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PROTEIN BINDING OF RIFAMPICIN TO BOVINE SERUM ALBUMIN AS MEASURED BY GEL FILTRATION

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SUMMARY

A "large zone–small zone elution method" with Sephadex G-25 has been developed for the study of the interactions of [^{14}C]rifampicin with bovine serum proteins. The method was demonstrated to be capable of yielding both qualitative and quantitative information about the binding reactions. However, its application requires a large amount of experimental work, and therefore it should be reserved for instances when other procedures are not applicable, *e.g.*, with highly lipophilic ligands.

INTRODUCTION

The use of gel filtration to measure the binding of low-molecular-weight ligands to macromolecular compounds has recently been reviewed by Ackers[1]. Four main procedures have been reported: the batch method and the Brumbaugh and Ackers method[2–4], which in principle are dialysis methods; the Hummel and Dreyer method[5], in which the gel column is equilibrated with a solution containing the ligand at a desired concentration and a small sample of a protein solution is than added to the column; and the Nichol and Winzor method[6,7], in which, to the column previously equilibrated with buffer containing neither the ligand nor the protein, a large sample of the protein–ligand mixture is applied, in sufficient volume to establish a series of plateaux. From the trailing plateau regions, the binding ratios of the ligand to the protein can be calculated.

There is, however, a fifth procedure, the "large zone–small zone elution method", which was introduced by Barlow *et al.*[8], who measured the change in elution volume of the a low-molecular-weight ligand brought about by developing with a solution of a completely excluded macromolecule. Clearly, while macromolecules that do not bind the ligand will not change the elution volume of the latter, those macromolecules which bind it very tightly will decrease the elution volume of the low-molecular-weight ligand down to the void volume of the column (Fig. 1).

The aim of this paper is to provide a theoretical outline for the calculation of the binding constants by the Barlow *et al.* gel filtration procedure, and to describe its application to the study of the binding of rifampicin (RAMP) to bovine serum proteins. As a control the method was validated by calibration with sodium warfarin, a less lipophilic ligand, which could also be standardized by equilibrium dialysis.

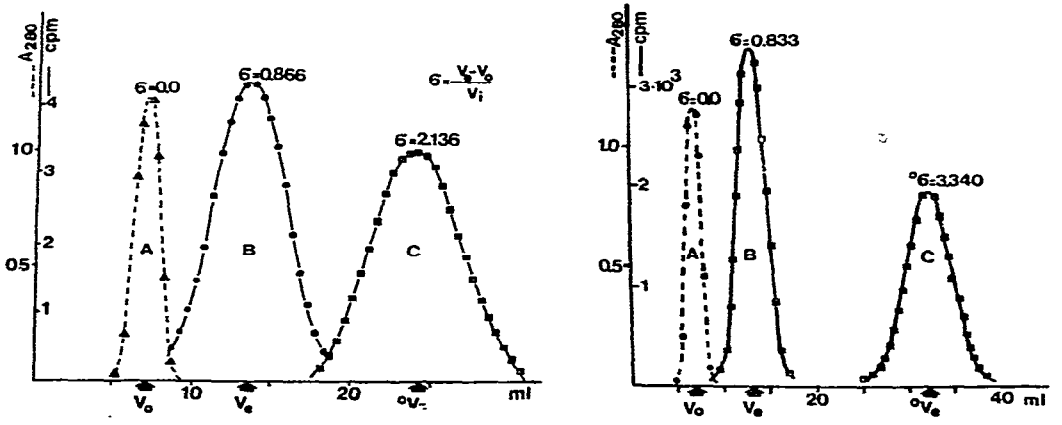


Fig. 1. Gel filtration on Sephadex G-25 (medium type) using 0.05 *M* sodium phosphate buffer, pH 7.4, at 22°. (A) BSA eluted with buffer (V_0); (B) sodium [^{14}C]warfarin and [^{14}C]RAMP eluted with buffer containing $1.5 \cdot 10^{-5}$ and $8.96 \cdot 10^{-5}$ *M* BSA, respectively (V_e); (C) sodium [^{14}C]warfarin and [^{14}C]RAMP eluted with buffer (^0V_e). Left: RAMP; right: warfarin.

