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# Study of the Interaction between Human Serum Albumin and Some Cephalosporins

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SUMMARY

Dialysis and microcalorimetric methods were used to calculate the binding parameters of some cephalosporins to human serum albumin (HSA) and to study the nature of the interactions involved in the binding process. Dialysis results agree with microcalorimetric data for cephapirin, cephradin, cefamandole, and cefazolin. Binding forces seem to be principally electrostatic. The parts of the drug molecule involved in HSA drug binding have been identified by high-resolution NMR. The major binding site for cephalosporins with high HSA affinity is thought to be the electron-rich heterocycle fixed on the methylene at position 3. Four classes of cephalosporin have been defined: (a) very weak affinity for HSA (cephalexin, cephradin); (b) moderate affinity (cephapirin, cefoxitin, and cefotaxime) in which binding to the protein involves the heterocycle substituent of the acetamide chain carbon atom; (c) strongly binding (cefamandole), in which binding to HSA is by means of the methyltetrazole ring; and, finally (d), cefazolin, with two classes of binding sites for protein, showing strong and moderate affinity.

## INTRODUCTION

The cephalosporins form a chemically and pharmacologically homogeneous group, but they are known to bind to serum proteins to differing extents. There are numerous reports (1–4) on the consequences of this binding on bacteriological activity, rates of renal excretion, and body distribution. The therapeutic efficacy of these drugs is therefore influenced by their protein binding behavior.

There are several reports on the ratio of cephalosporins bound to HSA[1] as determined by ultrafiltration techniques (1–4). However, published results, which in general report percentage binding, are not consistent. Furthermore, HSA binding parameters (equilibrium constant of binding and site number), the nature of the binding site on the drug, and the thermodynamic characteristics of the interaction have not been determined, in contrast to other drug groups such as benzodiazepines or penicillins.

Therefore, we have measured the binding parameters of some cephalosporins by equilibrium dialysis and have identified by proton NMR the drug sites involved. Moreover, in order to obtain more information about interaction forces involved, the thermodynamic parameters of complex formation were measured directly by a microcalorimetric method. The molecules chosen were cefalexin, cephapirin, cephradin, cefazolin, cefamandole, cefoxitin, and cefotaxime (Table 1).

[1] The abbreviation used is: HSA, human serum albumin.

## MATERIALS AND METHODS

*Reagents.* Chromatographically pure HSA was purchased from Sochibo (Koch-Light Products, Colnbrook, Bucks., United Kingdom). The absence of fatty acids which are known to change protein binding of many drugs was verified. The HSA was purified by charcoal treatment (5) and checked by extensive dialysis against EDTA, and gave the same results as the original product. The concentration of HSA was determined by absorbance measurement at 280 nm ($E^{\%}_{cm} = 5.3$).

Cephalosporins, used without further purification, were kindly supplied by Bristol, Paris, France (cephapirin); Lilly, Saint Cloud, France (cefatoxin, cefazolin, cefamandole); Merck Sharp & Dohme, Paris, France (cefoxitin); Roussel, Paris, France (cefotaxime); and Smith Kline & French Laboratories, Puteaux, France (cephradin).

*Experimental conditions.* All experiments were carried out at 37°. The ionic strength of the solutions was maintained at a constant value of 0.154 by means of a phosphate buffer, and the pH was maintained at 7.4.

*Dialysis technique.* Equilibrium dialysis was performed in a 2-ml macrocell using a rotative Dianorm apparatus, the two compartments of which were separated by a Diachema membrane (5000 D). At 37° 3½ hr were required to ensure that equilibrium had been reached. Concentrations of free drug ($C_f$) were determined by UV spectrophotometry (see Table 2 for wavelength used) in the compartment without HSA. Results

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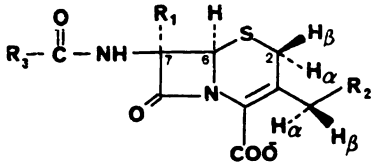
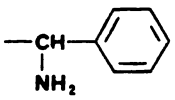
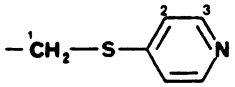
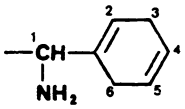
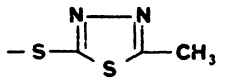
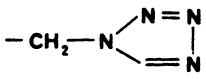
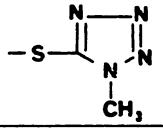
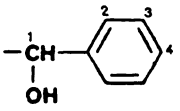
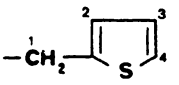
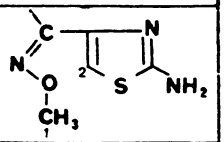
TABLE 1

*Structural formulae of cephalosporins studied*



| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| CEFALEXIN | H | H |  |
| CEPHAPIRIN | H | $CH_3-COO-$ |  |
| CEPHRADIN | H | H |  |
| CEFAZOLIN | H |  |  |
| CEFAMANDOLE | H |  |  |
| CEFOXITIN | $CH_3-O-$ | $NH_2-COO-$ |  |
| CEFOTAXIME | H | $CH_3-COO-$ |  |

were corrected for possible adsorption on cells and membrane by comparison with an experiment carried out without protein.

