RESTORATION OF VISUAL ACUITY AFTER OPTIC NERVE SECTION AND REGENERATION, IN *ASTRONOTUS OCELLATUS* AGASSIZ

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1960

ACKNOWLEDGEMENTS

I am indebted to Professor R. W. Sperry, my supervisor, for the original stimulus to perform this work, and for his guidance, advice, and criticism during its progress.

To Professor A. Van Harreveld, I wish to express my gratitude for continued encouragement and helpful advice.

I should like to thank Professor H. Autrum, of the Zoologisches Institut der Universität, München, whose generous hospitality permitted the continuation of these studies.

I am indebted to Professor Paul G. Hoel for suggesting the method of statistical analysis of results employed here; and to Dr. Allan M. Schrier, Dr. Harbans L. Arora, and Dr. Dietrich Schneider, for many valuable and stimulating discussions. Many others in Cal Tech and München have given generous advice, support, and encouragement.

I am grateful also to the National Science Foundation, and to the California Institute of Technology, for financial support during my graduate studies.

I am most of all indebted to my husband, Eberhardt, and to my parents, for their support and inspiration.

ABSTRACT

The visual acuity of a cichlid fish, Astronotus ocellatus Agassiz, was measured by means of a conditioned visual discrimination response. The average minimum separable visual angle was 5.3', as measured in 16 fish with at least one normal eye; this corresponded closely to the fineness of grain of the retinal receptor mosaic.

In 12 fish, acuity of one experimental eye was measured after the optic nerve had been transected and allowed to regenerate. The value for postregenerative acuity was then compared with a previous value for acuity of one normal eye in one and the same fish, in each case. Restoration of acuity by regenerative processes averaged 78.4%. This high figure shows that formation of functional, specific synaptic contacts probably does not occur on a chance basis.

INTRODUCTION

Regeneration of the optic nerve with recovery of vision was conclusively demonstrated in 1925 and 1926 by Matthey (1,2,3,4) for adult urodeles. After recovery, the experimental animals were able not only to detect light, but also were able to see small objects and localize them correctly. This recovery of response to visual stimulation suggests that there had been a high degree of regrowth of nerve fibers; and, further, that there had been good functional recovery of connections between nerve fibers and central brain areas. Since the experimental animals were able to localize objects in the visual field correctly, these experiments suggest that fibers from specific retinal areas must re-establish connections with corresponding visual areas in the brain.

Restoration of localization of objects in the visual field was later observed also by Stone (5), in urodeles, and in urodeles, anurans, and fishes by Sperry (6,7) and other workers (8,9,10,11). Furthermore, Sperry showed (12) that when the eye of amphibians and fishes is rotated 180°, the regenerating nerve fibers re-establish central relations according to their original, "normal" position in the eye, instead of the artificially created inverted position. After regeneration, the animals were able to perceive objects discretely (not as a blur), but directed their reactions in the wrong direction. These rotation experiments thus showed conclusively that learning was not responsible for the recovery of normal field orientation, and hence indicated the presence of orderly growth processes which regulated re-establishment of previous anatomical connections. Sperry concluded that

"The optic fibers differ from one another in quality according

to the particular locus of the retina in which the ganglion cells are located. The retina apparently undergoes a polarized, field-like differentiation during development, which brings about local specification of the ganglion cells and their optic axons. The functional relations established by the optic fibers in the brain centers are patterned in a systematic manner on the basis of this retinal specificity." (13)

To explain these and related results (14,15,16,17,18,19,20,21, 22,23), Sperry concluded (24) that

"The formation of synaptic connections must be regulated on some sort of chemo-affinity basis. We may picture the optic fibers, as they invade the optic lobe, encountering many different elements: capillaries, glia cells, axons of other afferents, and the numerous dendrites and cell bodies of the tectal neurons. Not all contacts made by the growing fiber tips result in the formation of synaptic endings. In most cases the growing tips continue to push on and beyond the various elements they encounter. It is only when a fiber happens to contact a nerve dendrite or soma, the chemical constitution of which specifically matches that of the invading fiber, that a specialized synaptic ending is formed and a further growth of that fiber tip ceases. For each retinal locus we assume a corresponding focal area in the optic lobe. Fibers arising from a given point of the retina have a predetermined, selective affinity for the neurons of the corresponding central locus."

The Marchi method has been used by Akert (25,26), Ströer (27,28), and Leghissa (29) to detect and localize degenerating optic tract fibers in the optic lobe of fish. Results of their studies were in agreement with results of behavioral (30,31,32,33,34) and electrical (35,36,37) investigations, which support the view that neurons from any given retinal area project to specific tectal regions. Recent electrical studies of Gaze (38) have also lent physiological support to the view that the regenerating nerve fibers form connections with the areas of their original tectal projection. When the optic nerve is cut and rotated 90°, the optic fibers form connections according to their "normal", original position, so that the projection of a given fiber is unchanged, but the projection of the artificial "dorsal" quadrant on the tectum,

for example, has been rotated 90° compared to the normal projection of fibers from the original dorsal quadrant. Thus its position in the eye has had no influence on the central projection of any given fiber, in these experiments on regeneration.

These results were qualitative, in showing that regenerating fibers form central connections in a specific way, but do not give quantitative information. However, the eye also seems to be a promising organ for the investigation of the extent of restoration effected by the processes involved in formation of central connections.

The retina is a mosaic of receptor cells communicating, via the neurons of the optic tract, with loci in the optic centers of the brain. For detecting small objects, the best performance is yielded (under optimal lighting conditions) by the cells in the foveal area (when present), where each retinal receptor may be connected, via one optic neuron, to the appropriate central locus. The actual performance of the eye in detection of small objects will be influenced by the light available (39,40), and by physical factors such as function of the light-focussing apparatus. One can predict a value for maximum visual acuity under optimal conditions from the simple geometrical factors of number, size, and arrangement of the retinal receptor cells, and the ratios between numbers of receptor cells, optic axons, and central loci, provided that no other mechanisms are present (as seems likely at least in lower vertebrates). In higher vertebrates (41), there are in addition dynamic neural mechanisms, such as physiological tremor (42,43), which may contribute to provide a higher value for visual acuity in actual tests.

However, if accessory factors remain constant, any decrease in the optimal ratio between optic axons and central loci should theoretically be detectable in measurements of visual acuity of an animal. By

measuring visual acuity before and after optic nerve regeneration, we might obtain a quantitative estimate of the efficiency of re-establishment of functional connections between optic axons and their corresponding loci in the optic tectum.

Visual acuity is defined in terms of the "minimum visible" or "minimum separable": "the visual angle subtended by the smallest object whose presence can be detected, or by the smallest separation which can be perceived between two objects" (44). In man, typical tests for determining this value employ detection of single small objects, resolution of gratings, or the detection of the fine separation between the two arms of the Landolt C. The best value obtained for acuity in man, using a single line as test object under conditions of optimal illumination, has been 0.5" of visual arc (45); more typical is 30", and 1' is defined as normal. Visual acuity is thus simply defined as the reciprocal of the minimum separable visual angle in minutes (hence normal acuity, by definition, is 1/1). Since the cones of the human fovea have a minimum diameter of about 1 μ (46), corresponding to about 12" of visual angle, the higher values for acuity actually measured must be attributed to other factors such as the effects of dynamic central and retinal neuronal mechanisms. Under conditions of optimal lighting, the experimentally established value for visual acuity of lower vertebrates and invertebrates has in general been found to correspond to that which would be predicted from the size and spacing of the retinal receptors (44,47,48,49,50). The morphological considerations predict a minimum separable visual angle of 2' 10" for the dove (Columba), for example; the value established physiologically is 2' 42". In the frog rods (Rana) the morphological value is 6' 48", the physiological value is

6' 53" (data summarized in (49)).

The behavioral tests for measuring the acuity of lower vertebrates are of two kinds. The first was developed by Hecht (47,48), who found that several animals will respond to the movement of stripes past the eye, with motions of their eye or body. Assuming that an animal will not respond when its eye is unable to resolve the stripes, he progressively reduced the size of the large moving stripes and the spacing between them, to see when the animal ceases to respond. With this method, Hecht measured the visual acuity of the honey bee and Drosophila, and obtained values which agree with the size of the receptor units in these insects (1° of visual angle, in bees). For fish, this method is of questionable applicability (50).

The second type of behavioral method used for acuity measurements depends on learning of discriminations between standard and variable visual stimuli. This type of test has also been used successfully for fish. Herter and others (for literature see Herter, 51) have shown that fish are able to learn to discriminate between visual patterns of considerable complexity. Many varieties of patterns have been used in these experiments. Herter once displayed to marvelling audiences, in Berlin, experimental fish which could be seen to search for food only in containers labeled "WURM", ignoring those labeled "LEER". This apparent ability of the fish to read reveals their high ability to distinguish and remember optical contours.

Rowley (52) used a simple visual discrimination test to measure visual acuity in Carassius auratus. The standard stimulus was a circle 3.0 cm in diameter; the variable stimulus was a circle of larger or smaller diameter. The threshold value for size discrimination was found to be a difference in diameter of 0.3 cm, when both stimuli were

viewed from an average distance of about 8 cm. This corresponds to a minimum separable visual angle of 2° 10', which is surprisingly large. However, the just-discriminable size difference measured might well be dependent on the absolute size of the test objects used, in application of the Weber-Fechner law (44). Therefore, this measurement would probably not supply a reliable measure of the minimum separable visual angle.

Brunner, investigating the changes in visual acuity with light intensity in minnows (Phoxinus laevis) (53), obtained a result for maximum visual acuity which corresponds more closely to the value expected on the basis of Wunder's (54) measurements of cone diameter. Brunner taught minnows to feed at a striped pattern, as opposed to a plain gray. Under optimum light intensity, they were able to detect stripes with a minimum spacing of 0.25 mm, at a distance of 8 cm. This corresponds to a visual angle of about 11' and a retinal image subtending about 6.25 μ. According to Wunder, the cone diameter of the minnow is about 5 μ, so the visual acuity in these fish corresponds well with the value predicted from the fineness of the retinal receptor mosaic. This type of method thus seems appropriate for testing the visual acuity of fish.

