were being affected by time in cold stress and recovery while the AB_2s were unaffected, however, in light of the above argument, this interpretation did not seem justified.

Figures 10 and 11 show this data reanalyzed with A and B waves (respectively) plotted separately. In Figure 10 the A_1s were consistently larger than the A_2s and there were differential effects on these for groups and for cold stress and recovery. A_1 for group D showed a greater effect due to cold stress and no recovery relative to A_1 for group L whereas A_2 for group D was relatively unaffected by cold stress or recovery while A_2 in group L animals was not recordable in late cold stress and showed the familiar recovery effect. This suggested that the rod system (A_1) was relatively more

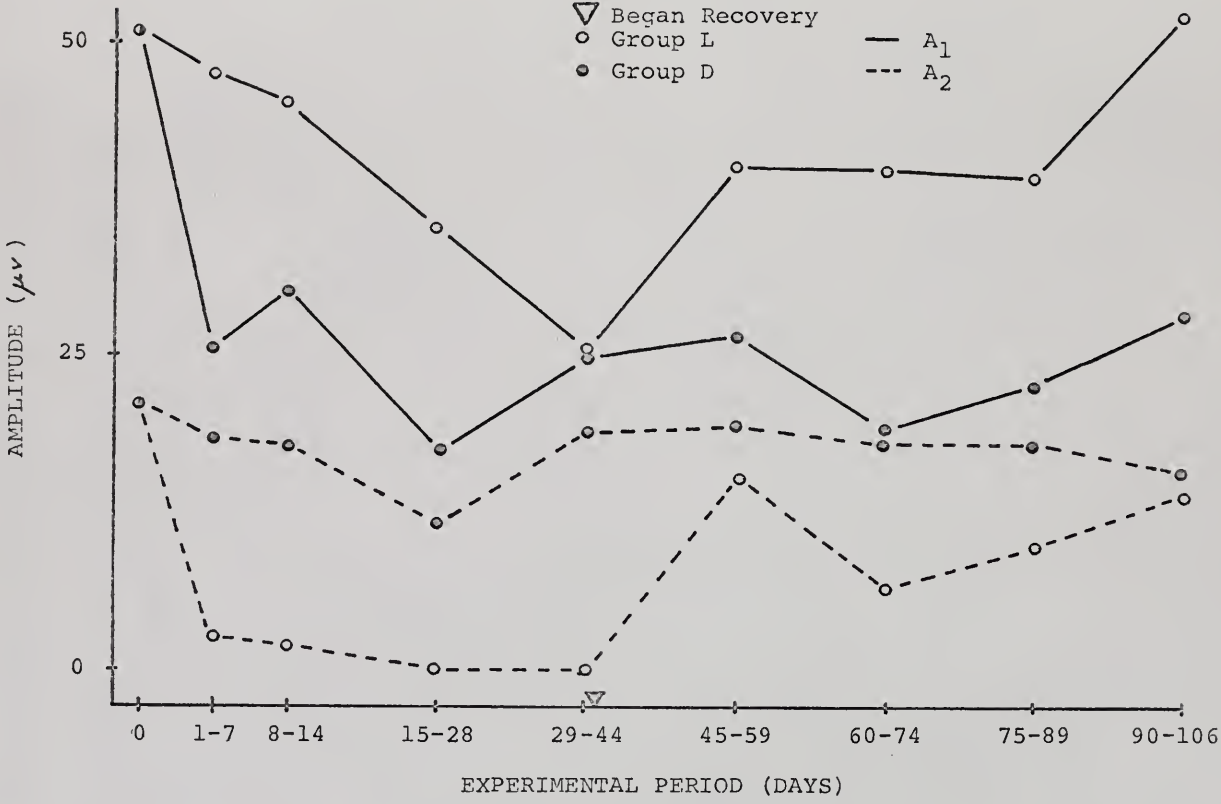

Figure 10.-- A Wave Amplitudes from Groups L and D under Scotopic Adaptation.

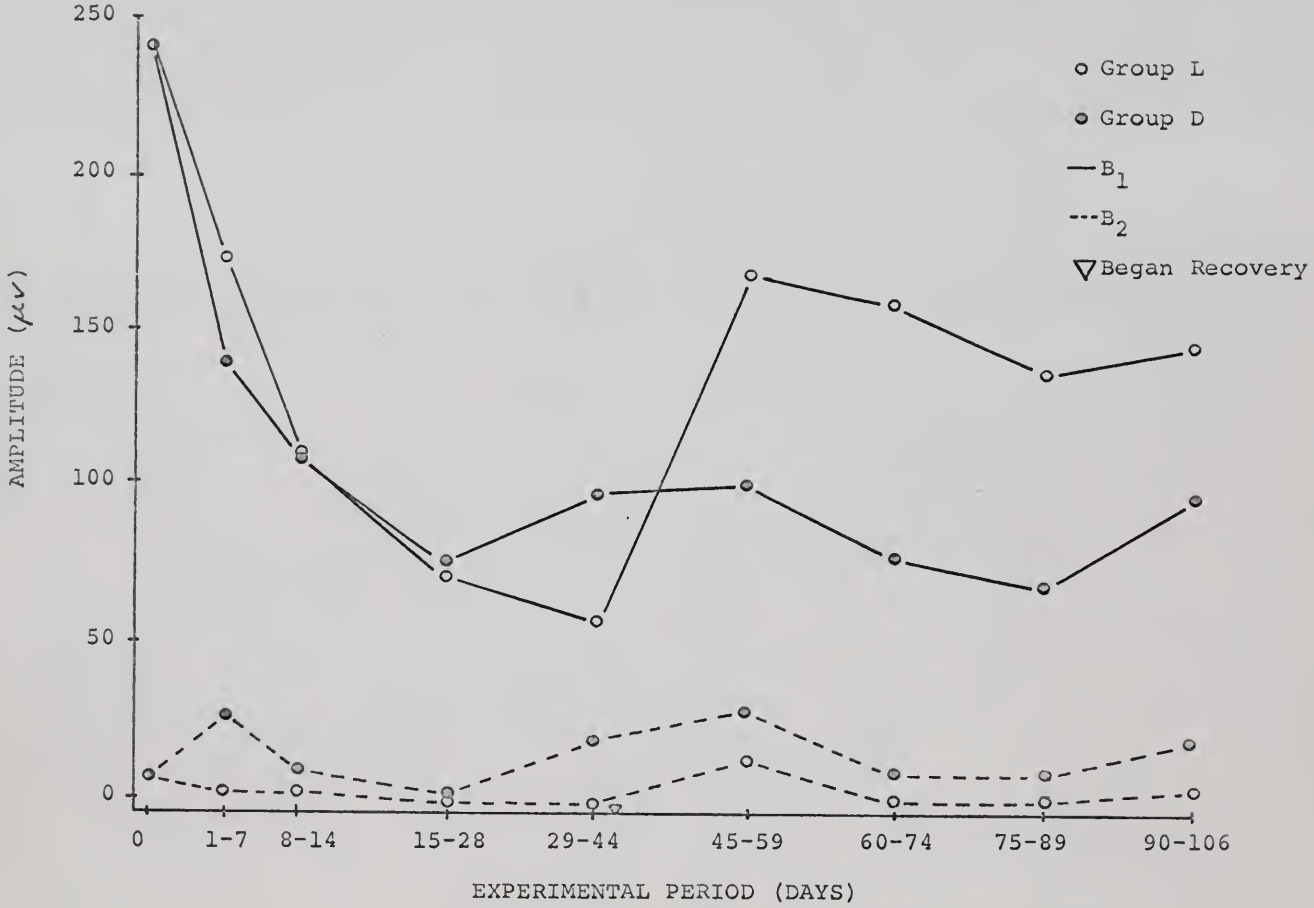

Figure 11.-- B Wave Amplitudes from Groups L and D Under Scotopic Adaptation.

affected by cold stress or was affected more rapidly for group D animals than group L. Cone function, reflected by A_2, appeared to have been only slightly affected or completely spared in group D animals but was totally lost in group L, then showed partial recovery. It was interesting that A_1 for group D animals approached and very nearly approximated A_2 through both cold stress and recovery, again suggesting that these animals lost only rod function. Group L animals, on the other hand, lost both rod and cone function but the latter disappeared earlier and more completely.

As seen in Table 6, summarizing the analysis of variance performed on the A waves separately, these observations were supported statistically. The significant ($p<.01$) main effect, component, further established the difference between the responses to the first and second flash. The significant group x component interaction gave additional weight to the observation that the amplitudes of the two responses were affected differently in the two groups, as described above.

An interaction of this type can sometimes mask one or both of the main effects contributing to it (Winer, 1962, pp 174-175). It seemed unlikely that the groups factor was masked since the two curves for each group were rather uniformly distributed above and below an approximate midpoint in Figure 10. Simple main effects were tested (not presented), however, and the group factor was insignificant at both levels of response.

TABLE 6

SUMMARY OF ANALYSIS OF VARIANCE:
A WAVES FROM GROUPS L AND D UNDER SCOTOPIC ADAPTATION

Source	SS	df	MS	F	Fc
Between Subjects	214.6493	102			
Group	1.3246	1	41.3246	<1	<1
Experimental Period	42.2982	8	5.2873	2.747**	1.765
Group X Period	9.2513	8	1.1564	<1	<1
Subjects within Groups	161.7752	84[a]	1.9246		
Within Subjects	441.7630	103			
Component (Flash)	250.1408	1	250.1408	192.446**	123.710**
Group X Component	60.4907	1	60.4907	46.538**	29.916**
Period X Component	18.7424	8	2.3428	1.824	1.180
Group X Period X Component	3.2023	8	<1	<1	<1
Component X Subjects	109.1868	84[a]	1.2998		

** $p < .01$ [a]correction for control group (Winer, 1962, 263-267)

The B waves were not separately analyzed since observation of Figure 11 indicated that the general form of the curves for B_1 and B_2 were so similar to those for AB_1 and AB_2 that reanalysis would have been redundant, offering no additional information, as well as offering similar problems in interpretation.

Figure 12 presents normalized curves for AB_1 under each of the three adaptation conditions considered thus far for group L. There was a clear tendency for observed cold stress losses to vary directly with adaptation level during recording. To the extent that this shift from photopic through mesopic to scotopic adaptation level could be expected to be accompanied by a corresponding shift from greater relative cone contribution to primarily rod contribution, the data in this figure further supported the conclusion that the cone or photopic system was affected more quickly than the rod or scotopic system. Since the scotopic and mesopic curves had not reached asymptote at the final period of cold stress, suggesting that additional amplitude loss would have occurred, no conclusions could be reached regarding terminal amplitude levels. However, it was noted that some signals were recordable throughout cold stress on the average. A few (3) individual animals failed to generate ERGs recordable at the cornea, under the conditions employed, from as early as day 6 but, with these exceptions, ERGs were recordable throughout the experiment. No differences were seen during recovery in Figure 12.

