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The binding of sulphonylureas to serum albumin

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The interaction of tolbutamide, glibenclamide, chlorpropamide and tolazamide with serum albumin has been examined. Glibenclamide, the most strongly bound of the four compounds, is bound to only one class of sites. The other three compounds are bound to at least two. The interaction between glibenclamide and albumin was independent of pH and increased markedly with decreasing temperature suggesting that a non-ionic mechanism is involved. In contrast, the overall interaction of tolbutamide with albumin showed little temperature dependence and, in addition, binding of both tolbutamide and chlorpropamide decreased with pH. These findings imply that the predominantly bound species is the anion. Binding parameters corrected for electrostatic effects were found to fit binding data for tolbutamide, chlorpropamide and tolazamide better than uncorrected parameters. Electrostatic correction of binding of glibenclamide is unnecessary.

Previous workers have established that the sulphonylureas, used in maturity-onset diabetes, are strongly bound to plasma proteins at therapeutic plasma concentrations (Wishinsky, Glasser & Perkal, 1962; Jackson, 1969). It is generally accepted that a high degree of binding may modify drug distribution (Scholtan, 1961, 1963; Rieder, 1963; Martin, 1965) and elimination (Krüger-Thiemer, Diller & Bunger, 1965; Inglott, 1972). There is also the possibility that other compounds present in the plasma, with similar binding tendencies, may competitively displace drugs from their binding sites. This may produce adverse side-effects, namely hypoglycaemia in the particular case of the sulphonylureas.

Displacement of sulphonylureas by other drugs has been demonstrated in human serum (Christensen, Hansen & Kristensen, 1963; Wishinsky & others, 1962) and in solutions of purified albumin (Büttner & Portwich, 1967; Judis, 1972). A detailed understanding of the strength and nature of the association of the sulphonylureas with serum albumin may assist in the interpretation of their pharmacokinetic behaviour and the prediction of possible adverse interactions. The present work reports an investigation of the interaction of tolbutamide, chlorpropamide, glibenclamide and tolazamide with serum albumin.

MATERIALS AND METHODS

Bovine serum albumin (BSA lot number 300–2060) and human serum albumin (HSA lot number 81c–13028) were both crystalline fraction V albumin obtained commercially from Sigma Co. HSA was found to contain some dialysable component which absorbed light in the ultraviolet region. Concentrated solutions were dialysed before use to remove this material and were then diluted to the required concentration. BSA contained no detectable dialysable material and was therefore used as received.

Tolbutamide and [^{14}C]glibenclamide were generously donated by Farbwerke

Hoechst. Chlorpropramide and tolazamide were kindly donated by Pfizer Laboratories and Upjohn Pty. Ltd., respectively. Tolbutamide was recrystallized from 95% ethanol, m.p. 127–128°, and the pKa was determined to be 5·3 ± 0·04 by potentiometric titration. [^{14}C]Glibenclamide was labelled similarly to that used by Christ, Heptner & Rupp (1969). The material which was radiochemically pure was diluted with unlabelled glibenclamide and recrystallized from absolute ethanol for use. The final specific activity was 0·84 mCi g^{-1}, m.p. 173°. The pKa of glibenclamide was estimated as 6·5 ± 0·03 by the solubility method of Albert & Serjeant (1962) and was identical to that obtained by Hajdu, Spingler & others (1969). Chlorpropamide was recrystallized from 95% ethanol, m.p. 127–129°. The pKa of 4·95 ± 0·04 was determined by potentiometric titration. Tolazamide was used as received, m.p. 171°.

METHODS

Binding of tolbutamide, chlorpropamide and tolazamide in all systems was determined in phosphate buffer of constant ionic strength (0·150) using the dynamic dialysis procedure described by Meyer & Guttman (1968). Concentrations of bound (D_b) and free (D_f) drug were calculated as described previously (Crooks & Brown, 1973). Dynamic dialysis is unsuitable for use with glibenclamide because the drug is strongly bound to membrane and to albumin and has a low rate constant for diffusion across the membrane. Thus to study binding at the low therapeutic levels, extended dialysis times and a very large membrane would be needed. Thus equilibrium dialysis was used. Good agreement between the two methods has been established previously (Meyer & Guttman, 1970; Crooks & Brown, 1973). Studies were carried out in sterile glass dialysis cells of 5 ml capacity.

