using VI*b*. TLC analysis after 30 sec. indicated only a very faint fluorescent spot of VI*b* remaining and a strongly fluorescent spot corresponding to VII*d*. The starting material disappeared completely in 3 min. After 15 min., two additional spots appeared corresponding to VII*c* and VI*a*. Finally, VII*e* was treated in the same manner as VII*d* and VI*b*. TLC analysis after 45 sec. revealed two spots, a faint one corresponding to VII*e* and a stronger spot corresponding to VII*c*. After 2 min., the starting material had disappeared and an additional spot corresponding to VI*a* appeared.

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Altered Bioavailability of Drugs in the Eye Due to Drug-Protein Interaction

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Abstract ☐ The biological activity of a number of ophthalmic drugs is influenced by drug-protein interaction in tissues and fluids of the eye. High concentration of protein in lacrimal fluid, in both normal and pathological states, coupled with a relatively rapid turnover of this fluid, which moves drug solution away from the eye, leads to a considerable loss in drug activity for drugs that bind to protein. High levels of protein, as occur in some pathological states, and a slower, but substantial, turnover rate of aqueous humor can also lead to significant drug loss and a decrease in drug activity for compounds that complex with proteins. The present study, utilizing both *in vitro* and *in vivo* experiments, shows that drug-protein interaction in tissues and fluids of the eye occurs and that this interaction has an enormous influence on drug bioavailability. Equilibrium dialysis experiments, using pilocarpine nitrate, sulfisoxazole, and methylprednisolone, demonstrated that extensive binding to proteins in tears, cornea, and aqueous humor does occur. In addition, pupillary diameter experiments, using pilocarpine nitrate as the test agent, illustrated the influence of drug-protein interaction on drug bioavailability. A discussion of drug binding in combination with tear and instilled fluid dynamics is presented. It is suggested that this phenomenon can be responsible, partly or wholly, for some reported anomalous observations associated with drug therapy in the eye.

Keyphrases ☐ Ophthalmic bioavailability—effects of drug-protein binding, equilibrium dialysis, pupillary diameter experiments in rabbits ☐ Drug-protein binding—effect on bioavailability of ophthalmic drugs, equilibrium dialysis and pupillary diameter experiments

Binding of drugs to proteins can greatly affect drug activity. When this interaction occurs in tissues and fluids that are turned over at appreciable rates, and from which drug absorption must occur, the problem becomes even more severe, since both free drug and its reservoir of bound drug are being lost. Lacrimal fluid in both normal and pathological states contains appreciable quantities of protein and is turned over at rapid rates, while aqueous humor in some pathological conditions also contains large amounts of protein and is turned over at a slower but still significant rate. One could expect then that drug protein interaction in eye tissues and fluids would reduce the amount of free drug to either act locally or reach the anterior chamber

of the eye and potentially to alter the amount of free drug in the anterior chamber, thus decreasing the apparent biological drug activity.

The purpose of this report is to show that protein binding of drugs to fluids and tissues of the eye can have an enormous influence on the biological activity of these drugs. It is our contention that many apparently anomalous, unexplained observations associated with drug utilization in the eye, including the difficulty in treating some disease states with conventional drugs, are explained partly or wholly by protein binding. For example, the relative unresponsiveness of uveitis conditions to steroid therapy may be due to drug–protein interaction since large amounts of protein are present in this disease state. In addition, this effect can play an important role in the total biological activity of some drugs used in ophthalmology.

Upon instillation of a drug into the eye, the drug mixes with lacrimal fluid and then is transported across several membranes until it reaches the target area. If the target area is assumed to lie posterior to aqueous humor, the drug, after mixing with tears, must cross the cornea and enter the anterior chamber, where it mixes with aqueous humor and is then transported to the target area. Scheme I illustrates the major fluid and tissue boundaries, in terms of protein content and turnover rate, that the drug contacts during its transport and distribution.

BACKGROUND

Lacrimal Fluid—The normal volume of tears present in the eye is approximately 7–10 μl. (1) in humans and about 7 μl. in rabbits (2). The turnover rate of tears in humans is about 16%/min. (1) under normal circumstances, whereas in rabbits it is 7.1%/min. (2). These turnover rates apply when the volume of fluid in the precorneal area of the eye is the normal volume. Upon instillation of fluid into the eye, the drainage of instilled solution proceeds at a rapid rate (2) until the volume is back to normal.

