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INVESTIGATIONS ON THE DRUG-PROTEIN INTERACTION OF CERTAIN NEW POTENTIAL LOCAL ANAESTHETICS

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ABSTRACT

Generally, plasma proteins owe their binding capacity to the presence of aminoacid units which enter into intra- and intermolecular hydrophobic bonding with a diverse range of endo- and exogenous chemical substances. The intermolecular interactions between the hydrophobic areas of drug molecules and those of plasma proteins play an important role in drug-macromolecular complex formation and stabilization. This largely accounts for the carrier capacity of proteins for lipid soluble drugs. Albumin may be particularly responsible for the binding of local anaesthetics in plasma, but another binding factor may be lipoproteins present in blood cell membranes. Thus, and due to the special importance of the drug-protein binding phenomenon and its influence on the biological response, this investigation has been commenced on the purpose of establishing whether the degree of drug-protein interaction could be correlated with the duration of action of certain new potential local anaesthetics. These are derivatives of 2-phenoxyethyldialkylamine hydrochloride. Equilibrium dialysis, being generally the most reliable of the various methods available, was chosen as a means of determining the extent of drug-protein binding, and bovine serum albumin (BSA) of molecular weight around 70,000 was employed. An ultraviolet assay method was used to measure the concentration of free, unbound local anaesthetic molecules in the protein-free compartment, once equilibrium had been attained. It was found that the affinity of the test compounds for BSA does not parallel their duration of action produced in the guinea pig intradermal wheal test. Moreover, the ability of BSA to bind these local anaesthetics appeared not to depend on the number of binding sites on the protein molecule, but rather on the proportion of unionised lipophilic species. This suggests that ionic forces probably play no essential part in the binding process.

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INTRODUCTION

In a previous study[1] conducted on certain novel local anaesthetics,[2] it was found that there was no correlation between the extent of drug-protein binding and the duration of local anaesthesia. However, the partition coefficients (Log P) were found to be related with the degree of binding. Similar results were reported by Buechi, et al.[3-5] In addition, the same authors[4] have shown that there was a positive correlation between the extent of drug-protein binding and both corneal anaesthesia and irritation, but not with conductivity anaesthesia.

The affinity of serum albumin for organic ions has been found to depend on the charge, structure, pH, and nature of the buffer solution, as well as the concentrations of both the drug and protein solutions.[1,6-11] Among molecules of similar size and structure, anions are bound much more strongly than cations.[12]

In the light of the paucity and scarcity of information on the local anaesthetics-protein interaction, and to confirm our previous findings, we felt it is necessary to perform further studies on the protein-binding of other new potential local anaesthetics prepared by the author.[2] These are derivatives of 2-phenoxyethyldialkylamine hydrochloride. The results of this study are reported in this communication.

METHOD

The technique of equilibrium dialysis was utilised for determining drug- protein interaction.[1] Various concentrations of duplicate 10 mL samples of the local anaesthetics in phosphate buffer (Bp, pH 7.0) were pipetted into 50 mL ground glass necked, conical flasks. Dialysis bags (Visking size 7-30/32 inflated diameter, supplied by Medicell International Ltd., London, UK, lengths of 15 cm being routinely used), tied at one end, were filled with protein solutions (10 mL) in the same buffer system, then tied at the other ends, the bags were immersed in local anaesthetic solutions in the conical flasks which were then tightly stoppered. The protein concentrations in the first and second sets of experiments were 2.8571×10^{-5} and 4.0×10^{-4} mol/L.

Duplicate control dialysis assemblies containing buffer but no protein (10 mL) were prepared to accompany each determination. Two further duplicate blank dialysis assemblies were also prepared, the first containing buffered protein solution (10 mL) inside the bag and phosphate buffer (10 mL) outside; while the second blank assembly contained phosphate buffer (10 mL) both inside and outside the dialysis bag. All the dialysis assemblies were allowed to equilibrate at 20±1°C for four days with constant

Table I. Some of the pertinet physicochemical and biological properties of the test compounds[2]

Compound	Log P	pKa	WT	% Unionised (pH 7.0)
10A	-0.46	8.88	1.5	1.29
19A	1.05	8.86	24	1.36
Procaine-HCl	0.27	9.00	0.5	0.99
Bupivacaine-HCl	-	8.10	2.5	7.35

Log P= partition coefficient in n-octanol / pH 7.4 phosphate buffer;
pKa= ionisation constant; **WT=** duration of action of the anaesthetic tested as 0.5 % **W/V** solution, in the guinea pig intradermal wheal test (h).