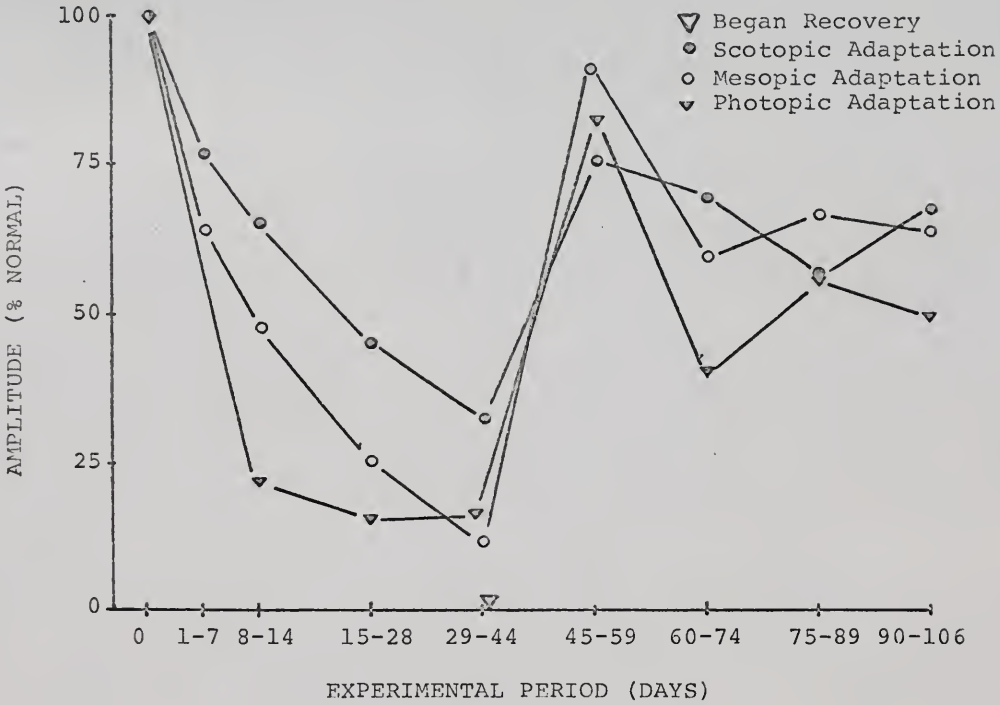

Figure 12.-- Normalized ERG Amplitudes from Group L Under All Adaptation Conditions.

Dark adaptation curves for AB_1 and AB_2 from group L are shown in Figure 13. The upper curve of each pair represents data for the AB_1s, the lower that for the AB_2s. Curves were fitted to the data points visually. Because of the wide intervals between samples during the dark adaptation process, the point of the shift from the photopic to the scotopic arms was usually missed, thus in these curves was extrapolated. Reference to the summary table (Table 7) of the ANOV of these data showed that all main effects and interactions were significant. Several of these effects were not of particular interest or could not be directly interpreted. The familiar change of ERG amplitude during dark adaptation, time in dark adaptation ($p<.01$), was expected and not of interest. The significant ($p<.01$) response effect indicated that the response to the second flash was severely reduced in dark adaptation. This effect could be seen clearly in control (cold stress period 0, Figure 13) animals. Rather than maintaining a stable photopic level as was expected, AB_2 showed a progressive attenuation, well correlated with increasing scotopic function in normal controls. Therefore, the significant response effect apparently resulted from normal functions unrelated to experimental variables under consideration, although this effect was somewhat enhanced during cold stress.

The significant ($p<.01$) cold stress effect reflected the change in maximum amplitude reached during dark adaptation. This effect could be seen in Figure 13 as a reduction

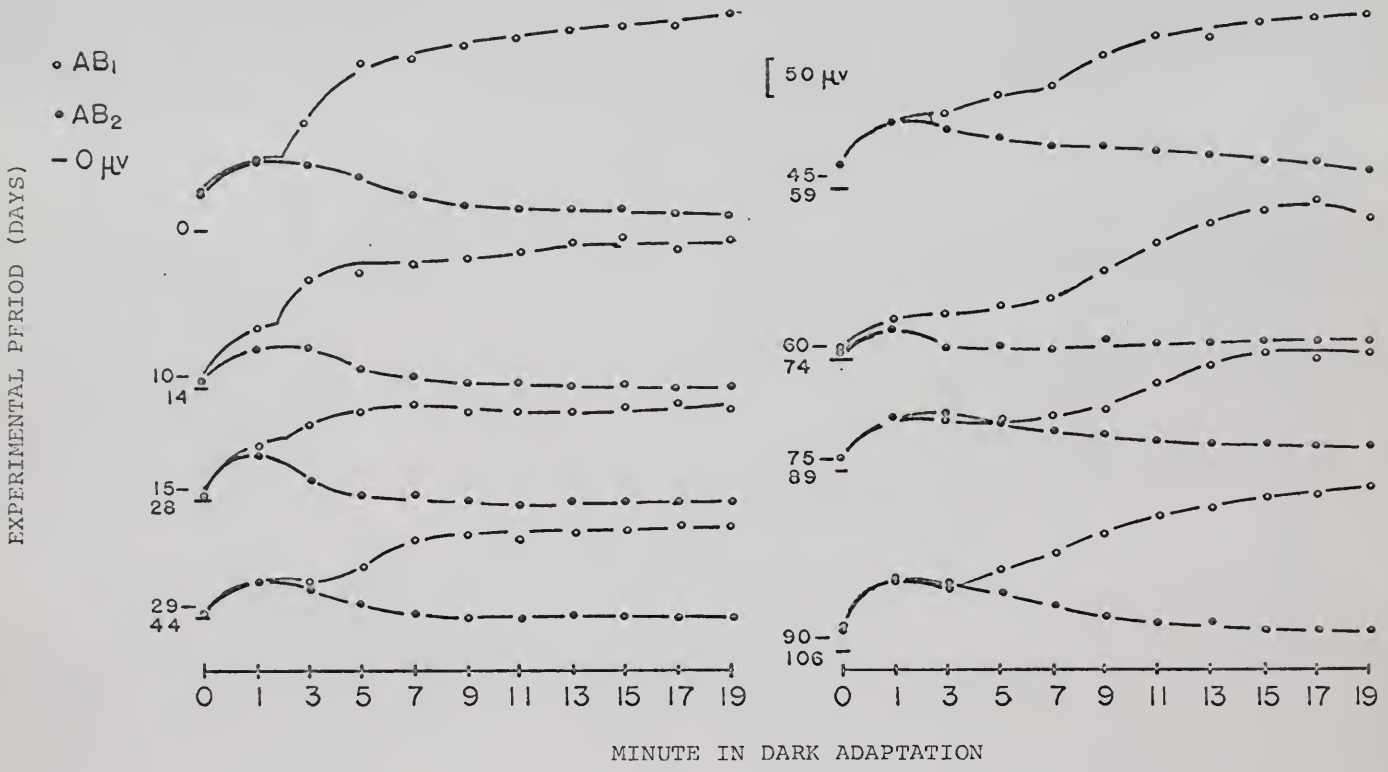

Figure 13.-- Dark Adaptation Curves from Group L.

TABLE 7

SUMMARY OF ANALYSIS OF VARIANCE:
ERGS FROM GROUP L THROUGH DARK ADAPTATION

Source	SS	df	MS	F	Fc
Between Subjects	10,909.4486	50			
Experimental Period	5,562.7268	7	794.6752	6.391**	5.202**
Subjects within Groups	5,346.7218	43	124.3423		
Within Subjects	39,975.5569	1071			
Minute in Dark Adaptation	326.4344	10	32.6434	8.399**	6.836**
Period X Minute	567.0436	70	8.1006	2.084**	1.696**
Minute X Subjects	1,671.2976	430	3.8867		
Response (Flash)	18,859.1800	1	18,859.1800	205.8869**	167.582**
Period X Response	1,932.3754	7	276.0536	3.014*	2.453*
Response X Subjects	3,938.7912	43	91.5997		
Minute X Response	6,399.6055	10	639.9606	128.5655**	104.668**
Period X Minute X Response	1,205.4025	70	17.2200	3.459**	2.816**
Minute X Response X Subjects	2,140.4267	4	4.9777		

*p < .05
**p < .01

in dark adaptation curves through cold stress and increase during recovery.

For reasons discussed above, with respect to the scotopic data, interactions involving the response effect cannot be interpreted directly. The significant ($p<.01$) period in cold stress and recovery x time in dark adaptation interaction indicated that the cold stress effect was stronger on some portions of the dark adaptation curve than others. This effect may have resulted from the tendency for the middle portion of the dark adaptation curves to be rather distorted during recovery. Thus, minutes 3 through 9 were selectively reduced during recovery but not selectively during cold stress. From Figure 13, it appeared that this distortion may have been the result of a delay in the development of the scotopic arms of these curves. This was particularly notable in the two intermediate periods in recovery (days 60-74 and 75-89).

Figures 14-16 present tracings of ERGs recorded from an animal on days 0, 15 and day 60. These records were chosen because they illustrated two peculiarities characteristic of dark adaptation records of a relatively large percentage of the animals recorded during cold stress and recovery. The first of these peculiarities was the abnormal increase in A wave amplitude during the first minute following extinction of the adaptation lights, sometimes accompanied by complete disappearance of B waves, as seen on day 15, and sometimes with relatively well developed

