J. Pharm. Pharmac., 1973, **25**, 125–130 *Received July* 17, 1972

Competitive binding of two drugs for a single binding site on albumin: a circular dichroic study

J. H. PERRIN AND D. A. NELSON

School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706, *U.S.A.*

Sulphaethidole has been previously shown to have two classes of binding site on bovine serum albumin (BSA). Only the primary binding site is capable of inducing optical activity in the drug. Drugs that bind to the same primary binding site as sulphaethidole, but do not become optically active themselves on binding, reduce the size of the circular dichroism signal in competitive binding studies with sulphaethidole. The binding constant for such antagonists can be calculated. It appears that a range of acidic drugs share the same primary binding site on bovine serum albumin; basic drugs do not compete for this site.

Many drugs are bound to some extent to serum albumin, and only the fraction unbound at any instant possesses biological activity (Brodie, 1965). The drugs appear to undergo a hydrophobic and an ionic or hydrogen bonding interaction with the protein (Perrin & Nelson, 1972). The equilibrium is interpretated in terms of numbers of binding sites per protein molecule (n) and the corresponding binding constants (k). Acidic drugs, including salicylates, sulphonamides, coumarin, anti-coagulants, phenylbutazone analogues, and the flufenamates, appear to be bound very strongly to one or two sites on the albumin molecule (Meyer & Guttman, 1968).

The concurrent administration of two or more strongly bound drugs may produce competition at the binding sites, so producing higher free concentrations and greater biological activity of both drugs than if the drugs were given alone. Untoward effects may be noticed if the doses are not adjusted correctly and considerable clinical attention has been given to this problem in recent years. The competition between acidic drugs appears to be particularly significant; for example, the administration of phenylbutazone or chlorophenoxyisobutyric acid (from clofibrate) concurrently with warfarin displaces warfarin from albumin (Solomon, 1970), and clinically serious haemorrhaging has been reported. Any technique that can give insight into this competition for binding sites and the nature of the binding is of value.

Classical methods of studying binding such as dialysis, ultrafiltration, gel filtration and partitioning can give information about competition after much mathematical manipulation. Techniques that give a more direct measurement of competition include spectrophotometry, where a probe molecule undergoes a metachromatic shift on binding (Klotz, Triwush & Walker, 1948; Moriguchi, 1968), or fluorescence, where the emission from the probe molecule shifts in a hydrophobic environment (Chen, 1967; Chignell, 1969). These investigations are frequently complicated by the probe molecule binding to more than one site; however, dansyl glycine, which binds to two sites on BSA (Chen, 1967), has been reported to bind to only one site on human serum albumin (HSA) (Chignell, 1969). In recent years several strongly bound acidic drugs such as flufenamic acid and phenylbutazone (Chignell, 1969), dicumarol

(Chignell, 1970; Perrin & Idsvoog, 1971), and sulphaethidole (Kostenbauder, Jawad & others, 1971), although inherently optically inactive, have been found to become optically active on binding to albumin. These phenomena appear to be predominantly due to strong hydrophobic interactions, and further investigations may lead to a clearer understanding of the mechanism of drug binding to proteins. With sulphaethidole binding to BSA, the induced circular dichroism was so large that quantitative interpretation of the data by the method of Scatchard, Coleman & Shen (1957) could be made. Dialysis investigation of the binding showed a single site of high affinity ($K = 1{\cdot}2 \times 10^5$ litre mol^{-1}) and three secondary sites of lower affinity ($K = 1{\cdot}0 \times 10^3$ litre mol^{-1}); however, optical activity in sulphaethidole was only induced by the primary site, and the CD gave $n = 1$ and $K = 2{\cdot}1 \times 10^5$ litre mol^{-1} for crystalline BSA. This technique thus offers an opportunity to study competitive binding at a single site on BSA. In these investigations, the competitive binding on BSA of sulphaethidole with drugs that do not have measurable induced Cotton effects at the concentrations employed were measured.