Table II. Equilibrium dialysis study on BSA-compound 10A system at pH 7.0 (albumin concn.= 2.8571×10^{-5} M)

Initial drug concn. outside dialysis bag(M)	Initial drug concn. outside dialysis bag at equilibrium(M)	Unbound drug concn. (X) outside dialysis bag at equilibrium(M)	%Bound	r	r/X
4.1899×10^{-3}	2.0400×10^{-3}	1.9150×10^{-3}	11.55	8.75	4.569×10^{3}
3.4178×10^{-3}	1.6700×10^{-3}	1.5550×10^{-3}	12.89	8.05	5.177×10^{3}
3.0964×10^{-3}	1.4650×10^{-3}	1.3850×10^{-3}	10.36	5.60	4.043×10^{3}
2.7042×10^{-3}	1.4000×10^{-3}	1.2850×10^{-3}	15.18	8.05	6.265×10^{3}
1.9076×10^{-3}	0.9300×10^{-3}	0.7850×10^{-3}	26.98	10.15	1.293×10^{4}
1.2103×10^{-3}	0.5600×10^{-3}	0.4150×10^{-3}	41.13	10.15	2.446×10^{4}

r= total moles of bound drug/total moles of BSA; r/X= intrinsic affinity of BSA for test anaesthetic agent

shaking. Thereafter, the absorbance of the phosphate buffer outside the dialysis bag was measured.

RESULTS

The chemical structures of the test compounds and the reference standard agents are shown in Fig. 1. In addition, some of their physicochemical properties and their duration of action[2] are presented in Table I. The results for each of the test drug-BSA equilibrium dialysis systems at pH 7.0 are shown in Tables II-VIII. The first column in each table shows the initial drug concentration outside the dialysis bag before equilibrium, while the second column refers to the initial drug concentration outside the dialysis bag after equilibrium had been attained. This was calculated by determining the concentration of the control system at equilibrium. The third column gives the unbound concentration (X) at equilibrium from analysis of the experimental dialysis assembly after considering the two duplicate blank assemblies. The other three columns depict the per cent of drug-albumin binding (% bound), the calculated values of r(i.e. the ratio of total moles of bound anaesthetic to total moles of protein), and r/X (the intrinsic affinity of protein for a particular compound) from the data of the second and third column. The concentration of bound drug is equal to the difference between the concentration in the second column and the concentration of the third column (X) which is the concentration of unbound drug outside bag

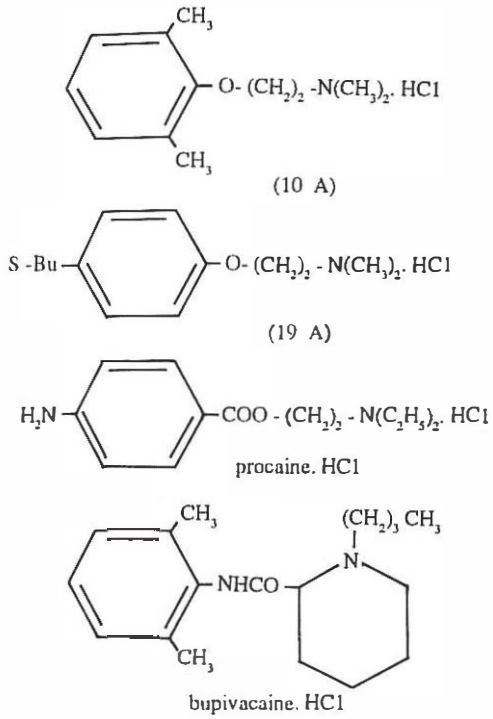

Fig 1. The chemical structures of the test compounds.

at equilibrium. All figures presented are everage of duplicate experiments. Procaine and bupivacaine were used as standard local anaesthetics. A comparison of the pH values before and after equilibrium dialysis showed that no change

