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BINDING OF A DIPHENHYDRAMINE ANALOGUE TO BSA

AN NMR INVESTIGATION

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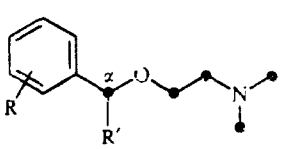
Abstract—In a study of the behaviour of diphenhydramine analogues with macromolecules, the interaction between the 4-methyl compound and bovine serum albumin (BSA) has been investigated with the aid of NMR spectroscopy: binding to BSA occurs primarily with the dimethylamino group; with time, a second type of binding, involving at least one of the phenyl groups, is observed; the binding is pH-dependent, reaching a maximum at pH = 4·3 and a minimum at pH = 5·3. To explain the increase at higher pH a second conformational change of the protein is assumed to occur.

THE ACTION of a drug is, as is generally accepted now, based on its ability to form complexes with specific macromolecules or to form nonspecific complexes with macromolecules at specific places in the body. As part of an investigation to explain the differences in *in vivo* behaviour between the diphenhydramine analogues with either 2- or a 4-methyl substituent (Fig. 1), we decided to investigate the nature of the



I;	R = H;	R′ = phenyl:	diphenhydramine*
II;	R = 2-CH_3;	R′ = phenyl:	orphenadrine†
III;	R = 4-CH_3;	R′ = phenyl:	*N*,*N*-dimethyl-2-[(*p*-methyl-α-phenylbenzyl)oxy]ethylamine‡
IV;	R = 4-CH_3;	R′ = pentadeuterophenyl	

FIG. 1

binding of these drugs to various macromolecules by using the NMR spectroscopic technique described by Jardetzky and coworkers.[1, 2] These authors added bovine serum albumin (BSA) to solutions of several penicillins and sulfonamides and measured the resulting changes in the linewidths of the resonance signals arising from the various

* Benadryl ® (registered by Parke-Davis); Benodine ®.
† Disipal
‡ Toladryl ® (registered by Parke-Davis; Neo-Benedine.

H-containing parts of the molecules. They were able to demonstrate that the phenyl group plays the major part in the binding.

In this preliminary paper we report findings regarding the binding to BSA of compound III, the most active antihistaminic from a series of alkyl-substituted diphenhydramines.[3]

In order to obtain the signals of the *p*-tolyl group undisturbed by those of the phenyl, we used the equipotent analogue (IV) in which the phenyl group is fully deuterated. Deuterium oxide was used as the solvent and the effects of changes in pH, in concentration of drug or of protein were determined as well as the changes occurring with time. In some of the experiments lysozyme and dextran (M = 80,000) were used for comparison.

Measurements were made on a Varian A60 NMR spectrometer using standard glass tubes (5-mm O.D.) and hexamethyldisiloxane as an external reference. The changes of the chemical shifts were negligible. Linewidths at half height ($\Delta\nu$), measured 3 hr after starting the preparation of the solution were corrected for instrumental linewidth (0·30–0·40 cps), and in the case of the phenyl and the methine signal, for broadening by spin–spin coupling, according to Jardetzky.[1] The obtained value $\Delta\nu_{corr.}$ was transformed into the relaxation rate $1/T_2$ according to the expression $1/T_2 = \pi\Delta\nu_{corr.}$

The pH values are actual meter readings, uncorrected for deuterium oxide.

Compound IV possesses four groups, *viz.* the phenyl group, the *p*-tolyl group, the oxygen and the positively charged tertiary nitrogen atom ($pK_a = \pm 9{\cdot}0$). On addition of

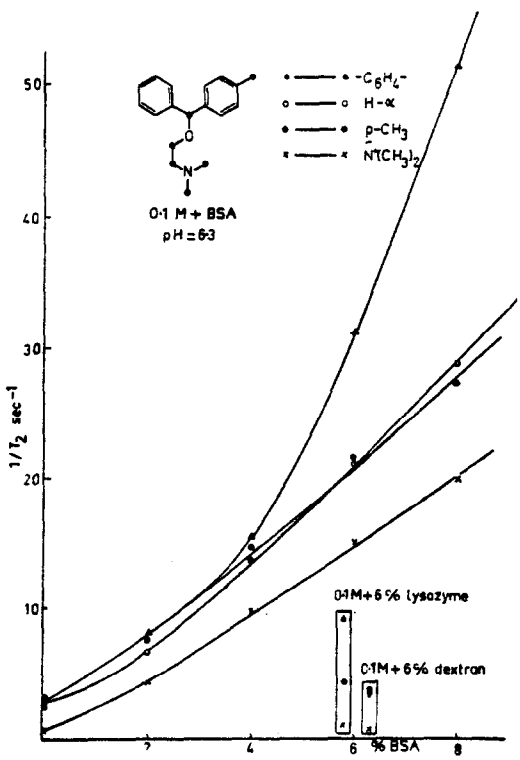


FIG. 2. Change of the relaxation rates $1/T_2$ of NMR signals of a 0·1 M solution of compound IV in deuterium oxide at pH = 6·3, on addition of BSA, lysozyme, and dextran.

albumin, broadening of the resonance signals of three of these—either of the group itself or of a neighbouring group—can be observed. This broadening is directly proportional to the total protein concentration if the drug concentration remains constant, binding is weak and the exchange of the drug molecule between the free and the bound state is rapid.[1]

In a series of 0·1 M solutions of compound IV and increasing percentages of BSA at pH 6·3, there is indeed a significant broadening of the resonance signals, although this is not strictly linear with the concentration of BSA in all cases (Fig. 2). This indicates that considerable binding to BSA takes place. Solutions containing dextran or lysozyme instead of BSA do not show this effect. The negligible broadening of the lines in the case of the very viscous 6% dextran solution also indicates that viscosity is not important in this phenomenon.