The results of the dialysis experiments were processed according to Scatchard's method (6). Each point is a mean of five experiments.

*Proton NMR.* Fourier transform proton magnetic resonance spectra were recorded on a Cameca spectrometer at 250 MHz. A 90° excitation pulse and an acquisition time of 2.6 sec were used. The transverse relaxation rate ($1/T_2$) values are calculated from the expression $1/T_2 = \pi\Delta\nu_{1/2}$, where $\Delta\nu_{1/2}$, the full-line width at half-maximal height, is the mean of several spectra, usually five (only those spectra showing excellent homogeneity were used). For broad signals an estimation of this quantity can be made by comparison with spectra of different line widths, previously simulated by the ITERCAL[2] computer program.

For each signal at the fast-exchange limit for a single class of sites on the macromolecule (7):

$$\frac{1}{T_{2\,\text{obs}}} = \frac{1}{T_{2\,\text{free}}} + \alpha\left(\frac{1}{T_{2\,\text{bound}}} - \frac{1}{T_{2\,\text{free}}}\right)$$

where $1/T_{2\,\text{obs}}$, $1/T_{2\,\text{free}}$, and $1/T_{2\,\text{bound}}$ are, respectively, the observed transversal relaxation rate and the relaxation rates of free and bound drugs and $\alpha$ is the fraction of total drug bound to HSA. In the case of small $\alpha$ values, i.e., $(S_T) \gg (P_T)$ (7), then

$$\alpha = \frac{nK_a(P_T)}{1 + K_a(S_T)}$$

[2] Program library supplied by Cameca (Courbevoie, France).