Table III. Equilibrium dialysis study on BSA-compound 19A system at pH 7.0 (albumin concn.= 2.8571×10^{-5} M)

Initial drug concn. outside dialysis bag(M)	Initial drug concn. outside dialysis bag at equilibrium(M)	Unbound drug concn. (X) outside dialysis bag at equilibrium(M)	%Bound	r	r/X
3.8825×10^{-3}	1.7700×10^{-3}	1.6200×10^{-3}	15.60	10.50	6.482×10^{3}
3.2855×10^{-3}	1.6300×10^{-3}	1.5500×10^{-3}	9.40	5.60	3.613×10^{3}
2.7870×10^{-3}	1.3800×10^{-3}	1.3800×10^{-3}	12.88	6.65	5.175×10^{3}
2.1210×10^{-3}	1.1100×10^{-3}	1.0300×10^{-3}	14.17	5.95	5.777×10^{3}
1.0411×10^{-3}	0.5200×10^{-3}	0.4680×10^{-3}	18.18	3.64	7.778×10^{3}
0.4213×10^{-3}	0.2100×10^{-3}	0.1850×10^{-3}	21.28	1.75	9.460×10^{3}

r= total moles of bound drug/total moles of BSA; r/X= intrinsic affinity of BSA for test local anaesthetic agent

Table IV. Equilibrium dialysis study on BSA-compound 10A system at pH 7.0 (albumin concn.= 4.0000×10^{-4} M and drug concn.= 1.5000×10^{-3} M)

Initial drug concn. outside dialysis bag(M)	Initial drug concn. outside dialysis bag at equilibrium(M)	Unbound drug concn. (X) outside dialysis bag at equilibrium(M)	%Bound	r	r/X
1.5000×10^{-3}	6.3500×10^{-4}	50000×10^{-4}	35.06	0.68	1.360×10^{3}
1.5000×10^{-3}	6.5000×10^{-4}	5.0000×10^{-4}	37.50	0.75	1.500×10^{3}

r= total moles of bound drug/total moles of BSA; r/X= intrinsic affinity of BSA for test local anaesthetic agent

Table V. Equilibrium dialysis study on BSA-compound 10A system at pH 7.0 (albumin concn.= 4.0000×10^{-4} M and drug concn.= 2.0000×10^{-4} M)

Initial drug concn. outside dialysis bag(M)	Initial drug concn. outside dialysis bag at equilibrium(M)	Unbound drug concn. (X) outside dialysis bag at equilibrium(M)	%Bound	r	r/X
2.0000×10^{-4}	9.5000×10^{-5}	7.0000×10^{-5}	41.67	0.12	1.786×10^{3}
2.0000×10^{-4}	9.0000×10^{-5}	7.30000×10^{-5}	35.40	0.10	1.370×10^{3}

r= total moles of bound drug/total moles of BSA; r/X= intrinsic affinity of BSA for test local anaesthetic agent

Table VI. Equilibrium dialysis study on BSA-procaine HCl system pH 7.0 (albumin concn.= 2.8571×10^{-5} M)

Initial drug concn. outside dialysis bag(M)	Initial drug concn. outside dialysis bag at equilibrium(M)	Unbound drug concn. (X) outside dialysis bag at equilibrium(M)	%Bound	r	r/X
3.8904×10^{-3}	1.9000×10^{-3}	1.8500×10^{-3}	5.13	3.50	1.892×10^{3}
3.3108×10^{-3}	1.6500×10^{-3}	1.6200×10^{-3}	3.60	2.10	1.297×10^{3}
2.4225×10^{-3}	1.0000×10^{-3}	0.9850×10^{-3}	4.83	1.75	1.777×10^{3}
2.0093×10^{-3}	0.8500×10^{-3}	0.8370×10^{-3}	3.68	1.12	1.338×10^{3}
1.5742×10^{-3}	0.6480×10^{-3}	0.6300×10^{-3}	5.36	1.26	2.000×10^{3}
0.0866×10^{-3}	0.4480×10^{-3}	0.4330×10^{-3}	6.48	1.05	2.425×10^{3}

r= total moles of bound drug/total moles of BSA; r/X= intrinsic affinity of BSA for test local anaesthetic agent

occurred during the course of the experiments. Experiments were conducted at room temperature (20 ± 1°C).