In addition to this ability to distinguish and remember optical contours, fish possess the capacity for optic nerve regeneration. Successful regeneration of the optic tract in various teleost fish has been reported by Sperry (19,20) and other workers (55,56,57), leading to the conclusion that the "ability of the optic nerve to regenerate and to restore vision after its complete transection seems to be widespread" among fish (58). In the work concerned with recovery of directional

vision, no attempts were made to measure the recovery of acuity; but because of their ability to make visual discriminations, fish would be appropriate subjects for such measurements.

A high degree of quantitative restoration of vision in operated fish was suggested by unpublished observations of Sperry and Deupree (59) and of Arora. Arora trained a cichlid fish, Astronotus ocellatus, to jump from the water to obtain food from a dotted plaque, as opposed to plaques of various shades of gray varying from white to black; the size of the dots was then progressively decreased until the fish was unable to distinguish between the two plaques. Fish with regenerated optic nerves performed so well that Arora was unable, with this method, to measure a difference in acuity between them and normal fish.

In the present investigation, the experimental subjects were the same species of cichlid fish. A type of discrimination problem like that typically used as a standard for measuring human visual acuity was applied for visual acuity measurements in the fish. Comparisons of acuity were made in each case between the normal eye and the eye with operated, regenerated optic nerve, in one and the same fish. It was hoped to obtain in this way a quantitative measure of the effectiveness of orderly restoration of functional central connections between retinal fields and central optic projection areas.

MATERIALS AND METHODS

Subjects

A tropical cichlid fish, Astronotus ocellatus Agassiz, was used in these studies. Subjects were kept in aerated three- to five-gallon tanks at a temperature of 26-28° C.; the tanks were cleaned and the

water partially replaced every 2-3 days. The fish were fed daily. The subjects tested ranged from 4 to 12 cm in length, the average for experimental cases being 6-8 cm. Twelve of the final group of subjects were from the same spawn.

Operative technique

Operations were performed under a binocular dissecting microscope. The fish was wrapped in wet gauze and held in a plasticine cradle which could be moulded to fit the individual fish and to hold it, firmly but gently, with the head in an elevated position. The fish were anesthetized in a 0.005% solution of MS222 (tricaine methane sulfonate, Sandoz Pharmaceuticals). During the operation, the anesthetic solution was dripped continuously into the mouth and over the gills through a tube leading from an elevated vessel. This kept the fish narcotized and at the same time supplied a flow of oxygenated water over the gill area during the operation. Sanitary precautions were observed but sterile procedure was found to be unnecessary.

To reach the optic nerve, a small piece of skin, with a part of the soft supraorbital crest, was removed above the eye. The eye was then gently extruded from the orbit and rolled slightly ventrad, bringing the optic nerve into view. In some preliminary cases, the optic nerve was then cut with fine scissors, leaving no bridge between the cut ends of the nerve; regeneration did not succeed in these cases. In four of the final 12 cases, fine jewelers' forceps were inserted between the nerve and the retinal blood vessels, and the optic nerve was repeatedly pinched until complete division of the nerve substance was visible. This could be accomplished without damaging the fine strands of ciliary nerve and the optic blood vessels supplying the retina,

while at the same time leaving the outer nerve sheath partially intact as a guide for the regenerating fibers. It seemed possible, however, that crushed remnants of former delicate connective tissue paths within the sheath could be used again, by growing tips of some of the severed nerves, as a path to their previous central projection areas. To avoid this criticism, the nerve sheath was slit lengthwise with a fine needle in eight subjects, and a small sharp knife was used to cut the nerve fibers, so that each part of the pleated nerve strand could be seen to be severed although the tough outer nerve sheath still remained intact (except for the lengthwise slit), and therefore bridged the separated stumps. This more drastic method insured complete interruption of all former nerve and connective tissue pathways.

After section of one optic nerve, the subject was trained to make the visual discrimination using the normal eye. Acuity was tested in the normal eye, which was then in turn blinded. By this time, vision had in most cases partially returned in the first-operated eye. The onset of return of vision was determined by the following behavioral criteria: (1) ability of the fish to see and react to objects presented outside the tank, and (2) prompt localization and seizure of food in the aquarium, even when the food was presented on the operated side (these fish were not able to detect food quickly using only olfactory or tactile cues, as they were forced to do when completely blind).

Training methods

For the present investigation special apparatus and testing methods were designed, comparable to devices used for discrimination training of mammals (60,61).

An opaque partition, shown in Figure 1, divided half of the fish tank into two alleyways. The test sample plaques were suspended in these alleyways. A movable plexiglas gate separated this divided half of the tank from the remainder. This gate was masked with plastic tape so that only a vertical three-centimeter medial strip was transparent; the fish was thus obliged to view both sample plaques from a central position before choosing one alleyway and its plaque. A light bulb clamped to the wall of the tank behind the fish provided a constant illumination on the plaques (250 foot-candles at the water surface) during the tests.

For the tests of acuity, the subject was required to discriminate between dotted and uniform-gray surfaces. A series of dots of graded sizes was obtained from the Graf-Tint Mfg. Co.; and a series of gray plaques, including gray values lighter and darker than those of the dotted samples, was prepared photographically. The dots used (with measurements of their diameter) are shown in Figure 2.

Gray plaques of varying intensities were distributed irregularly through various parts of the testing series. The tests made on different days and at different distances included grays of several intensities, distributed randomly so that the fish could not use simple light intensity cues as a basis for discrimination.

The pattern "targets" were mounted between thin 2" x 2" lantern-slide cover glasses, sealed with clear Tygon plastic sealer, and held in identical, interchangeable aluminum frames which could be suspended in the water at any distance from the fish.

Subjects were trained once a day, with 10-25 trials, and were not fed at other times. In preliminary training procedure, after they

Fig. 1: Diagram of testing arrangement. Dotted figures: position of fish, etc. at begin of trial. Solid: positions just before gate lifted.

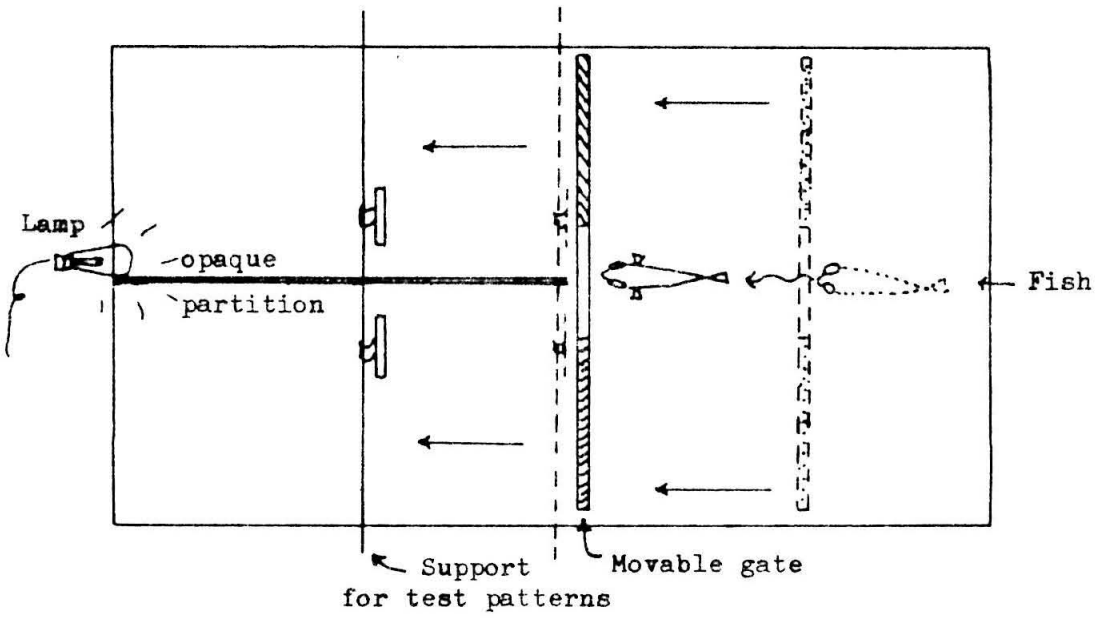
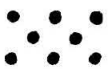
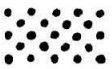


Fig. 2: Acuity test patterns

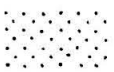
Dot 1
2.0 mm



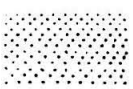
Dot 2
1.3 mm



Dot 3
0.6 mm



Dot 4
0.32 mm



Dot 5
0.13 mm

(too small for clear Ozalid reproduction)

had learned to take food from forceps, the fish were fed at the positive (dotted) plaque for 2-3 days. As the plaques became familiar, introduction of the food was progressively delayed until the fish started to snap at the plaque in anticipation; food was then given as a reward. During testing, food was not in the water, or visible to the fish; it was then presented immediately after the correct response had been made. There was no punishment for incorrect responses.

When the fish had learned to make a correct choice between the larger dots and gray, the size of the dots was progressively reduced. This could be accomplished, within a considerable range, by using the prepared series of dotted plaques. Since the available gradation of dot sizes allowed only a rough estimate of visual acuity, the final acuity tests were performed by another method. The same dotted plaque was used in all tests, and the grays were changed every 5-10 trials, as previously. The distance at which the fish must distinguish the dots from the gray was then varied, thus altering the size of the visual angle subtended by the dot on the retina. Accessory factors, such as opacity of the water and the difference in eye accommodation which may be necessary, may prevent an ideal absolute measurement of acuity with such a method. However, as these factors must influence the fish's performance to about the same extent in all trials, comparisons can still be made between the fish's performances in acuity before and after optic nerve section and regeneration.

In testing, the plexiglas gate was used to hold the fish at any desired distance from the end of the partitioned space. The two test plaques were then lowered into the water in the middle of the tank, at the end of the partition. Distances between gate and partition

(plaques) were marked both on the tank bottom and on the rim of the tank, so that they could be continuously and closely controlled. The fish was now permitted to view both plaques through the gate for 3-5 seconds, and then the plaques were moved slowly back into the two alleyways. The restraining gate, with fish following, was also moved at the same speed toward the end of the partition. At the partition, the gate was lifted, and the fish was permitted to enter one of the alleyways. If it approached and snapped at the correct pattern, it was rewarded with food. The interval between presentation of plaques and opening of the gate was timed, and constant for each fish. (It was necessary to show both plaques before they were moved into the alleyways, as a monocularly blinded fish when hungry showed a tendency to swim quickly toward the first plaque it saw, in case the plaque in the other alleyway was momentarily not in view. This was especially important in critical tests near the limits of resolving power.) A series of measurements showed that from the time of presentation of the plaques, through the coordinated movement backwards, to the opening of the gate, the error involved in distance measurement was not more than 0.3 cm. In the trials near the limits of perception, this meant an error on the order of 0.1' of visual angle, which will be seen later to be small, compared to the experimental uncertainty involved in estimating the minimum visual angle resolvable by the fish.