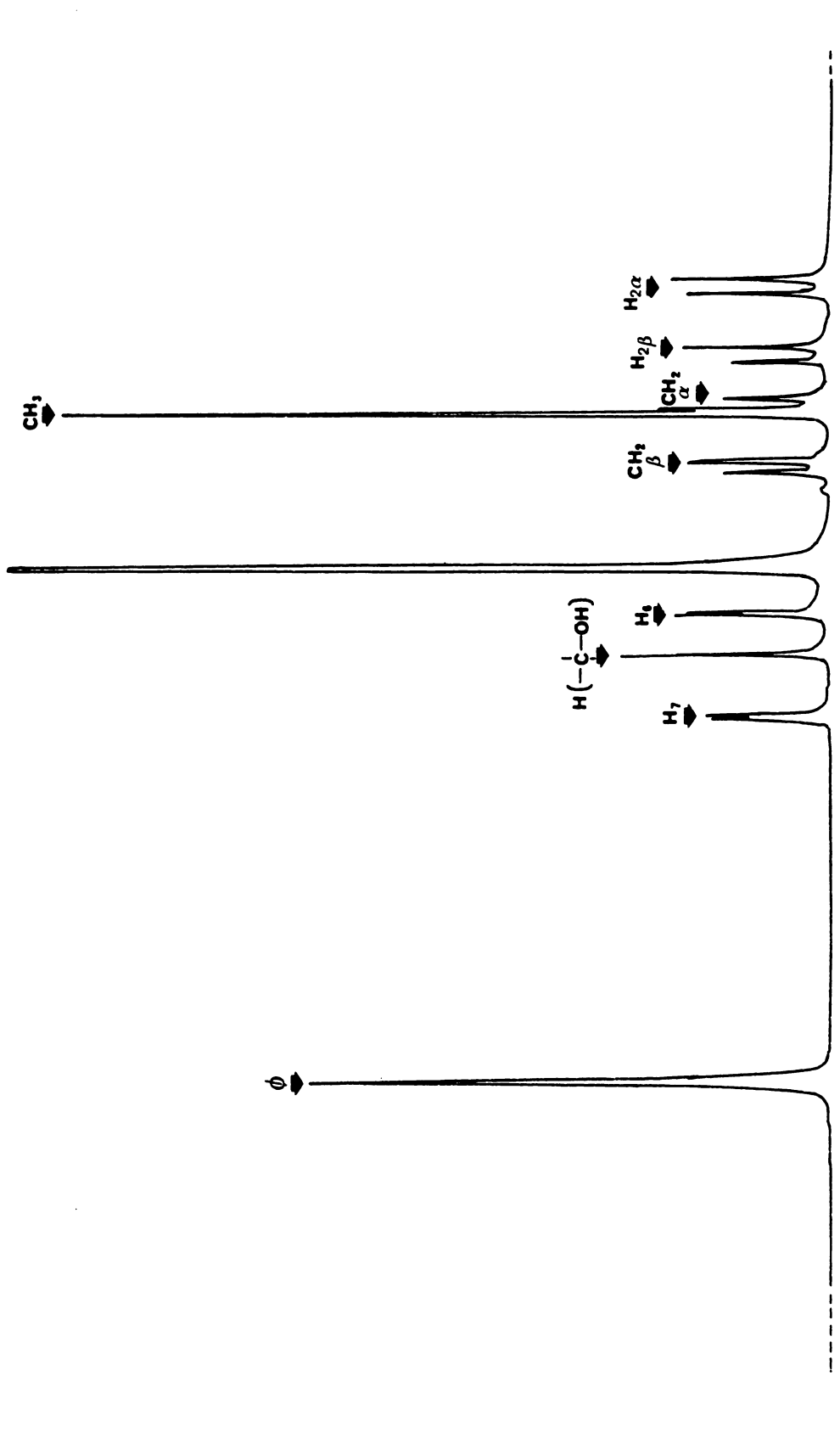



FIG. 1. *Cefamandole ($10^{-2}$ M) NMR spectrum*

where $K_a$ is the affinity constant, $n$ is the number of identical sites, and $(P_T)$ and $(S_T)$ are the total concentration of albumin and drug. For instance, Fig. 1 shows the spectrum for cefamandole and Fig. 2 shows the variations of $1/T_{2\,obs}$ for all observable protons as a function of concentration ratio at a constant concentration of protein ($5 \times 10^{-5}$ M). By using $n$ and $K_a$ values supplied by dialysis techniques, $\alpha$ is calculated at each ratio $(P_T)/(S_T)$. The linear graph of the function $1/T_{2\,obs}$, plotted versus $\alpha$, adjusted by the least-squares method, allows us to determine the quantity $1/T_{2\,bound}$ assignable to each proton and then the ratio $T_{2\,free}/T_{2\,bound}$.

The ratio $R = T_{2\,free}/T_{2\,bound}$ was determined for each cephalosporin and for all observable protons, and the error was calculated using the formula:

$$\frac{\Delta R}{R} \leq \frac{\Delta(1/T_{2\,bound})}{1/T_{2\,bound}} + \frac{\Delta(1/T_{2\,free})}{1/T_{2\,free}}$$

As the value of $1/T_{2\,bound}$ was large, the first term was ignored. In addition, we used the largest values of $\Delta(1/T_{2\,free})$ for each proton of the molecule, in order to make $\Delta R/R$ as large as possible.

*Microcalorimetric technique.* The flow calorimeter used was a LKB apparatus, Type 10700-1. It was set in a thermostatically controlled enclosure; all pumps were operated at a flow rate of 20 $cm^3$/hr. Under these conditions $10^{-3}$ cal can be measured to an accuracy of about 5% (8). In the case of a single class of binding sites on the protein, experimental data were processed by an iterative method previously published (9).

If $Q_i$ is the experimental heat measurement and $(PS)_i$ the calculated drug-protein complex concentration, $K_a$ is fitted so that the residue $R$, calculated by the least-squares method from the equation $Q_i = A(PS)_i + R$, is equal to 0; then $A$ is equal to $\Delta H$.

The other thermodynamic parameters $\Delta G$ and $\Delta S$ are calculated from the classical thermodynamic equations. In the case of cefazolin, we used a method called "Simplex" (10). Let us call $(PS_1)_i$ and $(PS_2)_i$ the complex concentrations referring to each of the two classes of sites calculated by assuming arbitrary values for their complex formation parameters $n_1$, $K_1$ and $n_2$, $K_2 \cdot n_1 K_1$, $n_2 K_2$ are fitted to obtain on one hand the least value of the residue $R$ in the equation $Q_i = A(PS_1)_i + B(PS_2)_i + R$ and on the other hand the minimal value of the sum of squares.

Figure 3 shows that the point-by-point addition of the theoretical curves ($A$ and $B$) obtained by this method produces a curve $C$ compatible with the experimental points.