Control assemblies were used to indicate the exact initial concentrations of the test compounds since twice the concentration of the compound outside the dialysis bag in a control set up at equilibrium would be, theoretically, equal to the initial concentration of the agent outside the dialysis bag for the experimental assembly. Two further duplicate blank dialysis assemblies were also run with every experiment to allow for the possible effects of both the relatively large charged protein molecules and the high concentrations of phosphate buffer that were used.

All the dialysis systems were allowed to equilibrate at room temperature for four days constant shaking. At the end of equilibration the absorbance of the phosphate buffer outside the dialysis bag was measured at the wavelength of maximum absorption (λ max) of each compound, i.e. 267, 273, 290 and 264 nm for compounds 10A, 19A, procaine, and bupivacaine, respectively. The concentration of the local anaesthetic was then determined from the calibration curve of each compound, which represented the concentration of free, unbound drug outside the dialysis bag, being equivalent to free, unbound drug inside the dialysis sac equilibrium. The absorbance was linear in the range of concentrations studied.

A specimen of calculations for compound 10A (Table II) is shown below:

Absorbance of drug in external phase in the absence of albumin= Absorbance of external phase - Absorbance of blank;

∴concentration of drug in external phase in the absence of albumin = 2.0400×10^{-3}

∴ No. of moles of drug in 10 mL of external phase
$= 2.0400 \times 10^{-5}$ M

∴ Total no. of moles of drug in the system= 4.0800×10^{-5}

Absorbance of unbound drug in external phase in the presence of albumin= absrobance of external phase - absorbance of blank

∴ Concentration of unbound drug in external phase in the presence of albumin
(X)= 1.9150×10^{-3}

∴ No. of moles of drug in 10 ml of external phase in the presence of albumin= 1.9150×10^{-5} M

∴ Total no. of moles of unbound drug in the system= 3.8300

∴ No. of moles of drug bound to albumin
$= (4.0800 \times 10^{-5}) - (3.8300 \times 10^{-5})$
$= 0.2500 \times 10^{-5}$

No. of moles of unbound drug in equilibrium with bound drug= 1.9150×10^{-5}

∴ Total amount of drug in equilibrium
$= (1.9150 \times 10^{-5})$
$= 2.1650 \times 10^{-5}$

∴ % Bound = $(0.2500 \times 10^{-5}) / (2.1650 \times 10^{-5}) \times 100$
$= 11.55\%$

r = Total moles of bound drug/Total moles of albumin
$= 0.2500 \times 10^{-5} / 0.028571 \times 10^{-5}$
$= 8.75$

∴ r/X = $8.75/1.9150 \times 10^{-3}$
$= 4.569 \times 10^{-3}$

DISCUSSION

The equilibrium dialysis method is one of the oldest, direct, reliable, and most widely used methods for measuring drug-protein binding. It provides information

Table VII. Equilibrium dialysis study on BSA-bupivacaine HCl system at pH 7.0 (albumin concn.= 2.8571×10^{-5} M)

Initial drug concn. outside dialysis bag(M)	Initial drug concn. outside dialysis bag at equilibrium(M)	Unbound drug concn. (X) outside dialysis bag at equilibrium(M)	%Bound	r	r/X
30.7640×10^{-4}	15.3500×10^{-4}	14.4000×10^{-4}	11.66	6.65	4.618×10^{3}
22.4210×10^{-4}	11.2000×10^{-4}	10.2500×10^{-4}	15.64	6.65	6.488×10^{3}
16.2300×10^{-4}	8.1000×10^{-4}	7.2000×10^{-4}	20.00	6.30	8.750×10^{3}
14.6290×10^{-4}	7.3000×10^{-4}	6.2000×10^{-4}	26.19	7.70	1.242×10^{4}
11.2460×10^{-4}	5.6000×10^{-4}	4.7000×10^{-4}	27.69	6.30	1.340×10^{4}
6.9060×10^{-4}	3.3000×10^{-4}	2.5000×10^{-4}	39.02	5.60	2.240×10^{4}

r= total moles of bound drug/total moles of BSA; r/X= intrinsic affinity of BSA for test local anaesthetic agent

