ABSTRACT

A COMPARATIVE STUDY OF WATER AND SODIUM PERMEABILITY OF LAKE TROUT AND RABBIT CORNEAS

by Henry Francis Edelhauser

Experiments with corneas *in vitro* have shown that water permeability of lake trout corneas is the same whether the data are based on the transfer of tritiated water from the epithelial to the "endothelial" bathing media or vice versa. In these experiments there was present an osmotic gradient of some 6.6 atmospheres which favored movement of water through the cornea to the solution bathing the "endothelium." Fish corneas were found to be impermeable to sodium ions in both directions. Water permeability constants and sodium flux for normal and denuded corneas indicate that the "endothelium" offers some resistance to the transfer of water and sodium into or across the fish cornea, but the major resistance to this movement is provided by the epithelial layer.

Correlations between water permeability, sodium flux and histopathological conditions of diseased corneas are noted. Corneas in the late stages of the disease are at maximal hydration *in situ*, whereas, the normal and early disease stages imbibe water *in vitro* from the bathing media. Corneal sodium ion concentration varied inversely with corneal hydration in diseased lake trout corneas.

For fish corneas the permeability constant for movement of sodium across the "endothelial" barrier in a cornea-aqueous direction is 0.029mEq/cm^2/hr some 16% lower than that observed in rabbit corneas. HTO and Na^{22} flux in this fish corneal perfusion study occurred independent of each other; whereas, with rabbit corneas osmotic flow of water occurred. Rabbit corneas had a 3 to 5 fold greater water exchange than fish corneas and they were found to actively transport sodium in the tear-aqueous direction; however, in the fish cornea no sodium flux occurs. An adenosine triphosphatase was identified in the basement layers of the corneal epithelium, stroma and corneal "endithelium" of fish.

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Dedicated to my wife, Barbara, whose
sacrifices and encouragements made
the completion of this dissertation
a reality.

TABLE OF CONTENTS

LIST OF TABLES

APPENDIX TABLES

LIST OF FIGURES

APPENDIX FIGURES

INTRODUCTION

In the movement to restock waters of the Great Lakes region with lake trout, which have been depleted by the sea lamprey, both state and federal hatcheries have increased their lake trout rearing programs. Concurrently with this rearing program in the state of Michigan, it has been observed by Allison (1963) and Hoffert and Fromm (1965) and others that hatchery-reared lake trout are susceptible to developing various types of eye abnormalities, such as severe corneal and lenticular lesions.

If the literature on corneal physiology of the past twenty years is examined, it will be found that corneas adapted to an aerial environment have excited enormous interest; in so doing they have caused a general disregard for the phylogenetically more primitive corneas which develop in an aquatic environment devoid of a tear film, and, in the case of the fresh water fish, exposed to a hypoosmotic medium. In such species physiological regulation is accentuated, for the tendency of water to enter the cornea and salt to leave it is greater than in the case of mammals where corneas are bathed with slightly hyperosmotic tears.

A comparative study of water and sodium permeability of the isolated normal and pathological lake trout cornea was undertaken. Also investigated were the roles of the "endothelial" and epithelial layers of the cornea, as related to water and sodium permeability. (The word "endothelium" is enclosed in quotation marks because it is not really endothelium, but mesothelium or mesenchymal epithelium.) Experiments using rabbit corneas were also performed in order to compare corneas of aerial environments to those from aquatic environments.

The regulatory mechanisms by which the fish cornea remains transparent are presently unknown. Physiological processes involved in maintaining water balance in connective tissues are fundamental; are of great importance in diseases of connective tissue in general, and are especially interesting in a situation where there is present a potential osmotic gradient of 6.6 atmospheres favoring the uptake of water by the cornea across the epithelial layer. The ultimate objective of this investigation was to acquire some understanding of how the transparency of the fish cornea is maintained.

Along with an experimental procedure whereby the composition of fluid on the two sides of a clamped in vitro cornea can be controlled, analytical methods were applied utilizing titrated water (HTO) and radiosodium (Na^{22}) in this study of corneal permeability. Using these tracers, permeability was quantitated in terms of microgram and microliter quantities. With a more recent isotope technique "dual labeling," one can investigate the simultaneous behavior of two ions, a procedure of considerable importance in studies of water balance. Using this technique HTO and Na^{22} were studied concurrently to see: (1) if the water movement is dependent upon the sodium movement (as is the case with most other tissues), (2) if their fluxes are of prime importance in understanding the maintenance of fish corneal transparency, and (3) if the increased osmotic pressure of the external environment had any effect. Data from this study permitted comparisons of the permeability characteristics of the aquatic cornea (normal and diseased lake trout) to that of mammalian cornea (rabbit) to be made.

LITERATURE REVIEW

Studies of corneal permeability have been mainly centered around mammalian species. This research dates back to 1853 and has been extensively reviewed by Maurice (1953) who compared the reported rates of penetration of ionized substances across the cornea and its layers with that of Na^{24}. In general, the evidence suggests that the substances in solution within the corneal stroma exchange by passive diffusion with those in the plasma at the limbus, and, within a size range of 10-25 Å those substances in the aqueous humor cross the "endothelium." Maurice postulated that there is a small co-existent active transport of some substances out of the cornea which accounts for the maintenance of its normal thickness. In a more recent review (Chapter 6 in "The Eye" edited by Davson), Maurice (1962) deals comprehensively with such corneal problems as the physical basis of transparency, hydration, permeability, nerve supply, and wound repair. Dohlman (1963, 1964) composed the annual review on the cornea and sclera for those respective years. The permeability of the cornea as it relates to ion and water movement in teleosts has been reviewed by Edelhauser (1964). Donn (1965, 1966) wrote the corresponding annual review on the cornea and sclera and in the most recent publication he described the physiology of the posterior corneal membrane extensively.

Water Permeability

Cogan and Kinsey (1942a) presented data on water transfer through the excised cat cornea in the aqueous to tears direction. This transfer results in establishment of an osmotic gradient by an intact epithelium. Their results showed no net transfer of water in the anterior to posterior direction because bullae developed and consequently epithelial damage occurred. Nevertheless, they inferred that deuterium oxide is transferred through the excised cornea by diffusion in both directions, since the fluid volume remained constant. Therefore, every molecule of heavy water that was transferred necessarily was replaced by a molecule of water coming from the opposite side of the cornea. They (Cogan and Kinsey, 1942b) proposed a theory of fluid movement within the cornea, where water is continuously abstracted from the anterior and posterior surface by osmotic forces.

Anseth and Dohlman (1957) reported on the influence of intraocular pressure on the water-binding capacity of the corneal stroma in isolated bovine corneas. They noted that "endothelial" damage causes considerable corneal swelling whereas epithelial damage has a lesser effect and can be explained on a purely physical basis of pressure. They observed that intraocular pressure counteracts the corneal inhibition of tear fluid, but not that of aqueous humor; therefore, they implied that the effect of hydrostatic pressure is probably responsible for the difference in swelling when the epithelium and "endothelium" are removed. Hedbys and Mishima (1962) have measured the resistance of the corneal stroma to the flow of water both along and across the collagen fibers of the cornea as well as with various degrees of hydration. In these investigations of flow along the stroma, a marked difference in thickness was generated by allowing a part of the corneal strip to swell and the adjacent part to dry. Water equilibrium along the strip was established by the water movement from the swollen to the dry part and the profile was evened out. By repeated measurements of thickness along the strip, they established the flow of water. Since the relationship between thickness and swelling pressure was known, the pressure gradient along the cornea was determined. This flow of water across the stroma was determined by pressing 0.9% NaCl or water through a cornea tissue tightly sealed in a holder between two porous stainless-steel filters and collecting the water in a capillary tube. They found the resistance to flow in the two directions to be similar at normal hydration, but for dried cornea the resistance was higher across the tissue than along it. The calculations show that the collagen fibrils alone offer only 1/50 of the measured resistance. They suggested that the interfibrillar substance is responsible for the high resistance to the flow of water in the corneal stroma.

Donn (1962) reviewed information on the movement of solutes and water across the cornea and summarized the recent work on water diffusion and "endothelial" pinocytosis of the mammalian cornea. Direct measurements (with tritiated water) of water movement across the *in vitro* rabbit cornea were made by Donn *et al*. (1963). They found the water flux across the rabbit cornea was the same in either direction. This movement of water across the cornea can be accounted for by diffusion alone, without any bulk flow; the loss of water from the aqueous into the tears under conditions of normal hydrostatic pressure is negligible; and the resistance of the epithelium and "endothelium" to the diffusion of water is comparable to that of the stroma.

Hedbys *et al*. (1963) have shown that when the cornea of a living rabbit is cannulated a negative pressure with respect to atmospheric pressure is recorded. When the normal intraocular pressure is accounted for, the resulting negative pressure would then correspond to the swelling pressure of the excised tissue. This proves that the stroma has a tendency to swell *in vivo* which normally must be countered by an active transport mechanism located in the limiting layers of the cornea. Mishima (1965) studied the water permeability of the rabbit cornea with reference to the superficial oily precorneal tear film. He found this layer to retard the evaporation of the tear film from the corneal surface; the "evaporation tonicity" of the precorneal tear film increases on opening the eye and the cornea becomes slightly thinner and vice-versa. The tonicity increase in open eyes results in an osmotic water flow from aqueous through the cornea to the tear film.

Information on corneal hydration as related to corneal metabolism, a possible water pump, and passive diffusion as a result of active ionic transport, has been reviewed by Edelhauser (1964).

Ionic Permeability

The effect of hydrostatic pressure on the transfer of sodium chloride through the excised cat cornea has been studied by Cogan and Kinsey (1942c). They found that the cornea is impermeable to 1 percent sodium chloride solution in the out-in direction with a hydrostatic pressure up to 400 mm Hg. but permeable in the in-out direction under various hydrostatic pressures. The permeability to sodium chloride is in one direction only and is controlled by the epithelium. Maurice (1951) investigated the permeability of the <u>in vivo</u> rabbit cornea to sodium ions. First Na^{24} was placed in a conjunctival sac and allowed to remain in contact with the corneal epithelium for various times at the end of which the cornea and aqueous humor were removed and their activities measured. It was then possible from these results to derive most of the permeability relationship between the cornea and its external media. Secondly, Na^{24} was injected into the blood and the concentration in the plasma, aqueous humor and cornea measured. Thirdly, radioactive salt was introduced directly into the anterior chamber and the changes in activity with time in the cornea and aqueous humor were determined. With the data obtained Maurice was able to calculate permeability constants for the cornea "endothelium," epithelium, entire cornea and blood-aqueous barrier. He concluded that the corneal epithelium has a finite but low permeability to inorganic ions in both directions (0.00080-0.0024 $cm.hr^{-1}$); the corneal "endothelium" has a much higher permeability to inorganic ions in both directions (0.072 $cm.hr^{-1}$) than does the epithelium; and this sodium extrusion resident in the "endothelium" accounts for the maintenance of corneal dehydration.

Potts (1954) reviewed literature dealing with the permeability of corneal layers as demonstrated by tracer experiments and stated that only with isotopic tracers have analytical methods become available allowing analysis of sub-microgram amounts of inorganic salts and water in ocular tissue.