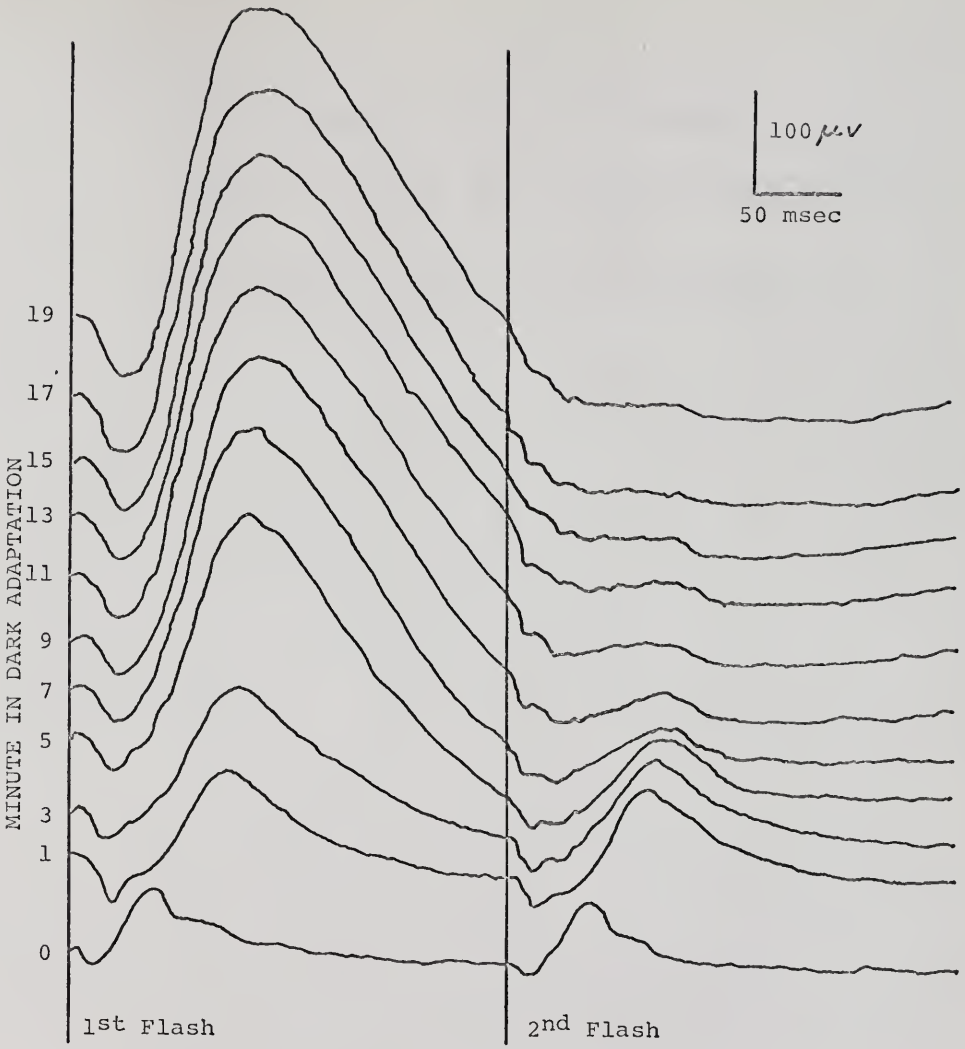

Figure 14.-- ERG Development During Dark Adaptation; Day 0.

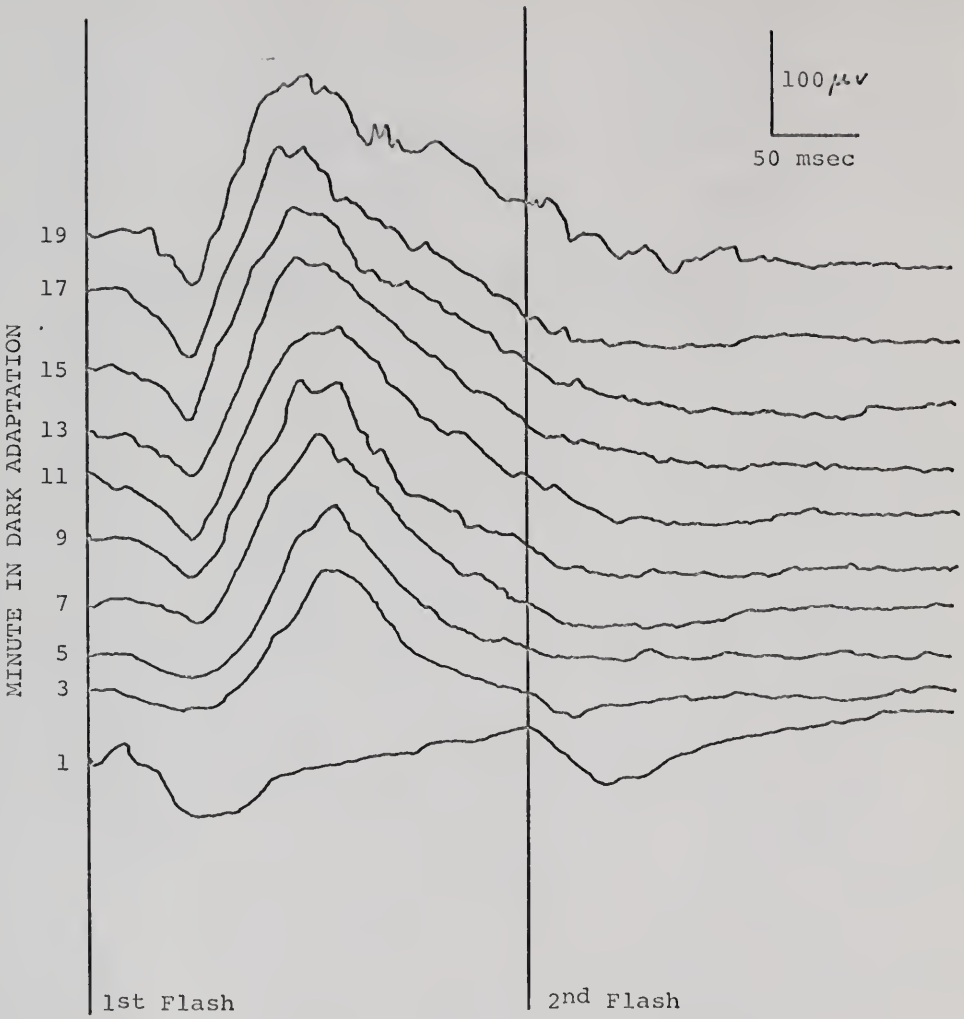

Figure 15.-- ERG Development During Dark Adaptation; Experimental Day 15.

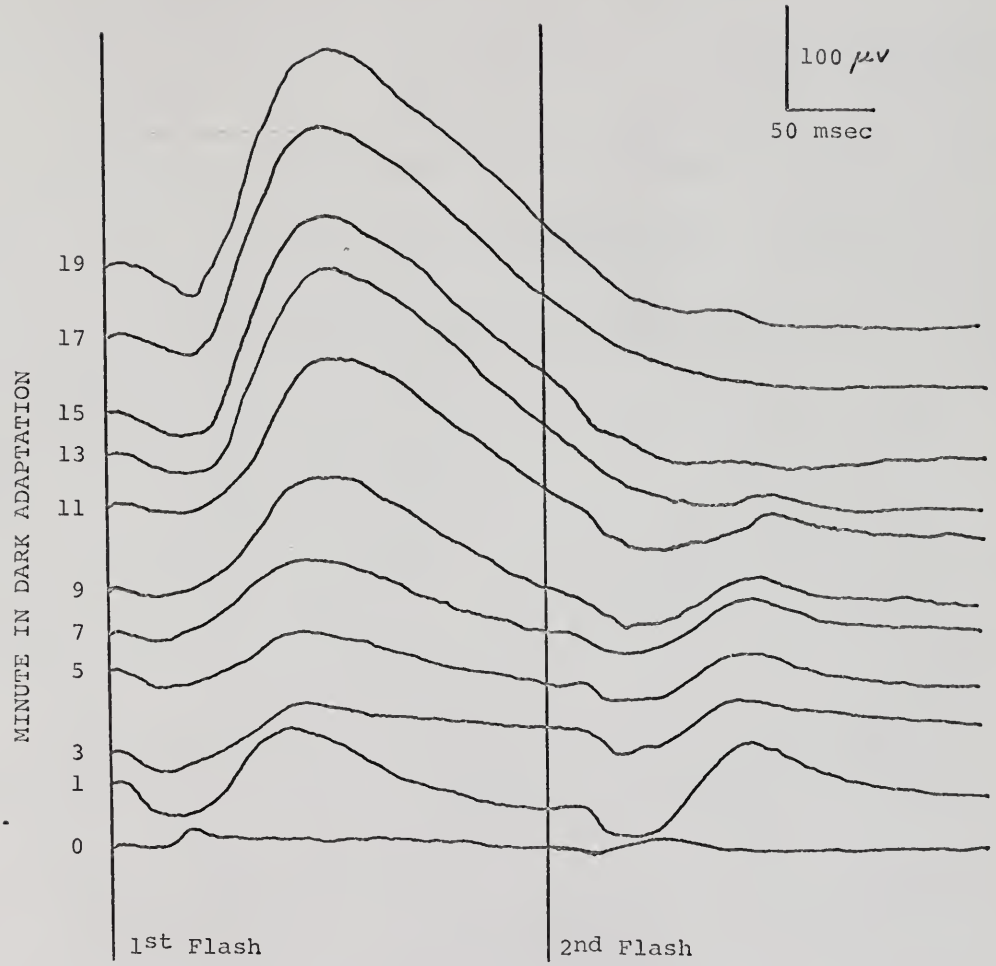

Figure 16.-- ERG Development During Dark Adaptation; Experimental Day 60.

B waves as on day 60. The second feature of these records was the long period of stability of B wave amplitude before the rapid increase beginning at about the ninth minute of dark adaptation in day 60 records. This rapid increase corresponded to the scotopic arm of the dark adaptation curves in Figure 13. The sluggish development of the scotopic B waves in the absence of diminished A wave activity suggested that the neural tissue subsequent to the receptors incurred permanent or long term functional loss as a result of cold stress.

Each of Figures 17, 18 and 19 presents the amplitude change during cold stress and recovery of the ERG evoked by the first flash at zero attenuation for animals in groups R, B and RB respectively. Each curve in these figures represents one of the three monochromatic stimuli utilized. Differential effects of environmental lighting conditions on sensitivity to any of the monochromatic stimuli would have appeared as inversions among the three curves for each group. No clear inversions were seen although there was a weak tendency for the curve for responses to the 430 nm stimulus to approximately equal those to the 630 nm stimulus for group R (Figure 17) during cold stress. In Figure 18 it could be seen that this was not the case for group B, as the responses to the blue, 430 nm, monochromatic stimulus were of consistently lower amplitude than those to the red, 630 nm stimulus. Group RB responses to the two stimuli were intermediate with respect to these relationships. The