Table VIII. Equilibrium dialysis study on BSA-procaine HCl system at pH 7.0 (albumin concn.= 4.0000×10^{-4} M and drug concn.= 1.5000×10^{-4} M)

Initial drug concn. outside dialysis bag(M)	Initial drug concn. outside dialysis bag at equilibrium(M)	Unbound drug concn. (X) outside dialysis bag at equilibrium(M)	%Bound	r	r/X
1.5000×10^{-4}	0.7450×10^{-4}	0.7020×10^{-4}	10.91	0.02	3.134×10^{-2}
1.5000×10^{-4}	0.7430×10^{-4}	0.7000×10^{-4}	10.94	0.02	3.143×10^{-2}

r= total moles of bound drug/total moles of BSA; r/X= intrinsic affinity of BSA for test local anaesthetic agent

not only on the amount of free and bound drug, but also on the number of binding sites involved on the protein molecule.[10] Moreover, the necessity to have an *in vitro* system comparable with the situation *in vivo* led to it being considered as the method of choice.[13,14] It consists mainly of two compartments separated by semi-permeable membrane through which only free, unbound drug molecules can diffuse. In practice, an aqueous phosphate buffer solution of BSA inside a dialysis bag is placed in a vessel containing a solution of the test drug, and the system is allowed to come to equilibrium. At the end of equilibration, measurement of the concentration of the drug in the outer protein free compartment will give a value equal to the concentration of free, unbound drug in the protein compartment. Thereafter, the amount of protein-bound drug and other parameters can be calculated (see results).

A Close look at the results shown in Tables II-VIII indicate that the degree of binding is inversely proportional to the concentration of the test compound, i.e. decreasing the concentration of the test agent increases the degree of drug-protein binding or per cent of bound drug whenever the concentration of BSA (2.8571×10^{-5} M) remains constant (see Tables II-V). On the other hand, the extent of drug-protein interaction is directly related to the concentration of the protein (compare Tables II-V and VI-VIII). Moreover, although both compounds 10A and 19A have widely different partition coefficients, their degrees of binding do not seem to vary greatly from each other. This can be attributed to the fact that both test compounds have approximately similar pKa values. Thus, the proportions of ionised to unionised lipophilic species of both of them are almost equivalent. Therefore, the ability of these agents to bind BSA does not depend on the number of binding sites on the protein, but rather on the proportion and partition coefficent of the unionsied lipophilic species present. In addition, since the partition coefficient of compound 19A is higher than that of compound 10A, the former test compound has exhibited a slightly higher degree of protein binding than the latter. Accordingly, ionic species and, in turn, ionic forces appear to have insignificant role in the drug-binding phenomenon. This is in accord with our previously reported results,[1] and parallels the reported behaviour of a series of parasubstituted acetanilides[15] as well as the present finding with respect to the two standard agents, i.e. procaine and bupivacaine.

Moreover, there has not been a correlation between the degree of binding and the duration of action of the assessed agents. The nature of the aromatic unclear substituent, i.e. size, shape and hydrophobic nature, appears to affect the extent of binding. Similar results were also reported by others.[1,6-11]

The intrinsic affinity r/X of BSA for all test compounds is inversely proportional to the concentration of the drug, i.e. decreasing the concentration of the assayed agent increases r/X. Thus, the interaction between BSA and the test agents may be non-specific involving, primarily, van der Waals forces and hydrophobic interactions and,

secondarily, hydrogen bonding. The drug-protein binding most probably involves a hydrophobic region of both the drug and BSA molecules. This has previously been reported for other compounds, i.e. 2-phenoxyethyldiakylamines,[1] para-substituted acetanilides,[15] penicillins,[16] dyes,[17] and hydrocarbons.[18] Such a property is consistent with the role of albumins as transport proteins. Furthermore, it would be appropriate to study the binding of these local anaesthetics to proteins or lipoproteins present in the vicinity of the injection site or the nerve fibres to ascertain whether specific binding to such proteins is, in some way, related to the duration of local anaesthesia as well, nor should the possibility of binding to macromolecules other than proteins be overlooked.

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