A significant feature of the tear composition is the presence of a relatively high concentration of protein. The total protein present in lacrimal fluid varies qualitatively and quantitatively depending upon the method of analysis, the method of obtaining tear samples, and the species of animal. A relatively conservative estimate (3) lists the albumin content of tears to be 0.4% for humans with a total protein content of approximately 0.7%, whereas for rabbits it is 0.3% albumin with a total protein content of 0.5% (4). Emo-
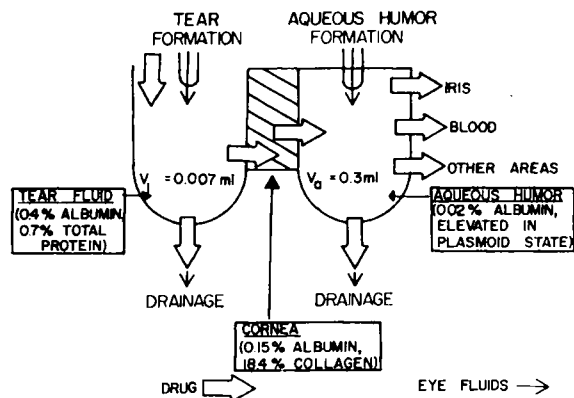
Scheme I—Pictorial representation of drug and fluid movement in a portion of the human eye. Protein concentrations in tears, cornea, and aqueous humor are listed together with the normal volume of tears and aqueous humor. Large arrows depict potential drug movement, and small arrows depict eye fluid movement.
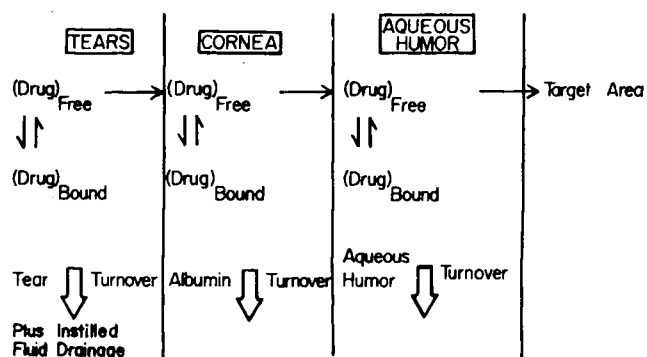
Scheme II—Potential rates and equilibria associated with drug movement through a portion of the eye

tional stress, irritation, and other factors greatly influence the protein content.

Cornea—The cornea is made up of about 80% water; the solid material present in the greatest amount is 18.4% collagen along with 0.15% albumin and globulin (5). Albumin diffuses from the peripheral capillaries toward the center of the cornea and is lost at the corneal surface to tears and aqueous humor. A transfer constant describing this turnover of albumin in humans was found to be 10–20%/day (6). Since the literature does not report this information for rabbits, their turnover rate was assumed to be similar.

Aqueous Humor—The volume of aqueous humor in humans is approximately 300 μl. with a turnover rate of approximately 1%/ min. (7, 8). These figures are comparable in rabbits (9, 10).

Aqueous humor is a much more dilute fluid than blood serum under normal circumstances. The most significant difference in solid content between aqueous humor and serum is the paucity of protein found in aqueous humor. Human serum contains approximately 7.0% total protein (2.8–4.5% albumin) (11), but the aqueous humor contains only 0.01–0.02% total protein (12). The blood serum of the rabbit contains 5.6% total protein of which 4.4% is albumin, whereas rabbit aqueous humor contains approximately 0.05% total protein of which 0.025% is albumin (12).

Aqueous humor composition, particularly the protein content, is a function of the integrity of the intraocular blood vessels, i.e., the blood–aqueous barrier. Various causative agents (e.g., mechanical means, responses to drugs, and certain disease states) may result in partial or complete breakdown of the blood–aqueous barrier. The most striking feature of this breakdown is the concomitant increase in protein concentration of aqueous humor. The fluid that develops, termed plasmoid aqueous humor of secondary composition, can, at the extreme, possess a similar protein composition to that of plasma, representing increases from 0.02 to 7% in man and from 0.05 to 5.6% in rabbits.

From the previous description, the binding of drugs to proteins was expected with subsequent loss due to turnover in the tears, cornea, and aqueous humor as well as other tissues. The relative importance of each tissue and fluid to drug loss is a function of the type of protein, protein concentration, drug concentration and affinity for the protein, and turnover rate of fluid and tissue.