In a series of solutions with 6% BSA and increasing concentration of the drug at pH 6·3, the linewidths decrease linearly, as required by theory[4] (Fig. 3).

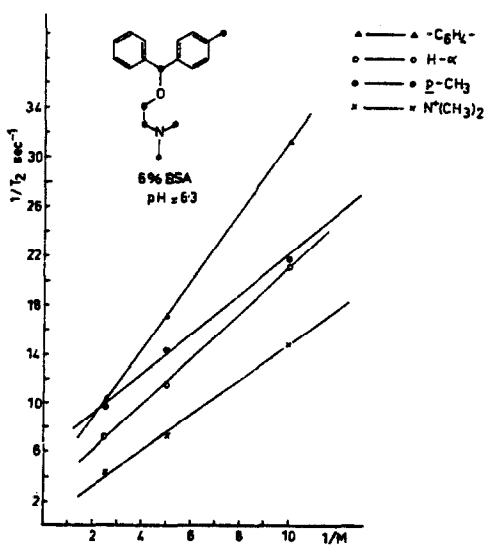


FIG. 3. The relaxation rates $1/T_2$ of NMR signals of compound IV, as a function of the inverse drug concentration at constant BSA concentration (6%) and pH 6·3.

Aging of the solutions causes an increase of the linewidths together with increasing viscosity and turbidity of the initially clear solutions. This phenomenon, which depends on the albumin–drug ratio, is probably the cause of the nonlinearity in Fig. 2. A solution of 0·1 M IV and 2% BSA at pH 6·3 shows only a slight change in the linewidths and remains clear (Fig. 4a). The same drug concentration with 4% albumin at the same pH produces a very turbid gel, within the first 12 hr, and there is a sharp increase in the linewidths (Fig. 4b). However, a solution of 0·4 M IV with 4% BSA remains almost unchanged. The formation of the gel is a reversible process, because, on addition of more drug, normal, very slightly turbid solutions are recovered.

Although the phenyl signal is the broadest one, the linewidth of the signal of the

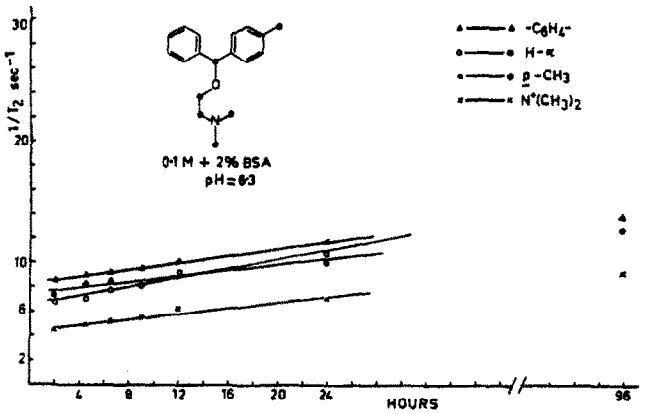


FIG. 4(a)

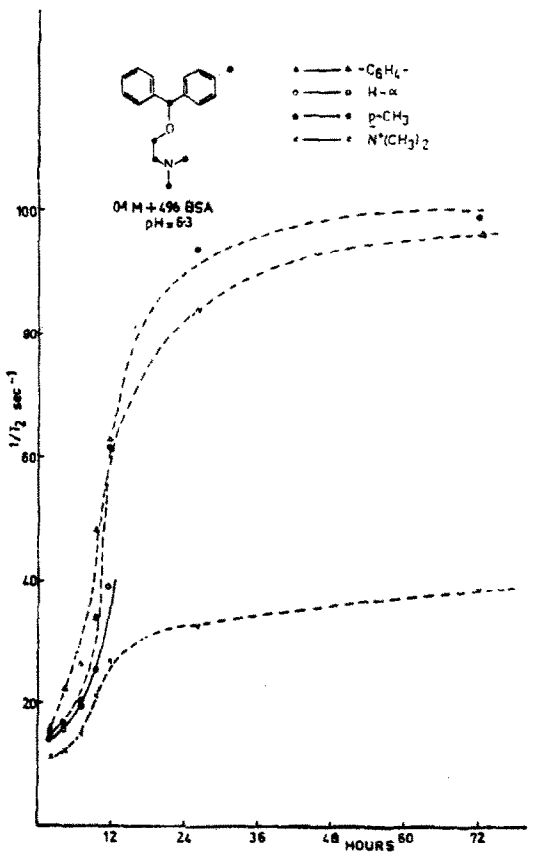


FIG. 4(b)

FIG. 4. Change with time of the relaxation rates $1/T_2$ of a 0·1 M solution of compound IV in D_2O, pH 6·3;
(a) in the presence of 2% BSA;
(b) in the presence of 4% BSA.