Analytical procedures. Tolbutamide, tolazamide and chloropropamide were estimated spectrophotometrically at 228, 228 and 231 nm respectively. For solutions containing protein a modification of the method of Alessandro, Emer & Abbondanza (1966) was used. [^{14}C]Glibenclamide was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Binding of the four sulphonylureas to HSA was determined at 37°. Scatchard plots are shown for tolbutamide, tolazamide and chlorpropamide in Fig. 1A and glibenclamide in Fig. 1B. Binding of both tolbutamide and of glibenclamide at several HSA concentrations is not protein concentration dependent. Binding parameters giving best fit to the data were estimated using the method of Hart (1965) as modified previously by Crooks & Brown (1973). A model of two classes of sites fitted data for the three drugs in Fig. 1A and the binding parameters are summarized in Table 1. The curves which were generated using these values (Fig. 1) demonstrate the agreement between the experimental data and the theoretical model.

In contrast, glibenclamide interacts with only one class of sites (Fig. 1B). Binding parameters were calculated by linear regression and are given in Table 2 together with values of the correlation coefficient.

Glibenclamide is clearly the most strongly bound. Using the binding parameters, the percent of drug free against total concentration in serum was calculated (Fig. 2). The therapeutic serum level of glibenclamide is less than 1 μg ml^{-1} (Christ & others, 1969). From Fig. 2, only 0·17% of the drug is free in this range which explains its relatively small volume of distribution (Fuccella, Tamassia & Valzelli, 1973).

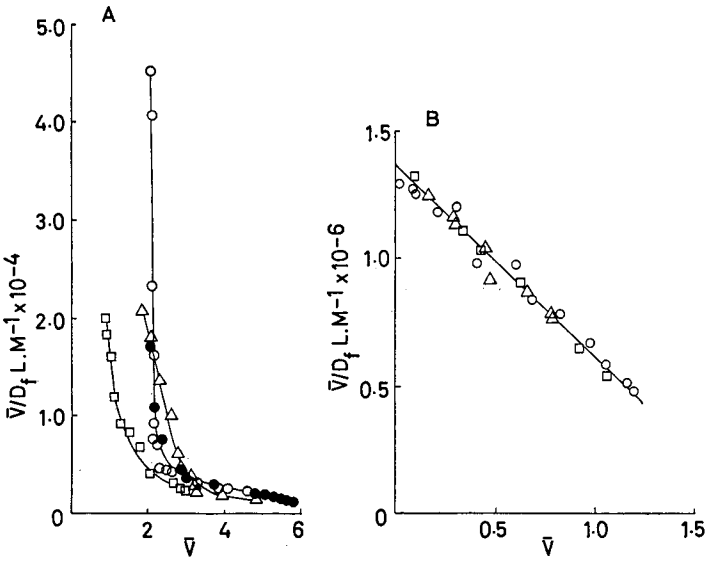

FIG. 1A. Scatchard plots for interaction of sulphonylureas with HSA at 37° and pH 7·4 in M/15 phosphate buffer. 1% HSA: □ tolazamide; △ chlorpropamide; ○ tolbutamide. 2% HSA: ● Tolbutamide.

B. Scatchard plots for interaction of glibenclamide with HSA at 37° in M/15 phosphate buffer: ○ pH 7·4 in 0·5% HSA; □ pH 7·4 in 1% HSA; △ pH 6·4 in 0·5% HSA.

Tolbutamide having a K_1 of $2{\cdot}2 \times 10^5$ molar^{-1} is 99% bound over the therapeutic range of 100–150 μg ml^{-1} (Fig. 2). Chlorpropamide and tolazamide are respectively 96 and 94% bound over the same concentration range. For the latter two compounds it might be expected that plasma binding would significantly influence distribution only at lower drug levels. Fig. 2 demonstrates the saturable nature of the binding sites with increasing drug concentration. This is particularly significant for drugs having a low n_1 value such as tolazamide. Doubling the serum level to 200 μg ml^{-1} increases percent free tolazamide from 6 to 14·6%. Binding of glibenclamide is much less sensitive to changes in the concentration level. A fifty-fold increase in concentration (1 to 50 μg ml^{-1}) only increases the free concentration from 0·17 to 0·3% free.

The binding of tolbutamide to HSA decreases with decreasing pH which suggests that the anionic species is most strongly bound. Elofsson, Nilsson & Ågren (1970)

Table 1. *Binding parameters for the interaction of sulphonylureas to 1% HSA at pH 7·4 and 37° in M/15 phosphate buffer.*

Sulphonylurea	n_1	K_1 molar^{-1} × 10^{-4}	n_2	K_2 molar^{-1} × 10^{-2}	Sums of squares of residuals*
Tolbutamide	2·27	21·86	8·21	1·71	1·88
Chlorpropamide	2·20	4·51	8·22	1·71	0·76
Tolazamide	0·97	8·67	3·15	14·90	0·20

*Residuals represent the differences between the experimental values of $\bar{V}$ and those calculated from the binding parameters.