THEORETICAL

Symbols

V_e, V_0 and V_i have their usual meanings and refer to the low-molecular-weight component. L and P are the molar concentrations of the low- and high-molecular-weight ligand, respectively. The macromolecular ligand is assumed to be totally confined in the mobile phase (*i.e.*, P^s and $^0L_b^s = 0$). Subscripts b, f and t refer to the bound, free and bound + free ligand, respectively. Subscript *ad* refers to the low-molecular-weight ligand not bound to the macromolecular ligand, which is reversibly retained on the stationary phase by a mechanism other than gel filtration or partition. The right-hand superscripts s and m refer to the stationary and mobile phases, respectively and the left-hand superscript zero refers to experiments carried out in the absence of a macromolecular ligand.

The chromatographic partition coefficients of the low-molecular-weight ligands are defined as follows:

$$^0\sigma = \frac{^0V_e - V_0}{V_i} = \frac{^0L_f^s + {}^0L_{ad}^s}{^0L_f^m} \qquad \text{(1a)}$$

$$\sigma = \frac{V_e - V_0}{V_i} = \frac{L_f^s + L_{ad}^s}{L_f^m + L_b^m} \qquad \text{(1b)}$$

(V_0 and V_t are constants intrinsically related to the column and are thus invariant throughout). The association constant, K_a, and the "combining affinity", C, between macromolecular and low-molecular weight ligands are defined as

$$K_a = \frac{L_b}{nL_fP_f} \tag{2a}$$

and

$$C = \frac{L_b}{L_fP_t} \tag{2b}$$

where n is the number of identical, non-interacting binding sites in the macromolecular ligand, each of them binding a single molecule of the low-molecular weight ligand.

Assumptions and the equations derived

We assume that: (i) the chromatographic (gel filtration, partition or "adsorption") isotherms are linear (*i.e.*, ${}^0L^s_{ad}/{}^0L^m_f = L^s_{ad}/L^m_f$ and ${}^0L^s_f/{}^0L^m_f = L^s_f/L^m_f$); (ii) the heights equivalent to a theoretical plate (HETP) are small and the interaction between low-molecular weight and macromolecular ligands is rapid (with respect to the chromatographic flow-rate), so that the system can be considered to be close to equilibrium in chromatographic experiments also; (iii) the macromolecule is totally confined in the mobile phase; (iv) the total concentrations of the two ligands do not change in the course of the experiment. It can be shown easily that

$$\frac{{}^0V_e - V_0}{V_e - V_0} - 1 = \frac{{}^0\sigma}{\sigma} - 1 = \frac{L^m_b}{L^m_f} = K_an\,(P^m_t - P^m_b) = CP^m_t \tag{3}$$

This equation can be used in the evaluation of batch experiments, in which all of the above assumptions hold; the L^m_b/L^m_f ratios as obtained from the values (*cf.* eqn. 1) at constant P_t but at variable L_t are plotted against the corresponding L^m_b values[9] (the magnitude of the latter is easily obtained by calculation, as L_t is known). From these Scatchard plots, one obtains both K_a and n in the usual way. Naturally, the L^m_b/L^m_f ratios yield directly the values of C, valid for the corresponding P_t values (*cf.* eqn. 3). In column operation, assumption (iv) above cannot be generally assumed to hold true, because in "small zone" experiments both P_t and L_t do change continuously during development. An exception is the so called "large zone" experiments, *i.e.*, when a large sample volume containing the low-molecular-weight ligand, with or without the macromolecular ligand, is run through the column and yields an emerging zone with a plateau region having both L_t and P_t values identical with those of the sample applied. The V_e and ${}^0V_e$ values are calculated from the mid-points of the front boundaries of the zone. The L^m_b/L^m_f ratios (obtained at constant P_t but at different L_t values) are easy to plot against the corresponding L^m_b values as their L_t values are known and remain constant in this area of the chromatogram. Again, eqn. 3 yields the CP_t values directly. Alternatively, the concentrations at the plateau in the trailing area of the chromatogram (which arise in the presence of the macromolecular ligand) enable the binding parameters to be calculated[6,7].