The various equilibria that occur when a drug is instilled into the eye are shown in Scheme II. Upon instillation, the drug is diluted with the lacrimal fluid and enters into a rapid equilibrium with the proteins. Only the free form of the drug is transported into the cornea. Drainage of the instilled solution plus lacrimal fluid turnover results in removal of both free and bound forms of the drug. When the drug enters the cornea, it also enters into a rapid equilibrium with the proteins. However, the turnover rate of albumin in the cornea is small enough so that removal does not present a problem unless the binding constant is very large or drug transport is very slow. Due to binding in the cornea, it would be expected that a lag time will exist before drug enters the anterior chamber and that the duration of the lag time will be proportional to the binding constant, concentration and diffusivity of drug, and thickness of the cornea. After entrance into the anterior chamber, the extent of protein binding in aqueous humor depends upon the condition of the eye and, therefore, the protein composition and content in the aqueous humor. Turnover of aqueous humor, which

is considerably slower than that of lacrimal fluid, results in drug loss. Naturally, the drug in these various fluids after parenteral administration is also subject to binding and loss.

EXPERIMENTAL

Adult, male, albino rabbits[1] were used as the experimental animals. No special pretreatment methods or diets were incorporated into the *in vivo* procedures.

Cellulose dialysis tubing[2] and crystalline rabbit serum albumin[3] were obtained from commercial sources.

Methylprednisolone[4], sulfisoxazole[5], tritiated pilocarpine nitrate[6], and N-1-naphthylethylenediamine dihydrochloride[7] were used; all chemicals were either USP or reagent grade.

Equilibrium Dialysis

Cells and Procedures—Two different sets of dialysis cells were utilized, depending upon the type of solution under investigation. Dialysis cells of identical half-cell chamber size of 10-ml. capacity were employed in studies to determine the binding capabilities of rabbit serum albumin. Unequal half-cell capacity cells were used for the tears, cornea, and aqueous humor studies. In the unequal half-cell experiments, the drug volume side was 5 ml. and the protein or tissue side was 1 ml.; cell thicknesses were adjusted so that differing volumes produced equivalent levels in the cells.

Protein solution or the tissue sample was placed in one half-cell chamber and drug solution was placed on the opposite side. The contents of the dialysis cells were equilibrated for 24 hr. at 25 ± 1.0° on a shaker[8]. Twenty-four hours under these conditions was sufficient for reaching equilibrium.

The equilibrium concentration of free drug in the protein-free dialysate was determined following equilibration. Assays were performed immediately after removal of the protein-free dialysate to avoid potential microbial growth which can occur upon standing. From measurement of the free drug concentration and after suitable blank corrections, the fraction of drug bound was determined.

Dialysis Membrane Preparation—Dialysis tubing was cut into strips and purified extensively prior to use (13).

Drug Solution Preparation—All drug solutions were prepared using Sørenson phosphate buffer, pH 7.38 (14). The buffer is isotonic, giving the solutions a sufficiently high ionic strength so that any discrepancies in distribution due to the Donnan effect should be minimal. Isotonic solutions include the drug solutions that were applied locally to the rabbit eye in the *in vivo* studies as well as the drug and rabbit albumin solutions employed in the *in vitro* dialysis studies.

Dialysis Solution—Crystalline rabbit serum albumin solutions, 2.89×10^{-5} M, were prepared in Sørenson phosphate buffer, pH 7.38.

Eight milliliters of protein solution was placed in one half-cell and 8 ml. of drug solution was placed in the opposite half of the dialysis chamber.

Corneal Tissue—The corneal tissues employed in the equilibrium dialysis binding studies were obtained from rabbits and were used fresh.

Dialysis studies on the cornea were initiated immediately following removal of the tissue, utilizing the entire intact cornea (without pulverization). The corneas were placed in the dialysis chamber with a small volume of buffer. Two corneas were placed in one-half of the dialysis chamber together with 0.5 ml. buffer solution, and 3 ml. drug solution was placed in the other half. The height of the liquids on both sides was the same. The corneas occupied an average volume of 0.092 ml. and had an average dry weight of 0.096 g. Calculation of the fraction of drug bound was carried out in the usual manner and included the unequal volumes in the half-cells, which were 3 ml. on the drug side and 0.592 ml. on the corneal side.