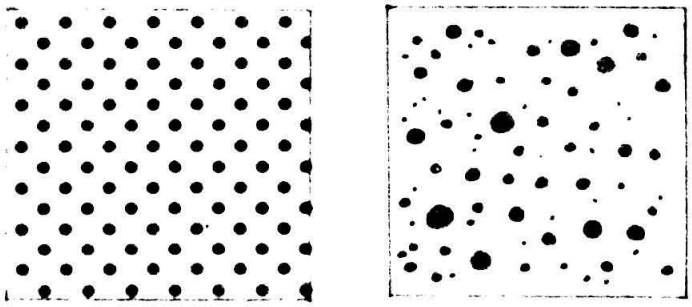
The two aluminum frames were used alternately for holding positive or negative plaques, in different groups of trials. The position of the two plaques was alternated randomly between right and left, with the restriction that the correct plaque appeared equally often in each of the two positions every 10 trials. Each fish was given 10-25

trials per day. Each day, dots of two to four different effective sizes were among those presented. But in initial trials, only larger dots were presented; and smaller dots were used regularly only later in the testing period.

Each fish was first trained to take food from the forceps, then either the right or left eye was blinded by one of the methods described above. During the period required for regeneration, the normal eye of the fish was tested for acuity. After an acuity value had been established for this eye, the normal eye was blinded in turn. In most cases, the first-operated eye had already recovered vision, and the fish could be tested immediately. The regenerated eye was then likewise used for a series of tests, with dot size being continuously decreased. As the fish was already familiar with the problem, the testing could progress quite rapidly, and thus possible acuity variations due to size or age of the fish could be eliminated. Acuity was tested continuously after first signs of return of vision in the regenerated eye, until no improvement in performance could be detected.

As an additional test, five fish were trained to distinguish a plaque with uniform, regularly-spaced dots from a plaque with different sizes of dots randomly scattered on a white background (Fig. 3). The "mottled" plaque was rotated 90° between trials. The speed of learning on this problem for three normal fish was then compared with that for two fish with regenerated optic nerves, to test whether optic nerve section and regeneration affected the fish's ability to distinguish a regular pattern from a random one (Fig. 4).

A mock operation was performed on two fish: the eyeball was extruded, as in the usual operation, but the nerve was not cut. These



Dot Mottle

Fig. 3: Patterns used for "mottle" vs. "uniform dot" problem.

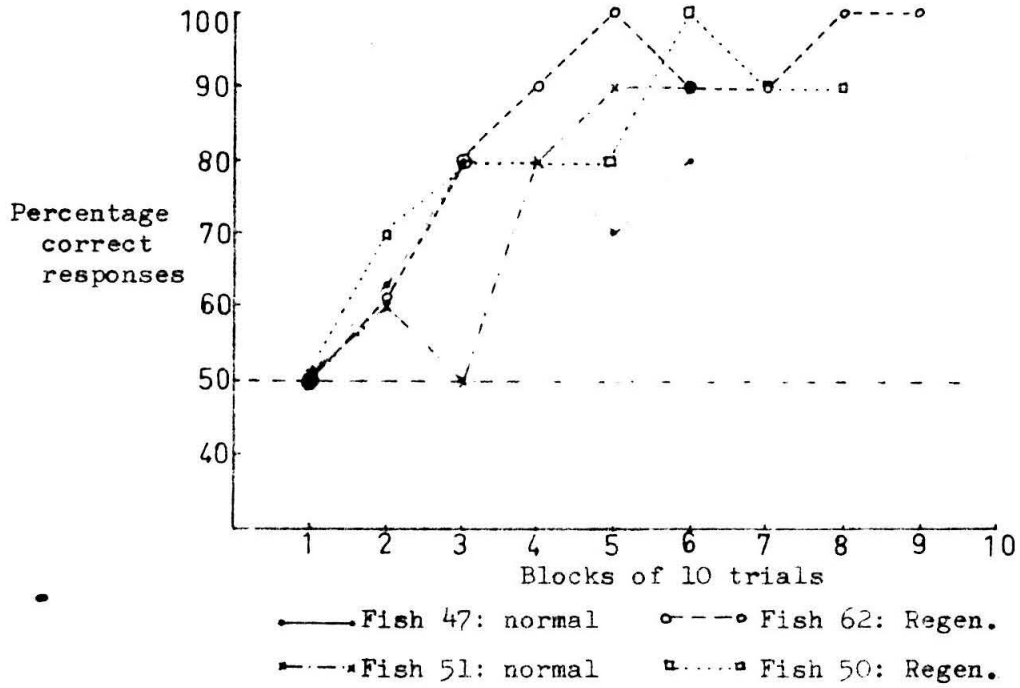


Fig. 4: Performance of individual fish on "mottle"-negative vs. "uniform dot"-positive problem.

fish were tested for acuity before and after the operation to determine whether the surgical procedures, exclusive of the optic fiber section, had any maladjustive effects on the eyeball or the focussing apparatus.

Histology

Measurements of the retinal receptor cells were made as follows: the eye was removed from an anesthetized or freshly killed fish and immersed in physiological saline. The sclera was then slit open, and the retinal cell layer separated from the pigment layer. The thin sheet of retinal tissue was placed in saline on a glass slide and observed under the oil immersion objective of a phase contrast microscope. Two fish, one 5 cm and the other 6.7 cm in length, were used; 84 and 117 cells were measured, respectively, with a calibrated ocular micrometer.

A protargol stain, performed according to the Bodian method (62), was applied to the regenerated optic nerve and brain of 13 fish, for general observation of the appearance of nerve fibers. In addition, longitudinal sections of one goldfish and one Astronotus optic nerve, after being pinched by the method described above, were stained both with protargol and with the azan method for connective tissue, in order to investigate the possible occurrence and extent of tissue bridges of connective tissue nerve sheaths.

RESULTS

Provided that some kind of bridge existed between the two stumps of the optic nerve, there was always regeneration. In the specimens examined histologically, the regenerated nerve was similar in appearance to the normal nerve; both appeared as relatively broad bands of nerve fibers, accordion-pleated into a bundle which is round in

cross-section. Longitudinal sections of freshly pinched optic nerves, stained with protargol and azan, showed some remnants of connective tissue within the nerve sheath. However, the degree of regeneration, as measured behaviorally by acuity tests, was about the same for nerves severed by the two methods, pinching and cutting within the sheath.

The data from the discrimination tests could be most simply presented in terms of a single curve for each eye, in which percentage of correct choices was plotted against the visual angle subtended by a dot. As observation showed that the fish responded correctly almost 100% of the time after the problem was learned (and when the dots were large enough to be seen easily), a criterion of 80% correct choices was arbitrarily adopted for assigning a visual angle representing the subject's visual acuity. An example of this type of curve is given for fish #74, for the normal (right) eye before operation, in Figure 5. The left eye had previously been blinded. When the test dots subtended a visual angle of 5.4' on the retina, the subject chose the correct plaque 80% of the time; thus, 5.4' would arbitrarily be chosen as a measurement of this fish's visual acuity, using one normal eye. When a similar curve is drawn later for the regenerated eye (the normal eye having been blinded in turn), one can compare the visual angles at which the fish performs with 80% correctness, using the normal or the regenerated eye, and thus arrive at an estimate of the extent to which visual acuity is restored during regeneration.

This method, however, fails to take into account learning on the part of the fish. After the fish has learned to take food from the dotted plaques, it will perform with nearly 100% accuracy as long as the dots are relatively large; but when suddenly presented with dots

near the limits of its visual resolution, its performance will drop to a chance level. Only gradually, as the test dots are slowly made smaller, will the fish learn to pay attention in order to make the fine visual discriminations demanded of it. Finally a point is reached where no more improvement can be elicited. If, now, all the trials are expressed in a single curve, the process of learning which has gone on during the testing period will have the effect of lowering the standard of performance below the maximum which the fish finally attains. This effect is especially deleterious in that it occurs primarily in the original learning, with the normal eye. Habits of attention learned by the fish while using one eye are available to the fish when it uses the newly regenerated eye, in agreement with results of Arora on transfer. Thus the normal eye is put at an unfair disadvantage in the comparison.

It is, however, difficult to separate the learning period from the period when performance is based alone on the morphological limits set to visual resolution.

A statistical method suggested by Professor P. G. Hoel can be applied to separate the learning period from the later trials.

Assume that the performance of the fish in resolving dots of a given visual angle improves at first, and then reaches a constant level which represents the true ability of the fish to resolve dots of this size.

Then the data could be expressed in terms of a series of percentages (of correct choices) at a given visual angle.

For example, in the case of fish # 80, the overall percentages of correct choices at the various visual angles tested are as follows:

Visual angle	Percent correct choices	
	Normal eye	Regenerated eye
11'	31/36 = 86%	31/38 = 81.5%
10.2'	20/20 = 100%	
8.8'	61/83 = 73.5%	110/132 = 83.3%
7.3'		104/134 = 77.5%
6.3'	62/72 = 86%	93/127 = 73.2%
5.5'	91/118 = 77%	
4.9'	23/38 = 60.5%	

The corresponding curves are shown in Fig. 6. Interpolated values for visual acuity, based on the usual arbitrary 80% criterion, are 6.6' for the normal eye and 7.8' for the regenerated eye.

Now the trial data were arranged as successive groups, of 20 trials each at each of the visual angles tested (provided enough trials were present to be worthwhile):

Normal eye:	Percentages, in successive groups of 20 trials						
Visual angle	First	Second	Third	Fourth	Fifth	Sixth	Seventh
8.8'	75%	65	70	85			
7.3'	75	75	55	70	60	80	90
6.3'	90	75	90	95			
5.5'	80	75	70	80	80	80	

Regenerated eye:	Groups of 20 trials							
Visual angle	1st	2nd	3rd	4th	5th	6th	7th	8th
8.8'	95%	70	80	95	85	85	80	95
7.3'	90	80	75	65	85	65	90	
6.3'	75	70	75	70	75	75		

These data are presented graphically in Figs. 7 and 8.