The "large zone–small zone elution method" (in which a small sample of

low-molecular-weight ligand is eluted with a solution of known and constant P_t) offers at least the following means of circumventing the limitations of assumption (iv). It is apparent from eqn. 3 that when $P_t^m \gg P_b^m$, and when P_t^m is known, the chromatographic data allow calculation of the product $K_a n$: a first plot of $([^0\sigma/\sigma] - 1)/P_t^m$ *versus* $1/P_t^m$ gives the $^0\sigma/\sigma - 1$ values at infinite P_t^m on the ordinate for a given load of low-molecular-weight ligand. A secondary plot of these intercepts *versus* the loads of low-molecular-weight ligand yields the product $K_a n$ for an extrapolated value of zero load.

Clearly, this procedure, in addition to requiring a large amount of experimental work, does not permit the calculation of K_a and n individually. It could be of some use, however, in these instances in which other procedures may, for some reason, not be applicable, if the macromolecular ligand is available in large amounts. The "large zone–small zone elution method" can also be applied to systems that have a low binding affinity.

EXPERIMENTAL

Materials

[38-^{14}C]Rifampicin (hydroquinone form) of specific activity 0.679 mCi/mmole was kindly supplied by Dr. R. White of Lepetit Laboratories, Milan, Italy. [^{14}C]-3-α-acetonyl[α-^{14}C]benzyl-4-hydroxycoumarin (warfarin) of specific activity 23.15 mCi/mmole, was purchased from the Radiochemical Centre, Amersham, Great Britain.

Bovine serum was furnished by Istituto Sieroterapico Milanese, Milan, Italy. Cohn's fractions I + III, II, IV and V were prepared from the same serum following the original method[10], and their purity was controlled electrophoretically. Bovine serum albumin (BSA) (Cohn's fraction V), 98% electrophoretically pure, was obtained from Armour Pharmaceutical Co., Chicago, Ill., U.S.A.

An Intertechnique SL 30 liquid scintillation spectrometer was used for radioactivity counting, with Instagel (Packard, Downers Grove, Ill., U.S.A.) as the scintillation cocktail.

Gel filtration

(A) Batch experiments. For each test sample, 0.5 g of Sephadex G-25 (medium type, 50–150 μm), weighed in a stoppered tube, was allowed to swell in 2.5 ml of 0.05 *M* phosphate buffer, pH 7.4, containing 10^{-3} *M* ascorbic acid (to maintain RAMP in the hydroquinone form), $1.5 \cdot 10^{-4}$ *M* albumin (molecular weight 67,000) and varying RAMP concentrations. The binding equilibrium was reached after vigorous shaking for 5 min. After sedimentation of the gel, the ligand concentration in the void volume (V_0) could be measured directly, while that in the inner volume (V_i) was calculated from the difference. For the calculation, the following constants were determined: $V_0 = 1.182$ ml, $V_i = 1.31$ ml and water regain (W_r) = 2.63 ml/g.

(B) Column experiments. Sephadex G-25 (medium type, 50–150 μm) swollen in 0.05 *M* sodium phosphate buffer, pH 7.4, with or without 10^{-3} *M* ascorbic acid, was packed in an LKB glass column (30 × 0.9 cm) after elimination of the fines. First the partition coefficients of the low-molecular-weight ligands were determined in the absence of the macromolecular component, then the column was pre-equilibrated with protein-containing buffer and was loaded with the ligand, dissolved in 0.3 ml of

the same solution containing 0.025 ml of ethanol. Elution was performed with the same buffered protein solution, and 0.5-ml fractions were collected.

An ISCO Model 312 micro-metering pump and a Gilson Model TDC 80 fraction collector were used as ancillary equipment.