Active transport of a substance has been proposed for the mammalian cornea and, strictly defined, requires its movement to be against an electrochemical potential gradient from one side of a membrane to another. The proof of such transport requires control of the composition of fluid and electrical potentials on the two sides of the membrane.
In the case of the cornea the necessary conditions have been achieved by clamping a freshly excised tissue from a rabbit between two chambers (a modified Ussing-Zerahn Chamber, Ussing 1949) so that the fluid could be circulated over each surface, Donn *et al*. (1959a, 1959b). The electrical potential was measured by using non-polarizable calomel electrodes, and it was found that a potential difference of 10 to 40 mv was

built up across the cornea with the outside of the cornea negative with respect to the inside. By removal of different layers it was found that the potential was generated by the epithelium (Potts and Modrell, 1957; Donn *et al.*, 1959b). These experiments demonstrated that sodium is actively transported across the epithelium into the corneal stroma and that the net flux of sodium is nearly equivalent to the neutralizing current.

Green (1965, 1966) measured ion fluxes which occurred during the first hour after excision of the rabbit cornea. He found that both sodium and chloride ions are actively transported across the cornea from the epithelial to "endothelial" surface. The location of this transport "pump" suggested by Green was in the epithelium. His idea of a chloride pump is consistent with that of Cogan and Kinsey (1924a). Active chloride transport by the cornea is also supported by the work of Zadunaisky (1966) who has shown that chloride ions are actively transported across frog corneas, and that this represents the only source of current across this "membrane." He measured the net rransport of chloride from the aqueous to the tear side of the cornea. Davis and Zadunaisky (1966) have proposed that potassium ions are required for this chloride transport and that both of these ions are involved in maintenance of corneal transparency.

Fish Cornea Water and Ionic Permeability

Aquatic corneal physiology has been studied very little except from the anatomical point of view (Walls, 1942a; Varbec, 1959). Varbec provided evidence for the existence of a doubted fundamental corneal "endothelium" of the teleost which can be clearly demonstrated in a flat preparation. Fish eyes are of physiological interest because they have transparent corneas adapted to media of extremely varied osmotic pressure. The cornea of the fresh water fish is constantly in contact with a hypoosmotic solution, whereas, for most salt water fishes, sea water is hyperosmotic with respect to their tissue fluids. In one group of marine fish, the elasmobranchs, the internal osmotic pressure is so high that their external environment is relatively hypoosmotic to the internal one.

In 1954 Smelser and Chen did a comparative study of the structure and hydration properties of corneas adapted to air and aquatic environments. They have shown that guinea pig and carp corneas are similar anatomically but have different hydration properties. In his Procter Award Lecture, Smelser (1962) reported on three types of aquatic corneas: carp-fresh water, scup-marine, dogfish (elasmobranch)-marine. He found corneas to be metachromatic indicating the presence of mucopolysaccharides but markedly less than mammalian corneas. Fish corneas were found to swell in a manner similar to mammalian corneas when placed in various physiological salt solutions; however, the degree of corneal swelling in distilled water was greater in the scup, which is adapted to life in sea water, and less in the carp which lives in a hypoosmotic medium. The elasmobranch cornea differs from those of all other species so far studied in that they do not hydrate in any solution ranging from hypoosmotic to hyperosmotic. These results led to the conclusion that the mechanism which keeps the teleost cornea dehydrated and transparent is diffusion of water from the cornea. This mechanism probably depends upon the colloid-osmotic pressure of aqueous-humor rather than on an active pump, which in turn would be dependent upon the metabolic activity of the epithelium or "endothelium" as is typical of mammals.

Cation concentrations and water content in the normal rainbow trout cornea, aqueous humor, lens, and vitreous humor were presented by Edelhauser _et_ _al_. (1965). The _in vitro_ rainbow trout cornea showed a limited permeability to both water and ions, and corneal ion concentration appeared to vary inversely with corneal hydration. Also postulated was an exchange of water across the trout cornea rather than a net movement which may have been caused by the osmotic gradient.

Allison (1963) reported that when control of the sea lamprey appeared imminent, production of lake trout in hatcheries was increased. Coincident with this increase, certain diseases of lake trout became evident. One disease, cataract, results in permanent damage to the eye. Another eye disease which occurs in hatchery-reared lake trout from the Harrietta, Michigan, hatchery is a corneal lesion. Pathological progression of this lesion has been categorized into stages and described by Hoffert and Fromm (1965).

MATERIALS AND METHODS

Experimental Animals

The two- to four-year-old (18-29 cm long) lake trout (*Salvelinus namycush*) used in these experiments were provided by the Michigan Conservation Department from their hatchery at Harrietta, Michigan. This age group was selected because their corneal diameter was one centimeter which was necessary in order to clamp the cornea for *in vitro* studies. Lake trout were transported from the hatchery in a galvanized metal tank lined with non-toxic paint and fitted with an agitator for aeration. In the laboratory they were kept in fiberglass lined tanks with continuous inflow of dechlorinated water at one end and an overflow spout at the other end of the tank. Conditions of 9°C and 15 hours light and 9 hours darkness were maintained each day.

Small rabbits of 0.5-1 kg. were used because their corneas had a diameter of one cm. which was desirable for the apparatus.

Apparatus

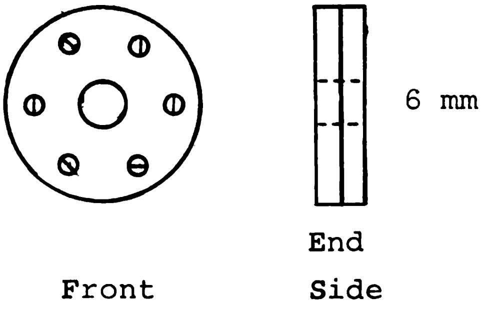
A technique for holding trout corneas *in vitro* between two chambers in such a manner to allow the fluid bathing each surface to be regulated has been previously described by Edelhauser (1964, 1965). In this apparatus excised corneas are held rigidly in place between two lucite blocks which contain the aforementioned chambers. The corneal clamps are of two types: number 1 with a diameter of 0.6 cm. and number 2 with a diameter of 1.0 cm. These clamps are illustrated in full size in Figure 1. Figure 2 illustrates the perfusion blocks for permeability studies on fish cornea conducted at 13°C. Figure 3 shows the perfusion blocks for similar studies conducted on the rabbit cornea at 35°C.

Experiments with Tritiated Water

The experiments in this series were conducted with the corneas clamped with holder number 1. In all experiments chemically defined tissue culture medium (TC199) obtained from Difco Laboratories, Detroit, Michigan, was used to bathe the "endothelial" surface of the corneas and tap water was used to bathe the epithelial surfaces. Depending upon which direction water movement was to be investigated, 0.3 ml of tritiated water (0.1 mc HTO/ml) was added to either the 3 ml of TC199 or the 3 ml of tap water. Four hours after the addition of tritiated water, 20 microliter samples of the two media were obtained, using disposable micropipettes (Drummond microcaps, Drummond Scientific Co., Broomall, Pa.), and transferred to glass vials containing 15 ml of a liquid scintillation counting solution. That part of each cornea which was exposed to the two media was dissected free and also placed in 15 ml of counting solution. The counting solution contained a primary scintillator (5 gm PPO; 2,5-

Corneal holder

Number 1. 0.6 mm diameter



Number 2. 10.0 mm diameter

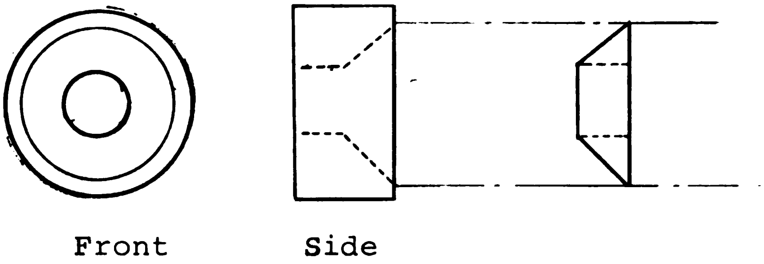


Figure 1. Corneal Clamps (actual size).

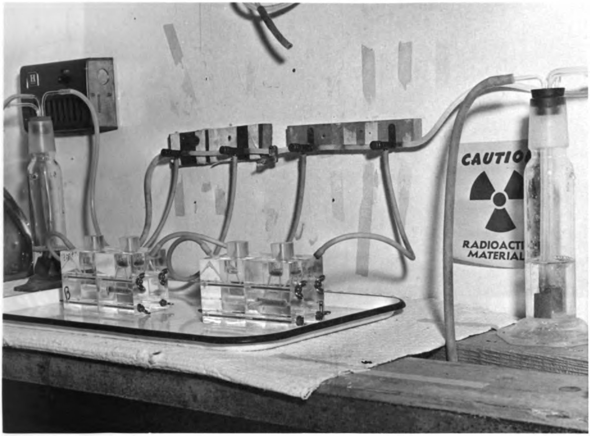


Figure 2. Pictorial view of fish cornea perfusion.

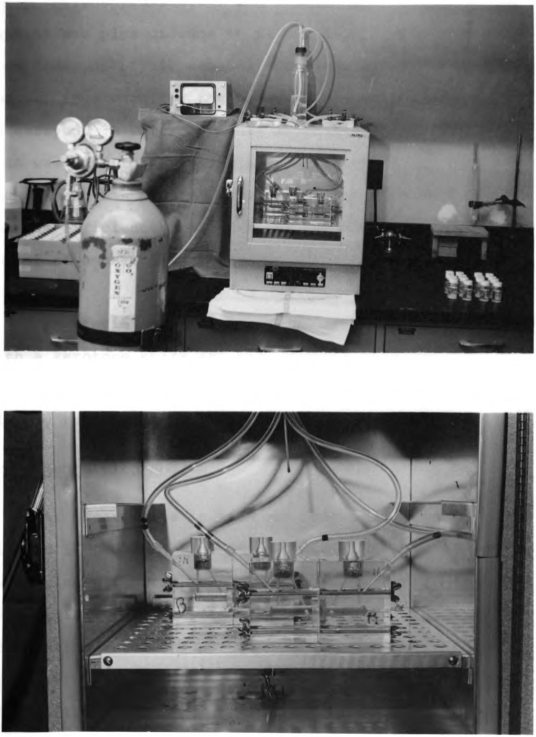


Figure 3. Pictorial view of rabbit cornea perfusion.

diphenyloxazole), a secondary scintillator (50 mg α -NPO; α-naphthylphenyloxazole), and a solvent system of 80 gm naphthalene plus dioxane to make 1 liter. Since dioxane is a good dehydrant, it removed the water and HTO from the cornea, thus enabling the activity within the corneas to be assayed. A Tricarb Liquid Scintillation Spectrometer 314A was used to assay the radioactivity in the samples. Each sample was counted three times for 10 minutes at a machine efficiency of 19 percent for tritium, and the average number of counts per minute was corrected for background activity.

In studies of the corneal layers, corneas were denuded with a kerotone knife and histological sections were made to verify that the epithelium and/or "endothelium" were completely removed by this procedure.

