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PROTEIN BINDING OF A SERIES OF RIFAMYCINS TO BOVINE SERUM ALBUMIN AS MEASURED BY THE "TWO-PHASE PARTITION" TECHNIQUE

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SUMMARY

A "two-phase partition technique" was developed and improved so as to be useful for the study of the interaction of proteins with low-molecular-weight ligands. The technique permits the macromolecule to be present almost entirely in one of the two aqueous phases without the aid of any physical solid interface, which is particularly useful in studies of lipophilic ligands. Compared with the other two-phase systems set up for binding studies, that used in this work takes advantage of the use of a more suitable medium, which permits a better protein partition even at very low ionic strengths. As an example of its usefulness, the measurement of the binding parameters of a series of rifamycins to bovine serum albumin is reported.

INTRODUCTION

For the determination of the binding parameters of low-molecular-weight ligands to macromolecules, the most commonly used methods (equilibrium dialysis, ultrafiltration or any techniques that utilize membranes) often cannot be applied to highly lipophilic ligands such as rifamycins, which are adsorbed on to and within the membranes and do not attain equilibrium. Instead, other methods such as gel filtration[1–4], spectrophotometric techniques[5] and two-phase equilibrium partition[6–8] have been demonstrated to be capable of yielding semi-quantitative or quantitative binding data.

In this work, the "two-phase system" consisting of poly(ethylene glycol)–dextran developed by Albertsson[9] was modified to make partition systems suitable for the study of the interaction of proteins with lipophilic ligands. As an example of its usefulness, the quantitative measurement of the binding parameters of a series of rifamycins to bovine serum albumin (BSA) is reported. The "two-phase partition method" was validated by calibration with sodium warfarin, a less lipophilic ligand, which could also be standardized by equilibrium dialysis.

THEORETICAL AND PRELIMINARY EXPERIMENTS

It is known that aqueous solutions of two different polymers may also separate into two phases[10–12]. In recent years, extensive use has been made of the "two-phase system" poly(ethylene glycol)–dextran (PEG–DEX), developed by Albertsson and Johansson as a separation technique for cell particles and macromolecules[9,13]. The system separates into two phases (the upper containing mostly PEG, the lower mostly DEX) with different negative charges, giving rise to an interface potential that permits the separation of macromolecules of different net charge[9].

Another characteristic of the system which directly influences the partition coefficient of hydrophobic low-molecular-weight molecules is the different polarities of the two phases, due principally to the different contents of hydrophobic groups ($-CH_2-$) in the two polymers (PEG 66%, DEX 8%).

However, whereas the difference in phase polarity is almost the only property that determines the partition of low-molecular-weight organic compounds, there are many factors that govern the partition behaviour of a macromolecule in the system: the relative concentrations of the two polymers, the nature of the partitioned macromolecule (size, conformation, isoelectric point), the ionic composition of the phases (pH, ionic strength, salts) and temperature[13].

Selection of the partition conditions

By taking into account the intrinsic properties of the PEG–DEX system, described by Albertsson[9], and the influence of some of the parameters known to affect the partition coefficients of BSA (K_{BSA}) between the two phases, we selected the experimental conditions most suitable for the protein partition, namely to confine albumin almost entirely in the lower phase.

BSA partition at different polymer concentrations. The partition coefficient of BSA was measured with various relative concentrations of the two polymers in an aqueous solution containing 0.02 *M* piperazine-N,N'-bis-(2-ethanesulphonic acid) sodium salt (PIPES) buffer, pH 7.4, at 22°. The results are summarized in Table I and indicate a decrease in K_{BSA} with increasing polymer concentration. Because of its lower value of K_{BSA} and its reasonable viscosity, the system containing 7% of PEG and 7% of DEX was selected for the further studies.

BSA partition in different buffer systems. The partition coefficient of BSA was

TABLE I

CHANGE OF K_{BSA} AS A FUNCTION OF POLYMER CONCENTRATION

PEG:DEX ratio (%, w/w)	K_{BSA}
6:6	0.262
6.5:6.5	0.193
7:7	0.093
7.5:7.5	0.042
6:7.5	0.163
6.5:7.5	0.132
7:7.5	0.093

measured for the system 7% PEG + 7% DEX in different buffer media, keeping the molarity (0.02), the pH (7.4) and the temperature (22°) constant.