Equilibrium dialysis

A Dianorm multi-equilibrium dialysis system (20 cells), (Innovativ Medizin, Esslingen, Zürich, Switzerland) was employed[11]. The operating conditions of the PTFE "macro"-cells were: total volume of half cell, 1.36 ml; dialyzing volume (V) = 1 ml; membrane surface area (A) = 4.52 cm^2; Q factor = A/V = 4.52.

The cells were gently rotated (20 rpm) about an axis perpendicular to the membrane in order to prevent denaturation and to help the equilibrium to be attained rapidly. Open Visking tubing, thickness 0.025 mm, was utilized after appropriate washing (with water, then 3:7 ethanol–water, water and 0.05 M sodium phosphate buffer, pH 7.4). The dialyses were performed at 22°.

RESULTS

Comparison of the "large zone–small zone elution method" with equilibrium dialysis

The method was compared with equilibrium dialysis by studying the interaction between warfarin and BSA. Under the conditions specified elsewhere[12], the product $K_a n$ for this compound is found to be $2.50 \cdot 10^5$ l/mole by equilibrium dialysis or $2.12 \cdot 10^5$ l/mole by the two-phase partition technique. At the same BSA concentration and at warfarin concentrations less than that of BSA (when $(P_t^m - P_b^m) \approx P_t^m$), the product $K_a n$ as determined by the "large zone–small zone elution method" was of $2.01 \cdot 10^5$ l/mole, in agreement with the values obtained by the other two techniques.

Test of linearity of isotherms and of equilibrium conditions

In the system studied in this work, assumption (iii) is known to be valid, serum protein being totally confined in the Sephadex G-25 mobile phase. The difficulties associated with L_t changing continuously during the chromatographic development [*i.e.*, assumption (iv)] were circumvented in the "large zone–small zone elution method" in the way described in the previous paragraph, *i.e.*, by extrapolating to $P_t^m \to \infty$ and to zero load of the low-molecular-weight ligand.

Concerning assumptions (i) and (ii) (linearity of isotherms and quasi-equilibrium conditions), convex isotherms or non-equilibrium conditions, or a combination of both, produce tailing zones. In fact, the RAMP zones obtained in the presence or absence of serum proteins were symmetrical and gaussian (Fig. 2). Specifically, the inflection point occurs at 0.607 of the peak height (h), where the peak width is 2 d (d = standard deviation); the width at 0.5 of the peak height corresponds to 2.354 d; the peak width at the baseline (w) has a value of 4 d.

Gaussian peaks were obtained with different ligands or different albumin concentrations. In addition, the number of theoretical plates was calculated along the respective HETP values, which were in fact found to be small.

In principle, convex isotherms plus non-equilibrium conditions produce opposite and thus potentially compensating effects on the shape of the zone. However,