Experiments with Sodium^{22}

A. Experiments in this series were conducted with corneas clamped in the larger holder number 2. The fish were killed by cutting off their heads, and the eye was protrused and held rigidly in this position using a pair of forceps pressed down on the outer edge of the ventral side of the eyeball. The sclera was pierced approximately 3 mm lateral to the outer edge of the cornea, and a neat cut was made around the sclera at this distance from the cornea. The cornea was placed in a petri dish in tap water and iris removed, then it was placed in the clamp and held stationary by pressure from the inside sleeve of the clamp pressing on the sclera. All experiments were conducted in a manner similar to that outlined for tritiated water except 0.2 ml of Na^{22} (1.0 μc/ml) was added to an increased volume of 6 ml of TC199 or 6 ml of tap water. After the addition of Na^{22}, two 100 microliter samples were taken hourly for 4 hours from the two media, using disposable micropipettes. One sample for counting was transferred to a five-dram plastic vial containing 10 ml of distilled water. The sodium content of the other sample was determined using a Coleman 21 flame photometer (Edelhauser, 1965). That part of each cornea which was exposed to the two media was dissected free, cut in half, and one half digested with 10 ml concentrated nitric acid for counting. Tissue water content of the other half was determined by drying and then, after ashing, corneal sodium was determined (Edelhauser, 1965). All Na^{22} samples from these experiments were counted using a two-inch thallium-treated NaI well type scintillation detector, Nuclear Measurements Corporation, Indianapolis, Indiana, Model PHA-1CA pulse height analyzer, and Model DS-1A decade scaler used as a slave scaler. Counting was done at the 5 percent level of error or less. Since counts per minute of Na^{22} and total amount of sodium ion in both media and cornea were known specific activity was calculated.

B. In studies of the corneal layers, the procedure described for tritiated water experiments was followed.

C. Corneal sodium and water concentrations: whole corneas of normal and diseased Lake Trout were excised and corneal sodium and water were determined as described by Edelhauser (1964).

D. Back diffusion: corneas from a single fish were excised and mounted in the recirculation blocks as described, and the same amount of Na^{22} was added to the TC199 which bathed the "endothelium." After a two-hour "perfusion," the cornea was uniformally labeled with Na^{22} since the specific activity of sodium in the cornea was approximately equal to that of the "endothelial" solution. Two-hour samples were taken of both media (199 and tap) then the media were removed and one cornea was dissected free and cut in half: one half for counting, the other half assayed for total corneal sodium as previously described. This cornea was used as a control for the amount of Na^{22} in the cornea at two hours. With the other paired cornea, the tap water and TC199 were removed from the recirculation blocks and the blocks rinsed. The cornea, which remained in the clamp, was rinsed with saline and then placed back in the blocks, 5 ml tap water and 5 ml TC199 without tracer were then added to the recirculating system. The back diffusion of Na^{22} from the labeled cornea was measured in separate experiments by determining the amount of Na^{22} in the bathing fluids after 0.25, 0.5, 1.0 and 2.0 hours of perfusion. Corneal Na^{22} was assayed in the usual manner.

Dual Labeling Experiments Using HTO and Na^{22}

Experiments in this series were conducted with corneas clamped in holder number 2 which allows more surface area of the cornea to be exposed between the bathing solutions. The fish cornea was excised and clamped as previously described. The rabbit cornea procedure was as follows: Rabbits were killed with an intravenous injection of sodium pentabarbital (Nembutal) given via the marginal vein of an ear. An eye was protrused and held rigidly in position using a pair of forceps inserted under the eyeball and resting on the outer edge of the orbit. The sclera was pierced and a neat cut was made at a distance of approximately 3 mm lateral to the outer edge of the cornea. The cornea and ring of sclera were immediately transferred to a petri dish containing TC199 solution at 35°C and the iris removed along with any connective tissue which remained attached to the sclera. The cornea was rinsed in the TC199 and immediately mounted in the clamp and placed in the recirculating blocks. TC199 was added to both compartments and maintained at pH 7.2 by using a 95 percent O_2:5 percent CO_2 gas mixture in the siphons. Although there is considerably more hydration of the rabbit cornea with TC199 than with "Kinsey" solution (Mishima, 1966), TC 199 was selected in order to have experiments similar to those with fish corneas. Temperature of the entire system was kept at 35°C. by conducting the experiments in a gravity convection oven (Figure 3).

Depending upon which direction water and sodium permeability was to be investigated 0.1 ml of HTO (100 µc/ml) and 0.2 ml Na^{22} (1.0 µc/ml) were added either to the 6 ml of TC199 in the "endothelial" (En-199) or epithelial (Ep-199) compartment. Samples were taken as described in the Na^{22} experiments except that samples and halves of corneas for counting were transferred to glass vials containing 15 ml of the liquid scintillation counting solution described in the experiments with tritiated water. The HTO and Na^{22} content of these samples were counted simultaneously with a Nuclear Chicago Mark I Liquid scintillation system (see Appendix A for procedure). The counting efficiency in this procedure was 30 percent for HTO and approximately 94 percent for Na^{22} and all counting was done at a 2 percent level of error or less. Using a channels ratio technique (Nuclear Chicago Mark I) the degree of quenching was essentially constant, therefore, no correction for quenching was made. With this counting scheme there is some overlapping of the Na^{22} spectrum in the channel peaked for tritium. The necessary corrections were made according to the detailed procedure outlined in Appendix A. Simultaneous counting for corneal HTO and Na^{22} was also possible without digesting the cornea. Since dioxane is a good dehydrant it removed the water and HTO from the cornea thus enabling the activity within the cornea to be assayed. Dioxane also removed some Na^{22} from the cornea but the majority of the Na^{22} remained within the cornea. With the corneal Na^{22}

distributed in this manner the Na^{22} activity was assayed and corrected for quenching. In order to justify counting cornea sodium in this manner for comparison with media sodium the following experiments were run: (1) When corneas were digested with Nuclear Chicago N.C.S.TM Solubilizer counted and corrected for quenching, the activity was similar to that received when corneas were not digested and located on the bottom of the vial. (2) When the cornea was suspended in the scintillation counting solution on a wire the counts were the same as if the cornea was resting on the bottom of the vial. (3) position of the cornea on the bottom of the vial had no effect on determination of corneal Na^{22}. (4) A Na^{22} solution on the bottom of a vial without fluors and scintillation counting solution and Na^{22} in dioxane without fluors gave five or six counts above background.

After the perfusion experiment was completed the cornea was removed from the clamp, and that part of the cornea exposed to the two media excised from the sclera and cut in half. One half placed in 15 ml of scintillation solution the other half weighed, dried and analyzed in the usual manner. Specific activities for Na^{22} and HTO in the tap water, TC199 and cornea were calculated.

Corneal Adenosine Triphosphatase

Lake trout corneas were excised fresh frozen, sectioned immediately with a cryostat microtome at 16 μ, sections mounted on slides and incubated. This method, without fixation, for fish cornea differs from that used for the rabbit cornea (Ehlers, 1965).

To demonstrate ATPase the method of Wachstein and Meisel (1957) found in Pearse (1960) was used with slight modifications. The principle underlying this method is that the phosphate ions, which the enzyme liberates from ATP, are trapped as lead phosphate. The lead phosphate is subsequently converted into black lead sulphide. Black or brown-black deposits thus indicate the site of enzyme activity. Differences between the method used and that of Wachstein and Meisel are: (a) ATP (disodium salt) was dissolved in 0.8 M saline; (b) the incubating medium was filtered before the sections were placed in it; (c) the fresh sections were incubated for 20, 40, and 60 minutes at 25°C and; (d) after incubation one quick dip in 1 percent yellow ammonium sulphide was sufficient to develop the deposits.

Control experiments were made on fish gut and cornea incubated without ATP.

Surface Area of Cornea

By using cornea holder number 2 and clamping the sclera rather than the cornea as hold number 1 does, the cornea of the fish and rabbit holds its spherical shape. The superficial area of the cornea was calculated using the following formula:

$$\text{curved surface} = 2\pi rh = \frac{\pi}{4}(4h^2 + c^2)$$

where r = radius; h = height; and c = chord length = 1.0 cm.

	Rabbit	Lake Trout
Radius	0.57 cm	0.69 cm
Height	0.30 cm	0.20 cm
Chord	1.00 cm	1.00 cm
Surface Area	1.06 cm^2	0.88 cm^2

One can observe from the calculated surface area that the rabbit cornea is more spherical than the fish cornea. When placed in identical clamps the rabbit corneas have an exposed surface area some 20 percent greater than for fish corneas.

RESULTS

I. Theoretical

It is convenient at this stage to formulate in a simple manner some of the diffusion relationships between the cornea and the fluids bathing the eye. This will help to establish terminology, to make evident some of the assumptions that have to be justified, and to emphasize numerical constants which must be evaluated.

Experiments with HTO - Equations

The Equation (3) used for the calculation of the permeability constant of the cornea to water, will indicate the time course of change in radioactivity in the labeled compartment when the opposite chamber has a finite volume rather than an infinite volume (Mishima, 1966). It does not take specific activities into account; therefore, the radioactivity measurements are converted directly into µl of water per hour that cross the cornea. It is assumed that there is perfect mixing in each chamber. Equation (3) does take into account any HTO that appears in chamber two and exchanges in the reverse direction from which it has come. Permeability constants were calculated over 4 hours since the diffusion rate is linear with time over this period.

In Figure 4, symbols are the following:

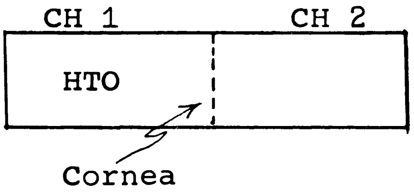


Figure 4

V = volume of chamber 1 = chamber 2 = 3 ml
A = area 0.28 cm^2
Kp = permeability constant
c_1 = conc. of HTO in chamber 1 at given time
c_2 = conc. of HTO in chamber 2 at given time
c_o = conc. of HTO in chamber 1 at time 0

Then $$\frac{dc_1}{dt} = \frac{-dV}{dtV} = \frac{-KpA}{V}(c_1 - c_2) \qquad (1)$$

Neglecting the HTO in the cornea (small volume)

$$C_1 + C_2 = C_o = \text{constant}$$

Therefore $C_2 = C_o - C_1$

Put this in equation 2:

$$\frac{dc_1}{dt} = \frac{-KpA}{V}(2C_1 - C_o) \qquad (2)$$

Transformation would go as follows:

$$\int_{C_o}^{C_1} \frac{dc_1}{2C_1 - C_o} = \frac{-KpA}{V} \int_0^t dt$$

$$\frac{1}{2}\left[\ln(2C_1 - C_o)\right]_{C_o}^{C_1} = \frac{-KpA}{V} t$$

$$\ln \frac{2C_1 - C_o}{C_o} = -2 \frac{KpA}{V} t$$

$$\frac{C_1}{C_o} = \frac{1}{2}\left[1 + e^{\frac{-2KpA}{V} t}\right] \qquad (3)$$

Experiments with Na^{22} and Dual Labeling Experiment Equations

The equations describing the exchange of radioactive ions between two compartments have been outlined by Ussing

(1949), Green (1965, 1966) and others. If $M_{Na(I)}$ is the total amount of Na^+ in the inside solution, $\Delta Na^{22}_{(o)}$ the amount of Na^{22} (CPM) in the total outside solution at end of time "t", $Na_{(I)}^{22}$ the amount of Na^{22} (CPM) in the total inside solution and A is the exposed surface area (cm^2) across which flux is occurring, then the flux (F) in time "t" is described by:

$$F = \frac{\left(M_{Na_{(I)}}\right)\left(\Delta Na^{22}_{(o)}\right)}{(A)\ (Na^{22}_{(I)})\ (t)} \qquad (4)$$

F is equal to the "endothelial" to epithelial rate of entry or flux of Na^{22} given as $\mu Eq/cm^2/hr$. The epithelial to "endothelial" flux of Na^{22} may be readily calculated by substituting the appropriate values in the equation. The flux calculated from equation (4) is total flux whereas equation (5) was used to describe the net flux of Na^{22} across the fish and rabbit cornea. The theory behind equation (5) can be found in Sheppard's (1962) book, where the solution yields an exponential relation and p (permeability constant) is constant.

