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Physico-chemical studies of analgesics. The protein-binding of some *p*-substituted acetanilides

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The binding of fifteen *p*-substituted acetanilides to bovine serum albumin is examined at pH 7·2. An excellent correlation is obtained between the binding enthalpy and Hammett's substituent constant, σ. This is interpreted to mean that the binding is non-specific in nature. A very good correlation is also obtained between the entropy of binding and σ, which suggests that the extent of hydration of unbound drug is a function of the charge separation within the drug molecule. Of the compounds examined, those that have been used clinically as analgesics possess the best thermodynamic properties, being neither so fully bound as to give low free drug concentrations in the blood-stream, nor so little bound that there is no sustained action.

Although the significance of protein-binding in relation to the activity of drugs is well-known, there has been no intensive investigation of the protein-binding of mild analgesics such as the acetanilides. The present study is concerned with the binding on bovine serum albumin (BSA) of some *p*-substituted acetanilides, including paracetamol and phenacetin.

EXPERIMENTAL AND RESULTS

Materials

The protein used was crystallized BSA obtained from Pentex Inc., with a moisture content of 3·2%. The acetanilides, obtained commercially or prepared by standard methods, were recrystallized to constant melting point.

Method

A dynamic dialysis method, similar to that described by Meyer & Guttman (1968) was used to determine the association constants of the binding of the acetanilides to BSA. The method has advantages over equilibrium dialysis in that it is much more rapid, and less subject to error, since a single run over a wide drug concentration range can yield the association constant.

A small bag was made from 3 cm flat width Visking dialysis tubing previously treated by heating (1 h: 90°) in deionized water and washing for one week. Into this was placed 10 ml of the solution of drug and BSA in Clark and Lubs 0·2 M phosphate buffer (pH 7·2). The bag was immersed in 200 ml of the same buffer, and the whole thermostatted; runs were made at 19°, 27° and 40°, provision being made for both solutions to be stirred. The concentrations of acetanilides used were 0·4 to 4·0 $\times 10^{-3}$ M, and those of BSA, 0·35 to 3·5 $\times 10^{-4}$ M.

Because of the large volume of the external solution, the concentration of drug in this exerts only a negligible effect on the dialysis, provided it is not allowed to rise

above about 1% of that in the dialysis bag. In this work, this was achieved by withdrawing, every 30 min, a 100 ml sample from the external solution and replenishing by the same volume of fresh buffer.

Each sample withdrawn was analysed spectrophotometrically at 19°, a temperature correction being applied for samples taken at 27° and 40°. The total amount of drug dialysed at any sampling time was thus determined, and by difference the total concentration (S_T) remaining in the dialysis bag obtained.

A dialysis run in the absence of protein showed that the diffusion was first-order with respect to the drug, and that adsorption by the dialysis bag was negligible (Meyer & Guttman, 1970). In the presence of BSA, a plot of log S_T against time was curvilinear, owing to the fraction of bound drug increasing as the total concentration in the internal solution decreased.

A computer program was written to obtain instantaneous rates of diffusion (slopes) at various times from this curvilinear plot. From a knowledge of the instantaneous rates, and of the rate constant obtained from the dialysis of drug in the absence of BSA, the concentration of unbound drug (S_F), and hence of bound drug, was calculated for various times. The number of moles of drug bound per mol of BSA ($\bar{\nu}$) was then calculated for these times, taking a value of 69 000 as the molecular weight of BSA. In a plot of $\bar{\nu}/S_F$ against $\bar{\nu}$ (Scatchard plot), the initial slope represents the association constant for the binding of drug to primary binding sites on the BSA molecule. Extrapolation to the abscissa gives the number of primary binding sites per BSA molecule. Fig. 1 illustrates Scatchard plots for three of the acetanilides at 19°. The second, lower slope represents binding to secondary sites, and was not

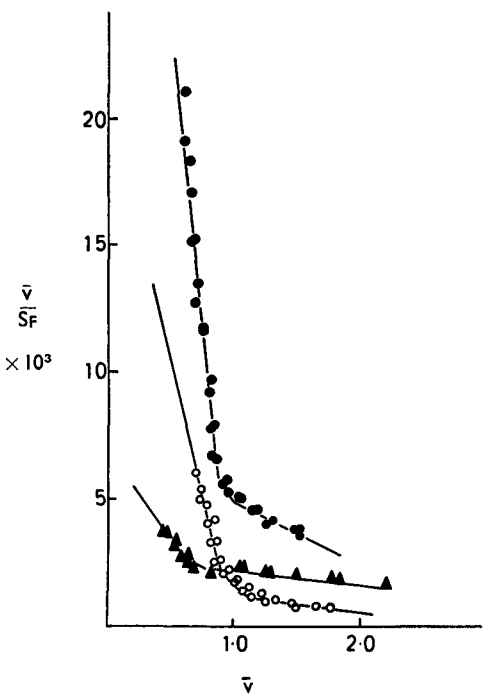

FIG. 1. Scatchard plots of the binding to BSA of acetanilide (—○—), *p*-aminoacetanilide (—▲—) and *p*-nitroacetanilide (—●—). The initial slopes for all 15 compounds intersect the abscissa at $\bar{\nu} = 1$, indicating a single primary binding site per BSA molecule.

considered in this work, since such binding does not generally occur until drug concentrations well above those used clinically are reached.