For a drug binding to a single site, the binding constant for the equilibrium $D + P = DP$ is:

$$K = \frac{[DP]}{[D]\,[P]}$$

where [DP] is the concentration of bound drug, [P] is the concentration of unbound protein, and [D] the concentration of unbound drug; for two drugs A and B in competition, the two binding constants K_A and K_B are

$$K_A = \frac{[DP_A]}{[D_A]\,[P]} \text{ and } K_B = \frac{[DP_B]}{[D_B]\,[P]} \quad .. \quad .. \quad .. \quad (1)$$

Eliminating [P], common to both equations, gives

$$\frac{K_A}{K_B} = \frac{[DP_A]\,[D_B]}{[DP_B]\,[D_A]} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

MATERIALS AND METHODS

Materials. Sulphaethidole, *N'*-(5-ethyl-1,3,4-thiazol-2-yl) sulphanilamide (Smith Kline and French) was recrystallized twice from water to give a melting point of 185–186°. The crystalline bovine serum albumin was obtained from Sigma Chemical Co. Trichloroacetic acid (Baker Analysed reagent grade from J. T. Baker Chemical Co.) was neutralized with sodium hydroxide and the sodium salt precipitated by ethanol and recrystallized from aqueous ethanol. Sodium thiopentone (Abbott); sodium warfarin (Endo Labs); tolbutamide (Upjohn); chlorpropamide (Pfizer); isopropamide iodide (SKF), pyridostigmin bromide and edrophonium chloride (Hoffman LaRoche) were used as supplied. Sodium sulphadiazine, sodium sulphathiazole, sodium sulphacetamide, sodium pentobarbitone, aspirin, sodium salicylate, sodium chloride, monobasic sodium phosphate, and dibasic sodium phosphate were all U.S.P., N.F., or analytical grade materials.

Method. All CD spectra were obtained using a 6002 attachment to a Cary 60 spectropolarimeter, using a slit programmed for a half-band width of 15Å. All solutions were prepared in de-ionized water containing a 0·054M sodium phosphate buffer, pH 7·4, made isotonic with sodium chloride, at 22°.

The concentration of BSA was $1{\cdot}45 \times 10^{-5}$M and of sulphaethidole $2{\cdot}523 \times 10^{-5}$M throughout the investigations. All solutions were scanned in 5 or 10 mm cells

from 350 to 245 nm where the ellipticity of the albumin became dominant. The concentrations of antagonist used were as high as possible; however, the combined absorbance of albumin, sulphaethidole and antagonist never exceeded 1·8 at the wavelengths of measurement.

RESULTS AND DISCUSSION

Fig. 1 shows a typical set of CD curves obtained when an antagonist displaces sulphaethidole from its primary binding site. The curves have a negative peak near 280 nm and a positive peak near 255 nm, in good agreement with the values reported previously (Kostenbauder & others, 1971). The small intrinsic optical activity of the BSA has been subtracted to give the induced ellipticities indicated. Increasing concentrations of salicylate decrease the size of both peaks in an apparently similar manner. Throughout these investigations a sulphaethidole concentration of $2{\cdot}52 \times 10^{-5}$M was used, and substitution into equation (1), using the binding constant of $2{\cdot}1 \times 10^{-5}$ litre mol^{-1} previously reported, gives a concentration of $1{\cdot}08 \times 10^{-5}$M sulphaethidole bound to the constant concentration of $1{\cdot}45 \times 10^{-5}$M