In Figure 5 and equation (5) the symbols are the following:

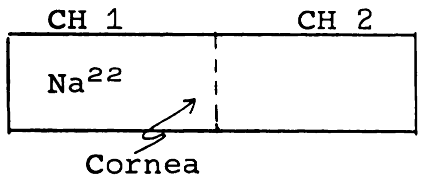


Figure 5

S = total amount of traced substance in the system measured in moles, grams, etc.

S_1, S_2 = amounts of S in chamber 1 and 2 respectively.

p = rate of exchange between compartments measured in microequivalents per hour.

a_1, a_2 = specific activities in chambers 1 and 2.

t = time in hours.

a(0) = specific activity of zero time in chamber 1.

A = area (fish 0.88 cm^2; rabbit = 1.06 cm^2)

$$\text{Then } \log (a_1-a_2) = \log a(0) - p \left(\frac{1}{S_1} + \frac{1}{S_2}\right) t \qquad (5)$$

Solve for p = μEq/hr

Then $\frac{P}{A}$ = $\mu Eq/cm^2/hr$

Equation to Correct for Backdiffusion of Sodium

The mechanism of exchange of corneal Na^+ with aqueous humor is very similar to the kinetics which have been widely studied by Sheppard and Martin (1950), and summarized by Comar (1955), for the exchange of potassium between plasma and erythrocytes. With fish corneas, under the experimental conditions described, the set-up may be considered as a closed two-compartment system since the corneal epithelium is impermeable to sodium ions. The process is represented schematically in Figure (6).

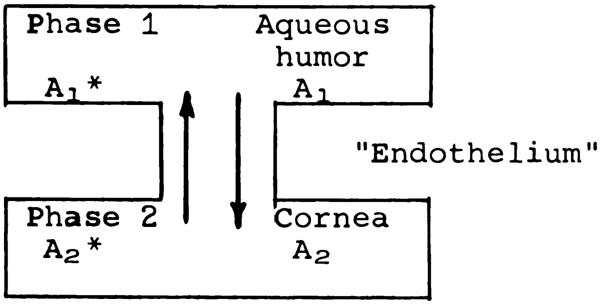


Figure 6

A_1 and A_2 = concentration of sodium in aqueous humor and cornea respectively.

A_1* and A_2* = concentration of labeled sodium in aqueous humor and cornea respectively at time "t".

A_o* = A_1* + A_2* = concentration of labeled sodium in aqueous humor (TC199) at zero time.

$\frac{A_1^*}{A_1} = S_1$ and $\frac{A_2^*}{A_2} = S_2$ are specific activities in aqueous humor and cornea respectively, at time "t".

p = rate of movement of Na^{22} from phase 1 to phase 2 and vice versa.

Equation (6) enables one to calculate the net flux of sodium into the cornea taking into account the exponential rate of loss of sodium from the cornea to the solution bathing the "endothelium."

The rate of movement of labeled Na^{22} out of phase 1 will be $-p\frac{A_1^*}{A_1}$ and the rate of movement into phase 1 will be $p\,\frac{A_2^*}{A_2}$. Therefore, the net movement of labeled Na^{22} may be represented as follows (Comar, 1955):

$$\frac{dA_1^*}{dt} = -p\,\frac{A_1^*}{A_1} + p\,\frac{A_2^*}{A_2}$$

converting to specific activities

$$\frac{dS_1}{dt} = -\frac{p}{A_1}\,(S_2 - S_1)$$

substituting for S_2 in terms of S_1 and integrating where

$S_o = \frac{A_o^*}{A_1}$

$$\frac{S_1}{S_0} = \frac{A_1 + A_2 e^{-Kp^{*} t \left[\left(\frac{1}{A_1}\right) + \left(\frac{1}{A_2}\right)\right]}}{A_1 + A_2} \qquad (6)$$

Solve for $Kp^{*} = \mu Eq/hr$

then $\frac{Kp^{*}}{A} = \mu Eq/cm^2/hr$

where $A = 0.78cm^2$ which equals the surface area.

Equation to Calculate Kp' from T1/2

A permeability constant for the corneal epithelium can be calculated if the specific activity and the rate of loss (T1/2) of corneal sodium are known (equation 7).

$$Kp' = \frac{0.693}{T1/2} \qquad (7)$$

Permeability constants

The permeability constants previously described are listed with their respective units.

constant		unit	isotope	equation
Kp	=	$cm^3/cm^2/hr$ $\mu l/cm^2/hr$	HTO	(3)
F	=	$\mu Eq/cm^2/hr$	Na^{22}	(4)
p	=	$\mu Eq/cm^2/hr$	Na^{22}	(5)
Kp^{*}	=	$\mu Eq/cm^2/hr$	Na^{22}	(6)
Kp'	=	$\mu Eq/cm^2/hr$	Na^{22}	(7)

II. Experiments with Tritiated Water

In all experiments the solution which bathed the "endothelial" surfaces of the corneas was very nearly equal osmotically (about 290 mOs/1) to trout plasma, whereas, that bathing the epithelial surface was tap water. Thus, in every case there existed across the cornea an osmotic gradient of some 6.6 atmospheres favoring an osmotic movement of water through the cornea to the solution bathing the endothelial surface. Permeability constants (Kp) were calculated according to equation (3).

Data presented in Table I show that the movement of tritiated water in either direction across the healthy lake trout cornea is equal irrespective of the existing osmotic gradient. There was a slightly greater increase in the permeability when the epithelia were removed than when the "endothelia" were removed. When both layers were removed the permeabilities were significantly increased above the value for intact corneas but were not significantly different from the permeabilities of corneas with the epithelia alone removed.

The triated water content of the corneas was determined and the concentrations of HTO in the corneas are expressed as CPM/mg of cornea. Data for normal intact corneas in the two experimental situations, A and B as given in Table I, indicate that the epithelium offers a greater resistance than the "endothelium" to entry of water into the cornea. With the

Table I. Permeability constants for water transfer and corneal tritiated water content of normal and denuded lake trout corneas <u>in vitro</u>.

Condition of Cornea	Number of data	Kp(3)** Permeability Constant ($cm^3/cm^2/hr$)	Corneal HTO Content (CPM/mg)
(A. With tritiated water in epithelial compartment at start of experiment.)			
Normal	6	0.16 ± 0.02*	535
Epithelium removed	6	0.52 ± 0.06	603
"Endothelium" removed	6	0.37 ± 0.02	651
Both layers removed	8	0.53 ± 0.03	827
(B. With tritiated water in "endothelial" compartment at start of experiment.)			
Normal	5	0.16 ± 0.01	916
Epithelium removed	6	0.47 ± 0.03	386
"Endothelium" removed	4	0.34 ± 0.06	570
Both layers removed	6	0.53 ± 0.03	410

*Standard error of mean
**Equation used for calculation of permeability constant

epithelia removed, the A-condition corneas showed expected high concentrations of HTO. The B-condition corneas had less HTO than normal controls presumably because of a greater loss of HTO into the epithelial compartment. We are unable to explain the high HTO content of A-condition corneas with the "endothelium" removed. As expected, B-condition corneas with the "endothelium" removed had higher concentrations of HTO than those with the epithelium removed. With both layers removed the B-condition corneas had less HTO than A-condition corneas again presumably due to a greater loss of HTO into the epithelial compartment by the former. With a single exception these data are consistent with the idea that corneal water is much more freely exchangeable with water in the "endothelial" than with water in the epithelial compartment.

Table II. Permeability constants, tritiated water content and hydration of normal and pathological lake trout corneas.

Stage of disease	No. of corneas	Kp(3)** Permeability Constant ($cm^3/cm^2/hr$)	Corneal HTO content (CPM/mg)	Wet weight of cornea (mg)
Normal	6	0.16 ± 0.02*	535	27.2 ± 0.9*
1	8	0.20 ± 0.02	577	27.1 ± 0.6
2	9	0.23 ± 0.03	725	29.3 ± 0.6
3	6	0.17 ± 0.02	676	38.8 ± 5.1
4	3	1.46 ± 0.02	738	43.1 ± 8.4
1-a	8	0.22 ± 0.02	978	25.6 ± 1.8

*Standard error of mean
**Equation used for calculation of permeability constant

With the exception of the values for Stage 3, those given in Table II indicate that as the corneal lesion progresses from Stage 1 through Stage 4, there is a tendency for the permeability of the cornea to increase. Viewed in the light of previous histological work, these results were not at all surprising. In all of these experiments tritiated water was placed in the epithelial or tap water compartment and permeability constants were calculated on the basis of appearance, after 4 hours, of HTO in the originally unlabeled compartment.

III. Experiments with Na^{22}

In all experiments corneas were subjected to the osmotic gradient as described under II above. Sodium fluxes were calculated with Eq. (4) and net permeability constants or fluxes calculated with Eq. (5).

A. Data presented in Table III show that there is no sodium flux in either direction across the normal intact fish cornea and that the specific activity of the cornea in A-condition is almost equal to that of the TC-199. Under B-conditions the specific activity of the cornea is extremely small compared to that of the tap water. This small specific activity could be due to surface absorption and does not necessarily indicate true uptake of Na^{22} by the corneas. There was a tremendous increase in the total Na flux when the epithelia were removed in condition A whereas in condition B the flux was very slight; however, the net flux in both conditions was very similar. This demonstrates the impermeable nature of the epithelium to Na^+. With this layer removed more sodium moves with than against the osmotic gradient. When the endothelia are removed there is a slight Na^+ flux, but no net flux which indicates that the corneal "endothelium" does to some extent control corneal Na^+; however, the

Table III. Average sodium fluxes over four hours; cornea and media specific activity; and corneal water of normal and denuded lake trout corneas *in vitro*.

Condition of Cornea	n	F(4)** Flux μEq/cm^2/hr	p(5)** Net Flux μEq/cm^2/hr	Specific Activity cpm/μEq			Cornea cpm/mg	Cornea % H$_2$O
				TC-199	Tap Water	Cornea		
A. Na^{22} in "endothelial" compartment								
Normal	9	0.0	0.0	87	0	78	9.2	81.5±1.5*
Epithelial Removed	11	32.1±2.8*	30.8±2.1*	83	74	112	6.7	86.1±0.6
"Endothelial" Removed	5	7.3±2.5	0.0	86	97	90	9.6	89.2±0.5
Both Layers Removed	4	44.9±5.6	59.3±5.2	71	69	82	6.3	83.7±1.2
B. Na^{22} in epithelial compartment								
Normal	8	0.0	0.0	0	2417	9	0.7	76.9±2.3
Epithelial Removed	10	2.5±0.4	23.9±0.4	11	555	125	5.5	86.9±1.2
"Endothelial" Removed	4	0.0	0.0	1	4377	7	0.6	86.8±1.0
Both Layers Removed	4	3.7±0.6	31.4±1.2	10	404	92	5.9	82.2±1.6

*Standard error of the mean

**Equation used for calculation of fluxes epithelium acts as the main barrier to preserve corneal Na. When both layers are removed the Na^+ fluxes were significantly greater than the fluxes across corneas with the epithelial alone removed.

The Na^{22} content of the cornea was determined and specific activities were calculated. With the epithelia removed both A and B condition corneas showed similar specific activities presumably because the specific activity of the tap water (condition B) was 4-fold higher than the TC199 in condition A, thus the probability of labeled Na^+ (condition B) penetrating the cornea was much greater. When the "endothelia" are removed in condition A the corneal specific activity is equal to the specific activity of the TC-199, but in the B condition the corneal specific activity is extremely small compared to the specific activity of the tap water.