The results in Table II show clearly that BSA has its lowest partition coefficient in the PIPES buffer. In fact, whereas the phosphate anion in the system has a partition coefficient of less than 1, both the anion ($R–SO_3^-$) of the PIPES buffer and the cation $[\overset{+}{H_2N}–C(CH_2OH)_3]$ of Tris, because of their organic nature, have partition coefficients above 1 (ref. 14). Consequently, while the phosphate and Tris ions would decrease the interface potential of the system, the PIPES ion will reinforce it, favouring the partition of BSA into the lower phase.

TABLE II

CHANGE IN K_{BSA} AS A FUNCTION OF THE BUFFER MEDIUM

Buffer (0.02 M, pH 7.4)	K_{BSA}
Tris–HCl	0.512
Sodium phosphate	0.826
PIPES (Na, K)	0.093

BSA partition at different buffer concentrations. Having selected the PIPES buffer as the best for decreasing the BSA partition coefficient, the influence of increasing molar concentrations on K_{BSA} was determined. As expected, while a considerable decrease in K_{BSA} is caused by initial small increases in buffer strength, higher concentrations do not affect the albumin partition coefficient more extensively (Fig. 1).

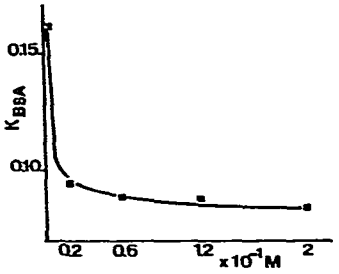

Fig. 1. Partition coefficient of BSA as a function of the molarity of the buffer.

BSA partition as a function of pH and temperature. As it is often useful, in order to predict the thermodynamic nature of a binding reaction between a low-molecular-weight ligand and a macromolecule, to measure the temperature and pH dependence of the interaction, K_{BSA} was determined as a function of these two parameters (Fig. 2).

As a consequence of the reduced dissociation of the albumin molecule, higher values of K_{BSA} were observed at lower pH; on the other hand, a temperature increase was shown to cause an increase in the K_{BSA}, which can be explained, according to the literature[15], by a reduced solubility of PEG.

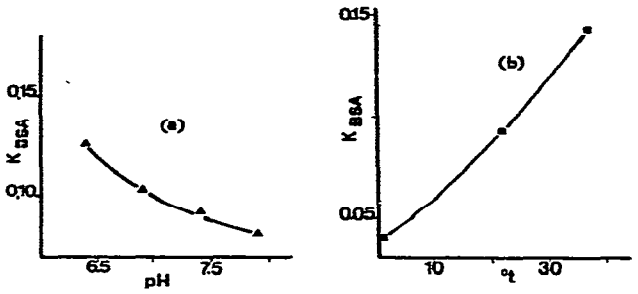

Fig. 2. Change in the partition coefficient of BSA as a function of (a) pH and (b) temperature.

Stated partition system. The preliminary investigations effectively demonstrated the possibility of utilizing the "two-phase partition technique" with the system PEG–DEX for the study of the interaction between rifamycins and BSA. The system adopted was the following: 7% PEG + 7% DEX in 0.04 *M* PIPES buffer, pH 7.4, containing 10^{-3} *M* ascorbic acid, at 22°, with $K_{BSA} = C^u_{BSA}/C^l_{BSA} = \approx 0.093 \pm 0.011$. The addition of ascorbic acid was necessary in order to maintain the rifamycins in their hydroquinonic form.

Mathematical solution

L and P are the ligand and protein concentrations. The superscripts u and l refer to the upper and lower phases and the subscripts f, b and t indicate the free, bound and total fractions of L or P, respectively.