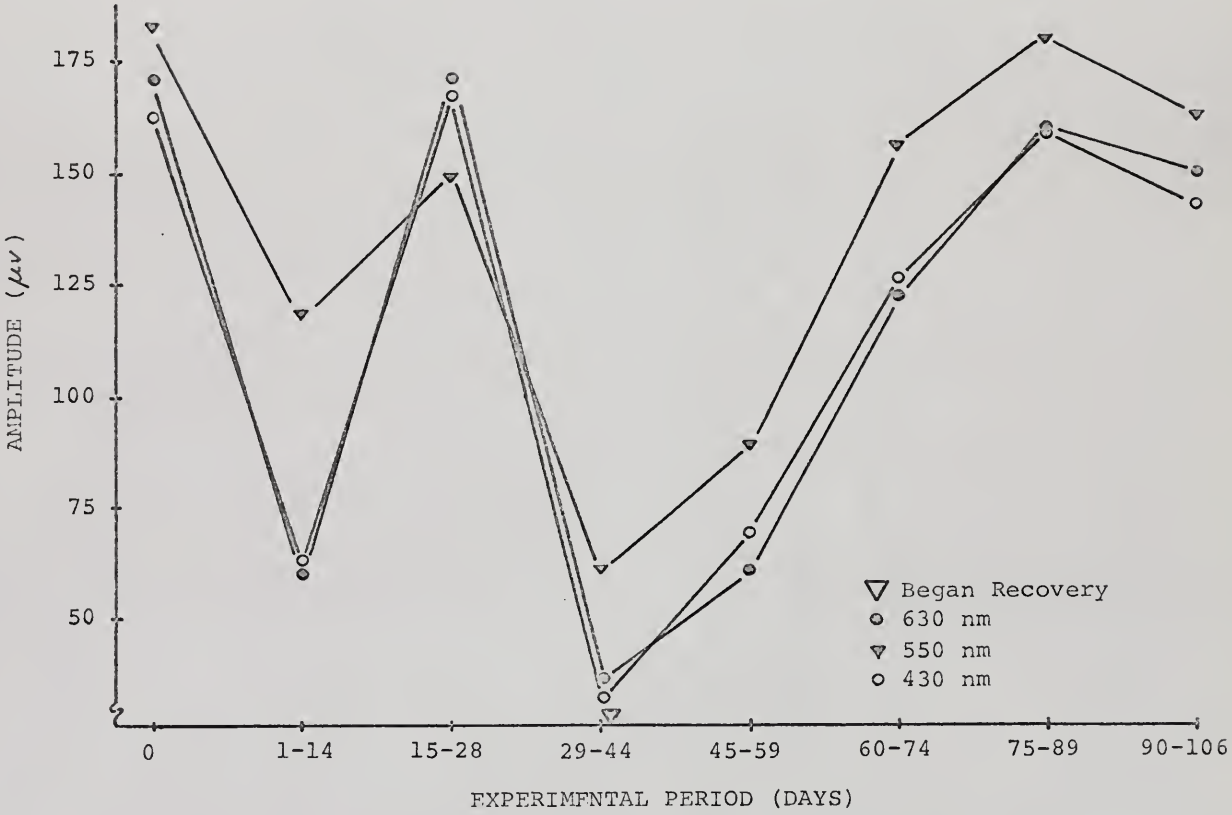

Figure 17.-- ERG Amplitudes from Group R to Each Monochromatic Stimulus.

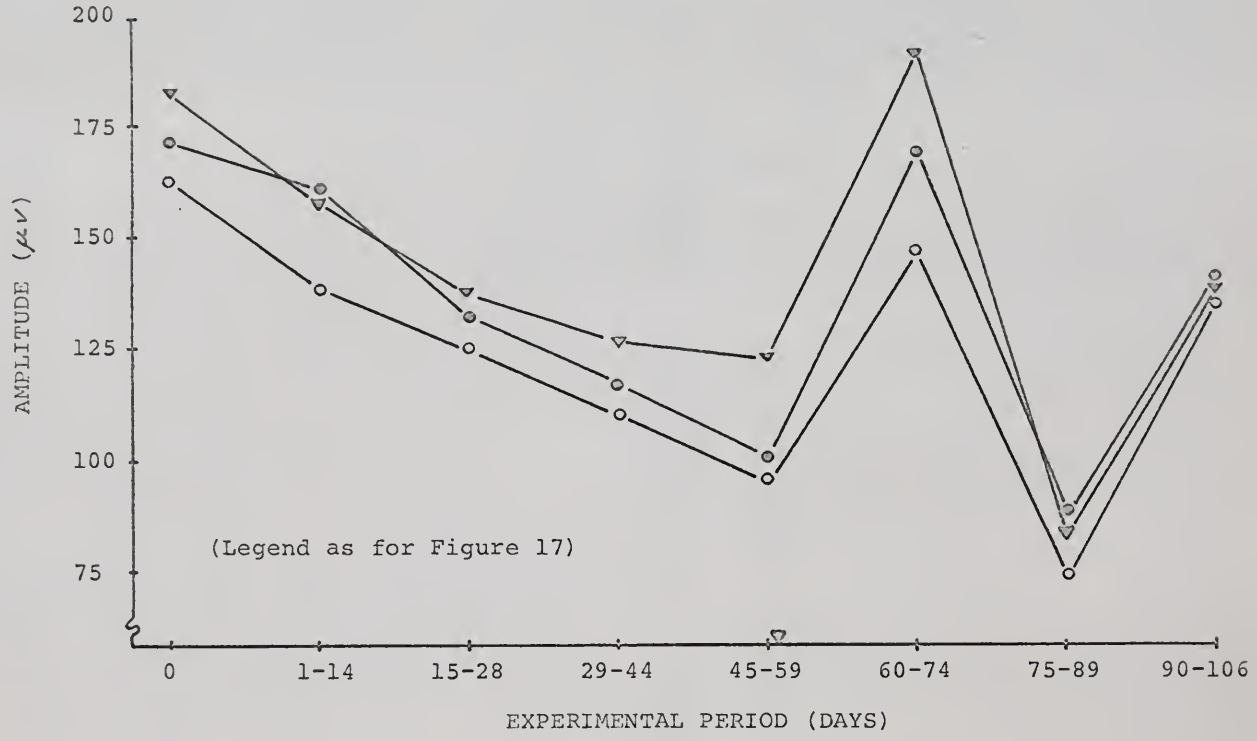

Figure 18.-- FRG Amplitudes from Group B to Each Monochromatic Stimulus.

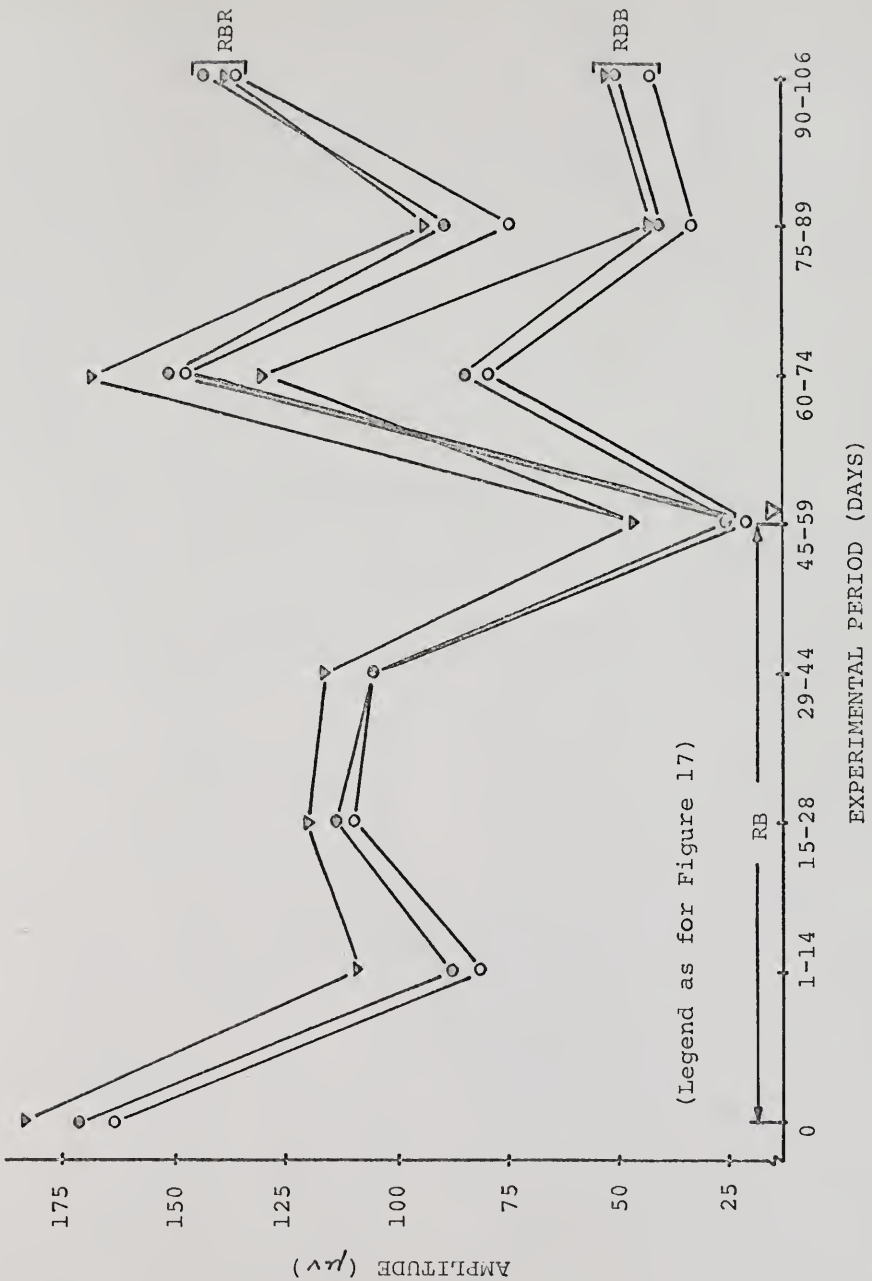

Figure 19.-- ERG Amplitudes from Groups RB, RBB and RBR to Each Monochromatic Stimulus.

differences seen for groups B and RB for the two stimuli during cold stress are not abnormal with respect to those seen in day 0, or normal animals, suggesting that if there was an influence due to wavelength composition of environmental lighting it was primarily an effect of red environmental light on ERG determined sensitivity to red stimuli. The statistical significance of this effect was estimated by calculating the mean difference between responses to the 630 and 430 nm stimuli throughout cold stress, then performing a t test on the difference between these means (Winer, 1962, pp 36-39) for groups R and B. These differences were not significant ($p>.10$). The effect seen in the curves therefore was, while fairly consistent, not of sufficient magnitude to warrant reaching a conclusion regarding the differential effect of spectrally restricted environmental light during cold stress on ERG determined spectral sensitivity.

Following the two weeks exposure to bright white illumination, group RB was divided and the two groups of fish were distributed between the other two environmental spectral lighting conditions for recovery. Group RBR, RB fish recovering under group R conditions, showed recovery whereas group RBB recovering with group B, appeared to show little or none. The difference between means for groups RBR and RBB was found to be significant (t test, $p<.05$), adding weight to the observations above. Apparently, recovery did not occur in group RBB animals to the extent that it did in RBR. Comparison of Figures 17 and 18 suggests that there

was a very weak tendency toward a similar difference between groups B and R. This was not tested statistically as the difference was small and the variance in the group B records during recovery suggested that testing was not warranted. The clear effect seen in the difference between groups RBR and RBB suggested that blue light during recovery tended to depress ERG determined recovery of function. The similar comparison between groups R and B in recovery, while adding little or no support, did not contradict this conclusion.

Several factors of note could be seen in the data taken from group RB and its two derivatives, RBR and RBB (Figure 17). Exposing group RB to bright white light on day 45 resulted in a drop in amplitude over the succeeding 14 days. Control animals subjected to this procedure without the influence of cold stress showed an insignificant change in ERG amplitude relative to normal controls ($p>.20$). The amplitude change with additional bright light, day 59, was tested against day 45 amplitudes and found to be ambiguous in this respect ($.10>p>.05$). Thus, there was no clear basis for a conclusion with respect to the effect of additional light in cold stress.

