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A fluorimetric investigation of the binding of drugs to lysozyme

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The binding of drugs in tears influences the pharmacoinetics of drugs used for ophthalmic purposes. The major proteins in human tears are albumin, globulins and lysozyme. Although there is much literature on the binding of drugs to albumin (Meyer & Guttman 1968; Vallner 1977) and lipoproteins (Vallner & Chen 1977) there is little on the binding of drugs to lysozyme (Chrai & Robinson 1976). This is partially due to the small molecular weight of the protein (14 400) making dialysis and ultrafiltration unreliable, due to leakage of the small quantities of protein through the cellulose membranes frequently used.

Lysozyme is a basic protein and is able to bind to anions of inorganic origin. It tends to associate into dimers and higher polymers, as the pH and concentration are increased and the temperature decreased. At the concentrations, ionic strength and pH of tears, it exists entirely as the monomer.

Three of the six tryptophan residues in lysozyme seem to be located near the binding site for substrates on lysozyme (Johnson & Phillips 1965; Phillips 1966). Residues 62 and 108 are readily accessible to specific oxidizing agents (Hayashi et al 1965; Imoto et al 1971) and appear to be in an aqueous environment whereas residue 63 is buried in the interior of the molecule (Hayashi et al 1965). This together with a low tyrosine content and minimal (Johnson & Phillips 1965; Phillips 1966) lysozyme to tryptophan energy transfer means that the fluorescence of emission of lysozyme is readily quenched by certain inorganic ions including nitrate and iodide (Altekar 1977) as well as by substrates such as tri- and di-*N*-acetyl-D-glucosamine (Lehrer & Fasman 1967). Such observations make the interaction of certain drugs and lysozyme by fluorescence a possibility.

Hen lysozyme, twice crystallized, batch numbers D2-3246 and E2-3359 were obtained from Schwarz/Mann, Orangeburg, New York. Solutions of lysozyme were aged for at least 6 h (Attallah et al 1968) and filtered through a 45 μm Millipore filter (Millipore Bedford, Ma). Sulphisoxazole, methyl paraben, ethylparaben and propylparaben, cortisone, hydrocortisone were all obtained from Sigma, St Louis, Mo, chloramphenicol from Aldrich, Milwaukee, WI; phenyl mercuric acetate and nitrate from Eastman Kodak, Rochester, N.Y. Sulphathiazole sodium from Pfaltz and Blauer, Stanford, CT, atropine sulphate and phenobarbitone from Mallinkrodt, St Louis, MO; chlorobutanol from Fischer, Fair Lawn, NJ and sulphathiazole from Nutritional Biochemical Corp., Cleveland, OH. Sulphaethidole was a gift of Smith Kline and French Laboratories, Philadelphia, PA. All other materials were reagent grade. Deionized water was used.

Fluorescence was measured at 22 °C using a Perkin Elmer MPF-44A spectrofluorimeter (Perkin-Elmer, Norwalk, CT). The excitation wavelength was 305 mm with a slit width of 6 nm and emission was scanned using a slit width of 7 nm. The emission peak was near 337 nm for lysozyme and lysozyme-drug solutions. The solutions were prepared in 0·1 M phosphate, the pH being adjusted with a Beckman Digital pH meter model 4500 (Beckman, Fullerton, CA).

Relative fluorescence was plotted as a function of drug concentration at fixed lysozyme concentration. The terminal slope was extrapolated to zero drug concentration, and the intercept reading subtracted from a given value on the terminal slope line, to correct for absorbance of the drug (Attallah & Lata 1968; Velick et al 1960). This value was then subtracted from the value on the experimental curve to give the corrected curve (Fig. 1). No drug caused a significant shift in the emission spectrum of the lysozyme. The binding constants (Table 1) determined at pH 7·4 are calculated assuming a 1 : 1 interaction, from the equation

$$L + D \rightleftharpoons LD$$

$$\text{and } K = \frac{[LD]}{[L][K]}$$

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Table 1. Binding constants for drug-lysozyme interactions at 22 °C assuming a 1:1 interaction. The constants are expressed ± 95% asymptotic confidence intervals. — indicates no observed quenching.