## RESULTS

*Dialysis.* Equilibrium constants and number of sites ($n$) for the seven cephalosporins studied are shown in Table 2. Scatchard's isotherms are plotted in Fig. 4, and the nonlinear isotherm of cefazolin is shown in Fig. 5.

*NMR.* $T_{2\,free}/T_{2\,bound}$ ratios and the calculated errors for each cephalosporin and for all observable protons are reported in Table 3. These results indicate that, in the case of cefamandole, the drug-binding site appears to be near the $R_2$ group and proton 6 of the cephem nucleus. On the other hand, with cephapirin, cefoxitin, and cefotaxime, it appears to be near the $R_3$ substituent and in some cases near the $2\alpha$, $2\beta$, or $CH_2\alpha$ protons when the $R_3$ substituent is not too bulky and its spatial conformation propitious as a consequence of acetamide chain mobility. Finally, drugs like cephradin, which bind very poorly, present no particularly high $T_{2\,free}/T_{2\,bound}$ ratio whatever proton is considered. In the case of cefazolin, which has two classes of HSA sites and probably two binding sites on its molecule, a mean effect is observed.

*Microcalorimetry.* Figures 3 and 6 show experimen-

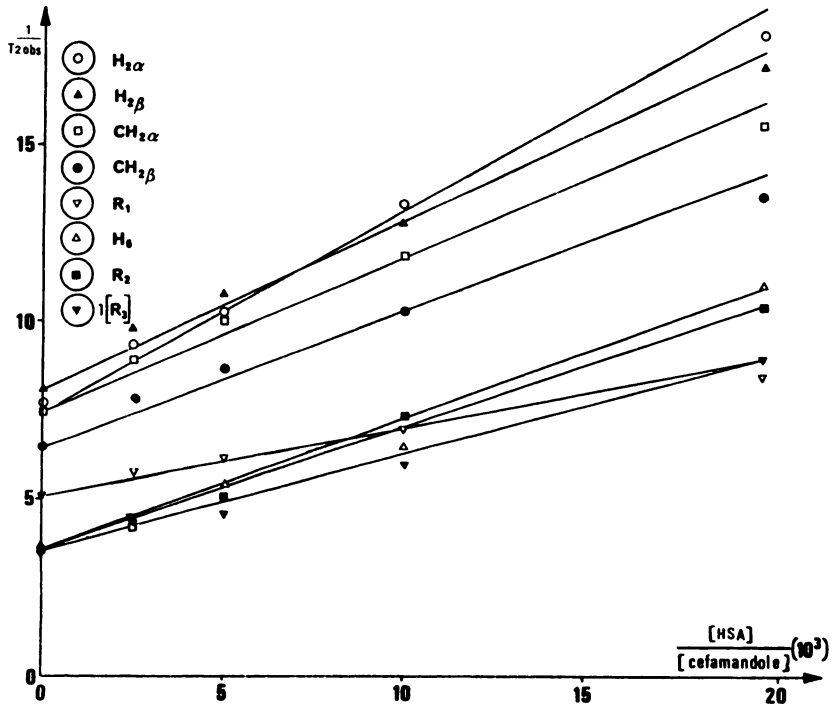



FIG. 2. *Cefamandole proton relaxation rates observed at* $5.10^{-5}$ M *HSA concentration*

