Binding of Sulfisoxazole to Protein Fractions of Tears

SUKHBIR S. CHRAI * **and JOSEPH R. ROBINSON** ˣ

Abstract □ By using equilibrium dialysis experiments, it is shown that sulfisoxazole binds extensively with albumin and α-globulin and to a lesser extent with γ-globulin and lysozyme, all of which are normal components of tears. The binding of sulfisoxazole to the various protein fractions quantitatively accounts for the overall binding of this drug to human tears. It is suggested that to predict the binding of drugs to proteins in lacrimal fluid, both in normal and pathological conditions, it is necessary to conduct binding studies of the drug with the major protein fractions of tears. Moreover, it is essential to know the composition of the major protein fractions of tears in both normal and various pathological conditions to conduct the necessary *in vitro* binding studies.

Keyphrases □ Sulfisoxazole—binding to protein fractions of human tears, normal and irritant induced □ Protein binding—sulfisoxazole to protein fractions of human tears, normal and irritant induced □ Lacrimal fluids—human, normal and irritant induced, binding of sulfisoxazole to protein fractions

It is well established that binding of drugs to proteins can substantially influence the bioavailability and biological activity of drugs in the body (1, 2). Recently, the problem of drug binding to proteins in ocular fluids and tissues was examined (2–4); it was shown that this interaction can significantly affect the bioavailability of drugs in the eye. This effect is perhaps more important in the eye than in other areas of the body because of the great rates of drainage and fluid turnover, *e.g.*, tear and aqueous humor turnover and instilled solution drainage.

All *in vitro* binding work thus far relative to the eye has been conducted using albumin as the protein. While albumin is the principal protein in tear fluid and aqueous humor, it is by no means the only protein. The purpose of the present study was to examine the drug binding potential of several proteins, which are known to be components of tears, in an attempt to gain a more quantitative picture of the drug binding capabilities of proteins in tears.

A significant feature of tear composition is the presence of relatively high concentrations of proteins. The total protein present varies qualitatively and quantitatively, depending upon the method of analysis, the method of collecting tear samples, the species of animal involved, and the pathological state of the eye. A relatively conservative estimate (5) lists the albumin content of tears to be 0.4% for humans with a total protein content of approximately 0.7%; for rabbits, the estimate is 0.3% albumin with a total protein content of 0.5% (6). Emotional stress, irritation, disease, and other factors greatly influence the protein content (7). Table I lists the reported (7) major protein components of both normal tears and irritant-induced tears.

Detailed studies (8–11) have been conducted on tear proteins and immunoglobulins in both normal and pathological cases in humans and animals. Sapse *et al.* (12, 13) identified serum albumin, ceruloplasmin, transferrin, immunoglobulins A and G, lysozyme, globulins, and specific tear albumin in human tears. Other proteins reported are plasmin (14) and tear lactoferrin (15). Pathological processes in the eye cause significant qualitative and quantitative changes in the protein pattern and a significant increase in the total proteins. None of the pathological conditions reported brought about the total disappearance of any of the major protein fractions. Therefore, changes in protein composition in pathological conditions would be expected to change the extent of drug–protein interaction for drugs that are susceptible to drug–protein binding.

EXPERIMENTAL[1]

Materials—Water was distilled from alkaline permanganate in an all-glass apparatus. Cellulose dialysis tubing[2] was purified according to the procedures described by Mikkelson (2). Crystalline rabbit serum albumin[3], α- and γ-globulins[3], lysozyme[3] (specific activity of 11,800 units[4]/mg), and sulfisoxazole[5] were used as received. All other chemicals were either reagent or analytical grade.

Sulfisoxazole Assay—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 the Bratton–Marshall reagent [*N*-(1-naphthyl)ethylenediamine] yields a product that displays an absorbance maximum in the visible region. The procedure described by Connors (16) was followed, except that the final dilution was taken to 25 ml rather than to 50 ml. Sulfisoxazole exhibited an absorbance maximum at 546 nm with a molar absorptivity of $2.85 \times 10^{-3}\ M^{-1}\ \text{cm}^{-1}$ at this wavelength. Beer's law was obeyed in the concentration range studied.