These corneal specific activities are as expected if we assume that the corneal epithelia controls the sodium flux. With both layers removed both condition A and B corneas had similar specific activities. Since condition B tap water specific activity was 4 times greater than the specific activity of TC-199 in condition A; these results are interpreted as indicating that there is a greater tendency for Na^{22} to move with the gradient rather than against it. Without a single exception these data are consistent with the idea that corneal sodium is more freely exchangeable with sodium in the "endothelial" than with sodium in the epithelial compartment. Hydration was greatest during the four hour perfusion of corneas with the "endothelium removed," less in those with the epithelium removed and least in those with both layers removed. Thus, hydration of the stroma is restricted and indicates that possibly corneal hydration in the fish is controlled by the epithelium and "endothelium" as in mammalian cornea.

B. In Table IV are data for sodium flux across Lake Trout corneas representative of the various stages in pathological progression of the corneal lesion described by Hoffert and Fromm (1965). Only in stages 3 and 4 is there a sodium flux across the cornea. This is consistent with previous histological work where one observes in the early stage 3 a definite erosion, thinning and general disorganization of the corneal epithelium. Once the corneal epithelium is disrupted (stage 3) an outward sodium flux will occur. Stage 4 corneas have a complete hole; therefore, there is nothing to prevent an outward flux of sodium. In all cases the cornea CPM/mg in the diseased eyes are lower than the normal indicating that these diseased corneas take up less radiosodium. This may be related to hydration of the cornea which will be considered in connection with Table V. We are unable to explain the high and variable specific activities of the diseased corneas unless these corneas have the ability to differentiate between Na^{22} and Na^{23} which is highly unlikely. In calculating a corneal specific activity there may be an

Table IV. Sodium fluxes; cornea and media specific activity; and hydration of normal and pathological lake trout corneas.

Stage of Disease	n	F(4)** Flux $\mu Eq/cm^2/hr$	Tc-199	Specific Activity cpm/μEq Tap Water	Specific Activity cpm/μEq Cornea	Cornea cpm/mg	Cornea % H_2O
Normal	9	0.0	87	0	78±3*	9.2	81.5±1.5*
1	5	0.0	78	0	120±12	7.4	81.8±0.6
2	5	0.0	79	0	152±49	8.2	85.3±1.4
3	5	5.8±1.0	79	67	109±17	8.3	85.4±0.4
4	3	96.3±0.9	68	61	151±53	5.1	86.1±1.2
1-a	5	0.0	78	0	185±65	8.1	85.4±1.0

*Standard error of mean
**Equation used for calculation of flux

Table V. Sodium and water content of diseased lake trout corneas.

Stage of Disease	n	% H_2O	Na mEq/l
Normal	9	75.9±1.5*	125±8.7*
1	5	79.0±2.3	126±6.9
2	5	83.8±2.3	103±8.9
3	2	85.6±1.6	91±5.7
4	9	85.3±1.2	90±6.8
1-a	6	80.4±1.2	113±6.8

*Standard error of mean

over-correction for hydration which would decrease values for the concentration of corneal Na^+.

C. Data in Table V indicate that as the corneal lesion progresses in the lake trout, increased corneal hydration occurs and simultaneously, corneal sodium concentration decreases. During a four-hour perfusion the normal, stages 1 and 1-a corneas hydrate 5.6 percent, 2.8 percent, and 5.0 percent, respectively, whereas stage 2 corneas hydrate slightly and stage 3 and 4 corneas do not hydrate at all. This indicates that stage 2, 3 and 4 corneas are at maximal hydration _in situ_, whereas, the normal, stage 1 and 1-a corneas are not hydrated maximally _in situ_ and they do imbibe water from the bathing media _in vitro_.

D. Figure 7 represents the reverse exchange-equilibration of labeled sodium between cornea and TC-199 (see also Figure 6). This semi-log plot of specific activity of the cornea vs. time was fitted for a straight line ($\hat{Y} = 1.7143 - 0.7816\ x$) and T1/2 was calculated. In the fish 1/2 of the corneal sodium exchanges with the TC-199 every 22.8 min. and the percent turnover per minute is 3.0 percent (see appendix for calculations). For rabbits, the exchange T 1/2 of the corneal stroma sodium is 14.0 min. (Maurice, 1951) and from this a percent turnover of 4.8% per minute can be calculated. Thus by comparison the fish cornea is able to retain its corneal sodium longer than the rabbit. The permeability constant Kp*

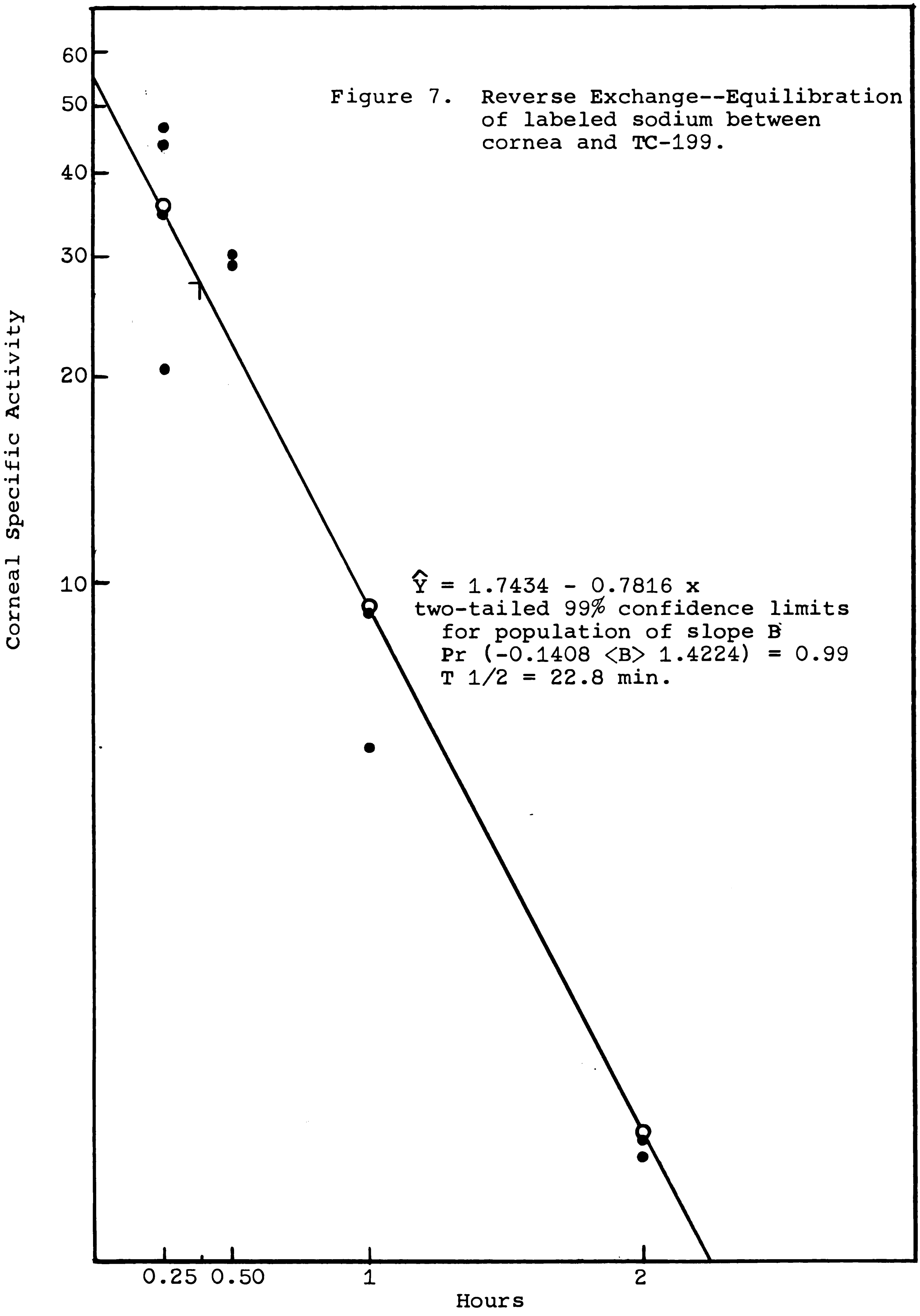


Figure 7. Reverse Exchange--Equilibration of labeled sodium between cornea and TC-199.

$\hat{Y} = 1.7434 - 0.7816\ x$
two-tailed 99% confidence limits
for population of slope B
Pr (-0.1408 <B> 1.4224) = 0.99
T 1/2 = 22.8 min.

for the fish corneal "endothelium" is 0.029±0.003 mEq/cm^2/hr calculated using equation (6) and corrected for backdiffusion. The Kp' calculated from the T 1/2 using equation 7, is 0.030 mEq/cm^2/hr. Since Kp* = Kp', it appears that simple diffusion and not active transport is involved in this exchange. No data were presented by Maurice (1951) and, a Kp* for the rabbit cornea "endothelium" using formula (6) could not be calculated; however, a Kp' was calculated from the T 1/2 yielding a value of 0.048 mEq/cm^2/hr. From the permeability constants (Kp* and Kp') it appears that the rabbit cornea "endothelium" has a greater permeability for sodium than the fish corneal "endothelium."

IV. Dual Labeling Experiments for HTO and Na^{22}

The results of an experiment with Na^{22} and HTO are given in Tables VI and VII and these tables should be viewed in light of each other. HTO and Na^{22} fluxes were calculated with equations (3) and (4) and net sodium flux calculated with equation (5). In the fish cornea there is no apparent movement of water drawn along osmotically with the movement of sodium ions; thus HTO flux and Na^{22} flux in this corneal perfusion study occur independent of each other. However, with rabbit corneas there is evidence of an osmotic flow of water. In Table VI condition B and C the flux of water through the rabbit cornea is significantly increased over condition A. Data presented under condition B and C in Table VII also show a flux of sodium across the rabbit cornea. These results are consistent with the previously proven active transport of Na^+ across the rabbit cornea epithelium in the tears to aqueous direction. It is possible that with active transport of Na^+ across the corneal epithelium one would expect an increased osmotic water flux in the tears-aqueous direction and maximally hydrated corneas as indicated by data for conditions B and C in Tables VI. In all cases the rabbit corneas had a 3 to 5-fold greater water exchange than fish corneas. Values for the water flux across the fish corneas (Table VI) are slightly lower than the values given in Table I. This could possibly be due to differences between the corneal clamps used. Corneal clamp 1 does damage the outer edge of the corneal disc, whereas, clamp 2 holds the cornea in place by clamping sclera tissue and does not touch the cornea _per se_.

The water flux across the fish and rabbit cornea is mostly an exchange with only a small net water flux found in condition B and C rabbit corneas. In condition A the specific activity of sodium in the fish cornea and cornea CPM/mg are much higher than comparable values for rabbit corneas, perhaps due to the greater exchange of water by the latter. Values for corneal specific activity and corneal CPM/mg are reversed in condition B, indicating the fish corneal epithelium maintains a greater impermeability to water than that of the rabbit. When one compares all of the data in condition A and B, it becomes apparent that water flux is impeded to a much greater

Table VI. Average permeability constants for water transfer over four hours; cornea and media specific activity; and water content of normal fish and rabbit corneas *in vitro*

Experimental condition	n	Kp(3)** Permeability constant $\mu l/cm^2/hr$	Specific Activity cpm/μl			Cornea	Cornea cpm/mg	Cornea % H_2O
			En-199[+]	Ep-199[+]	Tap			
A. HTO and Na^{22} in endothelial compartment at start								
Fish	4	102±5.6*	762	-	46	686	487	83.1±1.0*
Rabbit	4	340±30	584	168	-	399	327	81.2±3.8
B. Na^{22} in endothelial compartment HTO in epithelial compartment at start								
Fish	4	85±9	38	-	802	213	178	83.7±0.8
Rabbit	4	460±26	182	523	-	329	289	85.4±0.7
C. HTO and Na^{22} in epithelial compartment at start								
Rabbit	4	481±36	187	554	-	331	300	88.8±1.4

*Standard error of the mean

**Equation used for calculation of permeability constant

[+] EP = epithelium TC-199; En = endothelium TC-199

Table VII. Average sodium fluxes over four hours; cornea and media specific activity of normal fish and rabbit corneas.