As no changes in the phase volumes occur after addition of protein, the low-molecular-weight ligand partition coefficients in the absence (K_0) or presence (K_p) of the macromolecular component are defined as

$$K_0 = \frac{L^u_f}{L^l_f} \tag{1a}$$

and

$$K_p = \frac{L^u_f + L^u_b}{L^l_f + L^l_b} \tag{1b}$$

from which, assuming that K_{BSA} is not affected by the presence of the low-molecular-weight ligand, it is easy to derive the equation

$$\frac{L^l_b}{L^l_f} = \frac{K_0}{K_p}\left(1 + \frac{L^u_b}{L^u_f}\right) - 1 \tag{2}$$

From the Scatchard equation

$$\frac{L_b}{L_f} = K_a\,(nP_t - L_b) \tag{3}$$

from which

$$\frac{L_b^u}{L_f^u} = \frac{L_b^l(n\,P_t^u - L_b^u)}{L_f^l(n\,P_t^l - L_b^l)} \tag{4a}$$

or

$$\frac{(n\,P_t^u - L_b^u)}{(n\,P_t^l - L_b^l)} = \frac{L_b^u}{L_b^l\,K_0} \tag{4b}$$

where the ratio L_b^u/L_b^l is given by

$$\frac{L_b^u}{L_b^l} = \frac{n\,P_t^u}{n\,P_t^l} \cdot K_0 - \frac{L_b^u\,(K_0 - 1)}{n\,P_t^l} \tag{5}$$

Hence the L_b^u/L_b^l value will range between $(P_t^u/L_b^l)K_0$ and P_t^u/P_t^l as a function of the L_b^u value because, for $L_b^u \to 0$, $L_b^u/L_b^l = K_0(P_t^u/P_t^l)$ and, for $L_b^u \to n\,P_t$, $L_b^u/L_b^l \approx P_t^u/P_t^l$. As it is impossible to determine L_b^u in order to calculate the ratio L_b^u/L_b^l, we can use a relationship that approximates well to eqn. 5:

$$\frac{L_b^u}{L_b^l} = \frac{P_t^u}{P_t^l} \cdot (K_0 - K_p) \tag{6}$$

where, for $L_b^u \to 0$, $K_p \to 0$ and, for $L_b^u \to P_t^u$, $K_p \to K_0$. Therefore, from eqns. 2, 4 and 6, we obtain

$$\frac{L_b^l}{L_f^l} = \left(\frac{K_0}{K_p} - 1\right)\left(\frac{K_p P_t^l}{K_p P_t^l - (K_0 - K_p)P_t^u}\right) \tag{7}^*$$

Eqn. 7 permits the calculation of the ratio L_b^l/L_f^l in the lower phase where, with P_t^l known from the protein value and L_b^l calculated from the measured total ligand concentration in the same phase, it is possible to construct a plot L_b^l/L_f^l against L_b^l (Scatchard plot) for the determination of K_a and n.

Other workers, who previously developed a "two-phase partition system" for quantitative binding studies, in order to simplify calculations thought it preferable to consider, for all practical purposes, that the upper phase is free from binding proteins. However, it should be emphasized that the correction factors we introduced in calculations with eqn. 7 are small but not completely negligible, especially when the number of binding sites, n, is to be evaluated.

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* Eqn. 7, even though derived as an approximation, satisfies some of the requirements of the proposed partition model, *e.g.*, (i) for $K_p = K_0$, $L_b^l/L_f^l = 0$; (ii) when $P_t^u = P_t^l$, $K_p = K_0$ and L_b^l/L_f^l became indeterminable; (iii) when $P_t^u = 0$, $L_b^l/L_f^l = (K_0/K_p) - 1$, the same equation one derives when $L_b^u = 0$.

EXPERIMENTAL

Materials

[38-^{14}C]AF/AMP (rifampicin) (3-{[(4-methyl-1-piperazinyl)imino]methyl}rifamycin SV), specific activity 10.77 mCi/mmole, [38-^{14}C]AF/AETP (3-{[(4-ethyl-1-piperazinyl)imino]methyl}rifamycin SV), specific activity 15.29 mCi/mmole and [38-^{14}C]AF/ABDP (3-{[(4-*cis*-aminobenzyl-2,6-dimethylpiperazinyl)imino]methyl}rifamycin SV), specific activity 12.15 mCi/mmole, were used. The radiochemical syntheses were carried out by G. Sartori of Gruppo Lepetit, Milan, Italy. The same molecules, but unlabelled, were supplied by Dr. R. Cricchio of Gruppo Lepetit. The formulae of these compounds are given in Fig. 3.