Solution Preparation—Sulfisoxazole solutions were prepared by the addition of the drug to pH 6.24 Sørensen phosphate buffer. Protein solutions were prepared fresh by addition of an appropriate weight of protein to pH 6.24 Sørensen phosphate buffer solution.

Equilibrium Dialysis Studies—The methods used and dialysis cell descriptions were reported previously (3).

RESULTS

For reference purposes, the binding of sulfisoxazole to human lacrimal "overflow" tears is shown (3) in Fig. 1. The lacrimal fluid employed was obtained from healthy human eyes. Overflow tears were collected in the form of drops rolling from the eyelids during late hours of the day or as an accompanying phenomenon to yawning. It is clear from Fig. 1 that sulfisoxazole is extensively bound at the concentrations tested.

For therapy, a 4% solution of this drug is generally used. This concentration is well above the concentration range studied, although with tear turnover the concentration of drug is quickly reduced to encompass the range studied here. Thus, although protein binding of sulfisoxazole in ocular therapy would not appear to have a very significant influence on drug activity, the drug is a useful experimental probe for ocular drugs in general. Other ocular drugs, *e.g.*, steroids, are influenced much more by drug binding to proteins. It is important to recognize that the tears used in this

[1] All spectral measurements were carried out on either a Cary 14 or Cary 16 spectrophotometer.
[2] Union Carbide Corp., Chicago, Ill.
[3] Schwarz/Mann, Orangeburg, N.Y.
[4] A unit is defined as a decrease in absorbance at 450 nm of 0.001/min at pH 7.0 and 25°.
[5] Donated by Hoffmann-La Roche Inc., Nutley, N.J.

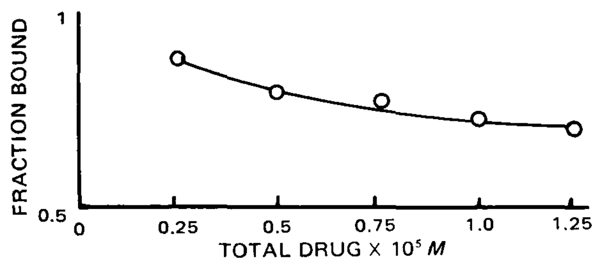

Figure 1—*Extent of protein binding of sulfisoxazole to human overflow tears. The data points are mean values from three separate determinations, and standard errors of the mean are within ±7% of the mean values (3).*

study (Fig. 1) were obtained from healthy eyes and hence were presumably low in protein content as compared with tears obtained from inflamed or diseased eyes.

As mentioned earlier, there are several protein fractions in human tears, estimated by some to be as many as 14 (12). Since it was not possible to obtain all of the different fractions of tear proteins, drug binding studies were conducted on serum albumin, lysozyme, and α- and γ-globulins. Binding of sulfisoxazole to these different protein components is shown in Fig. 2. The data are presented as a plot of fraction of drug bound *versus* the amount of drug present in the system.

The total amount of protein present in tears and the values reported (7, 12, 17, 18) for the different fractions are conflicting. To assess the binding capacity of each protein component at a fixed concentration of sulfisoxazole and its contribution to the total binding of drug in tears, it is assumed that the values reported by Brunish (7) are closest to the actual values. It is assumed that the binding capacity of a particular class of proteins is similar for a given drug irrespective of source; *i.e.*, specific tear prealbumin has the same binding properties as serum albumin in tears. The second assumption reduces the seven major components of tear proteins into three classes. These assumptions are necessary to reduce the complexity of the problem.

According to Brunish (7), protein concentration for normal tears is 0.6% with 0.23% albumin, 0.27% globulin, and 0.1% lysozyme. Upon instillation of drug solution, due to mixing of 7.5 μl of resident tears with 25 μl of instilled drug solution, these concentrations of albumin, globulin, and lysozyme in tears decrease by 4.3-fold to 0.053, 0.062, and 0.023%, respectively. If it is assumed that the fraction of drug bound is directly proportional to the concentration of the protein fractions (2, 3), it appears from Fig. 2 that the fraction of drug bound to albumin, globulin, and lysozyme at their diluted concentrations should be 0.2, 0.05, and 0.004, respectively, for 1×10^{-5} M sulfisoxazole. Thus, the fraction of 1×10^{-5} M sulfisoxazole bound to normal tears upon instillation of 25 μl is calculated to be 0.254.