N-methyl group changes by a factor of 25, as the BSA concentration increases from 0 to 6%, while the others change with a factor of 7·5–11 (Fig. 2). This means that this group is more stabilised by binding, than the others, presumably because of ionic interaction between the positive nitrogen atom and a negative group of the protein, probably a carboxylate group. The less pronounced relative broadening of the other—hydrophobic—parts of the drug might be caused by the ionic binding alone as a distant effect, or by a second type of binding to the protein.

The pH-dependence of the relaxation rates (Fig. 5) shows that, although the ionic binding is drastically diminished at low pH's, some binding of the hydrophobic parts of the molecule still occurs. Towards higher pH, the percentage of ionic binding increases with the increase of negative charge on the protein. It attains an optimum at about pH 4·3, where a drastic change in the binding capacity of the protein occurs, and passes through a minimum at pH 5·3.

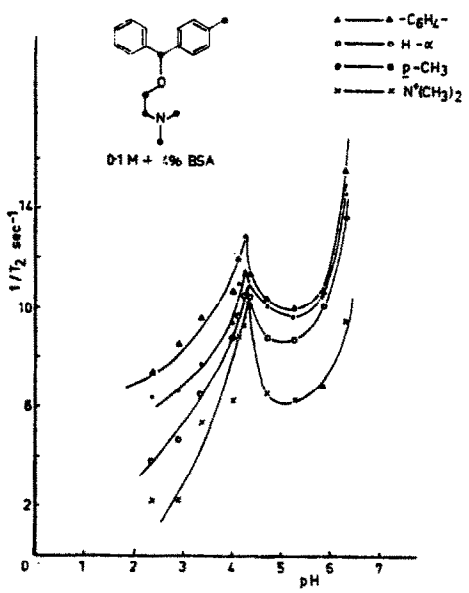


FIG. 5. The relaxation rates $1/T_2$ of compound IV (0·1 M) in the presence of 4% BSA as a function of pH.

The remarkable optimum at pH 4·3 must be ascribed to the well-known N-F transition of the protein.[4] In a recent investigation, Vijai and Foster[5] concluded that in the pH range below 4, where the protein is in the unfolded F form, all the carboxylate groups are freely exposed, and thus, as far as ionised, available for binding. At pH 4 the protein is converted into the N form, in which 40 per cent of thecar boxylate groups are masked, which reduces the binding capacity.

At pH values above the isoelectric point, binding increases again. Observations are limited, however, by the insolubility of the drug as the free base. This increase in binding has also been noticed by other authors, even with non-ionic compounds.[6, 7]

The explanation may be that the N form undergoes another unfolding process in this pH range, such that both hydrophobic and ionic areas become accessible to the

drug. The same suggestion was made by Leonard *et al.*[8] to explain the influence of pH on the optical rotatory dispersion of albumin. In contrast to the transition at low pH all the carboxylate groups are now ionised and are therefore potential binding sites. This increased ionic binding probably increases the organisation of the solution by allowing the cross-linkage of proteins with drug molecules between ionic and hydrophobic sites. This cross-linking causes aggregation of the protein molecules and the turbidity and related phenomena discussed above. The presence of hydrophobic interaction is supported by the effect of an increase in the ionic strength of the solution. This, as does an increase of the temperature, enhances the formation of the gel, which is in accordance with the tendency of hydrophobic binding. The loss of entropy, due to this organisation, will be fully compensated by the entropy gain of water molecules, expelled from the hydrophobic areas of both the drug and the protein.[9] Besides, interaction may occur between the unfolded chains of two protein molecules, held together by the drug.

At this stage of our investigation the results allow the conclusion that at therapeutical concentration 4-methyldiphenhydramine (III) in blood will to a considerable extent be bound to albumin in both an ionic and a hydrophobic way.

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REFERENCES

1. O. JARDETZKY and N. G. WADE-JARDETZKY, *Molec. Pharmac.* **1**, 214 (1965).
2. J. J. FISHER and O. JARDETZKY, *J. Am. Chem. Soc.* **87**, 3237 (1965).
3. A. F. HARMS and W. TH. NAUTA, *J. Med. Pharm. Chem.* **2**, 57 (1960).
4. J. F. FOSTER, in *The Plasma Proteins*, p. 179. Academic Press, New York (1960).
5. K. K. VIJAI and J. F. FOSTER, *Biochemistry* **6**, 1152 (1967).
6. A. WISHNIA and T. PONDER, *Biochemistry* **3**, 1377 (1964).
7. A. RAY, J. A. REYNOLDS, H. POLET and J. STEINHARDT, *Biochemistry* **5**, 2606 (1966).
8. W. J. LEONARD, K. K. VIJAI and J. F. FOSTER, *J. biol. Chem.* **238**, 1984 (1963).
9. G. NÉMETHY, *Angew. Chemie* **79**, 260 (1967).