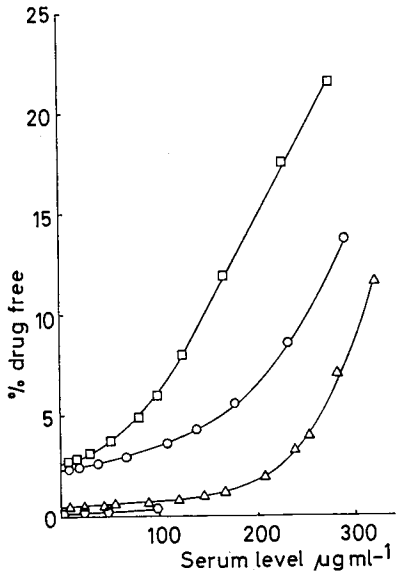

FIG. 2. Plot of % sulphonylurea free versus serum level generated from binding parameters assuming a serum albumin concentration of 3%. □ Tolazamide; ○ chlorpropamide; △ tolbutamide; ⬡ glibenclamide.

treated binding data for sulphonamides using an equation of the form:

$$\bar{V} = \frac{n_1 K_1 D_f \text{ ion}}{1 + K_1 D_f \text{ ion}} + \frac{n_2 K_2 D_f \text{ ion}}{1 + K_2 D_f \text{ ion}} \quad .. \quad .. \quad .. \quad (1)$$

where D_f ion is the free concentration of ionized species. A Scatchard plot of $\bar{V}/D_f$ ion against $\bar{V}$ showed good agreement of binding data obtained over a pH range 5·5 — 9·2. Data for binding of tolbutamide at pH 5·9 and 6·36 plotted in this way (Fig. 3A) agree well with those at pH 7·4 where tolbutamide is 99% ionized. Although not strictly comparable, the binding of chlorpropamide to BSA shows a similar type of pH dependence as that for tolbutamide to HSA (Fig. 3B). Scatchard plots of the anionic species at pH 5·38 and 6·4 are in good agreement with those at pH 7·4 where the drug is 99·9% ionized. Thus it appears that the anion of chlorpropamide also binds most strongly to BSA.

In contrast, binding of glibenclamide shows little dependence on pH (Fig. 1B). At pH 6·4 the binding varied little from that at pH 7·4 although percent ionized varied from 44 to 89%. The pH independent binding implies that unionized and

Table 2. *Binding parameters for interaction of glibenclamide with HSA in M/15 phosphate buffer at 37°.*

pH	HSA%	n	K molar^{-1} × 10^{-5}	Correlation Coefficient*
7·4	0·5	1·82	7·64	0·990
7·4	1·0	1·75	7·88	0·997
6·4	0·5	1·78	7·68	0·983

*Scatchard plot fitted by linear regression.

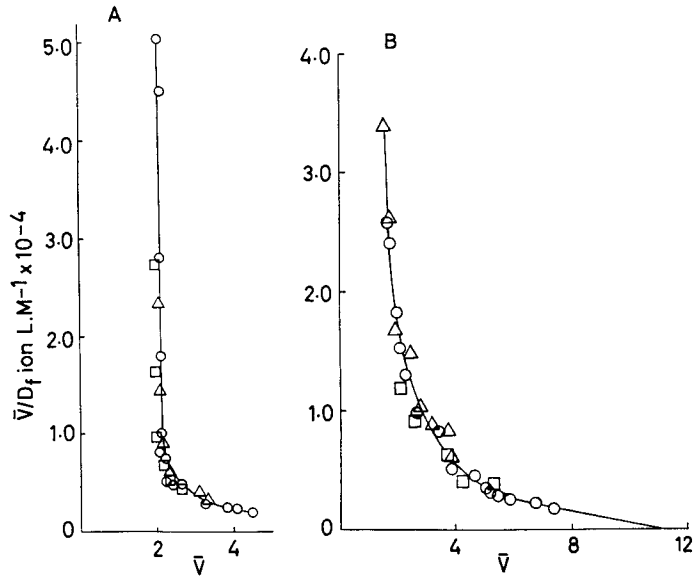

FIG. 3A. Plot of $\bar{V}/D_{f\ ion}$ against v for binding of tolbutamide to HSA at 37° in M/15 phosphate buffer of pH: □ 5·90; △ 6·36; ○ 7·37.