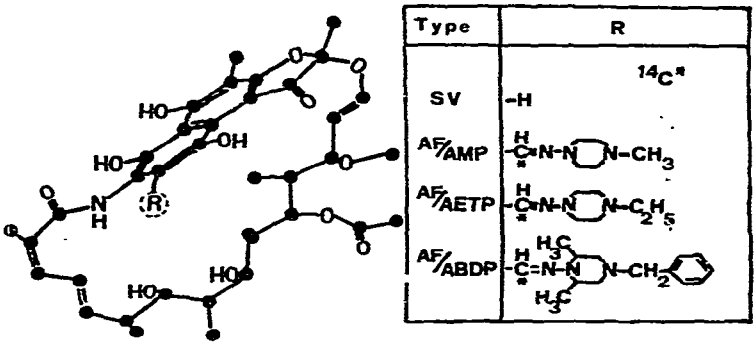


Fig. 3. Structures of rifamycins.

[^{14}C]-3-α-acetonyl[α-^{14}C]benzyl-4-hydroxycoumarin (warfarin), specific activity 23.15 mCi/mmole, was purchased from the Radiochemical Centre, Amersham, Great Britain. Unlabelled warfarin was obtained from Ferrosan-International, Malmö, Sweden. Piperazine-N,N′-bis-(2-ethanesulphonic acid) sodium salt (PIPES), grade A, was furnished by Calbiochem, Los Angeles, Calif., U.S.A., and adjusted to pH 7.4 with potassium hydroxide. Tris–HCl buffer, grade A, was purchased from Sigma, St. Louis, Mo., U.S.A., dextran T 500 (DEX) (molecular weight 500,000) from Pharmacia, Uppsala, Sweden, and poly(ethylene glycol) (PEG), molecular weight 6000, from Union Carbide, New York, N.Y., U.S.A. BSA, Cohn's fraction V, 96–99% electrophoretically pure, was purchased from Sigma. In all calculations a molecular weight of 67,000 for BSA was used.

Two-phase partition system

The partition systems were prepared in aqueous buffered solutions by dissolving weighed amounts of the two polymers. After equilibration, the two phases were separated by centrifugation for 5 min at 4000 *g*.

The partition experiments for the BSA were carried out with equal volumes of the two phases (2.5 ml), adding the protein to the lower phase and measuring the partition coefficient (K_{BSA}) spectrophotometrically at 280 nm. The protein-binding determinations, in the system 7% PEG + 7% DEX in 0.04 *M* PIPES buffer, pH 7.4, containing 10^{-3} *M* ascorbic acid, were performed by measuring radiochemically the

partition coefficients of the various rifamycins in the absence (K_0) or presence (K_p) of the macromolecular ligand. The K_0 values of the three rifamycin derivatives were as follows: AF/AMP, 2.58; AF/AETP, 2.61; and AF/ABDP, 4.02.

No changes in the phase volumes were observed after addition of protein. The binding experiments always required very short equilibration times (5–10 min of shaking), indicating that equilibrium was attained rapidly. Because of their low solubility in aqueous solutions, rifamycins were added to the partition system dissolved in 50 μl of methanol. This volume of solvent does not affect the binding reaction.

Equilibrium dialysis

A Dianorm multi-equilibrium dialysis system (20 cells) (Innovativ Medizin, Esslingen, Zürich, Switzerland) was employed[16]. The operating conditions of the PTFE "macro"-cells were: total volume of half-cell, 1.36 ml; dialyzing volume, 1.0 ml; and membrane surface area, 4.52 cm^2. Open Visking tubing, thickness 0.025 mm, was utilized after appropriate washing (with water, then 3:7 ethanol–water, water and buffer). The dialyses were performed at 22°.