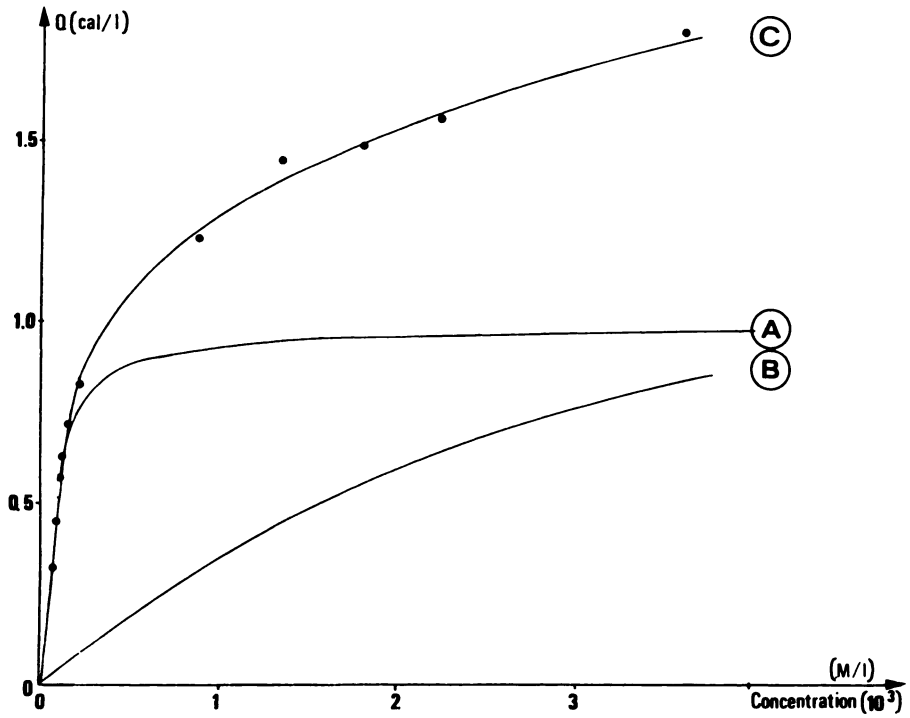



FIG. 3. *Cefazolin enthalpic titration curves*

*Q* is the experimental heat measurement. *Curve A* is plotted according to $n_1 = 0.7$ and $K_{a_1} = 20,000$ M$^{-1}$, *curve B* according to $n_2 = 1.9$ and $K_{a_2} = 300$ M$^{-1}$, and *curve C* is the sum of *A* and *B*; $P_t = 1.2\ 10^{-4}$ M).

tal heat measurements, and Table 4 gives equilibrium constants and thermodynamic parameters of the interaction ($\Delta G$, $\Delta H$, $\Delta S$) for each drug. Generally, values of constants supplied by the dialysis technique and by the microcalorimetric method are of the same order, except for the second class of binding site of cefazolin, for which, whatever the chosen experimental method, the equilibrium constant could not be accurately determined and was between 250 M$^{-1}$ and 1500 M$^{-1}$.

## DISCUSSION

*Dialysis.* Values of bound fractions calculated from our *n* and $K_a$ values (Table 2) are in good agreement with those previously published (1–4). Cefalexin does not bind to HSA, and the binding of cephradin is very weak ($K_a$ about 300 M$^{-1}$). Cephapirin, cefoxitin, and cefotaxime have only moderate affinity for HSA ($K_a$ around 1000–2000 M$^{-1}$). Finally, strong binding was seen for cefamandole with a single class of sites on protein ($K_a$ greater than 5000 M$^{-1}$) and for cefazolin with two classes of sites.

TABLE 2

*Binding parameters from dialysis experiments*

λ = absorbance wavelength. Bound ratio is calculated at $5.10^{-5}$ M drug concentration and $5.10^{-4}$ M HSA concentration

| | λ | $K_a$ | *n* |
|---|---|---|---|
| | *nM* | M$^{-1}$ | |
| Cefalexin | 262 | <100 | |
| Cephapirin | 257 | 1930 ± 200 | 2.4 ± 0.3 |
| Cephradin | 265 | 220 ± 80 | 2.4 ± 0.8 |
| Cefazolin | 370 | 20,000 | 0.7 |
| | | 1350 | 1.9 |
| Cefamandole | 268 | 6000 ± 500 | 2.0 ± 0.1 |
| Cefoxitin | 236 | 710 ± 100 | 3.3 ± 0.4 |
| Cefotaxime | 235 | 910 ± 100 | 2.2 ±0.2 |

*NMR.* It can be assumed that the broadening of the proton signals (Fig. 2) is produced by a specific interaction of cephalosporin with HSA, since $1/T_{2\,\text{obs}}$ decreases with increasing drug concentrations at constant protein concentration. The opposite effect would be expected if broadening were due to a nonspecific mechanism such as an increase in viscosity (6). In addition, the existence of a rapid exchange is supported by the low $K_a$ values, by the shape of the spectra, and by the effect of temperature on $1/T_{2\,\text{obs}}$ values (11): at a given concentration ratio, line width is increased as temperature is reduced.

It would be preferable, of course, to measure variation of longitudinal relaxation rate $1/T_{1\,\text{obs}}$, but these variations, unfortunately, are too weak and too dependent on viscosity to be taken into account (12).

In theory, the transversal relaxation rate of proton *i* is related to the correlation time $\tau_c$ by the equation (13)

$$(1/T_2)i = A\, f(\tau_c) \sum_j \frac{1}{r_{i,j}^6}$$

where *A* is a constant, $f(\tau_c)$ is a function of drug correlation time, and $r_{i,j}$ is the interatomic distance. Thus the ratio $T_{2\,\text{free}}/T_{2\,\text{bound}}$ allows for both variation of drug correlation time and changes in interaction, when binding takes place. The first effect involves a theoretically constant value of this ratio for each proton; the second results in an increase in the $T_2$ ratio, especially for protons that get close to the HSA macromolecule as a consequence of stronger or new interatomic interactions. Therefore, it is logical to investigate the drug-binding sites by comparing these $T_2$ proton ratios.