Now, let us split the total series of trials at a given visual angle into two groups--for example, into 2 equal halves, and assume tentatively that learning has occurred only during earlier trials, and has stopped, so that the second half of the trials do not include data

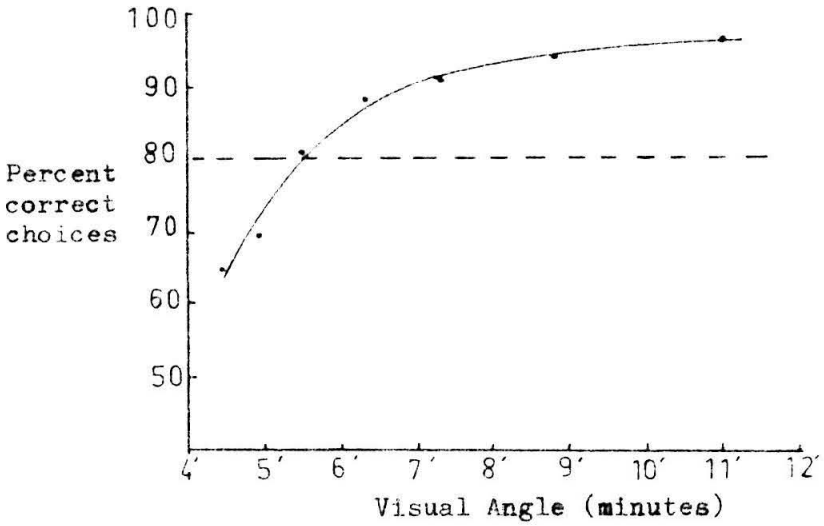


Fig. 5: Overall performance of fish #74, using normal eye.

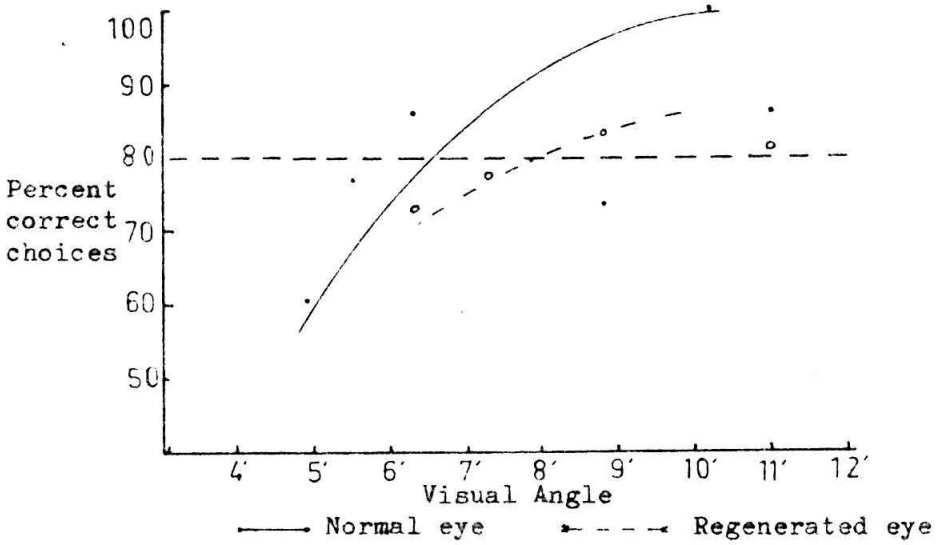


Fig. 6: Overall performance of fish #80; comparison of normal eye and eye with regenerated optic nerve.

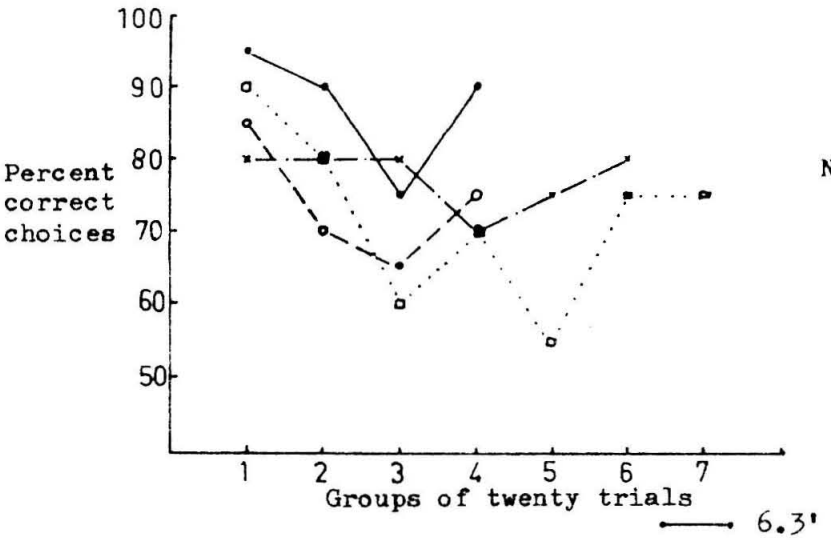


Fig. 7: Fish #80: successive tests of acuity at various visual angles. Normal eye.

NOTE: in Figures 7 and 8, the trials are numbered in reverse order: the last group of 20 trials is numbered "1", the next-to-last is "2", and so forth.

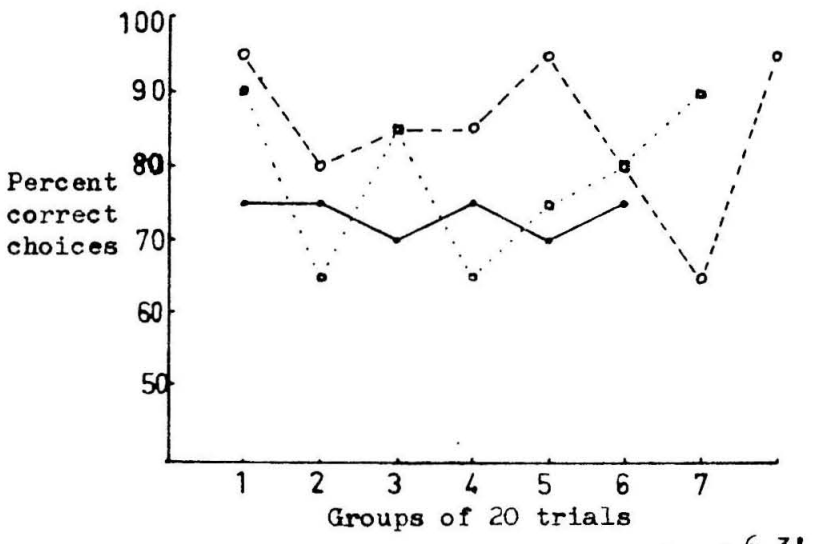


Fig. 8: Fish #80: successive tests of acuity at various visual angles. Eye with regenerated optic nerve.

collected during the learning period. We calculate the percentage for the last half of the trials, and assume that this is the true percentage for the fish at this visual angle, after learning has ceased. If this percentage is 85% or higher, one may use the Poisson approximation to calculate the probability of various numbers of failures in 20 trials.

In the trials at 6.3', using the normal eye, the average percentage of correct choices made by the fish is 92.5% during the last half of the trials. This means an average of 1.5 misses in each 20 trials.

The Poisson function $f(x) = \frac{e^{-n} n^x}{x!}$ indicates the probability of exactly x misses in 20 trials when the average number of misses per 20 trials is n. The probability of more than y misses is given by subtracting from 1 the sum of the probabilities for each number of misses from $x = 0$ to $x = y$: $F(y) = 1 - \sum_{x=y}^{x=0} f(x)$. The probability of more than 4 misses is 0.02, given an average number of misses $n = 1.5$. This is very small; hence, if we find any point among the first groups of trials where more than 4 misses have occurred, this point is incompatible with the assumption that learning has ceased, and therefore corresponds to the learning period.

In the second group of trials at 6.3', the number of errors made by the fish was 5. Since it is improbable that this could occur after learning has ceased, we assume that the fish was still learning at the time when this group of trials was made. If we now eliminate from consideration all trials previous to and including this group, we obtain the following data:

Visual angle	Percentage of correct choices	
	Normal eye	Regenerated eye
8.8'		92/105 = 87.5%
7.3'		97/125 = 77.5%
6.3'	37/40 = 92.5%	88/120 = 73.0%
5.5'	85/108 = 78.6%	
4.9'	23/38 = 60.5%	

Curves are shown in Figure 9. The revised percentages shown here for the regenerated eye were obtained by similar calculations:

Average percentage of correct choices in last half of trials at 8.8' was 86.2%. Average number of errors per 20 trials at 8.8' was 2.76. Hence the probability for more than 6 misses per 20 trials is only 0.02. Any point in the first half of trials where more than six errors per 20 trials occur, is incompatible with the thesis that learning is completed and a steady state has been reached where there is a probability of 0.978 that 6 or fewer errors will be made. Since this occurs in the second group of trials, all trials up to and including this group were omitted from consideration in compiling the revised percentages.

Using the usual arbitrary 80% criterion, visual acuity in this fish (as interpolated from these revised curves) is 5.6' for the normal eye and 7.4' for the regenerated eye.