Aqueous Humor—The primary aqueous humor samples were

[1] Klubertanz, Edgerton, Wis.
[2] Union Carbide Corp., Chicago, Ill.
[3] Schwarz/Mann, Orangeburg, N. Y.
[4] Gift of The Upjohn Co., Kalamazoo, Mich.
[5] Gift of Hoffmann–La Roche Inc., Nutley, N. J.
[6] Eastman Organic Chemicals, Rochester, N. Y.
[7] New England Nuclear, Boston, Mass.
[8] Eberbach.

obtained by making a single puncture into the anterior chamber of the rabbit eye. Depending upon the size of the rabbit, a volume of 0.2–0.3 ml. was obtained from each eye.

Pooled primary aqueous humor was used for dialysis studies. A volume of 0.2 ml. of aqueous humor together with 0.2 ml. of buffer was placed in one half-cell, and 1 ml. of drug solution was placed in the other half-cell. The height of the liquids was the same. Fraction of drug bound was calculated and included corrections for the unequal volumes as well as blank corrections.

Secondary or plasmoid aqueous humor was obtained by carefully removing primary aqueous humor and then removing the secondary at either a 1- or 4-hr. interval. Generally, the volume of the plasmoid aqueous humor obtained was in the range of 75 μl./eye.

Lacrimal Fluid—The lacrimal fluid employed in the equilibrium dialysis studies was collected from two adult, male human subjects. The lacrimal fluid from one subject was obtained by mechanically irritating the eye through placement of a thin strip of filter paper in the lower cul-de-sac. An overflow of tears was produced, which was collected as drops rolling from the lid. The majority of the tear samples came from the second subject where overflow lacrimal fluid was obtained without mechanical stimulation. The overflow tears were collected by the subject during late hours of the day as an accompanying phenomenon to yawning. Again, the lacrimal fluid in the form of drops was collected rolling from the eyelids. All lacrimal fluid samples were pooled and stored at 5° until used.

The volume and methods of dialysis, as well as the calculation, were the same as in the aqueous humor study.

Assays[9]—*Methylprednisolone*—Determination of methylprednisolone concentration was accomplished by direct spectrophotometric measurement. The compound was found to follow Beer's law in the concentration range studied, at an absorbance maximum of 248.5 nm. Methylprednisolone exhibits a molar absorptivity of 1.46×10^4 M^{-1} cm.$^{-1}$ at this wavelength in pH 7.38 aqueous Sørenson buffer.

Pilocarpine—The quantitative determination of pilocarpine nitrate was accomplished by utilizing its reactivity with hydroxylamine to form a hydroxamic acid that is subsequently complexed with ferric ion as previously described (15). The formed complex shows an absorbance maximum at 500 nm. and a molar absorptivity of 2.26×10^2 M^{-1} cm.$^{-1}$ at this wavelength.

Radioactive pilocarpine nitrate was assayed by liquid scintillation detection[10].

Sulfonamides—Quantitative determination of sulfisoxazole was accomplished by utilizing the primary aromatic amine character of the drug molecule. Diazotization of the primary aromatic amine, followed by coupling with Bratton–Marshall reagent (N-1-naphthylethylenediamine), yields a product that displays an absorbance maximum in the visible region (16). Sulfisoxazole exhibited an absorbance maximum at 546 nm, with a molar absorptivity of 2.52×10^3 M^{-1} cm.$^{-1}$ at this wavelength and obeyed Beer's law in the concentration range studied.

Miosis–Time Studies

Procedure—Anesthetized animals were used in these experiments. After onset of anesthesia, the test animals were placed in restraining boxes with the head elevated in such a manner that the lid line, upon closure, was approximately horizontal. This position, which is not the natural posture for the rabbit, was chosen to facilitate pupillary diameter measurements. Experimental testing of this animal position as compared to the normal position showed no difference in drug response, so the elevated position was used.

• Lighting and temperature in the test room were constant throughout the study. The entire test room was as isolated as conditions would permit to keep audiovisual stimuli to the test animal to a minimum.

The observer was hidden from the test animal by a cardboard shield containing a vertical slit through which measurements of pupillary diameter could be made. The observer, once inside the room, was not allowed movement in or out of the room until the experiment was complete. These precautions were found necessary for obtaining reproducible results.

[9] All spectral measurements were carried out on a Cary 16 spectrophotometer.
[10] Packard Illinois, model 2002.

Measurement of pupillary diameter (with an accuracy of ±0.1 mm.) was made with a cathetometer[11], positioned approximately 50 cm. from the test animal.