Consideration of the data from the three groups jointly produced two additional observations. The general form of these curves was very similar to that of the majority of the curves in preceeding figures showing data for other groups under various stimulation conditions. With the

exception of one datum point, days 15-28, in the group R data, the curves show an orderly decline during cold stress and less orderly increase in recovery. The initial overshoot in recovery noted in the data from groups L and D could also be seen in these data. A tendency for group B to show an orderly ERG amplitude decrease in cold stress and disorderly recovery and overshoot, with group R showing just the opposite suggested the possibility of complex effects of environmental stimulation. These tendencies were obviously weak and neither reliability nor statistical significance were demonstrated, therefore, no conclusions were warranted.

Morphology

Figures 20 through 22 present the results of cell counts from the ganglion cell, inner and outer nuclear layers and of ellipsoids, respectively. Individual curves in each of these figures were for central or peripheral loci for animals from group L or D. Data points represented means for animals sampled during the 14 day ($\pm$2) period indicated on the abscissa. Ordinate values were in terms of cells (or ellipsoids) per 10^{o} angle (geometric, not visual) on the histological specimens.

Clearly, there was no systematic variation in these curves which could be attributed to the experimental variables under consideration. In light of the obvious absences of effect seen in these figures, statistical analysis of these data was not undertaken.

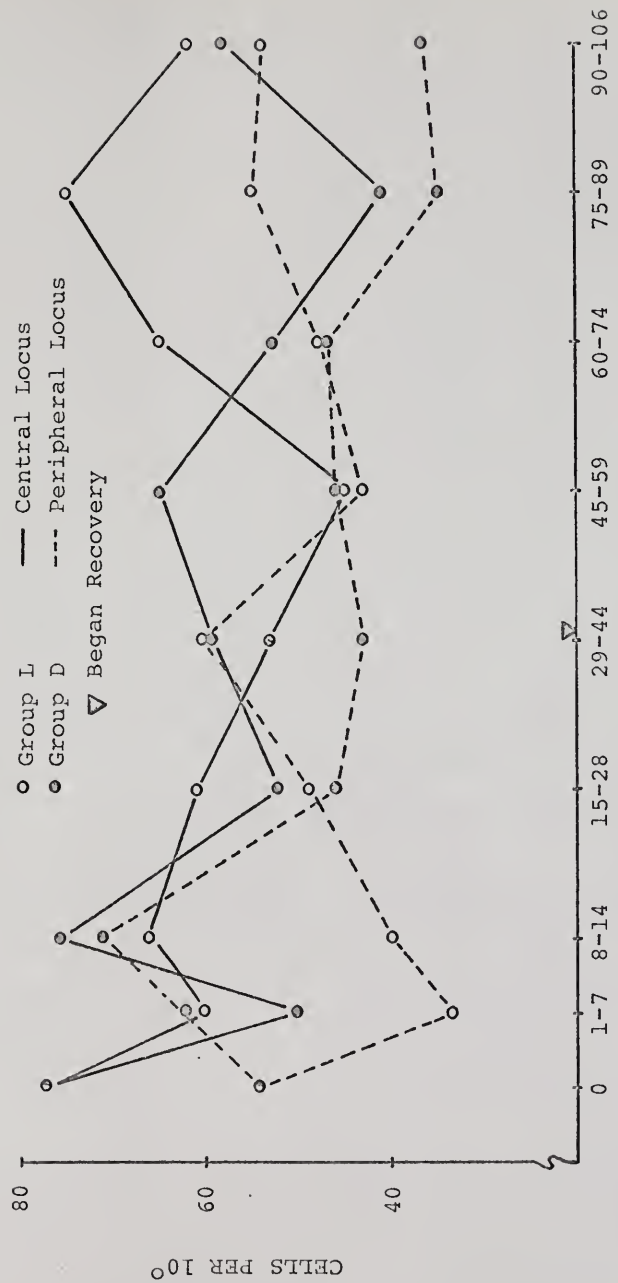

Figure 20.-- Ganglion Cell Frequency in Retinae of Groups L and D.

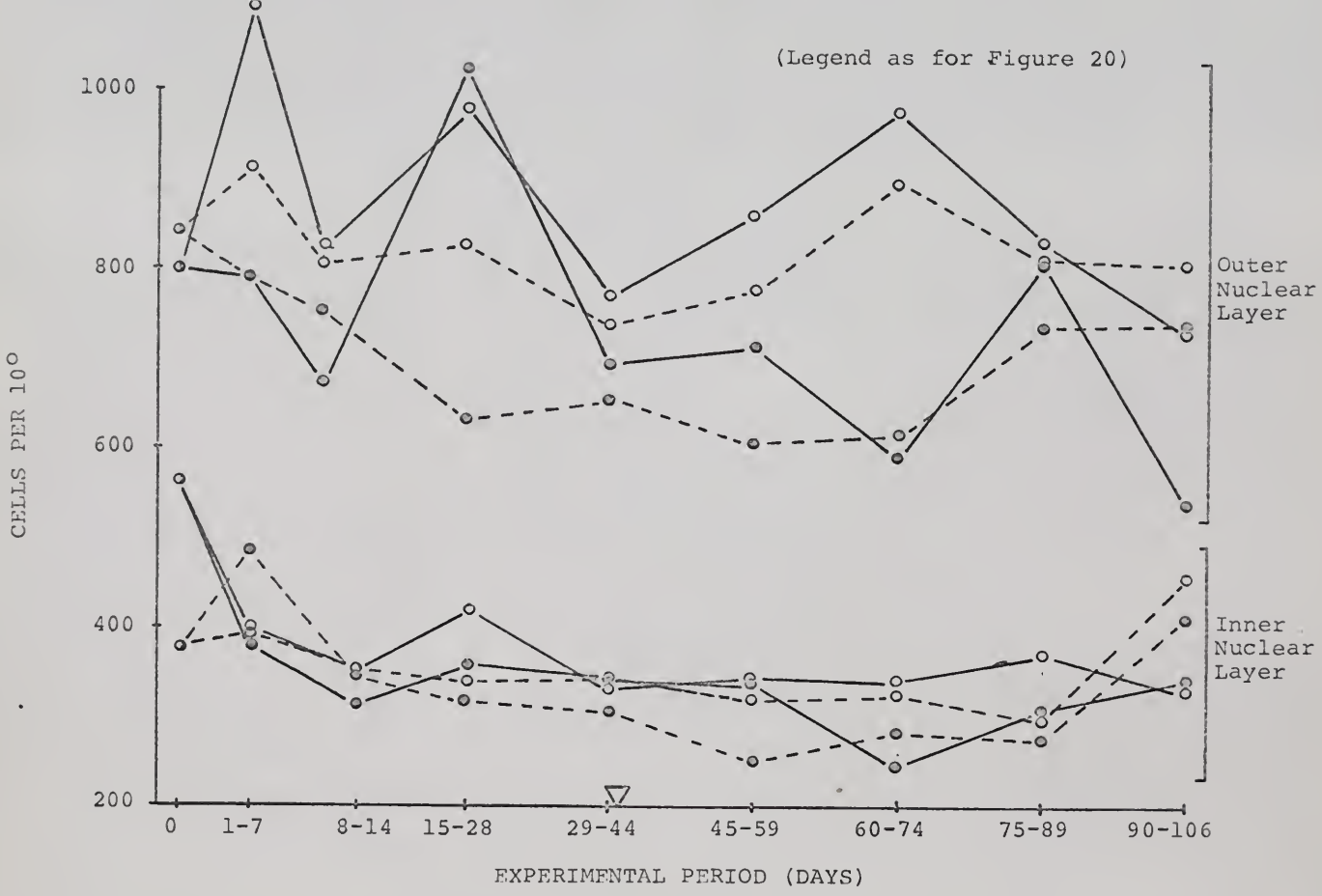

Figure 21.-- Cell Frequency in the Inner and Outer Nuclear Layers of Retinae from Groups L and D.

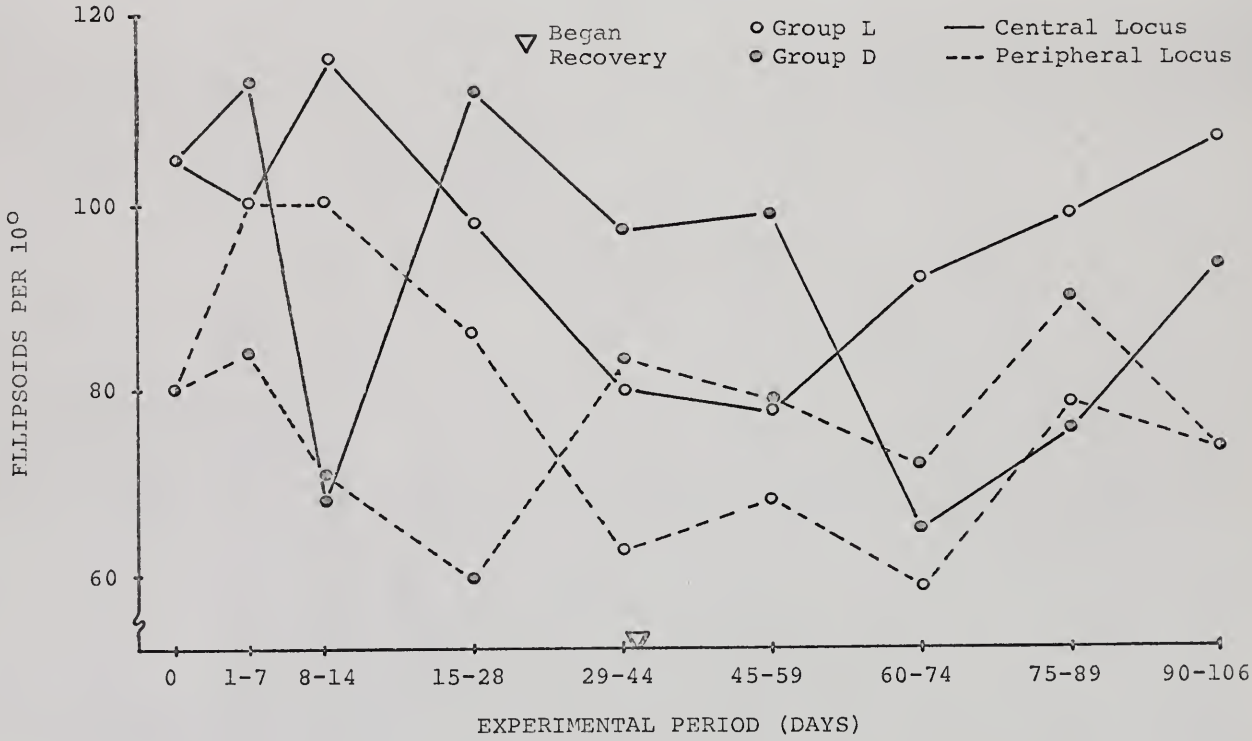

Figure 22.-- Ellipsoid Frequency in Retinae from Groups L and D.