Association constants obtained in duplicate runs at the three temperatures gave the thermodynamic parameters of association shown in Table 1.

That no serum albumin leaked from the dialysis bag was shown by the absence of colour development when the external solution was treated with 3% sulphosalicylic acid. The possibility of disturbance of the dialysis by a Donnan effect was examined by carrying out several runs at one tenth the normal buffer concentration; identical association constants were obtained at both buffer concentrations. Finally, solutions were checked regularly for pH changes during dialysis; none were found.

Table 1. *Thermodynamic constants of the association of* p-*substituted acetanilides with bovine serum albumin, and the Hammett constants of the substituents.*

p-Substituent	Association constant at 19° (litre mol^{-1})	$\triangle G$ at 19° (kJ mol^{-1})	$\triangle H$ (kJ mol^{-1})	$\triangle S$ (J mol^{-1} deg^{-1})	Hammett's *p*-substituent constant, σ
-H	21 500	—24·2	—15·2	+30·8	0
$-CH_3$	29 800	—25·0	—18·7	+21·7	—0·170
-OH	17 000	—23·6	—20·7	+10·0	—0·370
$-OCH_3$	16 100	—23·5	—19·8	+12·7	—0·268
$-OC_2H_5$	20 200	—24·0	—17·7	+21·4	—0·240
$-NH_2$	7 000	—21·5	—24·0	— 8·7	—0·660
-F	29 900	—25·0	—14·2	+36·8	+0·062
-Cl	62 500	—26·8	—12·5	+48·8	+0·227
-Br	105 500	—28·1	—11·4	+56·9	+0·232
-I	142 000	—28·8	—12·1	+57·3	+0·180
-CHO	26 000	—24·7	—10·8	+47·4	+0·265
-COOH	13 400	—23·1	— 9·0	+48·0	+0·450
$-NO_2$	48 600	—26·2	— 2·4	+81·2	+0·778
$=N-CH_3$,-H	18 800	—23·9	—14·9	+30·9	0
$=N-CH_3$,-OH	16 000	—23·5	—19·7	+13·0	—0·370

DISCUSSION

Fig. 2 shows a good rectilinear relation between the enthalpy of binding (ΔH) and Hammett's substituent constant, σ. A high enthalpy of interaction is thus associated with ability of a substituent to donate electrons, suggesting that the drug-BSA binding is a function of the electron density within the acetanilido moiety.

The close correlation of Fig. 2 also implies that the binding enthalpy is governed almost entirely by the electron-directing properties of the substituent, and not by any specific substituent property such as the ability to form hydrogen bonds. Hansch, Kiehs & Lawrence (1965) have also shown, in the case of phenol-BSA binding, that the size of the substituent similarly appears to play no significant part in the interaction.

The compounds examined include two *N*-methylated acetanilides, and although these lack an amino-hydrogen atom, they behave in a similar fashion to the simple *p*-substituted acetanilides. Since there is but a slight decrease of the binding enthalpy on *N*-methylation, this also suggests that hydrogen bonding of the acetanilide =N–H group contributes little, if anything, to the drug-BSA interaction.

At the pH used (7·2) the carboxyl group of *p*-acetamidobenzoic acid ($pK_a = 4{\cdot}2$) is 99·9% ionized. This has, however, no appreciable effect on the binding to BSA, so that ionic forces probably play no part in the binding.