B. Plot of $\bar{V}/D_{f\ ion}$ against v for binding of chlorpropamide to BSA at 37° in M/15 phosphate buffer of pH: △ 5·38; □ 6·36; ○ 7·37.

anionic glibenclamide both have similar affinities for the protein. This suggests a different mechanism of binding to that of tolbutamide and chlorpropamide.

To further investigate the interaction, the binding of tolbutamide and glibenclamide was examined at several temperatures. Tolbutamide represented the three sulphonylureas which undergo binding to two classes of sites on the albumin molecule. Varying temperature has little effect on the primary association constant, K_1, for tolbutamide (Table 3) suggesting that association to the first class of sites is ionic. Such temperature independent binding of ions has been demonstrated previously for sulphonamides (Davis, 1943), dodecyl sulphate (Putnam & Neurath, 1945), caprylate (Boyer, Ballou & Luck, 1947) and chloride (Scatchard, Scheinberg & Armstrong, 1950). In contrast the secondary association constant, K_2, is greatly affected by temperature. Decreasing temperature from 37 to 12° causes a 60% increase in K_2 (Table 3). This is characteristic of an exothermic drug-protein interaction. The large temperature dependence suggests that the secondary association is not ionic.

Table 3. *Binding parameters for interaction of tolbutamide with 1% HSA at pH 7·4 at varying temperatures including thermodynamic data for interaction with the second class of sites.*

Temp. °C	n_1	K_1 molar^{-1} × 10^{-4}	n_2	K_2 molar^{-1} × 10^{-2}	$\Delta G°$ kcal mol^{-1}	$\Delta H°$ kcal mol^{-1}	$\Delta S°$ e.u.
12	2·37	20·97	8·50	2·71	−3·21*	−3·14**	0·28
26	2·18	22·67	8·10	2·07	−3·22	−3·14	0·32
37	2·27	21·86	8·21	1·71	−3·22	−3·14	0·32

*−13·5 kJ mol^{-1} **−13 kJ mol^{-1}

Values of ΔG°, ΔH° and ΔS° were calculated using standard methods (Steinhardt & Reynolds, 1969). In the first class of sites ΔG° is high varying between $-6{\cdot}94$ kcal mol^{-1} (-28 kJ mol^{-1}) at 12° to $-7{\cdot}57$ kcal mol^{-1} (-31 kJ mol^{-1}) at 37°. It is entirely due to a large positive entropy change of 24·4 entropy units. In the second class of sites the contribution to ΔG° by ΔH° is very large (97%) while the contribution of ΔS° is small (Table 3).

Cho, Mitchell & Pernarowski (1971) reported a standard enthalpy contribution of 88% to standard free energy for the interaction of bishydroxycoumarin with albumin. It was proposed that the protein molecule possessed hydrophobic regions which were highly selective for the drug. The standard enthalpic contribution to ΔG° approaches 100% for an antibody antigen interaction (Karush, 1956).

In the present instance the magnitude of the standard enthalpy change is much smaller than that for the bishydroxycoumarin interaction and approximates that observed on the formation of hydrogen bonds. While it is difficult to further establish the mechanism of binding in the secondary sites, that the process is enthalpically driven suggests that ionic forces are not involved as they are in the first class of sites. It seems probable that tolbutamide molecules, whether ionized or not, bind to the secondary sites by a non-ionic mechanism. It is difficult to test this proposal because at reduced pH values where the fraction of unionized molecules is higher the solubility is much reduced and the value of D_f attainable is limited. Thus at pH 5·9, 24% of the drug exists in the unionized form but the free concentration is not sufficient to cause significant binding to the second class of sites. On this basis it would appear unnecessary to include a term for the binding of unionized tolbutamide in equation 1.

Data for glibenclamide (Table 4) indicate that the association constant increases dramatically with decreasing temperature to the extent that the value of K at 14° is twice that at 37°. This large temperature dependence suggests that, unlike tolbutamide at the primary sites, the interaction is not ionic. The enthalpic contribution to the standard free energy change is appreciable (47%) with an almost equal contribution from ΔS°. Thus it is likely that a large portion of the binding energy of glibenclamide is derived from non-ionic sources. Values of ΔH° and ΔS° (Table 4) are similar in magnitude to those observed previously for several *p*-substituted acetanilides (Dearden & Tomlinson, 1970) which were thought to bind to albumin solely by van der Waals forces. Furthermore, it has been suggested that the additivity of relatively weak Keesom, Debye and London forces may result in large binding energies, greater than for ionic bonds (Settle, Hegeman & Featherstone, 1971). This may explain the high association constant in relation to the other sulphonylureas.