Figure 23 presents the results of outer segment counts taken from these same specimens. Values on the abscissa and individual curves were as described above for Figures 20 through 22. The dependent variable (ordinate) in Figure 23 was the ratio of detectable outer segments to ellipsoids present in the portion of the histological section for which counts were made. That there was a decrease in this ratio during cold stress and an increase to normal, or near normal, in recovery was apparent in this figure. There was also a suggestion that group D animals showed somewhat less improvement than group L during recovery, although the difference was slight and obvious recovery did occur.

The statistical analysis (ANOV) summarized in Table 8 supported the observation that outer segments were being progressively lost during cold stress and were increasing in number during recovery. The locus and group effects were not significant, implying that differences seen in Figure 23 in recovery for the two groups were very weak and that there was little difference between the central and peripheral loci with respect to outer segment loss.

In Figure 24, these data have been averaged across retinal locus, normalized, and plotted along with normalized scotopic ERGs in order to allow direct comparison of functional and morphological data. The correspondence in form was remarkable for group L curves, however, the failure of group D ERGs to recover differed noticeably from the apparently full morphological recovery in this group. This effect could be

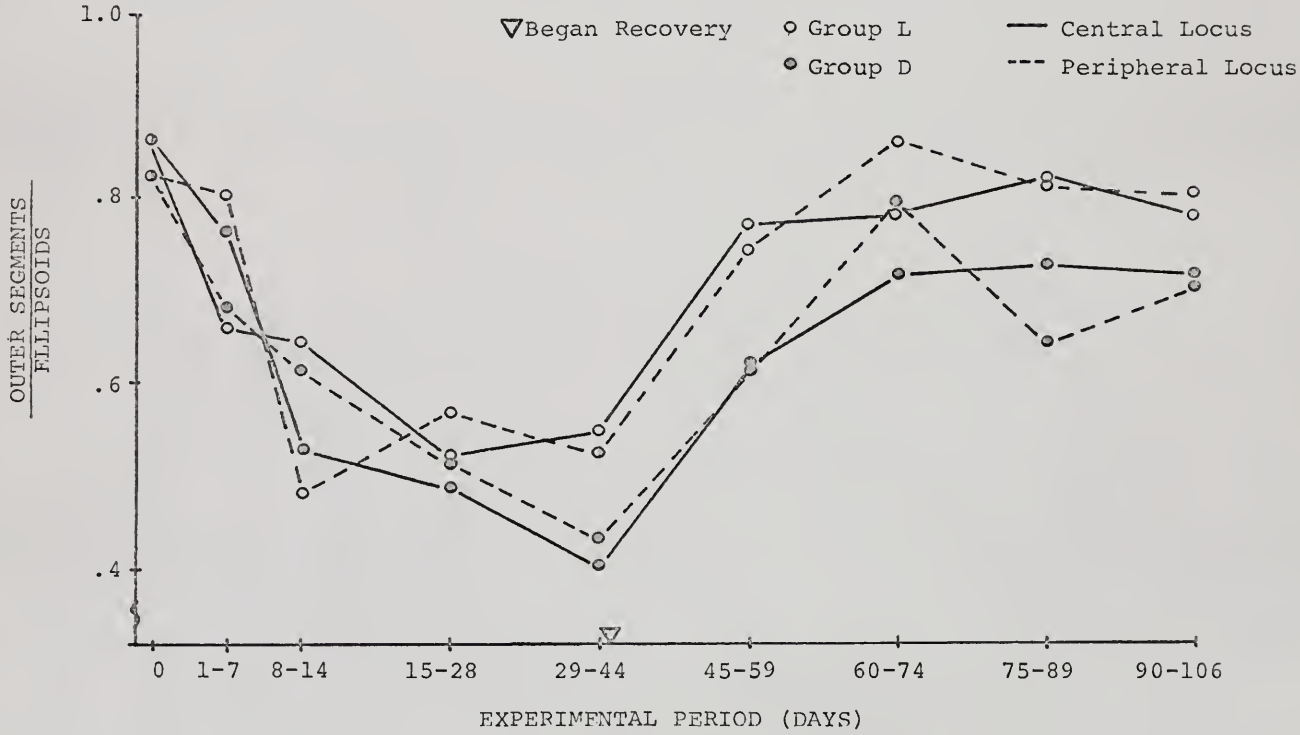

Figure 23.-- Outer Segment to Ellipsoid Ratios for Retinae from Groups L and D.

TABLE 8

SUMMARY OF ANALYSIS OF VARIANCE:
OUTER SEGMENT TO ELLIPSOID RATIOS

Source	SS	df	MS	F
Between Subjects	200.9285	45		
Group	5.8125	1	5.8125	3.630
Experimental Period	135.5546	7	19.3649	12.092**
Group X Period	13.1214	7	1.8745	1.171
Subjects within Groups	46.4400	29[a]	1.6014	
Within Subjects	26.3957	45		
Locus	.0423	1	.0423	<1
Group X Locus	.0061	1	.0061	<1
Period X Locus	3.1146	7	.4449	<1
Group X Period X Locus	.8085	7	.1155	<1
Locus X Subjects	22.4242	29[a]	.7732	

** $p < .01$

[a]correction for control group (Winer, 1962, 263-267)

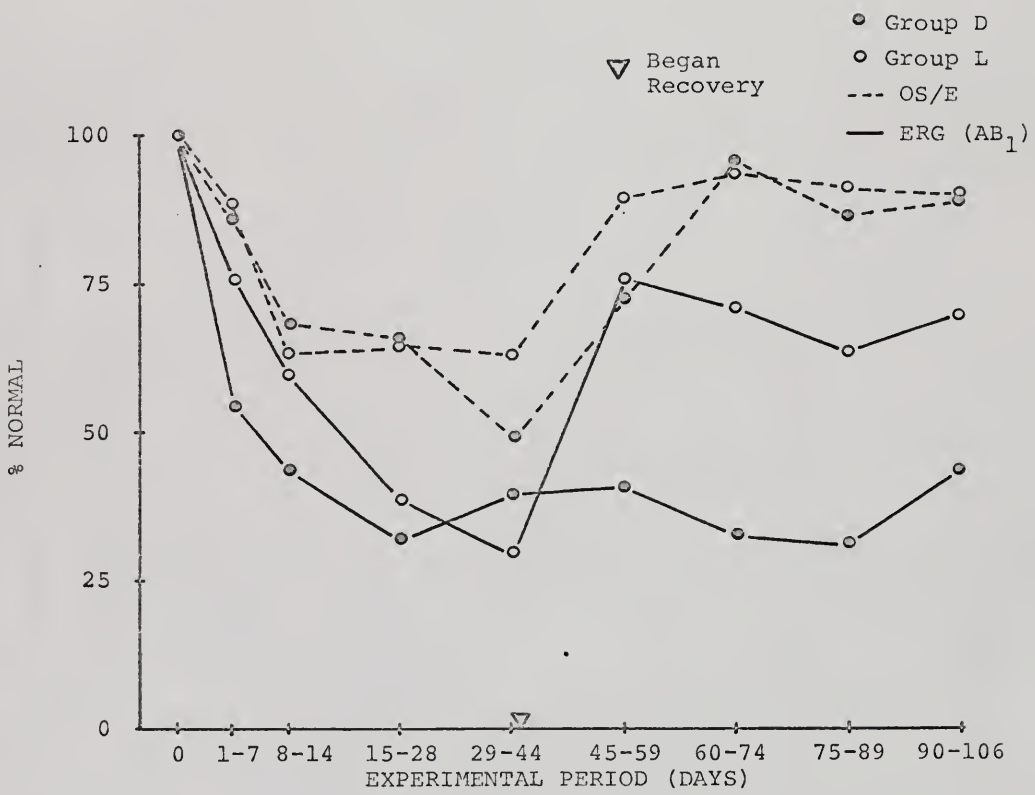

Figure 24.-- Normalized Outer Segment to Ellipsoid Ratios Compared to Normalized Scotopic ERGs in Response to the First Flash for Groups L and D.

seen to a lesser extent in group L as well. These comparisons strongly suggested a dissociation of structure and function in recovery which was further intensified by the absence of environmental illumination.

Outer segment to ellipsoid ratios, for animals in the spectral groups, for receptors in the migrated and unmigrated position are presented in Figure 25. For the purpose of generating these histograms, data were averaged across cold stress and recovery for each group. The relative outer segment to ellipsoid ratios for migrated and unmigrated cones in eyes from groups R, B and RB during cold stress were as would be expected if red environmental light was resulting in selective loss of cone outer segments of receptors maximally sensitive to red light. Testing the difference between the migrated mean outer segment to ellipsoid ratio for groups R and RB, both of which received red light during cold stress, against the mean ratio for migrated receptors from group B, which did not receive red light, indicated that this effect was significant ($p<.025$; t test, Winer, 1962, pp 36-39). A similar test for unmigrated cones, groups B and RB against group R, indicated no difference ($p>.10$). During recovery, only group RBB gave any indication of differential loss. Unmigrated ellipsoids had relatively fewer associated outer segments than migrated ellipsoids. This effect was not statistically significant ($p>.10$), however, when the mean difference was tested.

While the expected effect on migrated receptors for

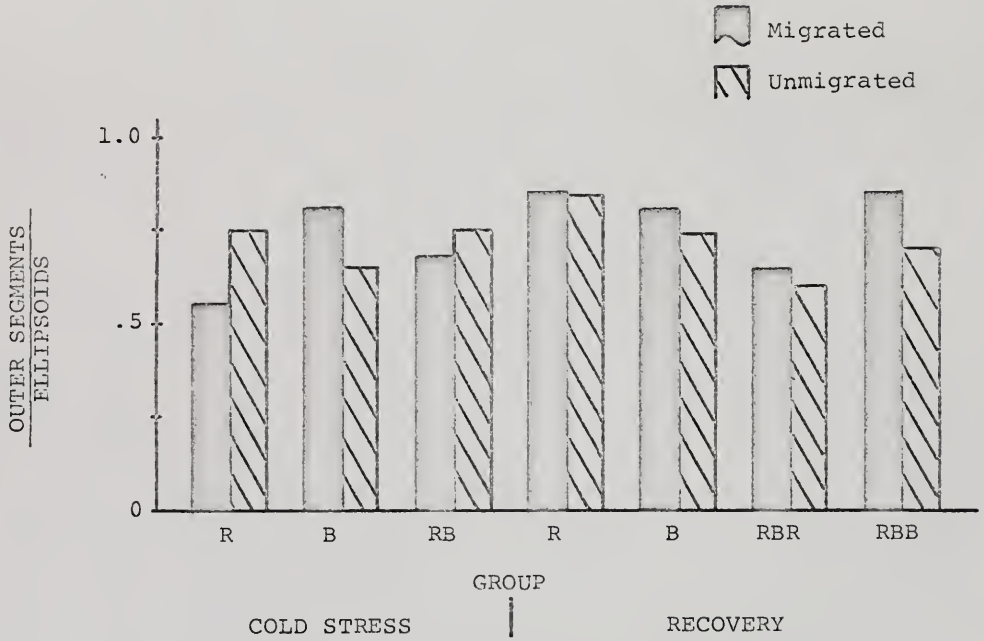

Figure 25.-- Outer Segment to Ellipsoid Ratios for Migrated and Unmigrated Cone Receptors for all Spectral Groups.

fish receiving red light during cold stress was fairly clear in the data, conclusions concerning its reality were judged to be unjustified. The histological sections from which these counts were made showed evidence of having been subjected to mechanical stress, probably in sectioning. The effect of this stress was to produce either shearing or tensil forces, or both, which acted primarily on the layer of the receptor inner and outer segments. This factor produced two effects on the receptors, disruption of the normal migrated-unmigrated positions and separating the outer segments from their ellipsoids. The former effect resulted in some degree of ambiguity in assigning a counted ellipsoid to one or the other of the two classes and the latter rendered it necessary to assign outer segments to ellipsoids. In conjunction, the effects sometimes reduced the judgments to sheer guesswork. While the majority of the sections did not produce problems in this respect the number which did was not insignificant. Under these conditions the possibility of spurious results could not be eliminated. These data were included, however, because of their suggestive nature with respect to avenues for future research and with respect to the present research.