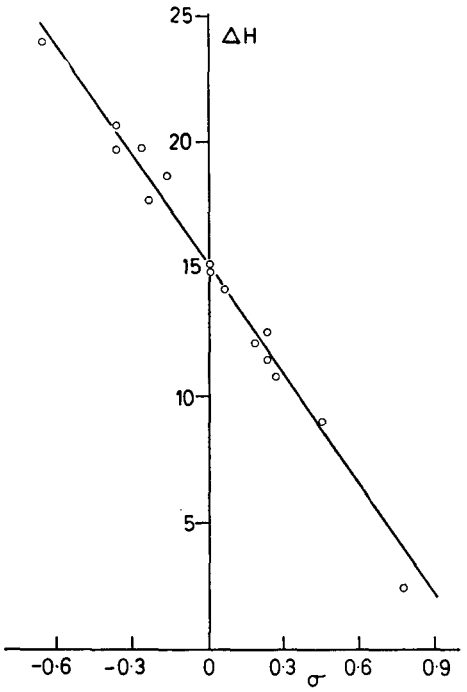

FIG. 2. Correlation of the enthalpy of substituted acetanilide-BSA binding, $\triangle H$, and Hammetts substituent constant, σ. The regression equation is $\triangle H = 14{\cdot}50\,\sigma + 15{\cdot}1$ kJ mol^{-1}, and the correlation coefficient is 0·992.

Interaction between acetanilides and BSA is thus non-specific, involving only van der Waals forces. Binding probably occurs to a hydrophobic region of the BSA molecule, as has previously been postulated for penicillins (Bird & Marshall, 1967), dyes (Weber & Young, 1964) and hydrocarbons (Wetlaufer & Lovrien, 1964); such behaviour is consistent with the role of albumins as transport proteins.

Table 1 shows that whereas the free energy of binding (ΔG) is negative in all cases, indicating spontaneous interaction, there is but little variation of ΔG amongst the different drug-BSA complexes. Variation of ΔH is therefore counteracted by opposing changes in the entropy of association, ΔS, and a good correlation in fact obtains between ΔS and σ (Fig. 3).

With the exception of *p*-aminoacetanilide, all the entropy changes on binding are positive. Although this would appear anomalous, since the association of drug and protein should result in an increase in the order of the system, a number of other factors must be considered. It has been suggested (e.g. O'Reilly, 1969) that hydration of both drug and BSA in the free state will reduce the entropy of the system. When drug-BSA association occurs, some at least of the bound water is released, thereby increasing the disorder, and thus the entropy, of the system. If this is correct, drug-protein binding should increase the total volume of the solution, and Lundgren (1945) has observed such an increase on the binding of alkylsulphonate anion by egg albumin. Using a value of $\Delta S = 20$ J mol^{-1} deg^{-1} to represent the loss of one water molecule (McMenamy & Seder, 1963) and assuming 'dehydration' to account for the bulk of the entropy gain on binding, then the binding of, for example, paracetamol involves half a water molecule, that of phenacetin involves one, and that of *p*-nitroacetanilide involves four water molecules.

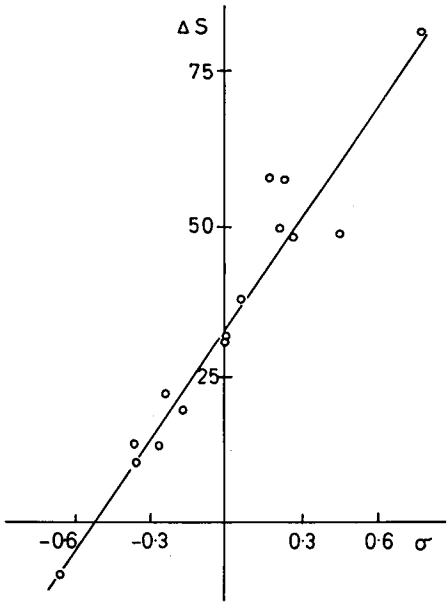

FIG. 3. Correlation of the entropy of substituted acetanilide-BSA binding, $\triangle S$, and Hammett's substituent constant, σ. The regression equation is $\triangle S = 61{\cdot}4\,\sigma + 31{\cdot}8$ J mol^{-1} deg^{-1}, and the correlation coefficient is 0·966.

Increased rotational and translational freedom in the protein, consequent upon association, could result from, for example, the transfer of interactive ability from an intramolecular to an intermolecular situation, or from the release of solvated water. Steinberg & Scheraga (1963) have shown that such an increase in intrinsic entropy can make a significant contribution to the overall positive ΔS of association.

Increased rotational freedom may also arise in the drug molecule. In the case of the acetanilides, rotational mobility around the aryl-N bond could increase on association owing to the release of solvated water (Aranow & Witten, 1960). Such an increase in entropy could probably occur only with a fairly non-specific type of binding, and might well be precluded by a more directional interaction such as hydrogen bonding. McMenamy & Seder (1963) consider that in such a case the opposite effect takes place, and that there is a decrease in intramolecular rotational (disorientational) freedom on binding. That this is not significant in the present case is shown by the fact that, using the equation of Flory (1953), the disorientation entropy per molecular segment (e.g. $-CH_2-$) is about 8 J mol^{-1} deg^{-1}. Thus if loss of disorientation occurs on binding, then the binding entropy of phenacetin should, other things being equal, be about 8 J mol^{-1} deg^{-1} more negative than that of *p*-methoxyacetanilide, whereas in fact it is some 9 J mol^{-1} deg^{-1} more positive.