Radioactivity measurements

Radioactivity measurements were effected by liquid scintillation counting with an Intertechnique SL 30 spectrometer, utilizing the scintillation cocktail Instagel (Packard, Downers Grove, Ill., U.S.A.).

Absorbances

Absorbance measurements were made on a Beckman Model DB-GT spectrophotometer.

Calculations

The binding parameters K_a and n were calculated according to the Scatchard equation[17]:

$$\frac{\text{Bound}}{\text{Free}} = K_a(n \cdot P_t - \text{bound})$$

were K_a is the association constant, n is the number of identical non-interacting binding sites per protein molecule and P_t is the protein concentration. K_{BSA} is defined as C^u_{BSA}/C^l_{BSA}, were C^u_{BSA} and C^l_{BSA} are the BSA concentrations in the upper and lower phases, respectively. The plots were constructed by the least-squares method.

The "combining affinity", C_{aff}, is given by

$$C_{aff} = \frac{\text{bound}}{\text{free}} \cdot \frac{1}{P_t}$$

The lipophilic indices, R_m, for the different rifamycins were determined by reversed-phase thin-layer chromatography as described by Pelizza *et al.*[18], according to Boyce and Milborrow[19] equation: $R_m = \log(1/R_F - 1)$.

RESULTS

Comparison of the "two-phase partition technique" with equilibrium dialysis

In order to evaluate the validity of the "two-phase partition technique" for the measurement of the binding parameters between low-molecular-weight ligands and macromolecules (BSA), a comparison was first made with equilibrium dialysis by studying the interaction between sodium warfarin and BSA. The results (Fig. 4) showed almost complete agreement between the two techniques and with the literature data[20]. The values of n as determined by either the "two-phase partition technique" or by equilibrium dialysis were fractional, but this is a common finding whether the low-molecular-weight ligand binds more than one site per protein molecule (*e.g.*, warfarin)[21]. It is a fact that when, *e.g.*, two binding sites are involved, the fractional value of n changes between 1 and 2 as a function of the experimental conditions used and of the nature of the two binding sites[22].

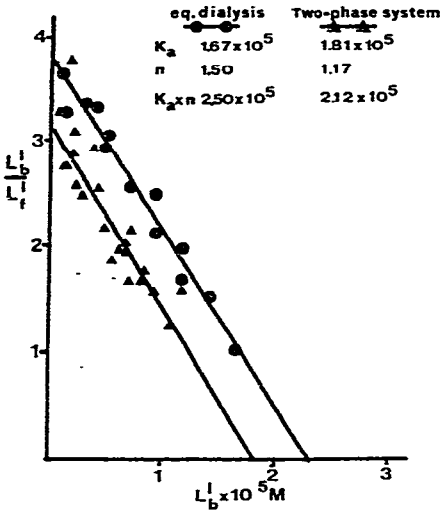
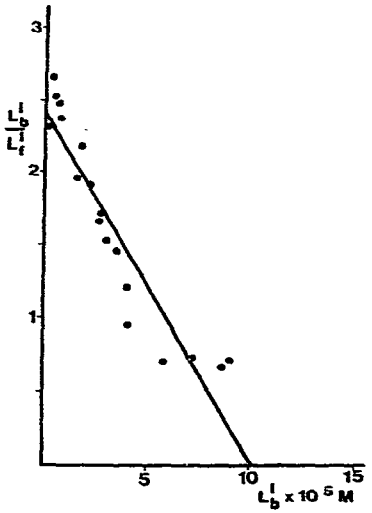


Fig. 4. Scatchard plots of the interaction between sodium [^{14}C]warfarin and BSA as measured by equilibrium dialysis and by "two-phase partition dialysis" (0.04 M PIPES, pH 7.4, 22°; albumin, $1.5 \cdot 10^{-5}$ M).

Measurement of the binding parameters of the complexes of rifamycins with BSA

The binding parameters K_a, n and ΔG for the complexes AF/AMP–BSA, and AF/AETP–BSA were determined by the "two-phase partition technique". The resulting Scatchard plots are shown in Figs. 5 and 6. The straight lines drawn in Fig. 5 and 6 do not fit completely the set of experimental points and the possibility of other less specific binding sites cannot be excluded. However, in order to study this hypothesis higher ligand concentrations must be tested, thus exaggerating the solubility difficulties.