The preceding criticism did not apply to data from groups L and D. Because there was no requirement that outer segments be attributed to a given ellipsoid, only that there be detectable outer segments, and because position was irrelevant, these effects did not affect those data.

DISCUSSION

On the basis of evidence presented in the preceding section, several conclusions seemed justified. First, the time course of the effect of cold stress and its withdrawal on the electrophysiological function of the retina and the structure of cone outer segments was orderly and has been partially defined. The evidence further suggested that this time course was different for rod and cone function, and that these differences were modified by environmental lighting conditions. Cone function deteriorated more rapidly with environmental light but was spared when animals were deprived of light. In general, rod function was lost under both conditions but appeared to suffer reduction more rapidly in the dark than in light. An additional effect of environmental lighting was seen in recovery. Animals in blue, following very bright light in cold stress, and those in the dark during recovery failed to recover. In this respect, it should be noted that, of the environmental lighting conditions other than dark, the blue light condition provided the least illumination.

Utilizing A and B wave components of the ERGs as indicators of receptor and neural (non-receptor) function, respectively, revealed that receptor function appeared relatively less affected than neural function, however, the latter showed better recovery. While the receptor-neural dichotomy has been assumed for the purpose of analyzing the

data from this experiment, Tomita has recently (1970) presented evidence, his own and that of others, showing that the A wave probably consists of two components. The first of these (distal P III), recorded in the receptor and outer nuclear layers, appeared to be clearly a receptor potential. The second (proximal P III) was unaccounted for within this region but was not localized. Additionally, chemical poisoning selective for neural tissue abolished the proximal P III but not the distal component. While this work clearly indicated that the A wave did not reflect purely reception and/or transduction, neither did it establish that the proximal P III component was of extra-receptor origin, since the region at which it was recorded apparently included the outer plexiform layer. Since this is the region of synaptic contact between cells in the inner nuclear layer and the receptors, the possibility of this site as the origin of the proximal component cannot be ruled out. In addition, the distal P III component appeared to account for the bulk of the A wave, although not all. The B wave, on the other hand, has been adequately localized in the inner nuclear layer and reflects activity of cells (probably bipolar) of that layer (Brown, 1968; Witkovsky, 1971), and is well correlated with psychophysical function (Witkovsky, 1971). Thus, practically speaking, use of the A and B wave components to estimate differential effects on receptor and neural function seemed justified even though the dichotomy may not have been absolute.

The effects of cold stress on dark adaptation were complex. The general effect was to reduce both the photopic and scotopic arms of the dark adaptation curves during cold stress and recovery. A number of peculiarities were seen in the individual records, two of which were pointed out. The very large A wave during the first minute of dark adaptation was very prominent throughout both cold stress and recovery. Brown and Watanabe (1965) reported that some A wave amplitude reductions, for example in light adaptation, may have been the result of decreasing B wave latency. That is, the positive B wave, being larger, obscured the A wave progressively, giving the appearance that A wave amplitude was being reduced. With intraretinal recordings, the A wave was seen to remain at constant amplitude whereas the conventionally recorded A wave showed reduction. This factor cannot account for the effect described in the preceding section, however, because this effect was seen with the B wave present. In addition, if this were the basis for the effect it would be expected to show up more clearly in the 0-minute records. While the explanation of this effect is obscure, it does not seem to lie in reduced B wave effects. Similarly, the sluggish development of the scotopic arm is difficult to explain. Simple amplitude reduction could be rather easily explained by proposing, for example, that a portion of the rods were permanently lost, however, the basis for delayed development of the scotopic activity was totally obscure. This effect, in conjunction with the

reduced amplitude generally, suggested that the effects of cold stress were more complex than originally thought, going beyond simple loss of receptor outer segments.

One rather surprising result of the present research was the failure to see systematic effects due to restricted spectral stimuli during cold stress and recovery. While group RBB displayed failure to recover, there were no detectable differential effects otherwise. Probably this was the result of the relative difficulty in detecting differences in monochromatic stimuli using the ERG as the criterion (Burkhardt, 1968). It was also possible that environmental light levels were not sufficiently high to provide the degree of dissociation required in order to distinguish differential effects with the relatively gross ERG as the dependent variable. This seemed unlikely as there was clear loss of ERG amplitude generally and this was also seen to occur in the dark with cold stress. The illumination levels in the aquaria containing groups R and RB were roughly equivalent to that for group L, further reducing the feasibility of light level as the critical factor. Use of monochromatic adaptation with monochromatic stimulation (e.g. Witkovsky, 1968; Burkhardt, 1968) would likely have provided added sensitivity to the electrophysiological procedure. In the present work, however, in which time was one of the variables, the threefold increase in time required to run spectral animals under this procedure was impractical.

One prominent feature of the present work was an obvious discrepancy with respect to previously reported results (Dawson et al., 1971; Hope et al., 1970; Dawson et al., 1969) of experimentation on cold stress effects on the goldfish retina. Comparison of results of the two studies indicated several important differences. The general effect of cold stress in the present work was much less severe than previously seen, both with respect to electrophysiology and structure. ERGs recorded in the present experiment never completely disappeared. The curves for these losses appeared to have reached asymptote at about 15% normal at the lowest. The earlier work suggested that no recordable electrical activity should have been present after the fourth day of cold stress. Additionally, electrophysiological recovery in the previous work was only to a level roughly equivalent to the amplitudes seen at maximum loss in the present study. Differences of roughly the same magnitudes were seen in outer segment loss, with only minimal recovery of these structures. Ellipsoid losses were also incurred by animals in the previous experiments but not in the present one. These differences indicated that cold stress effects in the present experiment were significantly ameliorated.

There were a number of differences in procedural and methodological factors, any or all of which could have contributed to the amelioration. The present study incorporated a much longer period of adaptation prior to final

temperature reduction. The ambient light levels were different, with the previous experiments conducted in an environment providing roughly 8.8 (vs. 2.0 in the present work) lumens / ft.^{2}. Temperature control in the previous work was more consistent within the cold stress range but, on occasion, as a result of mechanical difficulties, did rise briefly to normal room level. This never occurred in the present study because of the different means of cooling the water in the aquaria. In the present work, however, the daily temperature fluctuation was over the entire range defining cold stress, 4-8^{o} C.

The differences in recording procedure may have contributed to the differences in signal amplitude. Water used to respirate fish in the previous experiments was chilled to approximately the level of the water in the aquarium, or about 5-8^{o} C. Perfusing water in the present experiment was at room temperature, 20^{o}-22^{o} C. Fish in the earlier efforts were anesthesized prior to recording (M.S. 222, Finquel, Ayerst Laboratories) and injected with a neuromuscular blocking agent (Flaxedil, Davis and Geck).

Under the recording procedures utilized in the Dawson et al. experiments the anesthetic should have cleared the animals' systems sufficiently to allow its dismissal as a contributing factor. Infrequent use, in the present work, of the same neuromuscular blocking agent, always in the course of the recording procedure when movement artifacts became evident, indicated that this did not

result in detectable signal loss. Previously published data indicated that low temperature had a suppressing effect on electrophysiological signals, but this suppression was a joint function of acclimation level and recording temperature (Prosser, 1968). From those data, it appeared that in order to achieve a nerve block in fish acclimated to 5--8° C the perfusing water would have had to have been at about 0° C. Additionally, a control procedure, incorporated into the previous experiments, suggested that to 5°-8° C, the temperature of the perfusing water had no significant effect on ERG amplitudes, even without prior low temperature acclimation.

Thus, it appeared unlikely that recording procedures alone could have accounted for the differences in electrophysiology noted above, and certainly not for morphological effects. On the other hand, data recorded from the partial replications of these experiments (not shown) indicated that signal loss was intermediate to that seen in the original experiments and in the present work for group L animals. The savings seen in the replication subjects relative to those in the original experiments must have been attributable to one of the factors discussed immediately above or to possible seasonal variations. The original experiments were conducted in the late fall and late winter into early spring. The replications, on the other hand, were conducted in midsummer. Witkovsky (1968) has noted seasonal variations

in the ERG of the carp but they were in the opposite direction from those required to account for the noted anomalies.

The savings of animals in the present experiment relative to those in the replication were presumably the result of either environmental light level or temperature control. In general, the modest effects of manipulation of light level in the present experiment suggested that this factor could not be invoked to account for the discrepancy. For example, group RB animals, which received over 2000 lumens / ft^2, showed approximately the same terminal electrophysiological loss as did the replication animals, which received only 8.0 lumens / ft^2. This suggested that the differences must have been the result of adaptation period, daily temperature fluctuation or both. In this respect, casual observation suggested that a transition from lethargy to activity occurred at around 7° C. If one could assume that activity implied metabolic function, it may have been possible for the receptors to be partially sustained as a result of excursions above this temperature level. It should be noted that the long adaptation and daily fluctuations more nearly approximated natural environmental conditions than did the procedure in the replication. If the evolutionary history of the species had provided a protective mechanism (the survival value of such a mechanism is obvious) temperature variations of this sort could provide a handy triggering stimulus. Obviously, however, in spite of

the suggestions provided by the data, the disparate results remained an open question.