After positioning the animal in the restraining box, in line with the cathetometer and prior to drug instillation, pupillary diameter readings were made at 100-sec. intervals. This procedure was continued until the test animal had adapted to its environment as judged by the constancy of pupillary diameter (±0.1 mm. for 10 min.). The adaptation period varied from 15 to 30 min., and the initial diameter ranged from 6.0 to 7.8 mm.

Pipets[12] with blowout delivery were used to instill the drug solution and were checked for accuracy of delivery prior to use by weighing the delivered solutions.

Drug solutions were instilled onto the cornea so that they collected in the lower cul-de-sac. To prevent loss of solution during instillation, the lower eyelid was pulled slightly away from the globe of the eye to form a pocket. This particular form of solution instillation was followed at all times.

After instillation of drug solution, the lower lid was lifted back and forth over the cornea to mix the drug solution with lacrimal fluid. At no time was the lid ever massaged against the cornea so that only movement of the drug solution over the cornea occurred. To exert some degree of control in this method, the eyelid was lifted over the cornea exactly four times, during about 5 sec., in all cases. All procedures were performed in precisely the same manner and with the same time intervals.

After instillation of the drug solution, pupillary diameter was measured as a function of time. The other eye of the test animal served as a control. One hundred-second intervals were used for the first 50 min. of the experiment, 300-sec. intervals were used from 50 to 80 min., and 600-sec. intervals were used thereafter. Measurements were terminated when the measured diameter reached a constant value close to the initial value.

No experimental animal was used more than once, and each experiment was repeated at least four times.

Solution Preparation —*Pilocarpine Nitrate Solutions*—These solutions were prepared fresh and were discarded immediately after the experiment. The solutions were prepared by addition of pilocarpine nitrate to Sørenson phosphate buffer, pH 7.38. Solution pH was checked and adjusted where necessary[13]. The pH meter and electrode system were standardized against phosphate buffer as described previously (17). No attempt was made to sterilize the solutions. Isotonicity of solutions was maintained by addition of sodium chloride.

Anesthetic Solutions - Solutions containing sodium pentobarbital, 50 mg./ml., or sodium phenobarbital, 100 mg./ml., in a vehicle containing 20% (v/v) propylene glycol and 10% (v/v) ethanol were prepared. Both solutions were refrigerated between uses and were never kept for more than 2 weeks.

Anesthesia—It was found that a dose of 33 mg./kg. i.p. sodium pentobarbital and 100 mg./kg. i.p. sodium phenobarbital was sufficient in most cases to anesthetize the rabbits for 8–12 hr.

Miosis Study following Paracentesis--Anterior chamber paracentesis was accomplished by making a puncture incision at the limbus through the cornea into the anterior chamber and aspirating the aqueous humor. Extreme care was taken upon removal of the needle from the deflated anterior chamber to minimize damage to the surfaces exposed to the needle. After waiting for either 1 or 4 hr., the drug solution was instilled into the eye and the change in pupillary diameter was monitored as a function of time.

RESULTS

Demonstration of *In Vitro* Drug–Protein Interaction—The fact that many drugs are bound to proteins is well known (18). A demonstration of this interaction, using rabbit serum albumin as the protein and sulfisoxazole as the drug, is presented in Fig. 1. This particular form of data presentation, namely fraction of drug bound *versus* drug concentration, is generally used when the concentration and nature of the protein are unknown (13). Since an analysis of the various eye fluids and tissue used in this study, as to their types
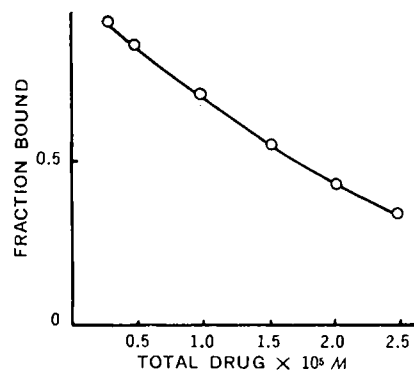

Figure 1—Equilibrium dialysis experiment on the extent of sulfisoxazole binding to 0.2% (w/c) rabbit serum albumin. Data points are mean values from four separate determinations, and standard deviations are within ±5% of the mean values.

and concentration of protein components, as well as their binding potential was not performed, the *in vitro* data are presented as a fraction of drug bound. Studies are currently underway to characterize these proteins and may be reported later.