Data and calculations for fish #81 (Figure 10) are as follows:

Visual angle	Percent correct choices	
	Normal eye	Regenerated eye
11'		95.5%
8.8'		87.5%
8.6'	100%	
7.3'	90%	68.5%
6.3'	91.9%	75.7%
5.5'	90%	
4.4'	70%	

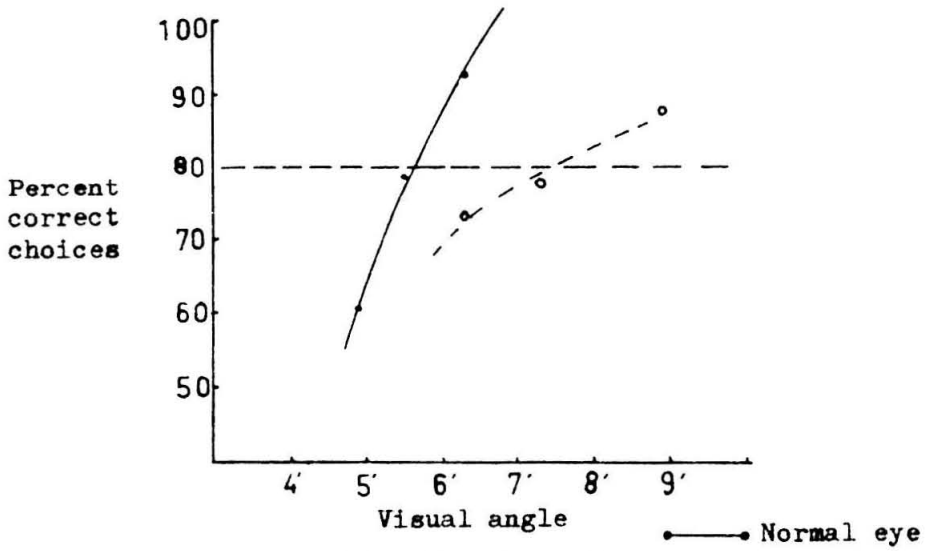


Fig. 9: Overall performance of fish 80, after data obtained during learning period have been eliminated.

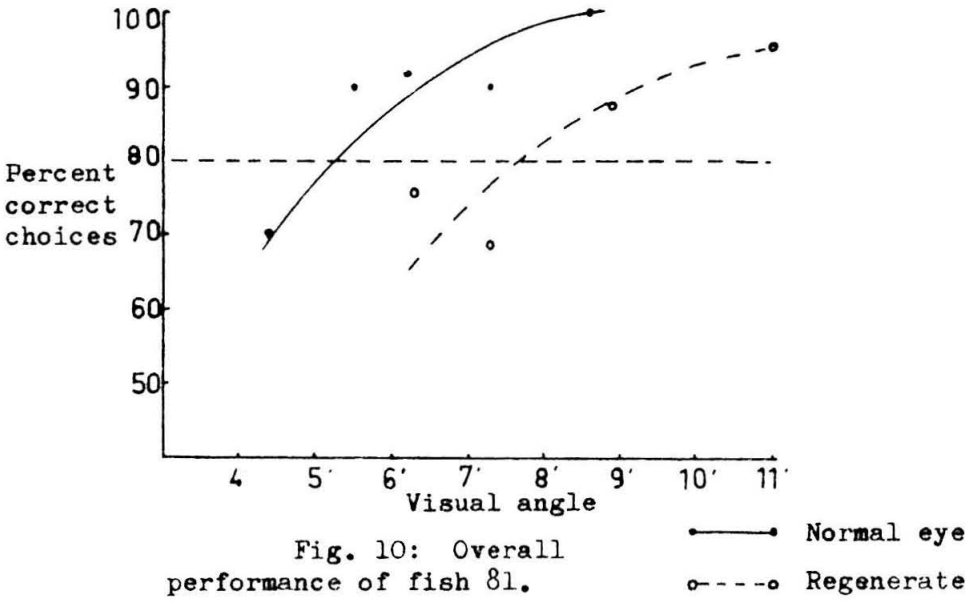


Fig. 10: Overall performance of fish 81.

Trial data (Figs. 11 and 12) arranged as successive groups of trials at each visual angle:

Normal eye

Visual Angle	Groups of 20 trials														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
6.3	95%	80	90	100											
5.5	80	90	95	90	100										
4.9	90	95	95	95	80	100									

Regenerated eye

6.3	80%	80	75	70	65	85	65	80	80	70					
7.3	85	75	85	75	75	80	75	85	80	75	80	70	80	80	75
8.8	85	85	85	85	85	95	80	90	90	85	90				

Calculations for normal eye: Average % for last half of trials at 5.5': 95%. Average number errors per 20 trials at 5.5': 1. After learning, there is a probability of only 0.02 that more than 3 errors will be made in 20 trials. However, in the second group of 20 trials, four errors were made; hence this period still includes learning.

Calculations for regenerated eye: Average % for last half of trials at 8.8': 88.3%. Average number errors per 20 trials at 8.8': 2.34. The probability is only 0.032 that more than 5 errors will be made in 20 trials. More than five errors were never made by the fish at 8.8'; hence, during the period in which a dot subtending a visual angle of 8.8' was being tested, scores are all compatible with the hypothesis that learning is complete. The revised data, omitting trials obtained while learning was going on, is as follows:

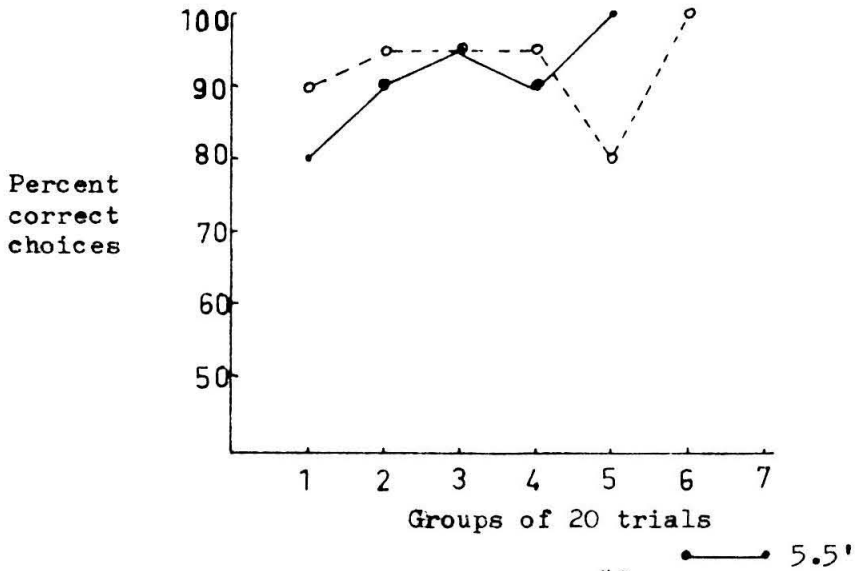


Fig. 11: Fish #81; successive tests of acuity at various visual angles. Normal eye.

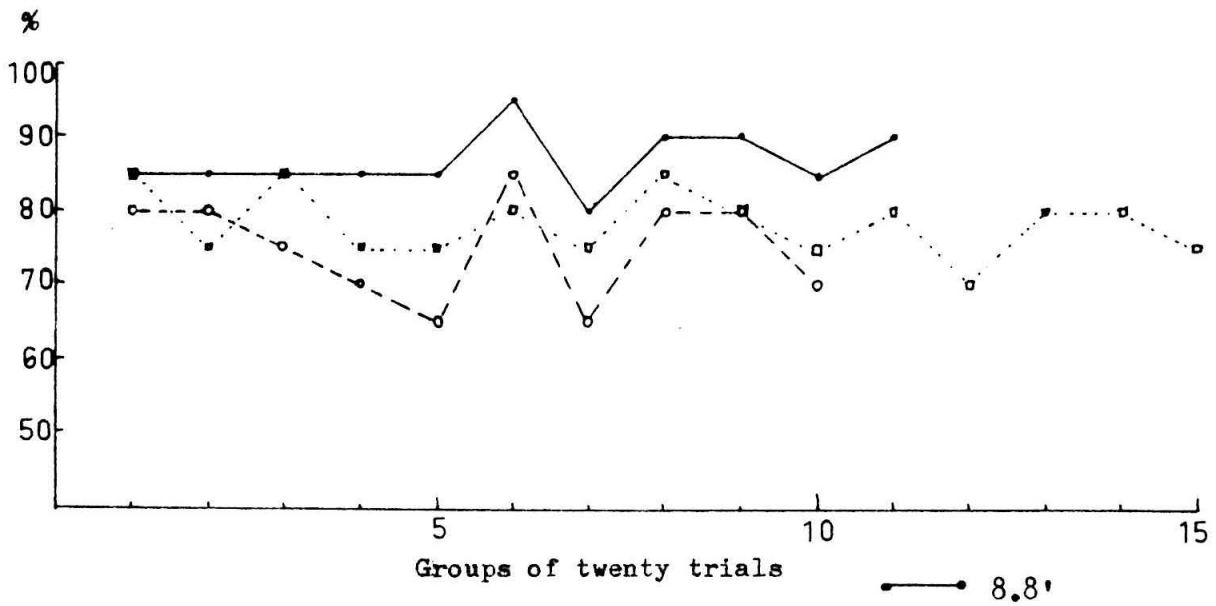


Fig. 12: Fish #81: successive tests of acuity at various visual angles. Eye with regenerated optic nerve.

Visual angle	Percent correct choices Normal eye	Regenerated eye
8.8'		86.5%
7.3'		79 %
6.3'	100 %	75.5%
5.5'	93.7%	65.5%
4.9'	91.6%	
4.4'	64.5%	

Revised estimates of visual acuity, based on curves shown in Fig. 13: 4.7' for the normal eye, 7.1' for the regenerated eye.

All data presented in this study were treated with this method (see Figures 14-27).

The visual angle subtended by a given object at a given distance was calculated according to the figures given by Brunner (53).

a) Acuity of Normal Eye

Table 1 summarizes the values for acuity for fish used in this study. Fish #87 had two values for the normal eye (a value with both eyes used at once, and the second value with only one normal eye); all other fish were using only one normal eye for the tests, the other eye having previously been blinded.

The average value for visual acuity in the normal eye of these fish was 5.3' of visual angle (S.E. 0.09).