One notable internal discrepancy in the results of the present experiment was the observation, in several cases, of functional adequacy in the face of significant morphological loss. The best example of this was seen in the data for group D in Figures 10 and 22. Figure 10 showed that the cone A wave (A_2) was spared throughout cold stress and recovery whereas cone outer segments were significantly reduced in number (Figure 22). While the basic mechanism underlying disappearance of outer segments in histological specimens is not known, the immediate and most obvious interpretation was that the outer segments had atrophied. This need not have been the case however. It was possible that the apparent loss of outer segments reflected differential effects on the efficacy of the staining reaction in histological processing, with these effects resulting from the influence of cold stress on metabolism. Under this hypothesis, the relationship of apparent outer segment loss need not be directly related to loss of receptor function since the biochemical substrate for the two need not be identical.

A more likely explanation of the anomalous results in the two sets of data might be that remaining cones were able to make a supranormal contribution to the ERG, as a result of weakening of the scotopic or rod system. Data from normal animals mentioned in the preceding section

suggested that the ERG evoked by the second of a pair of flashes underwent reduction in dark adaptation as the scotopic response to the first flash increased. This implied an inhibitory influence on the photopic (cone) system by the scotopic (rod) system. These effects were primarily on the B wave, however A wave reduction was clearly evident in many cold stressed fish. Brown and Watanabe (1965) have reported neural adaptation occurring between the receptors and inner nuclear layer in light adaptation. The effect of this neural adaptation was to reduce the B wave without reduction in the A wave. Tomita (1970) has reviewed evidence (see above) placing the proximal component of the A wave either in this region or central to it. Thus, since a portion of the A wave is apparently generated in regions where neural interaction has been established, and since scotopic inhibition of the photopic system has been suggested in the present data, it seemed feasible that remaining cones could have contributed disproportionately to the A wave if rods were lost or suppressed in cold stress. Such an explanation could perhaps also account for the peculiarities seen in the A waves of recovery dark adaptation curves as well.

Obviously, neither of the suggested explanations was really adequate. They did, however, suggest directions for additional research, particularly in the latter case.

Another point must be made, with respect to the present research, regarding the animals deprived of light during cold stress and recovery. Previous work dealing

with this variable has tended to indicate that the visual systems of adult animals were little affected by light deprivation (e.g. Hubel and Wiesel, 1970; Wiesel and Hubel, 1963; Legein and Van Hof, 1970). In general, light deprivation was shown to have its effect primarily during critical periods in early development. The effects seen in the present study did not seem explainable on this basis alone. Clearly, the time course of losses in cold stress for groups L and D animals was nearly identical. On the other hand, Legein and Van Hof (1970) observed losses in B wave amplitude with deprivation in adult animals but the effect was very small. The degree to which this small effect may have been magnified in recovering cold stressed animals was inestimable. It is possible that the mechanism producing the slight loss of B wave in the normal, robust, ERG can exert a greater effect on the presumably weakened cells generating the B wave, blocking its recovery. Clearly however, the two variables did interact in the present data. The general question of interactions between light level and cold stress offer promise for future research.

An additional question was raised by the rebound seen in some of the electrophysiological data from fish in early recovery. These effects suggested that cold stress affected several mechanisms, one of which recovered quickly and a second which lagged. One hypothetical example which would be consistent with the data would be that low temperature depressed conversion of foodstuffs into adenosine

triphosphate (ATP) (Lehninger, 1961) as well as the transport mechanisms which deliver foodstuffs to the cell. If, on return to normal temperature, the transport mechanism lagged ATP conversion the result might have been an initial increase in cellular activity until available materials were exhausted. Thereafter, cellular activity would have been dependent on the rate of transport of foodstuffs to the cell. The general effect would have been an initial increase in metabolism then a decrease to a subnormal level which might have been reflected in an overshoot or rebound in electrical activity such as was seen in the data.

There are, of course, other equally attractive (and speculative) biochemical mechanisms which might be invoked to account for the electrophysiological overshoot. On the other hand, a neural mechanism involving differential recovery rates for photopic and scotopic mechanisms would be difficult to justify. The conditions under which the rebound was seen were generally those which favored photopic function. An explanation of the rebound would therefore require that the photopic system recovered more rapidly than the scotopic system and the rebound reflected reduced suppression of the photopic system. Brown and Watanabe (1965) have reported that under similar adaptation conditions, favoring photopic function, that suppression was just the reverse of that required by a neural explanation of the rebound effect.

Several of the questions generated through consideration of the present research could be more adequately

treated, perhaps, if anything were known regarding the basic mechanisms, presumably biochemical, underlying the phenomenon. Thus far in the literature there have been two types of retinal research which may have applicability here.

Dowling's work with Vitamin A deficiency has shown that scarcity of this vital substance produced degeneration of outer segments and, eventually, entire receptor cells (Dowling, 1964). The author concluded that the effect resulted from the breakdown of opsin in the absence of retinene (Vitamin A derived). Normally, the two combine to form rhodopsin, the rod photopigment, which is more stable than opsin. Thus degeneration of the rod outer segment was seen as resulting from breakdown of opsin, with Vitamin A deficiency preventing the retinene-opsin linkage from forming by denying the outer segment the retinene component (Dowling, 1964).

When light impinged upon the rod outer segment, rhodopsin was broken into retinene and opsin, with the retinene or a portion of it being reconverted to Vitamin A which appeared in the pigment epithelium (Dowling, 1960). In dark adaptation the process was reversed with Vitamin A disappearing from the pigment epithelium at the same time that rhodopsin was increasing in the rod outer segments (Dowling, 1960). Presumably, since Vitamin A deficiency produced disruption of this process, the exchange system

between the outer segment and pigment epithelium provided fresh retinene thus retinene was being consumed in the process.

Research dealing with rod outer segment renewal has demonstrated that labeled amino acids, incorporated into proteins in the inner segment, moved into the base of the outer segment forming a disc which then was displaced along the outer segment by the formation of new discs and eventually expelled at the scleral, distal, end of the rod (Droz,1963; Young, 1965, 1966, 1967, 1969; Herron et al., 1969, 1971). Fractionation of outer segments has shown that the material containing radioactivity also showed the rhodopsin absorption spectrum, implying that the labeled protein was incorporated into the rhodopsin molecule (Hall et al., 1968; Bargoot et al., 1969).

One would presume then, on the basis of these lines of evidence, that opsin from the receptor cell combined with retinene from the pigment epithelium to form rhodopsin, the primary constituent of the rod outer segment. Presumably, general reduction in metabolism could, and probably did, interfere with both of these synthetic mechanisms. The necessity of retinene for outer segment integrity was obvious from Dowling's (1964) work. The constant turnover of opsin is presumptive evidence that this protein is consumed by the receptor and must be continually replenished. Thus, cold stress could act on either or both of these mechanisms to produce outer segment degeneration.

The data leading to this position have all come from investigation of rod receptors, while the present research has dealt exclusively with the morphology of cones. The basic visual pigment makeup of the two types of receptors is the same however, with both being chromoproteins consisting of the protein, opsin, and one of the aldehydes of Vitamin A_1 (retinene or retinal) and A_2 (3-dehydroretinal) (Dartnall, 1970). Thus one would expect that effects seen on rods would generalize to the cones. That full acceptance of this generalization is not justified is found in Young's (1969) observations that renewal as seen in rods did not occur in cones, with labeled proteins being found diffusely in cone outer segments in quantities too small to attribute to active incorporation. Similarly, Vitamin A deficiency is frequently referred to as night blindness (Dowling, 1964), implicitly restricting the effect to rods. On the other hand, retinal separation, which physically separates the outer segments from the pigment epithelium thus their source of Vitamin A, resulted in degeneration of both rods and cones (Kroll and Machemer, 1969b), suggesting that some analagous mechanism exists for the latter.

Dawson et al. (1971) have discussed the extremely high metabolic rate of retinal receptors and have related the metabolic requirements to the constant renewal of outer segment proteins discussed above. These considerations suggested protein synthesis as a more likely mechanism than Vitamin A exchange for explaining outer segment loss.

Results from the present experiment showed almost identical loss of outer segments for fish maintained in the light and dark. Recent publications by Noell (Noell et al., 1971; Noell and Albrecht, 1971) demonstrated that interactions between visible light and Vitamin A deficiency were such that Vitamin A deficiency did not result in outer segment degeneration when animals were maintained in the dark. Thus Vitamin A could be dismissed from consideration as the mechanism through which cold stress acted to produce outer segment degeneration.

Additional complications were raised by the present experimental results since it was shown that neural function in the inner nuclear layer was depressed (with no loss of cells from this layer) over and above that which followed outer segment loss. This result suggested that protein renewal was insufficient as an explanation of the effects of cold stress. It appeared more plausible that the basic effect of cold stress was on some metabolic function underlying both electrical function of cells of the inner nuclear layer and receptor protein renewal. One possibility could have been the ATP conversion system (Lehninger, 1961; Allfrey and Mirsky, 1961). Some support for this suggestion was offered by Dawson et al. (1971) who reported disruption of inclusion bodies of mitochondrial origin found in cone inner segments. Presumably, disruption of these mitochondrial structures could have resulted in failure of this energy

conversion system, thus resulting in loss of electrical activity as well as depression of protein synthesis.

While the preceding exposition was suggestive, the basic mechanisms of cold stress induced outer segment degeneration remain obscure, as do those underlying functional loss in the absence of detectable structural abnormalities. In general, the present research has, more than anything else, demonstrated that this phenomenon is much more complex than was expected from a hypothesis of simple loss of outer segments and associated function. In this respect it probably provided more problems than solutions, thus, perhaps its major contribution was in defining questions for future experimental attack.

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BIOGRAPHICAL SKETCH

George Marion Hope was born in Waycross, Georgia, January 24, 1938. He was graduated from Bryan County High School, Pembroke, Georgia, with honors, in June, 1956. In June, 1965, he received the Bachelor of Arts, with a major in Psychology, from Mercer University in Macon, Georgia. He was enrolled in the Graduate School of the University of Florida in September of the same year. He was a United States Public Health Service predoctoral fellow, graduate research assistant in the Department of Ophthalmology, and predoctoral fellow of the Center for the Neurobiological Sciences. He received the Master of Arts, with a major in Psychology, in December, 1967, and has matriculated for the degree of Doctor of Philosophy until the present time.

George Marion Hope is married to the former Dorothy Marie Hendrix and is the father of one son, Stephen Richard. He is a member of The Society of the Sigma Xi, The American Association for the Advancement of Science, The Southern Society for Philosophy and Psychology and The Association for Research in Vision and Ophthalmology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Wm. W. Dawson, Chairman
Professor of Psychology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

H.S. Pennypacker
Associate Professor of Psychology

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Robert L. Isaacson
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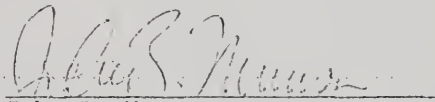

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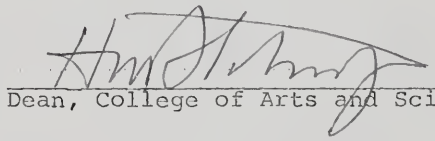
This dissertation was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June, 1971



Dean, College of Arts and Sciences

Dean, Graduate School