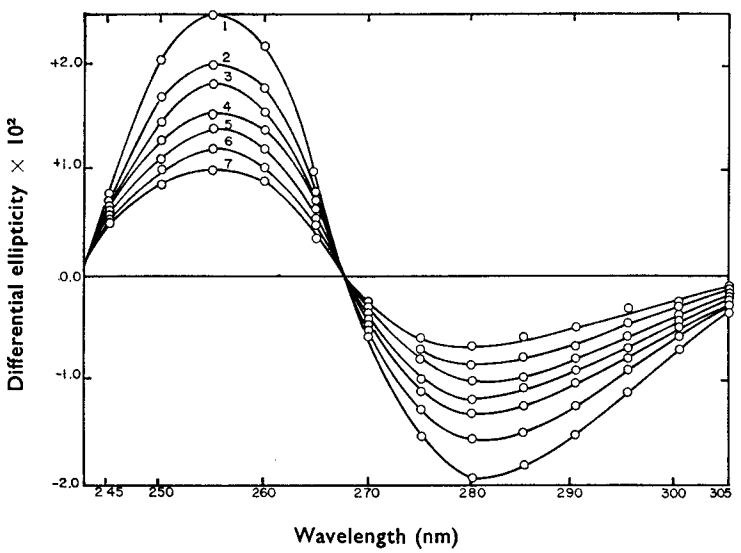

FIG. 1. CD curves for the binding of sulphaethidole to bovine serum albumin, antagonized by sodium salicylate. All measurements were made in 1 cm cells. BSA = $1{\cdot}45 \times 10^{-5}$M. Sulphaethidole = $2{\cdot}52 \times 10^{-5}$ M. Sodium salicylate concentrations (M): 1, 0·0; 2, $0{\cdot}53 \times 10^{-4}$; 3, $1{\cdot}05 \times 10^{-4}$; 4, $1{\cdot}58 \times 10^{-4}$; 5, $2{\cdot}1 \times 10^{-4}$; 6, $3{\cdot}15 \times 10^{-4}$; 7, $4{\cdot}2 \times 10^{-4}$.

BSA. This means that approximately 75% of the available primary binding sites on the albumin are filled with sulphaethidole when no antagonist is present. At these concentrations, approximately 1·5% of the total drug concentration is bound to the secondary binding sites and in the subsequent calculations binding at these secondary sites is ignored. In these investigations no antagonist was used that had a primary binding constant greater than $1{\cdot}0 \times 10^5$ litre mol^{-1} because such drugs may have an induced CD signal of their own and also are probably of limited solubility. The following procedure was used to calculate the binding constant of the antagonist from equation (2). The signal obtained with sulphaethidole alone was assumed to be proportional to the amount bound; any decrease in signal height

was assumed to be due to the displacement of sulphaethidole by an equivalent amount of antagonist. The concentration of bound sulphaethidole, $[DP_A]$, is given by

$$\text{concentration bound (no antagonist)} \times \frac{\text{peak height (antagonist present)}}{\text{peak height (antagonist absent)}}$$

Data from both the positive and negative peaks were averaged to give this figure, except for warfarin. The concentration of free sulphaethidole $[D_A]$ then equals the total concentration of sulphaethidole minus $[DP_A]$.

Substitution into equation (1) for A gives the free protein concentration, [P]. This enables the concentration of bound competitor, $[DP_B]$, to be calculated from $[P_T] = [DP_A] + [DP_B] + [P]$ where $[P_T]$ is the total protein concentration. The concentration of unbound competitor, $[D_B]$, is then given by total competitor concentration $- [DP_B]$. This enables an apparent binding constant, K_{app}, to be calculated for the competing drug B using equation (2). This is not a true binding constant for the competing drug at the primary binding site of the sulphaethidole as it ignores binding of the second drug at other binding sites. If, however, very low concentrations of B are used and B shares the same binding site as sulphaethidole, K_{app} approaches K_B for this binding site as the concentration of B approaches zero. Fig. 2 shows typical plots of K_{app} against concentration of B, and Table 1 shows values of K obtained from the intercept. Available literature values for the binding of the competing drugs are also shown in Table 1. Some of the values are for HSA; there is frequently a strong albumin species dependence for the binding constants and number of binding sites (O'Reilly, 1967). Comparison of the values of K obtained by the CD method with the literature values shows that, with the exception of the barbiturates, the binding constants from the two sources are of the same order of magnitude, suggesting that this is also the primary binding site for the other acidic drugs investigated. The three basic drugs investigated, isopropamide, edrophonium and pyridostigmine, even at concentrations thirty times greater, did not