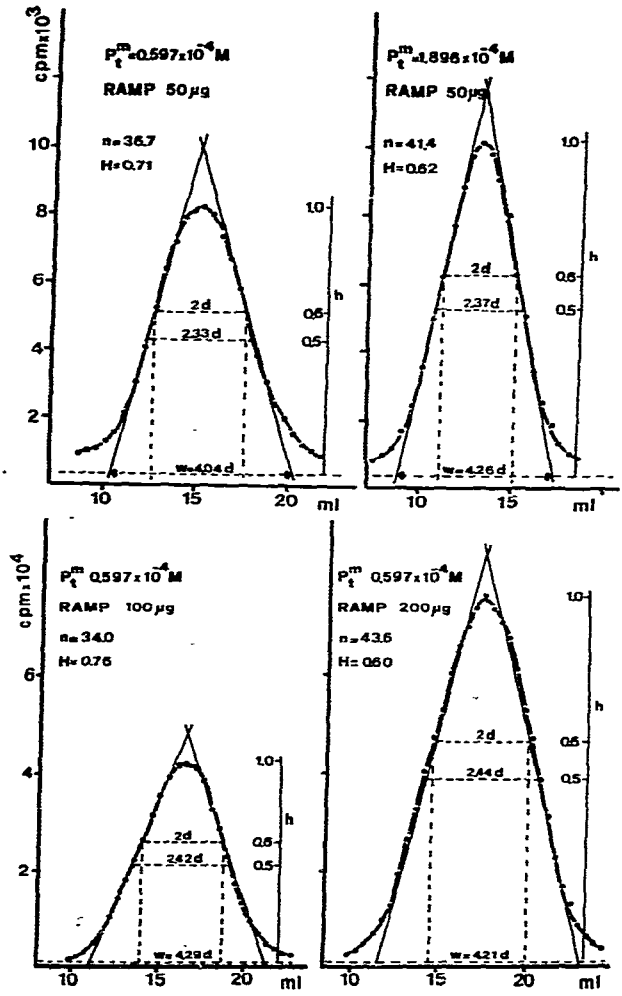
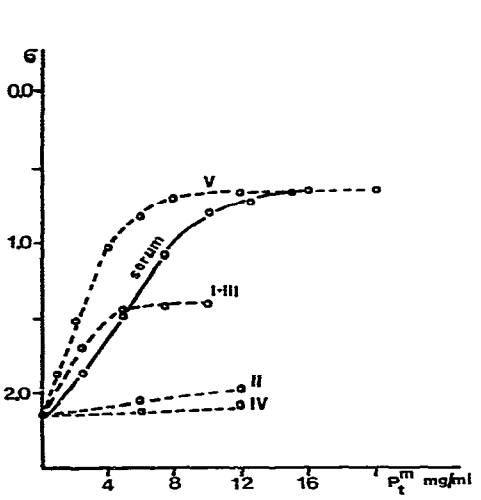


Fig. 2. "Large zone–small zone" experiments: graphical analyses of some elution curves of [^{14}C]-RAMP. The protein (BSA) concentrations and the RAMP loads are indicated, H is the HETP value (cm), n is the number of theoretical plates, h refers to the fraction of the peak height, d is the standard deviation and w is the peak width at the baseline.

it is most unlikely that the symmetry and the gaussian character of the zones in Fig. 2 could have arisen from combined compensating violations of assumptions (i) and (ii), because (a) identical symmetrical zones were obtained at widely different flow-rates and ligand concentrations and (b) the $K_a n$ values obtained by various methods were in reasonably good agreement (see below).

We conclude, therefore, that under the experimental conditions used, both assumptions (i) and (ii) held, which was of practical value as RAMP is easily adsorbed on to column materials. Thus, if it was adsorbed on to Sephadex G-25 at the concentrations used here ($^0\sigma > 1$) it did not interfere in the determinations of K_a or of $K_a n$, as adsorption with a linear isotherm was included when deriving eqn. 3.



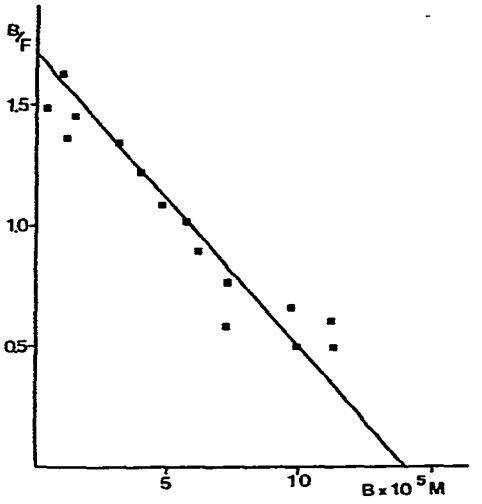


Fig. 3. Changes in the chromatographic partition coefficient of [^{14}C]RAMP (σ) with different protein concentrations of whole serum, Cohn fractions V (albumin), II (γ-globulins), I + III and IV. Eluent: 0.05 *M* sodium phosphate buffer, pH 7.4, containing 10^{-3} *M* ascorbic acid, 22°.