Experimental difficulties were encountered in studying the interaction between AF/ABDP and BSA, owing to the very low solubility of the compound in the partition system (less than $5 \cdot 10^{-6}$ M). Therefore, in order to evaluate the extent of



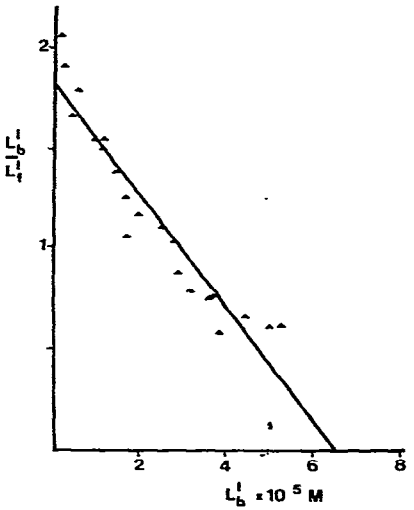


Fig. 5. Scatchard plot of the interaction between AF/AMP and BSA (1.37×10^{-4} *M*). Correlation coefficient = 0.938; $K_a = 2.35 \cdot 10^{-4}$ l/mole; $n = 0.75$.

Fig. 6. Scatchard plot of the interaction between AF/AETP and BSA ($0.92 \cdot 10^{-4}$ *M*). Correlation coefficient = 0.956; $K_a = 2.81 \cdot 10^{-4}$ l/mole; $n = 0.71$.

the interaction between AF/ABDP and BSA, a low concentration of the antibiotic (5 μg per 5 ml of the phase system) was used with increasing albumin molarity (from $5 \cdot 10^{-6}$ to $4 \cdot 10^{-5}$).

The results (Table III and Fig. 7) permit the binding reaction to be quantified in terms of "combining affinity", a parameter which, as $P_t \gg b$, corresponds with a good approximation to the product $K_a n$.

TABLE III

BINDING PARAMETERS FOR THE COMPLEX AF/ABDP–BSA

$P_t^l + P_t^u$ (*M*)	L_b^l/L_f^l	L_b^l (*M*)	L^l (*M*)	L^l/P_t^l
$5 \cdot 10^{-6}$	0.524	$0.22 \cdot 10^{-6}$	$0.2 \cdot 10^{-6}$	0.046
$1 \cdot 10^{-5}$	1.189	$0.34 \cdot 10^{-6}$	$0.29 \cdot 10^{-6}$	0.037
$2 \cdot 10^{-5}$	1.954	$0.46 \cdot 10^{-6}$	$0.23 \cdot 10^{-6}$	0.025
$3 \cdot 10^{-5}$	3.100	$0.72 \cdot 10^{-6}$	$0.23 \cdot 10^{-6}$	0.026
$4 \cdot 10^{-5}$	4.084	$0.74 \cdot 10^{-6}$	$0.18 \cdot 10^{-6}$	0.020
$6 \cdot 10^{-5}$	5.048	$0.80 \cdot 10^{-6}$	$0.16 \cdot 10^{-6}$	0.015
$8 \cdot 10^{-5}$	5.852	$0.75 \cdot 10^{-6}$	$0.13 \cdot 10^{-6}$	0.010

The binding parameters for the four rifamycins investigated are summarized in Table IV, which also includes their lipophilic indices in order to demonstrate the strict correlation between binding strength and the lipophilicity of rifamycins.

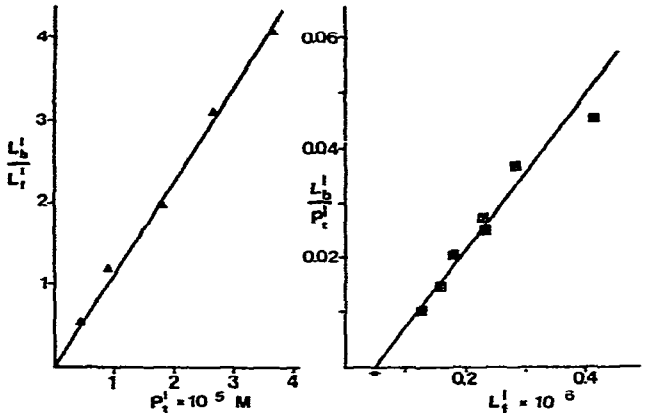


Fig. 7. Graphical estimation of the product $K_a\,n$ for the interaction between AF/ABPD and BSA. The data are those reported in Table III. The arrow indicates the concentration of the free ligand in the lower phase when no binding occurs.