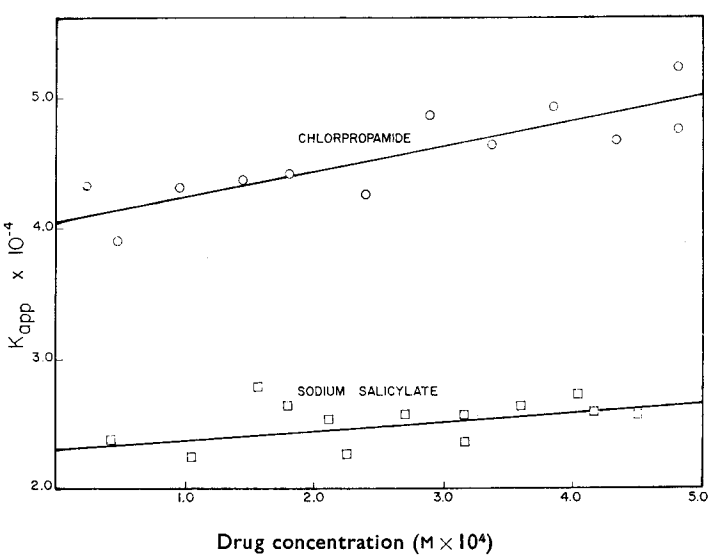

FIG. 2. Plot of K_{app} against drug concentration to give K for chlorpropamide and sodium salicylate.

Table 1. *Antagonist binding constants for the primary binding site of sulphaethidole on bovine serum albumin.*

Drug	$K_{obs} \pm 10\%$ litre mol^{-1}	Klit litre mol^{-1}	nlit	Experimental condition (lit)	Reference
Sulphathiazole	$4{\cdot}87 \times 10^3$ (1)*	$1{\cdot}26$–$6{\cdot}31 \times 10^3$	2	BSA, pH 7·4, 37 °C	Moriguchi & others, 1968
Sulphadiazine	No significant competition	$7{\cdot}94$–$79{\cdot}4 \times 10^2$	2	BSA, pH 7·4, 37 °C	Moriguchi & others, 1968
Sulphacetamide	No significant competition	$4{\cdot}2 \times 10^3$	1	BSA, pH 7·6, 5 °C	Klotz & Walker, 1948
Pentobarbitone	$1{\cdot}07 \times 10^3$ (1)	$2{\cdot}12 \times 10^5$	0·4	BSA, pH 7·4, 26 °C	Goldbaum & Smith, 1954
		$1{\cdot}60 \times 10^2$	21·6		
Thiopentone	$6{\cdot}0 \times 10^3$ (2)	$6{\cdot}0 \times 10^4$	1·25	BSA, pH 7·4, 8 °C	Goldbaum & Smith, 1954
		$6{\cdot}25 \times 10^2$	20·8		
Aspirin	$4{\cdot}52 \times 10^3$ (12)	$3{\cdot}5 \times 10^4$	0·04	BSA, pH 5·4, 4 °C	Davison & Smith, 1961
		$2{\cdot}0 \times 10^2$	4·2		
Sodiumsalicylate	$2{\cdot}30 \times 10^4$ (13)	$2{\cdot}5 \times 10^4$	0·72	BSA, pH 7·4, 4 °C	Davison & Smith, 1961
		$1{\cdot}5 \times 10^2$	5·2		
Chlorpropamide	$4{\cdot}04 \times 10^4$ (12)	$1{\cdot}08 \times 10^4$	1·65	HSA, pH 7·4, 40 °C	Judis, 1972
Tolbutamide	$6{\cdot}58 \times 10^4$ (10)	$4{\cdot}06 \times 10^4$	1·35	HSA, pH 7·4, 40 °C	Judis, 1972
Warfarin	$2{\cdot}20 \times 10^4$ (11)	$9{\cdot}2 \times 10^4$	0·99	HSA, pH 7·4, 27 °C	O'Reilly, 1967
	$4{\cdot}04 \times 10^4$ (11)				
Sodium trichloroacetate	$1{\cdot}31 \times 10^4$ (10)	$4{\cdot}62 \times 10^4$	1	BS mercaptalbumin pH 5·4, 25 °C	Scatchard & others, 1957
		$1{\cdot}93 \times 10^3$	8		
		$6{\cdot}4 \times 10^1$	18		
Isopropamide iodide	No competition				
Edrophonium chloride	No composition				
Pyridostigmin bromide	No competition				

* Number in parentheses is number of determinations used for competitions.