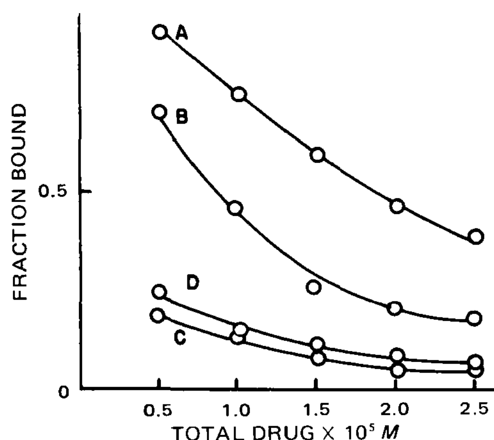

Figure 2—*Equilibrium dialysis experiments on the extent of sulfisoxazole binding to different protein components of human tears. Key: A, binding with 0.2% albumin; B, binding with 1.0% α-globulin; C, binding with 0.5% γ-globulin; and D, binding with 1.0% lysozyme. The data points are mean values from three separate determinations, and standard errors of the mean are within ±10% of the mean values.*

Table I—Protein Composition of Human Tears (7)

Type of Tear	Number of Subjects	Albumin, %	Globulin, %	Lysozyme, %
Normal	21	38.8	44.8	16.4
Irritant induced	12	19.3	54.0	26.7

It appears from Fig. 1 that the fraction of drug bound to total proteins in tears for 1×10^{-5} M sulfisoxazole is 0.7. This high binding value with total tears is due to the fact that the concentration of protein is 4.3-fold higher than the concentration of protein fractions assumed for reaching the calculated value of 0.254. After the necessary correction is made for the change of protein concentration of tears due to dilution with instilled drug solution, the experimental binding fraction value of 0.7 reduces to 0.161.

The difference in the experimental value of 0.161 and the calculated value of 0.254 is due to the fact that the tears used for the original study were obtained by stimulation and were thus low in protein content. Correction in the experimental value for the difference in protein content of normal tears and irritant tears yields an experimental value of 0.242—in excellent agreement with the calculated value of 0.254.

DISCUSSION

It is clear from the equilibrium dialysis studies that not only albumin but also other fractions of proteins do bind with drugs. According to Goldstein (1), γ-globulins appear almost exclusively to be highly involved in specific protein–protein interactions for immune reactions. Very few drugs have been shown to interact with α- and γ-globulins and those that do are poorly soluble in water. Based on the results from the present study, it appears that all of the protein fractions, including α- and γ-globulins, interact with the drug to some extent and the degree of binding varies from protein to protein, with albumin having the greatest binding for sulfisoxazole.

It is now apparent that the use of albumin to study the binding of a particular drug to a specific ocular tissue or fluid *in vitro* is not correct unless other proteins are included. However, for tears, using three fractions of protein, namely, lysozyme, albumin, and globulin, does apparently allow for the approximate quantitation of binding of drugs to the proteins in tears. Specifically with sulfisoxazole, the important components of tears are albumin and α-globulin insofar as binding is concerned.

As mentioned earlier, the values of the different fractions of tear proteins reported are conflicting. Newell (17) claimed that human lacrimal fluid contains 1–2% protein composed of 30% albumin, 40% globulins, and 30% lysozyme. Because of the different ratios of albumin to globulin and albumin to lysozyme, the different overall binding of drugs to human tear fluid will be different than the one reported here using the values of Brunish (7). Ridley (18) and Bonavida *et al.* (19) reported the total protein content to be about 0.7%. The former reported the albumin and globulin to be approximately 0.4 and 0.3%, respectively, with no lysozyme; the latter reported that 20–40% of the total protein is lysozyme. The calculation for the contribution made by each fraction to the overall protein binding by tears will be different if the calculations are done with the values of each fraction as reported by Ridley (18) and Sapse *et al.* (12).