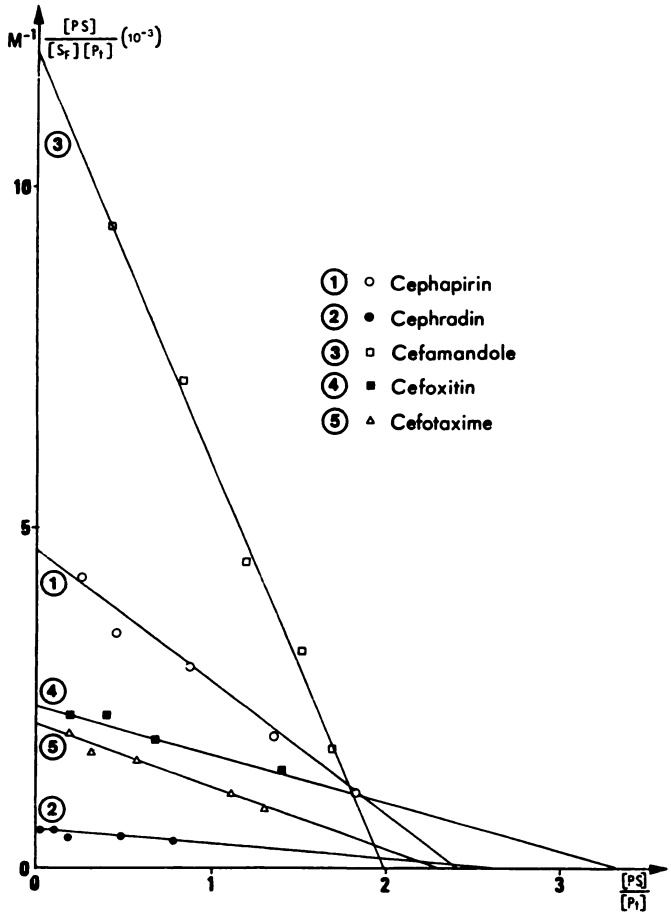



FIG. 4. *Scatchard plots from dialysis equilibrium measurements*
$S_F$ is the free drug concentration, *PS* the complex concentration, and $P_t$ the total HSA concentration.

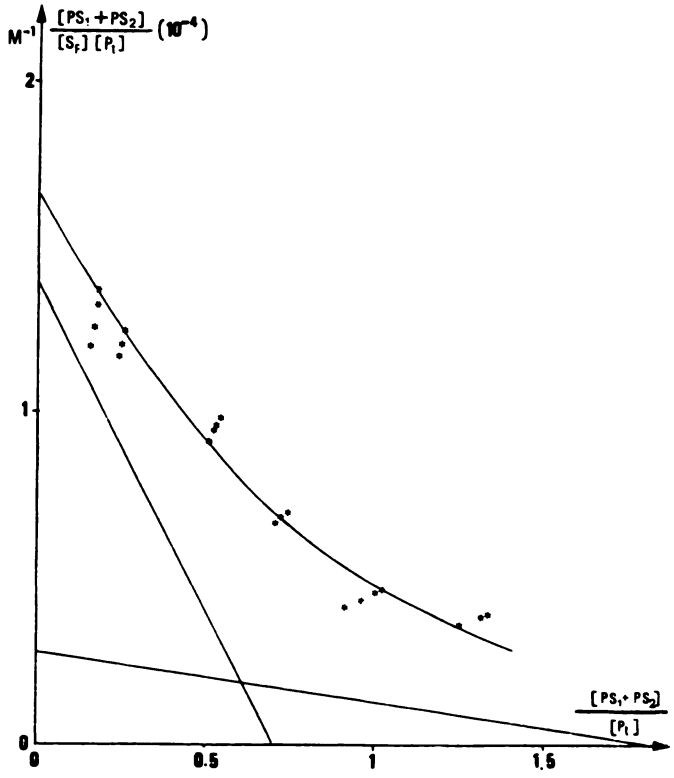



FIG. 5. *Nonlinear cefazolin Scatchard plot*
$S_F$ is the free drug concentration; $PS_1$ and $PS_2$ are the complex concentrations referring to the two classes of sites, respectively; and $P_t$ is the total HSA concentration.