Experimental condition	n	F(4)** Flux µEq/cm^2/hr	p(5)** Net Flux µEq/cm^2/hr	Specific Activity cpm/µEq			Cornea	Cornea cpm/mg
				En-199[+]	Ep-199[+]	Tap		
A. HTO and Na^{22} in endothelial compartment at start								
Fish	4	0.0	0.0	352	-	0	377	35
Rabbit	4	0.0	0.0	412	0	-	330	36
B. Na^{22} in endothelial compartment HTO in epithelial compartment at start								
Fish	4	0.0	0.0	344	-	0	275	33
Rabbit	4	1.19	0.0	372	2.2	-	294	44
C. HTO and Na^{22} in epithelial compartment at start								
Rabbit	4	3.43±0.001*	0.70±0.27*	6	356	-	21	3

[*] Standard error of the mean
[**] Equations used for calculation of fluxes
+Ep = epithelium TC-199; En = endothelium TC-199

extent by the epithelium in the fish cornea than in corneas of rabbits.

Data presented in Table VII shows that there is no movement of sodium in either the fish or rabbit in the aqueous-tears direction. In the reverse direction there is a sodium flux in the rabbit (active transport) giving rise to a corneal specific activity of 21. Under comparable conditions the specific activity of the fish cornea is only 0.7 (Table III) indicating no active transport of sodium.

In all experiments the permeabilities of the corneal endothelia of the fish and rabbit are much greater for water and sodium than the corneal epithelia.

V. Corneal ATPase - Histochemical

Localization of the enzyme activity: the basement layer of the corneal epithelium shows the highest ATPase activity (Figure 5, Plates A and B). There was also some diffuse enzyme activity within the stroma and the corneal "endothelium" of the fish shows some ATPase activity also. A very limited amount of activity was found in the corneal epithelium which is very different from mammals where most of the ATPase activity is located in the epithelium. Ehlers (1965) reported no ATPase activity in the stroma of rabbit corneas.

Controls were run (Figure 5, Plate C) where the corneas were incubated without the substrate ATP and gave a negative reaction. β-glycerophosphate in the same concentration as ATP

Figure 8

Plate A. Center of cornea XS
ATPase in fish cornea.
Note--strong reaction at the basal membrane.

Plate B. Limbal region of cornea XS
ATPase activity diffuse through stroma.

Plate C. Control section incubated without ATP.

Plate D. Control section incubated with β-glycerophosphate.

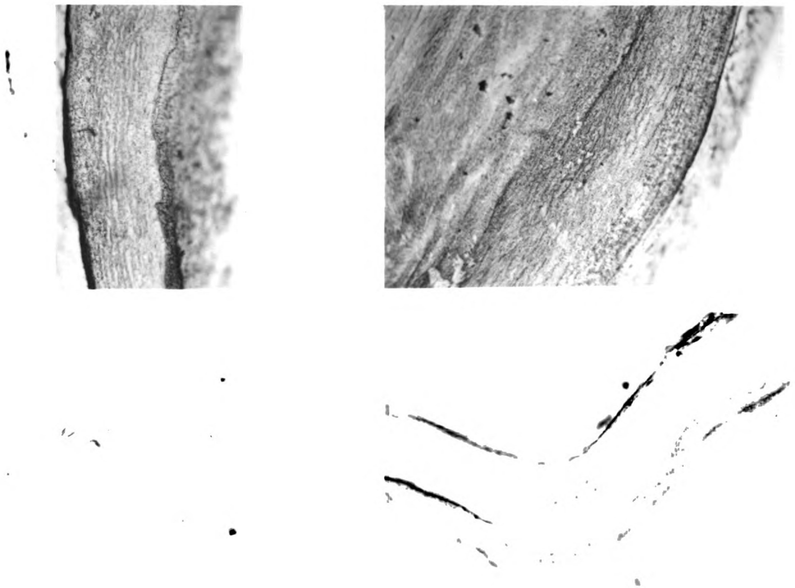


Figure 8

was used as the second substrate for localization of any non-specific alkaline phosphatases which might be present. A very slight reaction was seen (Plate D) but there was little difficulty in differentiating this reaction from the reaction of ATPase.

DISCUSSION

Data presented in Table I indicate that the permeability of normal lake trout corneas to water is the same irrespective of whether the values are based on the transfer of tritiated water from the epithelial to the endothelial bathing media or vice-versa. These results are similar to what has been observed with corneas of fresh-water rainbow trout. Permeability constants for both species of fish are of the same order of magnitude and are much lower than published values for corneas of terrestrial animals. Once again we are forced to believe that what we have measured represents an exchange of water across the cornea and not a net flow of water from one side to the other.

The data for mammalian corneas presented by Donn *et al*. (1963), are probably most comparable to those given here for trout, although conditions were different in the two experiments. Their experiments with rabbit corneas were conducted at 20-25° C., and the solutions (identical except for the presence of HTO in one) bathing both surfaces of the corneas were equal osmotically and were renewed by perfusion. In our procedure a small recirculated volume of fluid bathed each surface of the cornea. They applied a slight hydrostatic pressure to the endothelial surface, whereas, no hydrostatic pressure difference was maintained in our experiments. In the calculations of permeability constants, neither research group took into account the specific activity of corneal water. The assumption was made in both cases that the amount of HTO which appeared in the originally unlabelled solution represented the transfer of a certain volume of water as determined by the initial radioactivity of the labelled solution.

Donn _et_ _al_. (1963), reported that the movement of water across the rabbit cornea was equal in either direction. The permeability constants for intact rabbit corneas were some 300 percent higher (0.47 vs. 0.16 $cm^3/cm^2/hr$) than values presented herein for lake trout corneas. After removal of the epithelium, endothelium or both layers of the fish cornea the rate of transfer of water across the cornea increased. These denuded corneas had permeabilities comparable to those for intact rabbit corneas. Based on the permeability constants given in Table I, it has been concluded that some resistance to water transfer across the fish cornea is offered by the "endothelium," but that the major resistance resides with the epithelium. With the exception of a single datum (A-condition, "endothelium" removed) the values for the tritiated water content of the corneas after removal of different layers are consistent with the above conclusion. Donn _et_ _al_. (1963), on the other hand, showed that when the epithelium or the "endothelium" of the rabbit cornea is removed, the flux of water does not change greatly, indicating that these layers do not offer a large resistance to the passage of water.

The water permeability of lake trout corneas is the result of the interplay of many factors such as: presence of physical (anatomical) barriers, metabolism of the cornea as related to maintenance of the barriers, ion movement, effect of environmental (chemical) agents on physical barriers, osmotic pressure, intraocular pressure, and others. Some of these factors are implicated in the corneal lesion of lake trout which has been observed.

Stage 1 of the lake trout corneal lesion as described by Hoffert and Fromm (1965) is characterized by keratectasia, an increased anterior chamber and aqueous humor volume, and a possible thinning of the central area of the cornea. The movement of water across the cornea at this stage is not significantly different from that across normal corneas (Table II). Stage 2 corneas are characterized by a marked keratoconus-keratoglobus with pronounced changes in optical transparency. There are some degenerative changes in the epithelium including erosion of the superficial epithelium. It is at this stage when the effect of osmotic pressure apparently comes into play resulting in a great influx of water into the cornea causing it to become opaque. Various degrees of ulcerative keratitis characterize Stage 3. In this stage one also observes a prominent erosion and thinning of the corneal epithelium, an irregular thickening of Bowman's membrane, and a localized hyperplasia of the epithelium. The cornea shows a significant hydration and an increase in over-all thickness. Values in Table I indicate that more water gets into the cornea at this stage but less gets through into the "endothelial" compartment, hence the permeability constant is lower than that for the preceding stage. Permeability constants for Stage 4 corneas have little meaning since these corneas are characterized as having total perforations in their central areas. Solutions in the immediate vicinity of both surfaces of Stage 4 corneas contained considerable quantities of HTO and this probably accounts for the high HTO content of these corneas. Stage 1-a may be a continuation of Stage 1 or it may represent a second form of the onset of the lesion. In this stage the anterior chamber depth is normal and the cornea has a normal radius of curvature, but it takes on a milk cloudiness which may be diffuse or discrete. The normal cuboidally arranged basement epithelial cells are disorganized, and the epithelium does not remain attached to its base membrane. It is assumed that the increased permeability and hydration observed in Stage 1-a corneas is associated with this disruption of the basement layer of the epithelium.

It appears that as long as the epithelium and Bowman's membrane remain intact, there is only a limited movement of water into or across the lake trout cornea. Some of the changes noted in the permeabilities of pathologic lake trout corneas can be explained on the basis of destruction of the anatomical barriers to water transfer which are normally present in intact, healthy tissue. It is most probable that there are alterations in the metabolism of the pathologic corneas which may also affect their water permeability. Smelser and Chen (1954) report that the corneal epithelium of carp is rich in phosphatase and this enzyme(s) may be important in the maintenance of normal corneal hydration. We have positive histochemical evidence of the presence of various enzyme systems indicating the epithelium of lake trout corneas is quite active metabolically.

Data presented in Table III show that the normal lake trout cornea is impermeable to sodium in the "endothelial"-epithelial direction and vice-versa. This is of interest since the lake trout lives in a hypoosmotic environment conducive to loss of sodium to the surrounding water. Apparently there is a "barrier" within the cornea which maintains it impermeable and prevents a loss of sodium from the aqueous humor to the exterior. Histologically, the corneal epithelium consists of approximately five cell layers packed closely together (Edelhauser, 1964) and does not show any tendency of becoming hydrated, as would be expected of epithelium bathed with a hypoosmotic medium. Also, the corneal epithelium (basement membrane) cannot be regarded merely as a passive protective membrane in the fish, for it is rich in enzymes, including ATPase (Figure 5, Plate A) which suggests that it is an active metabolic tissue. Whether this barrier, which has an impermeable nature, contains an active pump mechanism as found in mammalian corneas (Donn et al., 1963; Green, 1966; and others) remains to be answered. However, when the epithelium is removed (Table III), there is a unidirectional net flux of sodium across the cornea, indicating that the impermeable barrier within the cornea is located either in Bowman's membrane or the corneal epithelium or both. After removal of the epithelium, "endothelium," or both layers of the fish cornea, the flux of sodium across the cornea increased. When the "endothelium" was removed, there was a slight sodium flux from the cornea, which is consistent with the water flux observed (Table I) and it can be concluded that the "endothelium" does offer some resistance to sodium and water movement. Possibly the resistance of the "endothelium" could be due to a mucinous substance layered on the "endothelium" similar to that Baramy (1957) observed in the owl. Thus upon removal of the "endothelium," this mucinous layer, high in hyaluronic acid, is also removed, and there results an increased sodium flux and water exchange across the fish cornea. Based on the permeability constants given in Table III, it can be concluded that some resistance to sodium flux across the fish cornea is maintained by the "endothelium" (in the TC-199 tap direction), but the major resistance to movement in both directions resides with the epithelium. Sodium flux across the fish cornea is more likely to follow the diffusion gradient (condition A) than to oppose the gradient (condition B). When a Na^{22} gradient was established by putting the Na^{22} on the epithelial surface compartment, only a small fraction of the labelled Na penetrated the cornea.