Although drug binding to blood proteins is well known, what is apparently not as well appreciated is that many other tissues and fluids of the body, such as eye tissues and fluids, also bind drugs and can greatly influence drug bioavailability. This influence is particularly important when the tissue or fluid is turned over at a rapid rate as is the case with tears. Figure 2 demonstrates the interaction of sulfisoxazole and methylprednisolone with the components of human tears, and it is clear that these drugs are extensively bound to the components at the drug concentrations tested. The ramifications of this binding to drug therapy can be appreciated by realizing that instillation of 50 μl. of 1% methylprednisolone solution, which is equal to about 0.5 mg. of drug, followed by dilution of the solution and removal through drainage and turnover, rapidly converts the drug concentration to levels equal to or below that indicated in Fig. 2.

It is important to recognize that the tears used in this study were obtained from healthy eyes by stimulation and, therefore, were low in protein content (19) as compared with tears obtained without stimulation or from inflamed eyes. Thus, binding of these drugs to "normal" tears or tears in an inflamed eye would be expected to be much greater than is indicated in Fig. 2.

In addition to tears as a potential site of drug-protein binding, due to their relatively high protein content, corneal tissue and plasmoid aqueous humor are also potential sites due to a rich supply of protein. In vitro dialysis studies of methylprednisolone and sulfisoxazole binding to corneal tissue, in the concentration range of 1-5 × 10⁻⁶ M, show very high drug binding. This is not at all surprising considering the high total protein content of corneal tissue. Likewise, binding of these drugs to plasmoid aqueous humor was also very extensive. It was earlier indicated that primary aqueous humor is low in protein content; as might be expected, the binding of sulfisoxazole and methylprednisolone to this fluid was negligibly small in the concentration range of 1-5 × 10⁻⁵ M. On
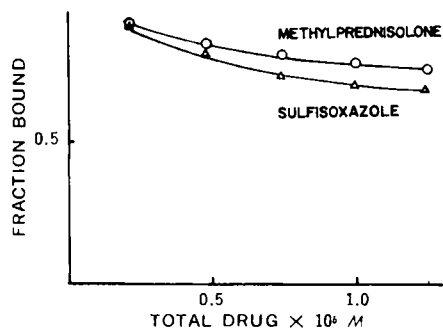

Figure 2—Equilibrium dialysis experiments on the extent of drug binding to human tears. Data points are mean values from three separate determinations, and standard deviations are within ±7% of the mean values.

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[11] Central Scientific Co., Chicago, Ill.
[12] Scientific Products, Evanston, Ill.
[13] All pH measurements and adjustments were made on an Orion model 801 digital pH meter, using a Beckman E3, wide range, glass electrode.
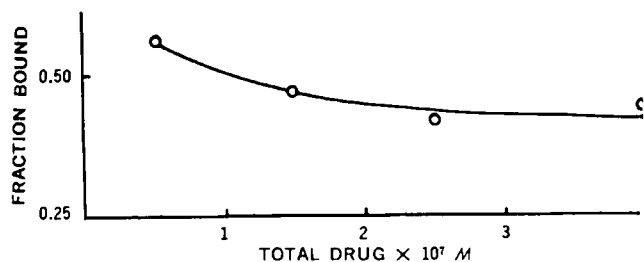
Figure 3—*Equilibrium dialysis studies of tritiated pilocarpine nitrate and plasmoid aqueous humor. Data points represent the mean values from two determinations.*

the other hand, plasmoid aqueous humor is extremely rich in protein; equilibrium dialysis studies with sulfisoxazole and methylprednisolone to this fluid showed almost complete binding in this same concentration range.

Since both sulfisoxazole and methylprednisolone are intermediate in their binding capabilities to albumin [sulfisoxazole is 5×10^3 M^{-1} (20) and methylprednisolone is approximately 10^4 M^{-1} (13)], an additional drug with a low binding constant was chosen to demonstrate the extent of drug binding to plasmoid aqueous humor. Pilocarpine nitrate is a miotic drug used extensively in ophthalmology. It has a binding constant of less than 100 to rabbit serum albumin[14] (21).