Table. 4. *Thermodynamic data for the interaction of glibenclamide with HSA in M/15 phosphate buffer.*

Temp. °C	n	K molar^{-1} × 10^{-5}	ΔG° kcal mol^{-1}	ΔH° kcal mol^{-1}	ΔS° e.u.
14	1·70	13·84	−8·06*	−3·70**	15·7
25	1·78	10·80	−8·24	−3·70	15·7
37	1·82	7·64	−8·38	−3·70	15·1
45	1·76	7·50	−8·55	−3·70	15·3

* 33·5 − 34·5 kJ mol^{-1}. ** 14·5 kJ mol^{-1}.

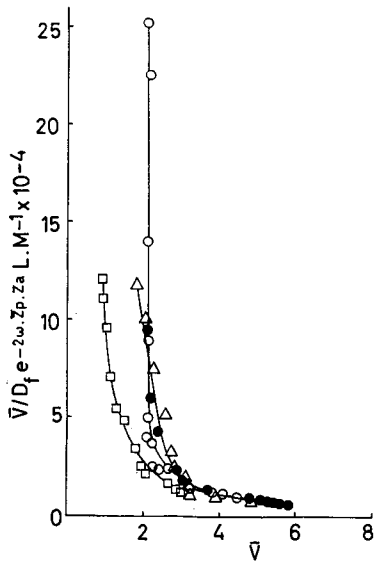

FIG. 4. Plot of $V/D_f e^{-2-2\omega Zp Za}$ against ν for the sulphonylureas in HSA at 37° and pH 7·4 1% HSA: □ Tolazamide; △ chlorpropamide; ○ tolbutamide; 2% HSA: ● tolbutamide.

In summary, glibenclamide appears to have a different binding mechanism to tolbutamide, and perhaps chlorpropamide, where binding apparently involves ionic forces analogous to the sulphonamides (Davis, 1943; Elofsson & others, 1970; Clausen, 1966). These findings may be significant with regard to competitive displacement from serum proteins.

As it appears that tolbutamide and chlorpropamide are bound as anions, it is probably more relevant to express the binding data for these drugs according to an equation which takes into account electrostatic effects. Binding of anions may be described using the Debye-Huckel-Born theory of electrostatic interaction (Scatchard & others, 1950; Steinhardt & Reynolds, 1969; Edsall & Wyman, 1958) by an equation of the form:

$$\bar{V} = \sum_{i=o}^{i=i} \frac{n_i k_i^{\circ} D_f \cdot e^{-2\omega \bar{Z}p.Za}}{1 + K_i^{\circ} D_f \cdot e^{-2\omega \bar{Z}p.Za}} \qquad (2)$$

where K_i° is the intrinsic association constant for sites in the 'i'th class, $\bar{Z}_p$ is the charge on the albumin molecule, Za is the charge on the drug anion and ω has the meaning described previously (Edsall & Wyman, 1958).

Data for tolbutamide, chlorpropamide and tolazamide to HSA were plotted as $\bar{V}/D_f e^{-2\omega Zp Za}$ against $\bar{V}$ (Fig. 4). $\bar{Z}_p$ was taken as −20 (Edsall & Wyman, 1958) in the absence of bound drug, Za was −1 and ω was calculated as 0·03. After electrostatic correction the Scatchard plots remained curved implying the existence of multiple classes of sites (Fig. 4). The plots were resolved into two classes of sites as described previously. Sums of squares of the residuals between experimental and theoretical values of $\bar{V}$ were calculated assuming D_f to be an independent variable (Table 5). The electrostatically corrected binding parameters gave a better fit to binding of the three drugs compared with the corresponding values determined

Table 5. *Binding parameters determined from data treated for electrostatic effects on the binding of sulphonylureas to 1% HSA at pH 7·4.*

Sulphonylurea	n_1	K_1^0 molar^{-1} × 10^{-4}	n_2	K_2^0 molar^{-1} × 10^{-2}	Sums of squares of residuals*
Tolbutamide	2·27	85·04	8·22	6·65	1·48
Chlorpropamide	2·20	18·49	8·22	6·52	0·45
Tolazamide	1·02	33·81	3·20	58·04	0·15

*The residuals represent the differences between the experimental values of $\bar{V}$ and those calculated from the binding parameters.

from the uncorrected binding parameters shown in Table 1. This might be expected as chlorpropamide and tolbutamide appear to be bound as ions. The linearity of the $\bar{V}/D_f$ against $\bar{V}$ plot for glibenclamide (Fig. 1B) suggests that an electrostatic correction is not necessary for this drug.

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