The values of corneal specific activity, corneal CPM/mg, and percent corneal water are consistent with the previous section for water movement and with the above conclusions. Cogan and Kinsey (1942a) have shown similar results for the cat cornea when its layers were denuded. Maurice (1951), Green (1966), and data given in Table VII show that the normal rabbit cornea possesses a limited permeability to Na in both directions. The fish cornea is impermeable to sodium in both directions. The low rate of water exchange across the cornea (Table I) and impermeable nature of the fish cornea to sodium explains why Smelser and Chen (1954) found the excised fish cornea to swell less than mammalian corneas when placed in isotonic and hypertonic sodium chloride solutions.

The lake trout corneal lesions have been briefly discussed in the section on water permeability. Values in Table IV indicate that sodium diffuses across the cornea only in stages 3 and 4. A cornea denuded of its epithelium permits an outward diffusion of sodium. Thus it is feasible to conclude that the stage 3 cornea does have epithelial damage. This supports previous histological work done by Hoffert and Fromm (1965). Corneal hydration appears to be a very important phenomena in these diseased lake trout corneas. Table V indicates that the normal, stage 1 and 1-a corneas are able to maintain an equilibrium condition with relation to corneal hydration and sodium ion concentration. It appears that in stage 2, 3, and 4 corneas the mechanism which maintains the cornea dehydrated has been lost for they are at maximal or near-maximal hydration in situ. When the data for corneal hydration (after a four-hour perfusion) given in Tables IV and V are compared, it is obvious that the normal, stages 1 and 1-a corneas do hydrate, but do not reach the maximum hydration observed in those of stages 2, 3, and 4. This indicates the presence of a hydration control mechanism in the normal, 1 and 1-a corneas. According to Smelser and Chen (1954), the resistance to hydration in fish cornea is due to the organization of mucoids (polymerization), electrolyte control and thickness of the epithelium all of which act together to inhibit the penetration of water. A theory has been suggested by Donn (1966), Smelser (1962), and Hedbys (1961) where a control of stroma sodium ion is needed to maintain a physiological pH and to neutralize the negative charges furnished by acid stroma polysaccharides. When for some reason the homeostasis of the cornea is altered as in the various stages of the lake trout corneal lesion, and the corneal sodium ion decreases, the negative charges on the mucopolysaccharide are not neutralized. The negative charges repel each other and cause the nucopolysaccharides to uncoil, allowing the cornea to take up more fluid. Even though this is still a theory, it may be the case in this lesion. In stages 2, 3, and 4 corneas, where hydration is maximal, the critical point could have been reached between ionic balance and mucopolysaccharide uncoiling; therefore, no control of corneal hydration can be maintained.

In the pathological progression of this corneal lesion (Hoffert and Fromm, 1965), stage 1-a has been questioned as to its place in the chronological progression. Data presented in Tables IV and V generally support the classification; however, sodium flux data, cornea CPM/mg, corneal water, and corneal sodium ion concentrations all indicate that stage 1-a is a continuation of stage 1 rather than a second form of the onset of the lesion.

According to Donn (1966) the mammalian "endothelium" interferes with corneal swelling in two ways: it provides some sort of barrier decreasing the access of aqueous humor to the corneal stroma, and its metabolic activity, aside from maintaining the barrier property, acts on the stroma in some poorly understood way to keep it dehydrated. Our data also indicates the fish corneal "endothelium" acts similarly for when the "endothelium" was removed, the water transfer, sodium flux, and corneal hydration all increased.

The barrier function of the "endothelium" does not stem from any unusual impermeability of this membrane. There is no great resistance to the diffusion of inorganic ions which have been studied in the mammal, and lack of resistance appears to be similar in fish. In fish, one half of the sodium in the stroma exchanges across the "endothelium" with that of the aqueous humor every 22.8 minutes, whereas in the rabbit $T\frac{1}{2}$ is 14 minutes. It can be concluded that the fish corneal "endothelium" is of the order of 16 percent less permeable to sodium than the rabbit. In view of such a large sodium movement across the "endothelium," it would appear that most of the movement of these ions is by way of a system of relatively large, uncharged pores as suggested by Maurice (1961).

Since all of the corneal permeability studies to date in other laboratories have been on mammalian corneas, rabbit corneas were used in order to compare fish and rabbit corneal permeability under nearly identical experimental conditions.

From the results of experiments using two-labeled compounds, it is evident that there are differences in permeability between mammalian and aquatic corneas. Corneas of all species which have been studied, except the elasmobranch reported by Smelser (1962), imbibe water from the surrounding media after damage to the limiting cellular membranes or after excision. With this in mind one would expect the fish cornea to exhibit a greater permeability to water than the rabbit cornea under the conditions of the experiments reported here, but this is not the case. As illustrated by data in Table I and VI the exchange of water is 3 to 5 fold higher in the rabbit. These data for water exchange across rabbit corneas are similar to what Donn *et al*. (1963), reported. Apparently the epithelial "barriers" in fish do not house the same mechanism as they do in mammals. A metabolic "pump" for active transport of sodium into the cornea was found in the fish, but it was very evident in the rabbit. Nevertheless, both epithelia and "endothelia" are of great importance in maintaining corneal deturgescene in fish because if they are removed, the exchange of water across the cornea is increased, and it imbibes water. Possibly these layers, by virtue of their being relatively impermeable to water, may passively limit entrance of water into the corneal stroma from the aqueous humor or the external fresh-water.

It is known that damage to the corneal epithelium drastically increases corneal permeability to water and ions (Maurice, 1953, 1962) and utmost care was taken to avoid any damage to the corneas in the present experiments. Values for the unidirectional fluxes and net fluxes of sodium across the rabbit cornea (Ep. to Endo. 3.43 ± 0.001 and net 0.70 ± 0.27 $\mu Eq/cm^2/hr$) are higher than those reported by Donn *et al*. (1959), Ep. to Endo. 0.28 and net 0.17 $\mu Eq/cm^2/hr$; and Green (1966), Ep. to Endo. 0.87 ± 0.06 and net 0.57 ± 0.003 $\mu Eq/cm^2/hr$.

Green stated the most plausible explanation for the discrepancy between his and Donn's data was that his solutions bathing either side of the corneas were well stirred, whereas, in Donn's investigation they were not. Proper stirring is important to minimize the diffusion barrier to solutes offered by the presence of the so-called "unstirred layers."

When investigating membrane permeability with isotopes, these barriers may offer a great resistance to solute entering and leaving the tissue (Green, 1965). There is no significant difference between the net flux that Green reported and that given in Table VII, but the unidirectional flux recorded in Table VII is much higher than what Green reported. Since the net fluxes are the same, it is believed that circulation in the system used here was sufficient; however, there are differences between the experimental set-ups which may account for this unidirectional flux difference: (1) TC-199 was used instead of Ringer's solution, (2) Green recorded a short-circuit current every ten minutes, and we did not. It is generally accepted that when a membrane is short circuited, the membrane potential and permeability characteristics of the membrane are altered for a given period of time after short-circuiting. Since this was not the case in the present study on the rabbit cornea (Table VII), the difference between the two reported unidirectional fluxes may be due to this short circuiting technique. Apparently short-circuiting had no effect on the net flux of sodium across the cornea.

The method employed (Wachstein and Meisel, 1957) appears to be the one best suited for histochemical localization of ATPases. However, it reveals only a small part of the total enzyme activity (Pearse, 1960), and it is not known if some ATPases (e.g., Na-K-activated) are more completely inhibited than others. With sodium β-glycerophosphate as a substrate in the incubating medium no reaction was observed. The enzyme demonstrated with ATP as a substrate cannot, therefore, be a non-specific alkaline phosphatase. An active transport of sodium across the mammalian corneal epithelium is known to occur (Maurice, 1962), and this transport was found to be inhibited by ouabain (Lambert and Donn, 1964). Ehlers (1965) was unable to demonstrate ouabain inhibition of the corneal ATPase, and he stated the method of Wachstein and Meisel is probably unsuitable for quantitative analysis of this enzyme. The histochemical technique as used in the rabbit and fish studies was for localization and not quantitation of ATPase.

A most interesting comparison pertaining to corneal sodium permeability in those animals which have been studied has been made. In the rabbit cornea there is a net sodium flux in the direction of outside-in. This net flux has been attributed to the active transport across the epithelium (Donn _et al_., 1959, and Green, 1966). Data in Tables III and VI show that there is no flux or net flux of sodium across the freshwater fish cornea; the cornea is impermeable enabling adaptation to its aquatic environment. Recently Zadunaisky (1966) worked on the bull frog cornea which has a nicitating membrane to prevent tear loss while in an aerial environment or submerged in a hypoosmotic medium. He found in the frog cornea that sodium fluxes measured with Na^{22} indicated that no net transport of this cation occurs; fluxes in both directions were similar. However, these corneas were found to actively transport chloride in an in-out direction while sodium moved passively.

Corneas of these three animals each handle sodium ions in a different manner in order to adapt to their environment, and normally each of these species maintains a transparent cornea. In the absence of more conclusive data, it is unwise to attempt to formulate a concept, that phylogenetically, fish and amphibia have more primitive corneas than mammals.

SUMMARY AND CONCLUSIONS

The investigation of the water and sodium permeability of the isolated normal and pathological lake trout cornea has shown the following:

1. The water permeability of lake trout corneas, *in vitro*, was found to be the same whether the data are based on the transfer of tritiated water from the epithelial to the "endothelial" bathing media or vice versa. This occurred despite the presence of an osmotic gradient which favored movement of water through the cornea to the solution bathing the "endothelium."
2. The fish cornea was found to be impermeable to sodium ions in both directions despite the presence of a gradient which favored a sodium flux through the cornea to the solution bathing the epithelium.
3. Permeability constants and sodium flux for normal and denuded corneas indicate that the "endothelium" offers some resistance to the transfer of water and sodium into or across the fish cornea but the major resistance to this movement is provided by the epithelial layer.
4. Correlations between water permeability, sodium flux, and histopathological condition of diseased corneas are noted.

5. Corneal hydration in fish, as in mammals, is controlled by the epithelium and "endothelium" since removal of these layers resulted in stromal hydration. Stage 2, 3 and 4 corneas are at maximal hydration *in situ*, whereas, the normal, stage 1 and 1-a corneas do imbibe water *in vitro* from the bathing media.
6. Corneal sodium ion concentration varied inversely with corneal hydration in these diseased lake trout corneas.
7. The permeability constant of sodium for the "endothelial" barrier from cornea to aqueous in fish is 0.029 $mEq/cm^2/hr$ which is 16% lower than that observed in rabbit corneas.
8. In the fish cornea there is no apparent movement of water drawn along osmotically with the movement of sodium ions. HTO movement and Na^{22} flux in this corneal perfusion study occurred independent of each other; however, with rabbit corneas osmotic flow of water does occur.
9. Rabbit corneas had a 3 to 5 fold greater water exchange than fish and they actively transported sodium in the tear-aqueous direction, whereas, in the fish cornea no sodium flux occurred.