TABLE IV

BINDING PARAMETERS FOR THE INTERACTION OF RIFAMYCINS WITH BSA, TOGETHER WITH THEIR LIPOPHILIC INDICES (R_m).

Rifamycin	K_a *(l/mole)*	n	$K_a\,n$ *(l/mole)*	$\Delta G = RT\,lnK_a \cdot n$ *(kcal/mole)*	R_m*
AF/AMP	$2.35 \cdot 10^4$	0.75	$1.75 \cdot 10^4$	5.727	−0.293
AF/AETP	$2.81 \cdot 10^4$	0.71	$2.0 \cdot 10^4$	5.805	−0.244
AF/ABDP	—	—	$11.11 \cdot 10^4$	6.510	0.340

* Data from Pelizza *et al.*[18].

DISCUSSION

The difficulties related to the study of the interactions between macromolecules and highly lipophilic low-molecular-weight ligands, *e.g.*, rifamycins, by traditional methods that utilize membranes, *i.e.*, equilibrium dialysis or ultrafiltration, have been demonstrated to be partially overcome by the "two-phase partition method" described here. The technique, which was developed on the model of Albertsson's two-phase system, limits the macromolecule almost totally to one of the two aqueous phases without the aid of any physical interface and can be treated theoretically as a normal dialysis, as the small errors in the calculations due to the non-quantitative partition of the macromolecule can be minimized mathematically.

Compared with the previous "two-phase partition system" described by Shaubhag *et al.*[7] and Von Schoultz *et al.*[6] for binding determinations, the partition system developed here has, in our opinion, an advantage in the use of PIPES buffer, at a low molar concentration, instead of 0.1 *M* KSCN in 0.05 *M* phosphate buffer, to concentrate the protein into the lower phase. On the other hand, an additional improvement to the system might be to use dextran 40 or 70 (see Von Schoultz *et al.*[6]) instead of dextran 500 in order to concentrate the protein even more into the lower phase.

The reliability of the system was compared with equilibrium dialysis for the interaction between [^{14}C]warfarin and BSA and almost complete agreement was found. Its application, which is particularly useful in studies of the binding of lipophilic ligands, has also been shown to be experimentally simple, requiring neither long equilibration times nor complicated instrumentation, giving reproducible results and being applicable under different experimental conditions.

There are two limitations to the method: the partition system must be adapted for each macromolecule to be studied, and the method cannot always be utilized with non-homogeneous macromolecular systems, even though examples of binding studies in such systems (plasma) by a "two-phase equilibrium partition technique" have been reported[6,7]. Its application, although of general use, should be reserved for instances when the traditional methods are not applicable.

With regard to the results, all of the rifamycins studied were demonstrated to interact with BSA, with association constants between 10^4 and 10^5 l/mole.

The number of binding sites per protein molecule measured for AF/AMP, and AF/AETP was close to but always less than 1, which may be tentatively explained by a partial dimerization of the BSA under the experimental conditions used, as its concentration was above 10^{-5} *M*. The results for AF/AMP and AF/ABDP were also in good agreement with previous work. In particular, the binding strength of AF/AMP to BSA had previously been determined by a gel filtration procedure[4] and that value agrees with the value calculated here. The $K_a n$ values calculated for the complex AF/ABDP–BSA agrees with the results of other workers[23] obtained by a modification of the spectrophotometric technique of Klotz.

Finally, it was observed that the binding affinity seems to be related to the lipophilicity of the ligand, which suggests a hydrophobic interaction. Additional thermodynamic investigations are needed in order to demonstrate whether this suggestion is correct.

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