compete with sulphaethidole and probably do not share the same binding site as the acidic drugs. The literature values for pentobarbitone must be considered suspect because of the lack of data obtained at the critical low drug concentrations. The resultant excessive extrapolation of the Scatchard plot gives n = 0·4 (Goldbaum & Smith, 1954). Of the other sulpha drugs investigated, only sulphathiazole showed significant competition for the binding site; in this case only one value of K could be obtained because of the high absorbance of the drug. The work of Moriguchi, Wada & Nishizawa (1968) suggests that sulphadiazine, sulphathiazole and N^1-acyl sulphonamides are bound to the same binding sites on BSA. They give no data for sulphacetamide, but comparison with structurally related compounds suggests that the literature value of K (Klotz & Walker, 1948) is too high. The fact that little or no antagonism was found, even using the competitor-to-drug ratio of greater than 25 to 1 with sulphadiazine and sulphacetamide may be due to their low binding constants rather than to the possibility that they bound at other sites. The value of K obtained for salicylate seems to be in good agreement with the literature value but this is not so with aspirin; however, the literature quotes a value of n = 0·04 and aspirin binding may be complicated by acylation of the albumin by the aspirin molecule (Hawkins, Pinckard & Farr, 1968). With warfarin, the height of the two CD peaks was repeatedly not reduced to the same extent on the addition of antagonist. The higher value of K was obtained from data at 280 nm and the lower value from data at 255 nm. Although the value of $4{\cdot}04 \times 10^4$ litre mol^{-1} is nearer the literature value, the absorption of the solutions was much greater at this lower wavelength, and hence this result is considered less reliable.

The previous investigations showed that CD detected only a single binding site for sulphaethidole on BSA. The current work suggests that a range of acidic drugs share this primary binding site with sulphaethidole. Assuming this to be so then the binding constant of those drugs that do not have optical activity induced into them by BSA can be calculated. The method, like those involving fluorescent and

spectroscopic probes, offers the advantage of speed and freedom from (a) adsorption on semi-permeable membranes, (b) hydrolysis of the drugs, and (c) bacterial growth, over the more frequently used techniques of dialysis and ultrafiltration.

REFERENCES

BRODIE, B. B. (1965). *Proc. Roy. Soc. Med.*, **58**, 946–955.

CHEN, R. F. (1967). *Arch. Biochem. Biophys.*, **120**, 609–620.

CHIGNELL, C. F. (1969). *Mol. Pharmac.*, **5**, 244, 455–462.

CHIGNELL, C. F. (1970). *Ibid.*, **6**, 1–12.

DAVISON, C. & SMITH, P. K. (1961). *J. Pharmac. exp. Ther.*, **133**, 161–170.

GOLDBAUM, L. R. & SMITH, P. K. (1954). *Ibid.*, **111**, 197–209.

HAWKINS, D., PINCKARD, R. N. & FARR, R. S. (1968). *Science*, **160**, 780–781.

JUDIS, J. (1972). *J. pharm. Sci.*, **61**, 89–93.

KLOTZ, I. M., TRIWUSH, H. & WALKER, F. M. (1948). *J. Am. chem. Soc.*, **70**, 2935–2941.

KLOTZ, I. M. & WALKER, F. M. (1948). *Ibid.*, **70**, 943–946.

KOSTENBAUDER, H. B., JAWAD, M. J., PERRIN, J. H. & AVERHART, V. (1971). *J. pharm. Sci.*, **60**, 1658–1660.

MEYER, M. C. & GUTTMAN, D. E. (1968). *Ibid.*, **57**, 895–918.

MORIGUCHI, I. (1968). *Chem. Pharm. Bull. (Tokyo)*, **16**, 597–600.

MORIGUCHI, I., WADA, S. & NISHIZAWA, T. (1968). *Ibid.*, **16**, 601–605.

O'REILLY, R. A. (1967). *J. clin. invest.*, **46**, 829–837.

PERRIN, J. H. & IDSVOOG, P. (1971). *J. pharm. Sci.*, **60**, 602–604.

PERRIN, J. H. & NELSON, D. A. (1972). *Life Sci.*, **11**, 227–283.

SCATCHARD, G., COLEMAN, J. S. & SHEN, A. L. (1957). *J. Am. chem. Soc.*, **79**, 12–20.

SOLOMON, H. M. (1970). *Proceedings of the Fourth International Congress on Pharmacology*, **4**, 169–172.