Figure 3 shows a fraction bound *versus* concentration plot for pilocarpine nitrate and plasmoid aqueous humor. At low drug concentration and presumed high protein concentration, there is considerable binding of the drug. Note that the drug concentration used in this study is considerably lower than with studies involving sulfisoxazole; this is a reflection of the smaller binding constant of pilocarpine nitrate to the protein. There is, of course, the possibility that pilocarpine nitrate is very highly bound to a specific protein that is present at low concentration, but this cannot be resolved until studies are completed on protein types, concentration, and drug affinity in plasmoid aqueous humor.

These *in vitro* results, using drugs that represent a range of binding affinities from 10^2 to 10^4 M^{-1}, show that there is extensive drug binding to the components of tears, cornea, and plasmoid aqueous humor. This binding can have a large or small influence on drug bioavailability, depending on the extent of binding and rates of turnover or removal of these drugs containing fluids and tissue.
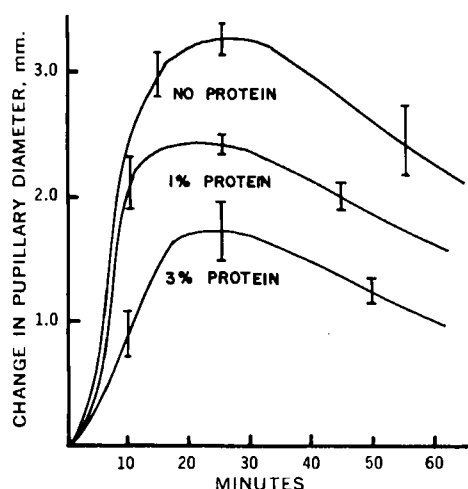

Figure 4 —*Miotic activity of 50 μl. of 2.5×10^{-2} M pilocarpine nitrate in the presence and absence of added rabbit serum albumin. Each experiment was conducted at least three times, and the vertical bars show the standard deviation at random times. Each line was drawn on the basis of approximately 30 experimental points.*
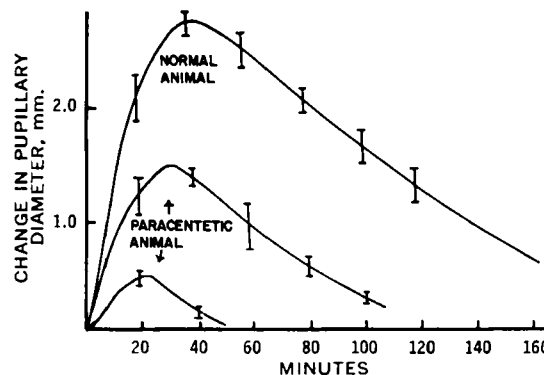
Figure 5—*Effect of plasmoid aqueous humor on the miotic response to pilocarpine nitrate as compared to normal aqueous humor. Fifty microliters of 1×10^{-2} M drug was instilled into the eye, and each line represents the mean of at least three determinations. The bottom line represents addition of drug 1 hr. after paracentesis, and the middle line corresponds to drug addition 4 hr. after paracentesis. Vertical bars are standard deviations at random points on the curve, and each curve was drawn on the basis of 15-40 experimental points.*

Demonstration of Drug–Protein Binding Influence on Drug Bioavailability—Pupillary diameter studies were used to assess the influence of protein binding on drug bioavailability. Pilocarpine nitrate was used as the test agent, and the change in pupillary diameter as a function of time and drug concentration was used as an index of drug activity.

The *in vitro* dialysis experiments using tears and various drugs showed that extensive binding of drug occurred. If drug binding to proteins in tears is responsible for a loss in drug, it should be possible to increase this loss by further addition of the proper protein to tears. Addition of protein to tears is difficult, but the same results should be obtained by placing the protein in the drug solution prior to administration into the eye. Rabbit serum albumin was added to the drug solution, and the resultant effect on drug bioavailability, as judged by response to the drug, is shown in Fig. 4. When lower drug concentrations were used in this experiment, the entire miotic activity of the drug could be suppressed by addition of rabbit serum albumin to the drug solution. This diminished response bears out the expectation that drug binding influences bioavailability through drug loss. Albumin was selected as the protein only for convenience. It is possible that other proteins normally present in tears would have a larger affinity for the drug and yield more dramatic results.