The importance of the results of the present study will be more significant in pathological conditions. In most diseases of the human eye, the lysozyme content is reported to decrease tremendously (20). Even if the total protein content is kept constant in pathological conditions, the decrease of lysozyme (20) and the increase in serum albumin (8) increase the overall binding of the drug to lacrimal fluid. Serum albumin is a relatively minor component of nonstimulated tears whose level rises after mechanical stimulation in certain disease states (8). In rabbits, tear lysozyme comprises only 1% of the total tear protein, which is reported as 1.3% (20). Since the total protein is greater in rabbit tears than in human tears and the lysozyme content is greater in human tears

than in rabbit tears, the change in binding capacity in disease states for rabbit tears is expected to be greater than in human tears.

Thus, based on the results of the present study, it is clear that to study the influence of drug–protein interaction on drug bioavailability in the eye, it is important to know: (*a*) the binding capacity of each major fraction of tear protein, and (*b*) the changes that occur in the major fractions during the disease state. This information will be of great help in predicting the loss of drug in pathological conditions due to drug–protein interaction.

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* Present address: Schering Corp., Bloomfield, NJ 07003

[x] To whom inquiries should be directed.

Phosphorus–Nitrogen Compounds XIX: Distribution of ^{32}P and Effect of an Active Oncolytic on Intracerebral Leukemia in Rodents

L. A. CATES [x] and M. B. CRAMER

Abstract ☐ *P,P*-Bis(1-aziridinyl)-*N*-1-adamantylphosphinic amide displayed oncolytic activity against intracerebral and intraperitoneal L-1210 leukemia. Administration of the isotope-labeled compound to rats shows ^{32}P distribution to the brain.

Keyphrases ☐ Organophosphorus compounds—adamantyl substituted, synthesis, oncolytic activity and tissue distribution screened ☐ Distribution, tissue—studied, adamantyl-substituted organophosphorus compounds ☐ Oncolytic activity—adamantyl-substituted organophosphorus compounds screened

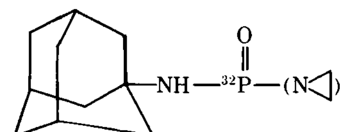
Of 19 adamantyl-substituted organophosphorus compounds synthesized and tested for anticancer activity (1, 2), only *P,P*-bis(1-aziridinyl)-*N*-1-adamantylphosphinic amide (NSC 166199, I) displayed oncolytic properties. Closely related to triethylenephosphoramide, I possesses a 1-adamantylamino moiety in lieu of one aziridinyl group. This modification produces a marked change in physical properties, with I having very low water solubility compared to triethylenephosphoramide, which is extremely soluble in water. The lipophilic nature of I suggests it may have the ability to overcome the blood–brain barrier and produce anticancer effect in the central nervous system (CNS). The eradication of leukemic cells in the CNS is a major goal of modern cancer chemotherapy.

To ascertain the ability of I to affect intracerebral leukemia, this agent was administered intraperitoneally to mice injected intracerebrally and intraperitoneally with L-1210 leukemia cells for survival time determination. The ^{32}P compound (II) of I was synthesized and administered intraperitoneally to rats to estimate its distribution to certain tissues and organs, including the brain.

EXPERIMENTAL

Chemistry—A mixture of 0.14 g (0.9 mmole) of ^{32}P-phosphorus oxychloride (4.47 mCi) and 10.3 g (67 mmoles) of phosphorus oxychloride in 50 ml of ether was added dropwise with stirring and cooling (0–5°) to a solution of 22.0 g (146 mmoles) of 1-aminoadamantane in 250 ml of ether. After standing for 24 hr, the reaction mixture was filtered and the filtrate containing *N*-1-adamantylphosphoramidic dichloride (3) was used for the *in situ* preparation of II.

The ethereal solution was added dropwise with stirring to a solution of 18 g (420 mmoles) of aziridine in 50 ml of ether, with a re-



II