Drug	K M^{-1}	ΔG cal mol^{-1}	ΔG J mol^{-1}
Sulphathiazole	21 000 ± 200	− 5850	− 24 500
Sulphaethidole	13 000 ± 200	− 5600	− 23 400
Sulphisoxazole	7 000 ± 100	− 5200	− 21 760
Methyl *p*-hydroxybenzoate	15 000 ± 500	− 5650	− 23 650
Ethyl *p*-hydroxybenzoate	15 000 ± 500	− 5650	− 23 650
Propyl *p*-hydroxybenzoate	16 000 ± 1000	− 5700	− 23 860
Chloramphenicol pH 5·0	32 000 ± 1000	− 6100	− 25 500
Chloramphenicol pH 6·2	30 000 ± 500	− 6065	− 25 400
Chloramphenicol pH 7·4	30 000 ± 500	− 6065	− 25 400
Chloramphenicol pH 8·0	38 000 ± 1000	− 6200	− 25 900
Cortisone	—	—	—
Hydrocortisone	—	—	—
Phenylmercuric acetate	—	—	—
Phenylmercuric nitrate	—	—	—
Phenobarbitone	—	—	—
Atropine sulphate	—	—	—
Chlorobutol	—	—	—

The concentration of drug bound [LD] at any given concentration was estimated from the percentage of quenching, allowing the free protein concentration [L] and the free drug concentration [D] to be estimated from the respective total concentrations. The molecular weight of lysozyme was taken at 14 400 (Raftery & Dahlquist 1969). The binding constants (K) were then determined by an iterative least squares procedure. The relative fluorescence against drug concentration plot (Fig. 1) and the computer analysis of the binding curves at high drug protein ratios were strongly indicative of a 1:1 interaction.

The only previous report in the literature of drug-lysozyme interaction estimated from dialysis data a binding constant of 600 for the sulphisoxazole-lysozyme interaction (Chrai & Robinson, 1976).

At the start of the investigation it was anticipated that anionic drugs would interact with the basic protein; phenobarbitone did not quench the fluorescence of lysozyme but the three sulpha drugs did. The weakest acid of the three, sulphathiozole with a pK_a of 7·12 (Suzuki et al 1970), had the highest affinity constant (21 000 M^{-1}) (Table 1) whereas the strongest acid, sulphisoxazole with a pK_a of 5·0, has an affinity constant of only 7000 M^{-1}. Chloramphenicol, neutral over the pH range investigated (Table 1) had a very high affinity constant of 30 000 M^{-1} which is essentially independent of pH over the range 5–8. Other neutral molecules like cortisone and hydrocortisone did not quench the fluorescence but the *p*-hydrobenzoate esters had affinities for the lysozyme which was independent of the size of the ester grouping. In all cases a residual fluorescence of about 20% remained at the saturation of the lysozyme by ligands, suggesting that the fluorescence from tryptophans 62 and 108 are completely quenched (Imoto et al 1971).

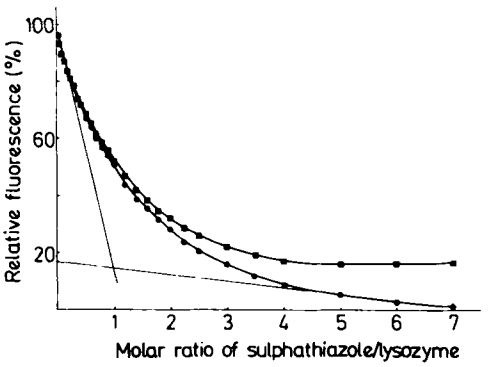

FIG. 1. Plot of relative fluorescent emission at 337 nm as a function of the sulphathiazole to lysozyme ratio. The lysozyme concentration is 1×10^{-4} M. ● is the experimental curve and ■ is the corrected curve (Attallah & Lata 1968). The intersection of the terminal slopes is at a molar ratio of 1·0.

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