The measurements of fresh fish cones, in physiological saline, gave the following results: the mean diameter of all cells measured was 2.92 (S.E. 0.04) μ. Lens diameter in these fish was about 2 mm; about the same as in Phoxinus. Brunner (53) has calculated that 1 minute of visual angle subtends 0.58 μ in these fish. In fish eyes of this size a single cone, therefore, corresponds to about 5' of visual angle. The actual cone dimensions are thus close to those predicted

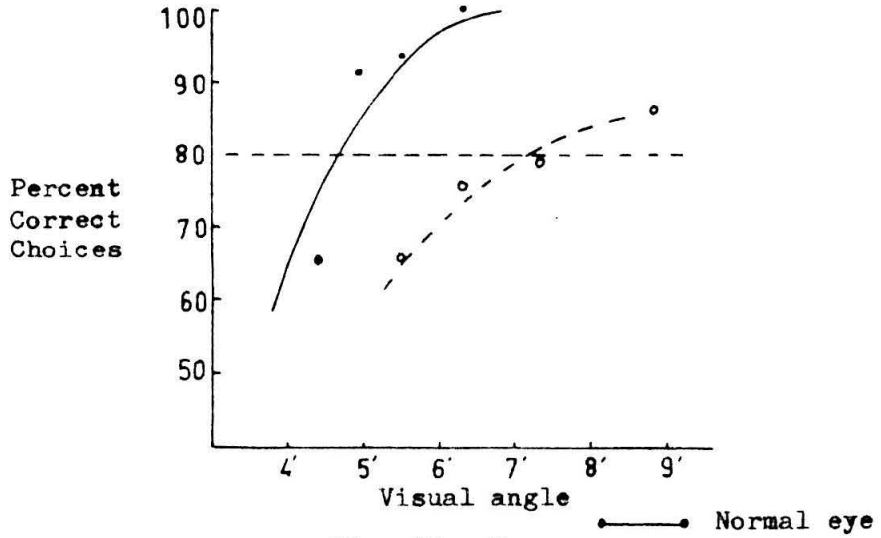


Fig. 13: Overall performance of fish #81, after omission of data obtained during learning period.

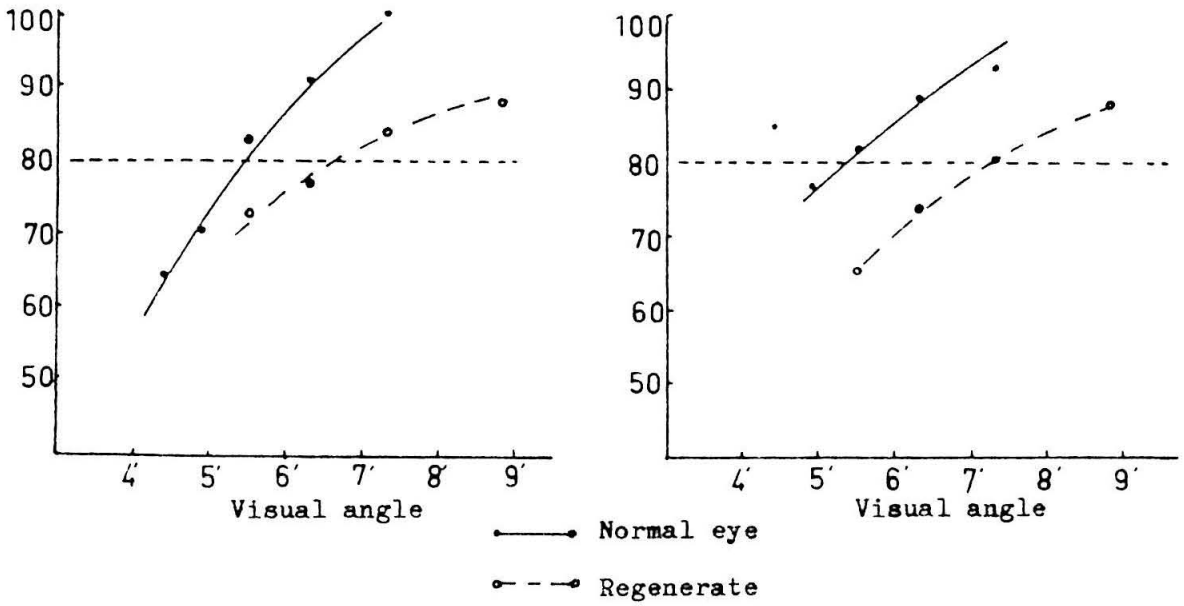


Fig. 14: Overall performance of fish #74, learning period eliminated.

Fig. 15: Overall performance of fish #75. Learning period eliminated.

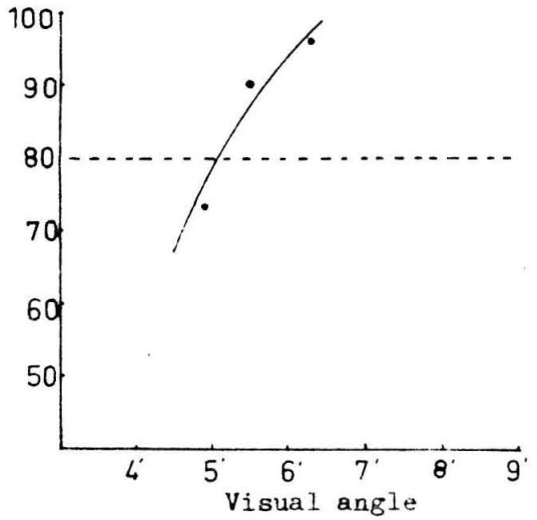


Fig. 16: Fish 76.

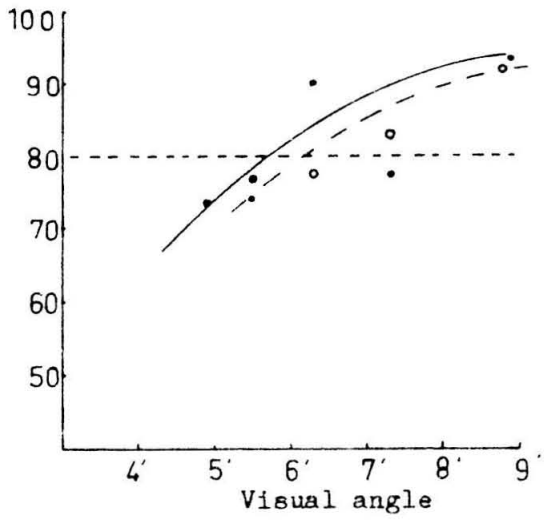


Fig. 17: Fish 78

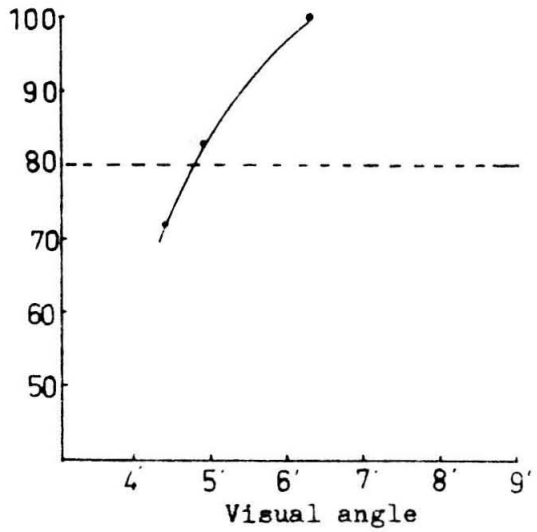


Fig. 18: Fish 79.

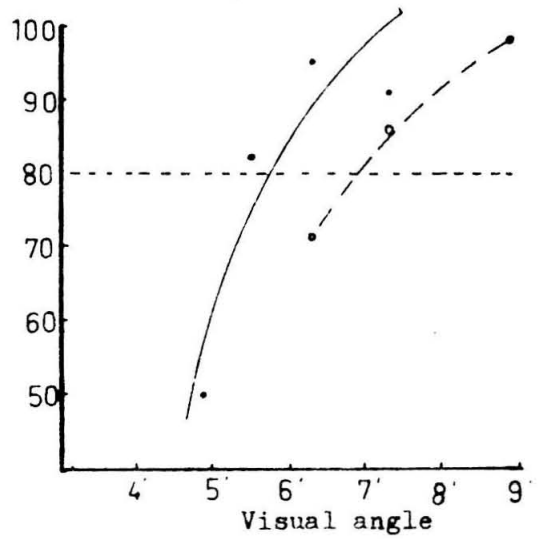


Fig. 19: Fish 82

Figs. 16-19: Overall performance curves.
Learning period omitted.

——• Normal eye o- - -o Regenerate

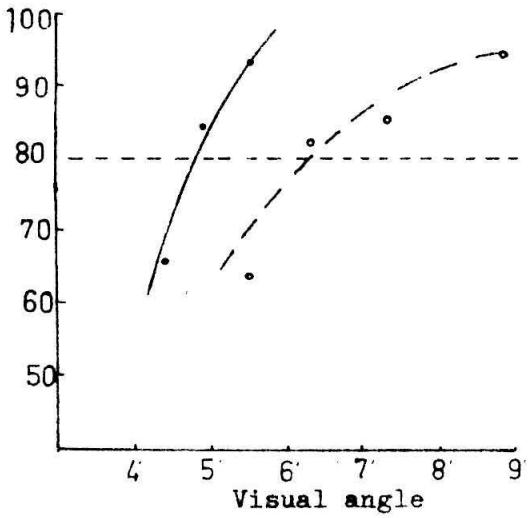


Fig. 20: Fish 84.

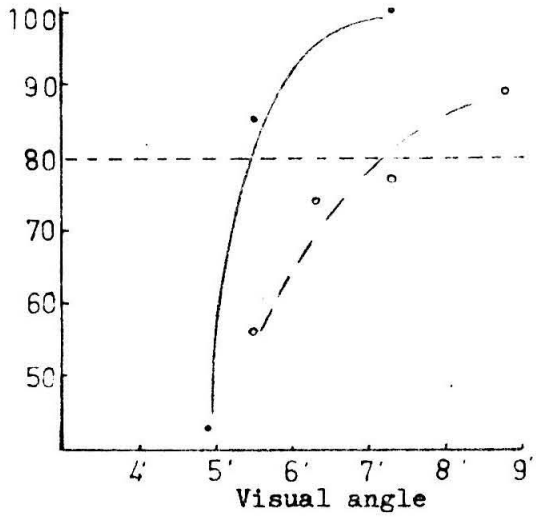


Fig. 21: Fish 85.