From these results, it is possible to classify the cephalosporins into four groups; (a) molecules with weak binding affinity (cephradin, cefalexin); (b) molecules with moderate binding affinity situated near the $R_3$ substitu-

TABLE 3

*Free and bound relaxation times and $T_{2\ free}/T_{2\ bound}$ ratios for each measurable proton signal of cephalosporins*

$\Delta R$ was calculated as explained in text. The highest $\Gamma(1/T_{2\ free})$ value found for cephapirin was 0.57 $sec^{-1}$; for cefamandole, 0.33 $sec^{-1}$; for cefoxitin, 0.43 $sec^{-1}$; and for cefotaxime, 0.66 $sec^{-1}$.

| | $2_\alpha$ | $2_\beta$ | $CH_{2\alpha}$ | $CH_{2\beta}$ | 6 | $R_1$ | $R_2$ | $R_3$ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 1 | 2 | 4 |
| | | | | | *sec*$^{-1}$ | | | | | |
| **Cephapirin** | | | | | | | | | | |
| $1/T_{2\ free}$ | 8.40 | 7.83 | 6.31 | 6.73 | 5.96 | 7.55 | 4.44 | 5.84 | 5.68 | |
| $1/T_{2\ bound}$ | 268 | 329 | 270 | 122 | 96 | 52 | 98 | 277 | 71 | |
| $T_{2\ free}/T_{2\ bound}$ | 32 ± 3 | 42 ± 3 | 43 ± 4 | 18 ± 2 | 16 ± 2 | 7 ± 1 | 22 ± 3 | 47 ± 5 | 13 ± 2 | |
| **Cephradin** | | | | | | | | | | |
| $1/T_{2\ free}$ | 7.10 | 9.80 | | | 3.80 | 5.75 | 5.31 | | | |
| $1/T_{2\ bound}$ | 220 | 217 | | | 57 | 46 | 122 | | | |
| $T_{2\ free}/T_{2\ bound}$ | 31 ± 3 | 22 ± 2 | | | 15 ± 3 | 8 ± 1 | 23 ± 3 | | | |
| **Cefamandole** | | | | | | | | | | |
| $1/T_{2\ free}$ | 7.38 | 8.05 | 6.48 | 7.46 | 3.69 | 5.11 | 3.61 | 3.61 | | |
| $1/T_{2\ bound}$ | 208 | 165 | 163 | 195 | 183 | 74 | 170 | 104 | | |
| $T_{2\ free}/T_{2\ bound}$ | 28 ± 2 | 21 ± 1 | 25 ± 2 | 26 ± 2 | 50 ± 5 | 14 ± 1 | 47 ± 5 | 29 ± 3 | | |
| **Cefoxitin** | | | | | | | | | | |
| $1/T_{2\ free}$ | 6.28 | 7.66 | 6.28 | | 4.40 | 4.71 | | 4.32 | | 5.18 |
| $1/T_{2\ bound}$ | 284 | 127 | 106 | | 57 | 80 | | 397 | | 65 |
| $T_{2\ free}/T_{2\ bound}$ | 45 ± 3 | 17 ± 1 | 17 ± 2 | | 13 ± 2 | 17 ± 2 | | 92 ± 9 | | 11 ± 1 |
| **Cefotaxime** | | | | | | | | | | |
| $1/T_{2\ free}$ | 7.62 | 7.73 | 6.55 | 6.20 | 4.52 | 5.52 | 4.25 | 4.28 | 2.63 | |
| $1/T_{2\ bound}$ | 231 | 249 | 117 | 81 | 85 | 79 | 81 | 94 | 145 | |
| $T_{2\ free}/T_{2\ bound}$ | 30 ± 3 | 32 ± 3 | 18 ± 2 | 13 ± 2 | 19 ± 3 | 14 ± 2 | 19 ± 3 | 22 ± 4 | 55 ± 14 | |