Conformational changes in either drug or protein on association could also give rise to entropy changes. Discussing the binding of warfarin to human serum albumin, O'Reilly (1969) argued that such changes were unlikely since, for example, they would involve the breaking of hydrogen bonds within the albumin molecule, and hence would give rise to a positive ΔH; experimentally, however, he found ΔH to be negative. Any new conformation, however, could also permit hydrogen bond formation; thus the net ΔH of the conformational change alone might be close to

zero. Helmer, Kiehs & Hansch (1968) have reported changes in the optical activity of proteins on association with small organic molecules, indicating that conformational changes do indeed occur. It may be that these changes are associated with binding to secondary sites, for there is some evidence that such sites are generated by the initial binding to primary sites (Klotz & Ayers, 1953).

Drug-protein interactions will thus in general be accompanied by a net increase in entropy; of particular interest in this work is the good correlation between ΔS and σ for the interaction of acetanilides with BSA. The slope of the regression line is positive, entropy change becoming more positive as the electron-withdrawing ability of the substituent increases. There appear to be three possible explanations for this. Firstly, a compound tightly bound to BSA (high negative ΔH of association) will have a lower entropy when bound than will a compound that is loosely bound, because the freedom of movement of the former will be less. Secondly, the greater the electron-withdrawing ability of the substituent, the lower is the aryl-N bond order, and hence the greater is the rotational mobility around that bond upon association, i.e. when water of solvation, which restricts such mobility, is released.

Thirdly, since the acetamido-group itself is electron-donating, an electron-withdrawing substituent (positive σ) in the *para*-position will increase the charge separation in the molecule, whilst an electron-donating substituent (negative σ) will decrease it. It is suggested that the extent of solvation depends on the magnitude of the charge separation—that is, on the dipole moment. Thus *p*-nitroacetanilide, with the highest dipole moment of all the compounds examined, is solvated to the greatest extent, and hence yields the greatest positive entropy change on binding to BSA.

It is probable that all three factors mentioned above contribute towards the observed relation between ΔS and σ. We believe, following McMenamy & Seder (1963), that dehydration on binding makes the greatest contribution to the entropy of binding; hence the variation of extent of solvation with σ probably makes the greatest contribution to the relation between ΔS and σ.

Of the compounds examined, acetanilide, *p*-methoxyacetanilide, *p*-ethoxyacetanilide (phenacetin) and *p*-hydroxyacetanilide (paracetamol) have been or are at present used in medicinal preparations. The last three named all possess electron-donating substituents and have fairly high binding enthalpies which lie in the same region as those of other classes of drugs. Thus, for binding to serum albumin, values for a series of indole derivatives lie between -8 and -40 kJ mol^{-1} (McMenamy & Seder, 1963), for warfarin sodium $-14{\cdot}5$ kJ mol^{-1} (O'Reilly, 1967) and for a series of *p*-hydroxybenzoic acid esters $-0{\cdot}8$ to -16 kJ mol^{-1} (Patel, Sheen & Taylor, 1968). Binding enthalpies within the range found in this work are sufficiently low for ready dissociation of the drug-BSA complex at body temperature.

The association constants found for the four "commercial" acetanilides are sufficiently low to give fairly high free drug concentrations in the bloodstream over relatively long times. An association constant as low as that found for *p*-aminoacetanilide would give very high initial free drug concentrations, and would be suitable for the relief of acute, transient pain, assuming that formation of active metabolite was sufficiently rapid not to be the prime factor governing relief. There would, however, be a rapid decrease in the free drug concentration, as the relatively high binding enthalpy would mean a slow release of bound drug.

On the other hand, an association constant as high as that found for, say, *p*-bromoacetanilide would give rise to very low free drug concentrations. Although this would

give prolonged action, the free drug concentration could well be below the therapeutic minimum. A related factor here is that a compound with high affinity for a hydrophobic surface generally has low aqueous solubility. Thus, with a compound like *p*-bromoacetanilide, a low solubility would further decrease the concentration in the bloodstream. For comparison, the molar aqueous solubilities at 25° of the six *p*-substituted acetanilides discussed immediately above are: –H, 0·0416; –OH, 0·0771; $-OCH_3$, 0·0709; $-OC_2H_5$, 0·0430; $-NH_2$, 0·1061; –Br, 0·00074.

In conclusion, it may be said that thermodynamically the four "commercial" acetanilides are among the best of the simple *p*-substituted acetanilides for the relief of pain.

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