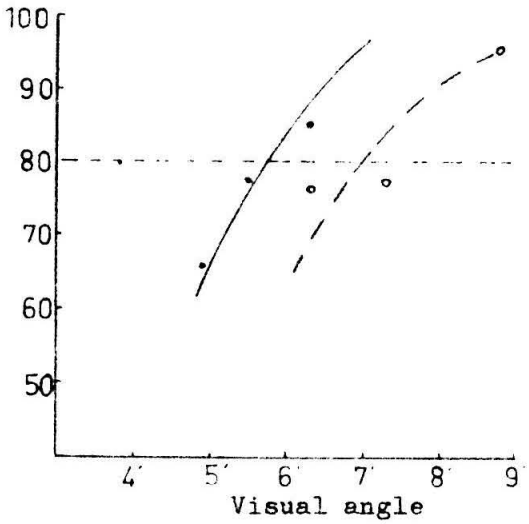


Fig. 22: Fish 86.

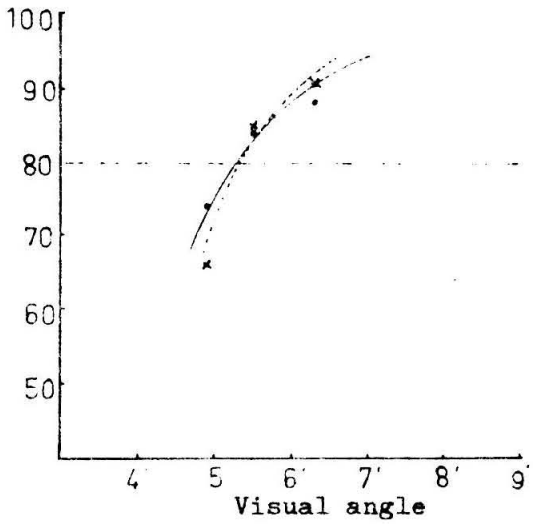


Fig. 23: Fish 87.

x·····x Both eyes

Figs. 20-23: Overall performance curves.
Learning period omitted.

——— Normal eye — — — Regenerate

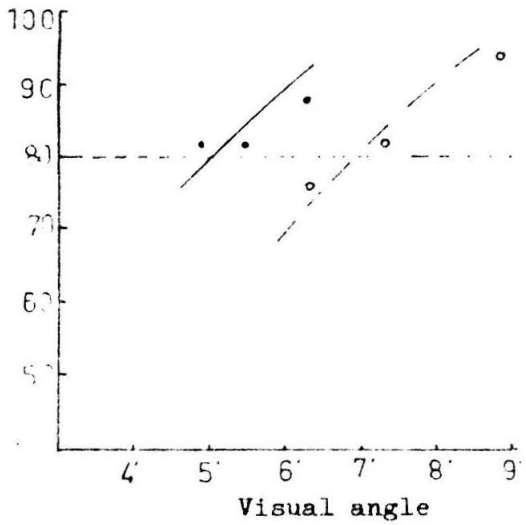


Fig. 24: Fish 89.

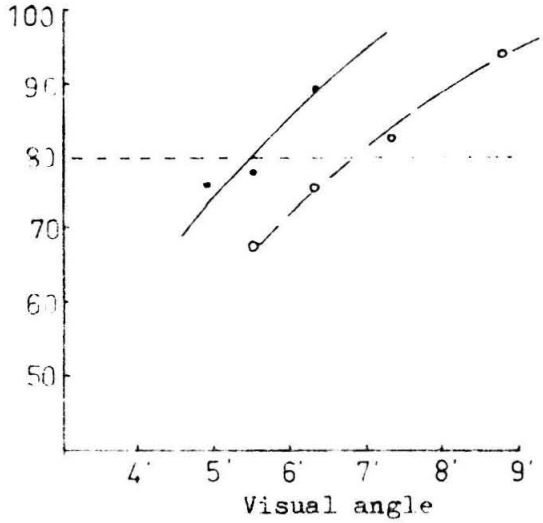


Fig. 25: Fish 90.

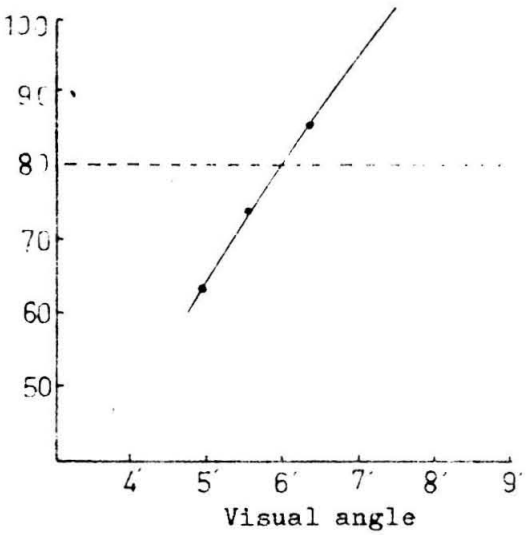


Fig. 26: Fish 91.

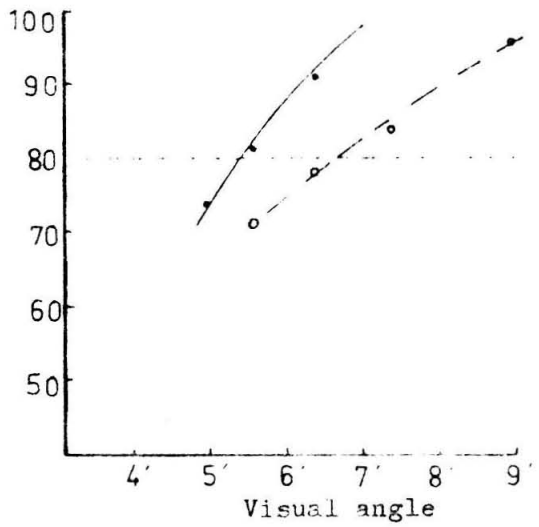


Fig. 27: Fish 92.

Figs. 24-27: Overall performance curves. Learning period omitted.

——— Normal eye - - - - Regenerate

from the upper limits of acuity measured experimentally.

TABLE 1

Fish No.	Eye	Acuity (minutes of visual angle)
74	R	5.5'
75	R	5.3'
76	L	5.1'
78	R	5.7'
79	L	4.7'
80	L	5.6'
81	L	4.7'
82	R	5.7'
84	R	4.8'
85	L	5.5'
86	R	5.7'
87	Both eyes	5.3'
	Left eye	5.3'
89	R	5.0'
90	L	5.5'
91	L	5.9'
92	R	5.3'
	Average:	5.3' ± S.E. 0.09

b) Restoration of Acuity after Regeneration

Several factors might operate to lower the acuity of an eye after the operation involved in blinding, aside from the pure effects of interruption and regeneration of the nerve fibers themselves. It was conceivable that a mechanical disturbance (distortion of the eye-ball, or damage to ocular muscles) could have occurred. Also, the ciliary nerve supply might have been accidentally interrupted and not successfully repaired by growth processes.

It is generally stated that fish eyes at rest are myopic (50) and the eye at rest is focussed for a distance of about five inches.

Accommodation in fishes is thus for far vision; as the tests were performed at a short distance, focussing would therefore seem to be a relatively unimportant factor. Unlike accommodation in mammals, focussing in fishes is achieved by retraction of the lens (63,64) or by distortion of the eyeball by contraction of fibers in the ciliary body (65). In either case the nervous impulses responsible are supplied by the ciliary nerve. Branches of this nerve run through the orbit separately from the optic nerve (63) and might conceivably be damaged during the operation. For this reason, a mock operation, including all manipulations except the actual cutting of the optic nerve, was performed on two fish, after they had reached a plateau in test performance. Following the mock operation, the performance of these two fish was not impaired.

The time required for regeneration varied with size and age of the fish. In one group of four 3½-month old fish of the same spawn (uniform 4.5 cm in length) the first signs of visual recovery were observed in each case 28-29 days after operation. In older fish, of varying size although from a single spawn, there was more variation; larger fish generally required longer for regeneration.

In the majority of fish observed in this study, regeneration of the operated eye was complete and may have been so for one to two weeks before the normal optic nerve was severed. This was of no consequence to acuity comparisons, since (a) transfer was not being tested, and (b) performance in acuity tests of a fish with two normal eyes was indistinguishable from that of the same fish (#87) with only one normal eye.

Since, however, the first signs of vision can be detected reliably only in a fish which has previously been completely blind (it reacts first to light, then to food and the feeding situation), the values

obtained for regeneration time were only rough estimates. The mean time for recovery of vision in the operated eye, based on these estimates, was recorded for 19 fish, between six and eight cm in length: 77 ± 29 days.

No abnormality was noted in the behavior of the experimental animals after regeneration of the optic nerve. They were able to localize food accurately and promptly, and responded correctly to the test situation. A series of tests was performed pre- and post-operatively with the "mottle" discrimination problem, to test for the quality of restored vision. Normal and operated (regenerated) fish were trained to discriminate between uniform dots and randomly-arranged dots of various sizes (see fig. 3). If the restoration of central connections were not orderly, and if various areas of the eye differed significantly in degree and quality of restoration, it might be expected that the fish could not distinguish a uniform from a mottled pattern. The learning curves of both groups of animals are shown (Fig. 4); speeds of learning for the two groups did not differ significantly. This is a gross indication that restoration of central connections is orderly, and probably fairly evenly distributed among all areas of the retina.

For visual acuity determinations, data were treated as for the normal eye. In most cases, little or no effect of learning was to be found in these data.

In fish #84, the normal optic nerve was cut before regeneration had been completed on the operated side. The fish was then completely blind. The first signs of vision in the first-operated eye then appeared 9 days later, 64 days after the original transection (by cutting). At 69 days after operation, the fish was taking food from the forceps; on successive days it responded correctly to the test situation,

and finally, 100-110 days after the original operation, the fish was giving a performance in acuity tests which was not surpassed in a total of 500 subsequent trials. As explained under "methods", the finest dots were not used at first in the testing series. So, in addition to the two-week lapse of time between first signs of vision and beginning of testing, another week elapsed between beginning of testing and the achievement of this high degree of performance. This three-week period is not long, relative to the total time required for regeneration. Thus it appears that under favorable conditions a high degree of restoration of acuity may be achieved rapidly.