10. An adenosine triphosphatase was identified in the basement layer of the corneal epithelium, stroma, and corneal "endothelium."

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APPENDIX

Procedure for counting tritium and sodium^{22} simultaneously.

The Mark 1 Liquid Scintillation system was peaked for HTO and Na^{22} with the following settings:

isotope	channel	attenuator	upper discriminator	lower discriminator	differential integral switch
HTO	A	A-507	9.9	0.0	L-U
Na^{22}	B	C-000	9.9	9.2	L-∞
30% channel A	C	A-926	1.9	0.0	L-U

With channel C set at 30% channel A one is able to have a calculated quench correction made by the Mark 1.

To account for the amount of Na^{22} found in the HTO channel the following procedure was used. A standard curve (see curves I & II) was made by diluting Na^{22} through the range of 0.00002µc to 0.2µc. These various solutions were then counted (see table A) and the counts per minute plotted on the ordinate against a channels ratio (channel B Na^{22} cpm/channel A Na^{22} cpm in tritium channel) which are plotted on the abscissa. Total counts of Na^{22} in channel A (tritium channel) are calculated by dividing the total counts of Na^{22} in channel B by the ratio B/A obtained from the standard curve of cpm channel B vs. ratio B/A (curves I & II). Channel A total counts minus calculated Na^{22} counts in channel A gives HTO counts in channel A corrected for Na^{22} overlap (see sample calculation).

Sample calculation for sodium^{22} correction in the tritium counts.

(1) $$\frac{B}{A} = \frac{\text{channel B Na}^{22}\text{ cpm}}{\text{channel A Na}^{22}\text{ cpm}} = K$$

(2) Take sample Na^{22} counts in channel B read them off standard curve to get B/A.

Example: HTO counts channel A = 51284

Na^{22} counts channel B = 197207

(a) find ratio of 196207 Na^{22} cpm from standard curve = 44.1 = B/A

(b) calculation

$$\frac{B}{K} = \frac{196207}{44.1} = 4440 \text{ cpm}$$

(c) subtract Na^{22} counts from HTO counts

$$\begin{array}{r} 51284 \\ -\underline{\ 4440} \\ 46844 \end{array}$$

46844 cpm = HTO counts in channel A corrected for Na^{22}

Table A: Data from Mark 1 Liquid Scintillation system used for standard curve for dual labeling experiment.

	Bottle number	Na^{22} µc	Channel A HTO cpm	Channel B Na^{22} cpm	Ratio B/A
Curve I					
	A	0.2	8333	383725	46.04
	B	0.1	4423	196679	44.22
	C	0.09	3960	176787	44.63
	D	0.08	3271	155882	43.65
	E	0.07	3125	136150	43.57
	F	0.06	2740	116807	42.65
	G	0.05	2285	97048	42.47
	H	0.04	1801	77402	42.16
	I	0.03	1444	58273	40.36
	J	0.02	973	37909	38.95
	K	0.01	531	19739	37.20
Curve II					
	M	0.002	127	3695	29.02
	N	0.00155	108	2839	26.26
	O	0.0010	83	1892	22.77
	P	0.0005	61	1019	16.64
	Q	0.0002	47	476	10.02
	R	0.0001	42	275	6.57
	S	0.00008	39	219	5.57
	T	0.00004	38	147	3.82
	U	0.00002	33	128	3.92
	V	0.00000	35	77	2.24

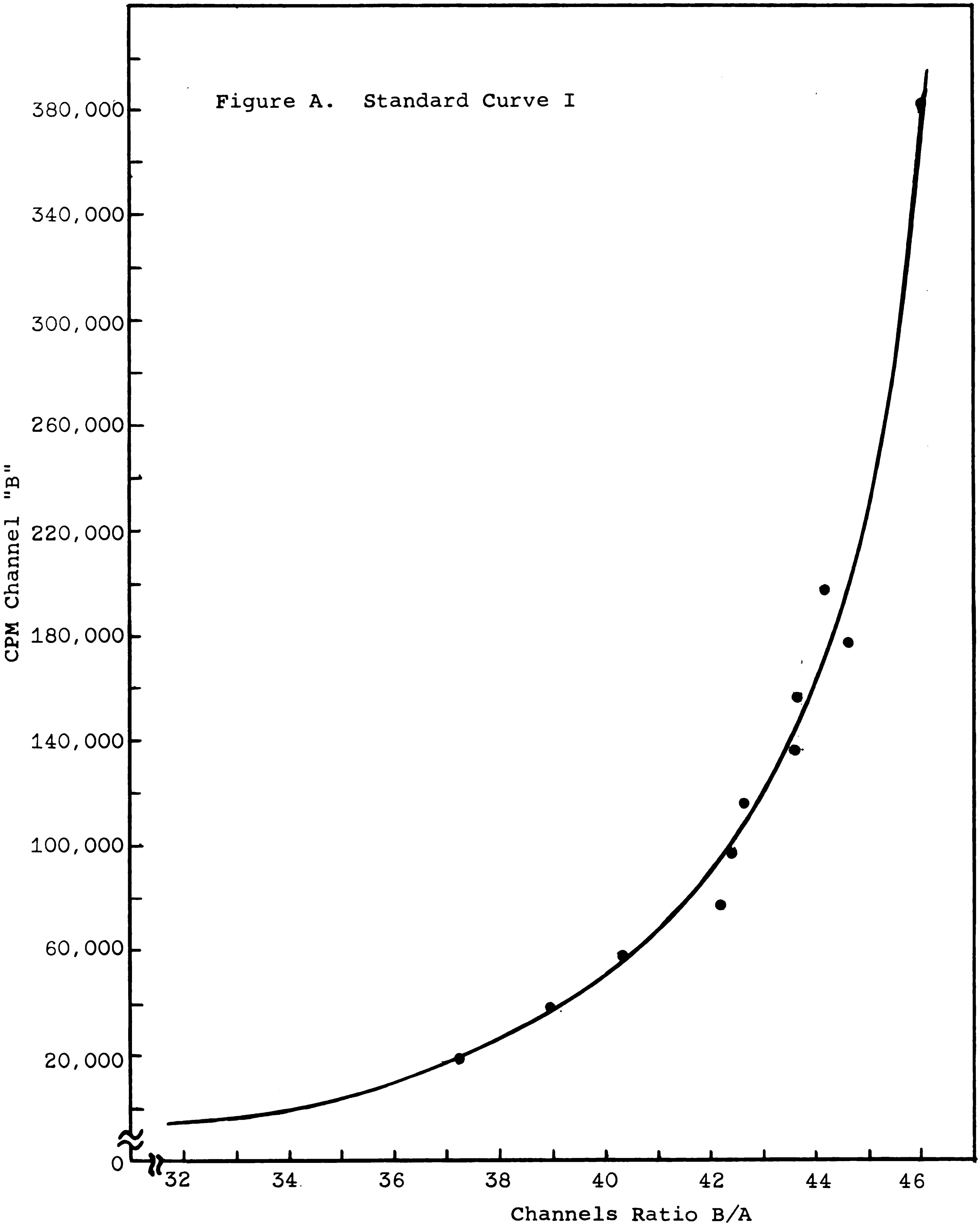

Figure A. Standard Curve I

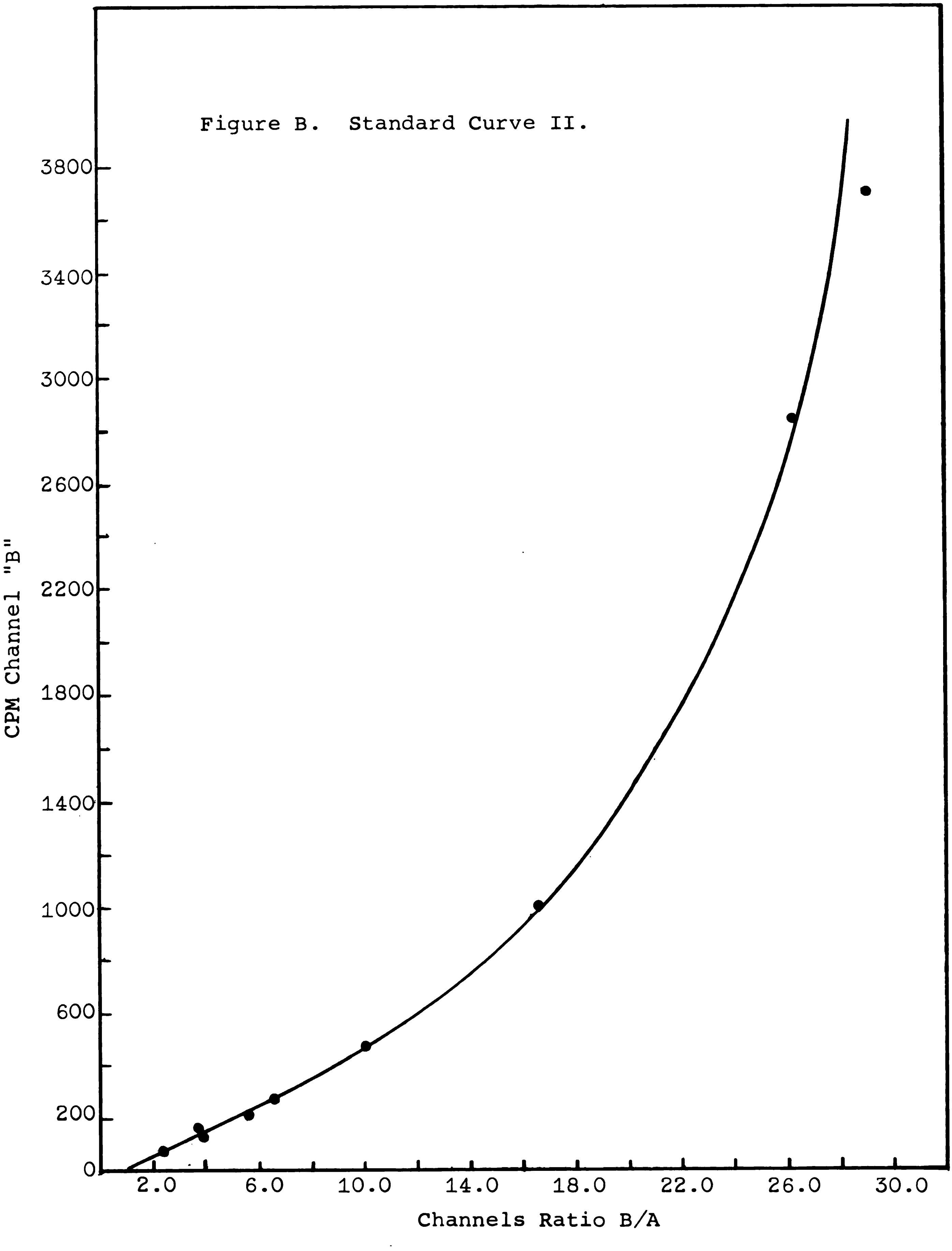
Figure B. Standard Curve II.
3800
3400
3000
2600
2200
1800
1400
1000
600
200
0
CPM Channel "B"
2.0
6.0
10.0
14.0
18.0
22.0
26.0
30.0
Channels Ratio B/A

Table B: Percent turnover of Na^{22} in fish and rabbit corneas.

	T1/2	$Kp = \frac{0.693}{T\ 1/2}$	e^{-Kt}	$1 - e^{-Kt}$
Fish	22.8 min.	0.03039	0.97005	0.030 = 3.0%
Rabbit	14.0 min.	0.0495	0.95170	0.0480 = 4.8%