Albumin, by itself, was found not to have any effect on pupillary diameter, although it does have surface activity and this property might have two additional effects that should be considered in evaluating the results. The first effect might be to promote drug transport, since a number of surface-active agents are known to have this effect in the eye (22, 23). This would cause an *increase* in drug bioavailability due to a faster penetration of the drug through the corneal tissue; if this was a factor in the present study, it was clearly swamped out, as judged by the *decrease* in biological response. A second effect, which might account for a decreased response, would be a lowering of the surface tension of the instilled solution and hence an increase in the rate of solution drainage. Data from this laboratory on drainage rate studies (2), as well as the fact that many of the tested surface-active agents are without effect in influencing corneal absorption of drugs[15] (22, 23), suggest that a reduction in surface tension of the instilled solution and a corresponding increase in drainage rate are not the mechanisms for the decreased drug response when albumin is added to the drug solution. Thus, the result from this experiment is attributed principally to drug–protein interaction and its concomitant effect on drug loss.

The magnitude of the drug–response change (Fig. 4) is considerable. From dose-response curves generated for pilocarpine nitrate (13), the addition of 3% protein to the drug solution is equivalent to a 75-100-fold reduction in drug concentration, as judged by

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[14] As determined by ultracentrifugation and by equilibrium dialysis studies using tritiated pilocarpine nitrate. These results will be the subject of a separate publication.

[15] Additional studies from this laboratory that are part of another report (13).

biological response in the absence of added protein. Considering the relatively small binding constant for pilocarpine nitrate to albumin, as compared with some antibiotics and steroids, the potential effect of this interaction with other drugs becomes apparent.

If it is possible to depress the bioavailability of drugs by addition of protein, it should be possible to increase the biological activity by competitively inhibiting the interaction. Addition of appropriate substrates to inhibit drug–protein binding can increase the free-drug concentration and, hence, the drug bioavailability. In the case of pilocarpine nitrate solutions, it is possible to increase miotic activity in healthy albino rabbits by more than a factor of 10 through addition of suitable substrates (13). Furthermore, the influence of added protein in depressing the miotic activity of pilocarpine, described earlier, was fully reversible in the presence of a competitive inhibitor. A full account of this work is given in a separate paper[15], but mention is made here to further support the contention of drug–protein binding in eye fluids and tissues as an influencing factor in drug bioavailability.

It was also of interest to determine if the presence of higher concentrations of protein in aqueous humor could be responsible for a decrease in drug activity. Paracentesis was carried out on several animals to form a protein-rich secondary aqueous humor, and the activity of pilocarpine nitrate was determined in these animals (Fig. 5). Instillation of pilocarpine nitrate solution 4 hr. after paracentesis caused a significant drop in the biological response to pilocarpine nitrate, and an even further reduction in activity occurred if the drug was instilled 1 hr. after paracentesis. At the 4-hr. point, the aqueous humor is returning to its normal state and is not as rich in protein as at the 1-hr. point.

The dramatic decrease in drug response, shown in the paracentesis experiment, could be largely attributed to trauma incurred by withdrawing aqueous humor. If trauma to the iris or any portion of the tissue responsible for pupillary activity occurred, it returned to normal within 12 hr. but nevertheless could account for a portion of the results in this experiment. When the result of the *in vivo* paracentesis experiment is evaluated together with the results from the *in vitro* dialysis experiment, using plasmoid aqueous humor and pilocarpine nitrate, it seems reasonable to assume that a sizable portion of the loss in drug activity in the paracentetic animal was due to drug–protein interaction and not trauma.

DISCUSSION

An important facet of drug binding influence on ophthalmic drug bioavailability is the turnover rate of eye fluids and tissues, as well as drainage of instilled solutions when these are used as the delivery system. Chrai *et al.* (2) showed that instilled solutions are very rapidly drained from the eyes of rabbits. For example, approximately 50% of a 50-μl. instilled drop will drain from the eye of a rabbit within 30 sec. postinstillation and better than 84% will be lost within 3–4 min. These values are even larger in humans and, according to Maurice[16], most instilled solution in humans is lost within 1–2 min. In addition to drainage of instilled solution, one must also be concerned with turnover of tears, since this results in both dilution and removal of the drug.

The smaller turnover rate of albumin in the cornea suggests that drug binding to this tissue would have less of an influence on total drug availability, although it can influence the rate of transport and the time lag before drug appears in the anterior chamber.

Aqueous humor also has a smaller turnover rate than tears but can nevertheless influence drug bioavailability if the binding constant is large or if protein content is large.

Other portions of the eye such as the sclera and conjunctiva can and probably do bind drugs and are responsible for a decrease in drug bioavailability. The present study was concerned principally with components of the eye that are constantly being turned over and are at the same time rich in protein.

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