In table 2, the data for the regenerated eyes, and the percentage of restoration of acuity, are summarized. The average percentage

TABLE 2

Fish	Normal Acuity	Operation	Post-op acuity	Percent Restoration
74	5.5'	cut	6.6'	83.3
75	5.3	pinch	7.2	73.5
78	5.7	cut	6.2	92.0
80	5.6	cut	7.4	75.6
81	4.7	cut	7.1	66.1
82	5.7	cut	6.9	82.6
84	4.8	cut	6.3	76.1
85	5.5	cut	7.2	76.4
86	5.7	pinch	7.0	81.4
89	5.0	pinch	6.9	72.5
90	5.5	pinch	6.8	81.0
92	5.3	cut	6.6	80.3
				Average %
				78.4 ±
				S.E. 1.9

of restoration of acuity was 78.4%. The average percentage of restoration in fish whose optic nerve was pinched was 77.1%; in cases where the nerve was cut, leaving the sheath intact, average restoration was 79.05%. Thus fish with operations performed by the two methods did not significantly differ in the extent of recovery achieved.

DISCUSSION

When level of performance of the fish was plotted as a function of the number of trials, an initial improvement in performance was observed, followed by a plateau which was then maintained for long periods of time (500-700 trials in some fish, over 3-4 months). The initial improvement in performance in tests of normal-eye acuity was not a function of size or age of the fish, for the following reasons: (1) The curve reached a plateau which was maintained over a period of weeks or months, although the fish continued to grow. (2) When training was interrupted for two weeks, values obtained after resumption of testing corresponded to the normal course of the curve; that is, either points remained on the plateau level, or showed an improved level of performance compatible with previous rate of improvement. (3) Individual fish showed different rates of improvement of performance. (4) No correlation was observed between age (or size) and the final level of performance achieved in acuity tests. This improvement in performance, therefore, did not seem to represent a true improvement in acuity. It may have represented, rather, an improvement in the fish's ability to concentrate on the problem and an increased attention to the difficult task of resolution presented by the smallest dots and the variable gray negative plaques. A statistical method was described for separating results from the learning period from the final results presented.

In agreement with the findings of Arora on interocular transfer, the fish showed little or no learning period when performing with the regenerated eye alone, after the trained normal eye had been blinded.

In this study, it has been assumed that visual acuity will depend upon a retinal mosaic of nerve cells, reporting to specific central tectal neurons or groups of neurons. It has been assumed that if this mosaic is made coarser, by eliminating some of the neurons, acuity will be decreased; and so a comparison of acuity in a normal eye and eye with regenerated optic nerve should give an estimate as to the extent of restoration of the retinal mosaic.

Cone diameter is not the only determining factor in visual acuity. Acuity may be less than predicted from receptor diameter, if there is multiple innervation; and it may be more, if certain kinds of neural networks are in operation, as in man. For example, in man (66) a shadow which causes a 5% decrease in the light falling on a receptor may be detectable. Even in this case, however, the receptor size has some importance, because the relation of cone size to the highest optical gradient in a moving pattern may determine whether the pattern is detected. Spacing of the receptors determines the rate at which different cells respond to a pattern moving at a given speed across the retina; and if this spacing is changed, visual resolution should be affected.

It may be objected that the central nervous system might react to neural signals indicating a <u>changed</u> excitation, as one cell and then another is affected by the light-dark alternation. In this case, regeneration might restore visual resolution without perfect restoration of an organized mosaic. Leaving the question of spatial organization aside momentarily, it would appear that a certain <u>minimum</u> number

of "changed excitation" reports must be received by the CNS before it will react as if to the information "dotted pattern". The size of the dots required to exceed this threshold in the CNS is then a measure of how many cells are available to report "changed excitation", as compared with that established on the basis of the neurons of the normal optic nerve.

Assuming the simplest relation between acuity and retinal mosaics in the fish, the acuity before and after optic nerve section and regeneration has been measured. Acuity after regeneration was slightly decreased, with respect to the normal eye. Is this decrease significant, or can it be considered to denote practically 100% recovery?

Two factors would be expected to lower the degree of restoration of visual acuity below the maximum theoretically attainable, 100% of the original acuity:

First, a certain percentage of the nerve fibers may fail to penetrate the scar tissue formed at the point of transection, or may reverse their direction of growth on the way; some ganglion cells might fail to regenerate at all; and some regenerating fibers might never form connections in the optic lobe. These factors make it improbable that 100% regrowth would be attained in most cases. More small nerve fibers can be counted in a regenerating peripheral nerve trunk than in the original stump (67), because each axon sends out a group of fine fibers, only part of which will establish functional connections. (Thus fiber counts cannot be used to determine how many ganglional cells send axons which successfully reach the optic lobe.) This multiple sprouting might conceivably compensate for part of the losses by blockading scar tissue or reversal of direction of growth.

Second, after part of the nerve fibers have successfully grown to the optic lobe and spread fan-like through the surface layers, the appropriate connections must be found and formed between optic axons and cells in the lobe. As Sperry has inferred, nerve specificities must somehow operate to ensure that the connections which are formed and continue to function are those compatible with previous patterns of optic projection and existing patterns of central nervous organization. The efficiency with which such processes might operate is not known.

The number of connections finally successfully formed should then be reflected in the percentage of restoration of visual acuity, as measured by behavioral tests.

We may imagine two mechanisms which might operate to restore central nervous connections in such a way that the animal shows recognition of discrete objects and accurate spatial localization (or else the systematically disoriented response patterns analyzed in cases of experimentally rotated eyeballs). These two mechanisms would differ with respect to the efficiency of restoration which they effect, and so could be distinguished experimentally by a quantitative measurement such as visual acuity.

a) The optic axons might, upon entering the optic tectum, form synaptic connections randomly with whichever central neurons happen to lie in the path of their growth. Only those connections which by chance happened to correspond to the old terminations, however, would be functional (or behaviorally observable). Thus the number of successfully restored connections would be determined purely by the number of regenerating fibers which by chance made the proper central connections.

The animal might then still manifest appropriate behavioral orientation; but as only a small fraction of the connections would participate in behavior, one would expect a marked reduction in the animal's visual acuity, as compared with normal.

b) The optic axons might enter the tectum at random, as above; but in the course of growth, each axon might give rise to a large number of exploratory processes. Many of these growing fiber tips would encounter an unfavorable chemical milieu; others, however, would push into more favorable regions and direct the growth of the entire fiber into this direction. If a fiber then happened to contact a nerve dendrite or soma of the appropriate specific chemical constitution, a functional synaptic ending would be formed. Only then would fiber growth cease. No long-range chemical guidance need be postulated here; nevertheless, if each fiber is thus able to sample a relatively large area of tectum throughout its course of growth, if growth continues until synaptic connections are formed, and if synaptic connections are formed only when the specific chemical conditions are favorable, a much larger fraction of fibers would be expected to form the appropriate functional central connections by this "searching" mechanism than by the postulated mechanism (a). In this case, one would expect to measure a fairly high order of restoration of visual acuity, in addition to the gross behavioral orientation already previously observed.

Considering the many possibilities for loss of fibers, a 78% restoration is high, under these experimental conditions, and seems to indicate that almost all optic nerve fibers which have reached the optic tectum are able to make specific functional central connections. Thus we are forced to assume that there exists a mechanism which provides a better efficiency in establishment of functional synapses than that

provided by mechanism (a).

If nerve fibers actually "search" for their specific destinations, as pictured under mechanism (b), then we must inquire whether the construction of the experiment allowed sufficient time for this process to achieve its maximum degree of restoration. Since nerve fibers may enter the optic tectum at random, growth processes may continue for a considerable length of time before the nerve fiber has successfully made contact with an appropriate central neuron.

The distance from the back of the retina to the caudal tip of the optic tectum, in a 9 cm fish, is at most 20 mm. It is known that peripheral regenerating nerves may have a rate of growth of 0.2-1.4 mm per day (68,69,70,71) but the regeneration time in a 6-8 cm fish was 77 $\pm$ 29 days (based on behavioral criteria); in 9 cm fish, about 100 days. This could allow time, in these experiments, for a slow advance of the growing nerve fiber through tectal tissue, sending out many exploratory fibers to sample the surrounding chemical milieu, and to atrophy or else guide fiber growth in a favorable direction.

If time were a severe limiting factor, one might have expected to observe a steady, noticeable improvement in performance during the course of 3-4 months of post-operative testing. As mentioned above, this improvement occurs to only a slight extent, when it occurs at all. It may be that the majority of fibers grow synchronously toward the optic lobe, reach it almost simultaneously, and then are growing through the tectum and "searching" also at the same time. The tectum is small, in terms of possible growth rate; and one might imagine that most fibers succeed in making connections within the space of 1-2 weeks. Thus, when vision becomes apparent behaviorally, many of the connections

which are to be formed are already present, and most of the remaining connections will be formed within a short period of time thereafter--too short to be reliably measured by slow psychological testing methods.

It may be questioned whether measurement of visual acuity can ever give an overall picture of the ability of the optic fibers to find their specific central connections, if one assumes that the measured visual acuity is always that of the restricted retinal area with the finest receptor mosaic and most successful central innervation. The comparison is perhaps being drawn between areas of maximum acuity in pre- and post-operative eyes; it may be that some retinal areas remain unrepresented, and never find central connections. These "holes" might be overlooked by behavioral tests; post-operational measurements of acuity might thus be based on relatively small, isolated islands of the retina where a high degree of recovery had been achieved.

Behavioral clues hint that this objection is probably not a serious one. Visual reactions of the fish with regenerated nerves, to objects approaching from all directions, could not be distinguished from those of normal fish; they were also able to learn the "mottle" problem just as readily as normal fish. The breeding and fighting behavior of post-operational fish, when mature, was identical with that of normal fish (72). Even if such restricted "islands" of high resolving power should exist, they would not alter the main conclusion which is to be drawn from these experiments; that there exists a very fine pattern of overlapping gradients of nerve specificities, which is operative in formation of connections between receptors and central associative areas--as shown in those parts of the eye with 78% recovery of visual acuity.

Mechanism (b) does not postulate long-range chemical guidance. But when functional synaptic connections are formed, they are determined and regulated by cellular specificities (perhaps of a chemical nature). A type of "searching" mechanism, such as that described above, would provide the high degree of specificity in synaptic arrangements found to conform to the neuronal patterns already laid down in the course of individual embryonic development; and it could provide a high degree of restoration of detailed vision, such as that observed in these experiments.

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