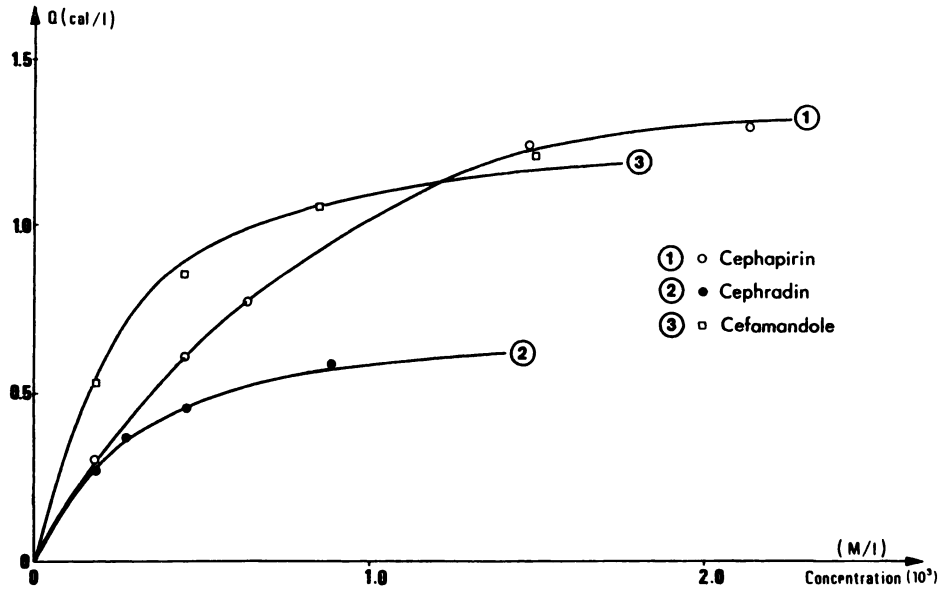



FIG. 6. *Microcalorimetric experimental heat measurements (Q) and theoretical curves for cephapirin, cephradin, and cefamandole* Theoretical curves are plotted according to the parameters of Table 4. $P_t = 1.2\ 10^{-4}$ M.

ent (cephapirin, cefoxitin, cefotaxime); (c) molecules with fairly strong binding with one class of protein sites, in which the heterocycle nucleus $R_2$ is certainly involved (cefamandole); and, finally, (d) a molecule (cefazolin) with two binding sites and the two heterocycles $R_2$ and $R_3$. Because there are two binding sites, a mean effect is observed. Moreover, if $\alpha_1$ and $\alpha_2$ are the bound fractions for the two classes of sites, then (14):

$$\frac{1}{T_{2\,\text{obs}}} = \frac{1}{T_{2\,\text{free}}} + \alpha_1\left(\frac{1}{T_{2\,\text{bound}_1}} - \frac{1}{T_{2\,\text{free}}}\right) + \alpha_2\left(\frac{1}{T_{2\,\text{bound}_2}} - \frac{1}{T_{2\,\text{free}}}\right)$$

$\alpha_1$ must be small (see above) and thus $\alpha_2$ is very small. This fact introduces important errors in the deduced $1/T_{2\,\text{bound}_2}$ value and therefore in the $1/T_{2\,\text{bound}_1}$ value. Therefore, this NMR method is not readily applicable to molecules with two classes of sites.

*Microcalorimetry.* All of these interactions are enthalpically driven with a negative $\Delta H$ value, but the negative $\Delta S$ value is thermodynamically unfavorable. The negative $\Delta H$ and $\Delta S$ values observed for the cephalosporin-HSA complex suggest that the interactions are dominated by electrostatic rather than by hydrophobic forces.

TABLE 4

*Thermodynamic parameters of formation of cephalosporin-HSA complex*

| | $K_a$ | $\Delta H$ | $\Delta G$ | $\Delta S$ |
|---|---|---|---|---|
| | $M^{-1}$ | *Kcal/M* | *Kcal/M* | *e.u.* |
| Cephapirin | 1,400 ± 80 | −7.4 ± 0.2 | −4.5 ± 0.1 | −9.3 ± 0.5 |
| Cephradin | 300 ± 30 | −6.6 ± 0.6 | −3.5 ± 0.1 | −9.8 ± 1.6 |
| Cefamandole | 8,800 ± 600 | −5.8 ± 0.8 | −5.6 ± 0.2 | −0.4 ± 2.0 |
| Cefazolin | | | | |
| $K_{a_1}$ | 20,000 | −12.25 | −6.1 | −19.9 |
| $K_{a_2}$ | 300 | −7.6 | −3.5 | −13.2 |

*Binding sites.* In the class of sites of high affinity (cefamandole and cefazolin) the interaction seems to take place close to the $R_3$ heterocycle, which is substituted by an electron-donor methyl group and which in space can be near the $H_6$ proton. These methylthiadiazole or methyltetrazole nuclei would probably bind to cationic sites of HSA by means of electrostatic forces.

In the case of binding sites of moderate affinity observed for cephapirin, cefoxitin, and cefazolin, the absence of an additional substituent like $NH_2$ or OH on the methylenic group of the acetamide part of the molecule seems to be important. A substituent would indeed induce, probably by "charge effect," a decrease in the interaction which takes place near the $R_3$ cycle; in particular, proton substitution by $NH_2$ would create an unfavorable positive charge at pH 7.4.

## CONCLUSION

The binding of cephalosporins to HSA is one of the major factors in their pharmacokinetics (3, 4), and our results show that it is possible to predict this binding by considering, first, the electron-rich heterocycle fixed on the methylene at position 3 and, second, the proton substituents of the acetamide chain.

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