Fig. 4. Batch experiments: Scatchard plot of the interaction between [^{14}C]RAMP and BSA ($1.5 \cdot 10^{-4}$ *M*). *B* and *F*, concentrations of bound and free RAMP, respectively. The plot was constructed by the least-squares method; correlation coefficient = 0.923.

Identification of the serum fraction complexing with rifampicin

A preliminary investigation was carried out to identify the fraction of the bovine serum proteins involved in the interaction with RAMP. For this purpose, total serum and Cohn's fractions I + III, II, IV and V were tested for their ability to decrease the partition coefficients of RAMP in gel filtration experiments.

As illustrated in Fig. 3, the results indicate that fraction V (albumin) is the main component binding RAMP, although fraction I + III (mainly α- and β-globulins) also showed some affinity.

The small changes in the partition coefficients of RAMP observed with fractions II and IV were tentatively attributed to albumin contamination. The saturation phenomena observed at high protein concentrations has already been described by Barlow *et al.*[8] for all of the ligands investigated.

The mechanism of this phenomenon is not clear. A possible hyphothesis may be an interference on the primary site by low-affinity binding sites occurring at high protein concentration.

Measure of the association constant of the albumin–rifampicin complex

Gel filtration. (A) Batch experiments were carried out using $1.5 \cdot 10^{-4}$ *M* albumin and RAMP concentrations varying between $1.21 \cdot 10^{-5}$ and $10.90 \cdot 10^{-4}$ *M*. From the ratio L_b/L_f, the bound fraction could be derived arithmetically from the total values, and the Scatchard plot fitted as shown in Fig. 4.

(B) Column experiments were performed as described under Experimental by eluting four different amounts of the antibiotic in the presence of four different al-

TABLE I
GEL FILTRATION: COLUMN EXPERIMENTS
$V_0 = 7.0$ ml; $V_t = 7.79$ ml; ${}^0V_e = 23.64$ ml; ${}^0\sigma = 2.136$.

P_t^m (BSA)	RAMP load (μg)	V_e (ml)*	σ	L_b^m / L_f^m	$\frac{{}^0\sigma}{\sigma} - 1/P_t^m$ (mole^{-1})	Primary plot: intercept values for $P_t \to \infty$ (mole^{-1})
2 mg/ml						
(0.298 × 10^{-4} M)	50	18.58	1.486	0.436	$1.436 \cdot 10^4$	$0.800 \cdot 10^4$
	100	19.50	1.605	0.330	$1.107 \cdot 10^4$	
	200	20.00	1.669	0.279	$0.936 \cdot 10^4$	
4 mg/ml						
(0.597 × 10^{-4} M)	50	15.50	1.091	0.956	$1.601 \cdot 10^4$	$1.210 \cdot 10^4$
	100	16.32	1.196	0.784	$1.313 \cdot 10^4$	
	200	17.42	1.337	0.596	$0.999 \cdot 10^4$	
	400	18.25	1.443	0.479	$0.802 \cdot 10^4$	
6 mg/ml						
(0.896 × 10^{-4} M)	50	13.75	0.866	1.464	$1.634 \cdot 10^4$	$1.505 \cdot 10^4$
	100	14.50	0.963	1.216	$1.357 \cdot 10^4$	
	200	15.50	1.091	0.956	$1.067 \cdot 10^4$	
	400	16.70	1.245	0.714	$0.797 \cdot 10^4$	
8 mg/ml						
(1.194 × 10^{-4} M)	50	12.50	0.706	2.023	$1.694 \cdot 10^4$	$1.750 \cdot 10^4$
	100	13.20	0.796	1.681	$1.408 \cdot 10^4$	
	200	14.25	0.931	1.292	$1.082 \cdot 10^4$	
	400	15.50	1.091	0.956	$0.801 \cdot 10^4$	

* Average of two experiments.

bumin concentrations ("large zone–small zone elution method"). From the column constants (V_0, V_t) and the experimental data (V_e, ${}^0V_e$), it was possible to derive from eqn. 3 and by means of the above plots the $K_a n$ value for the RAMP–albumin complex (Table I and Fig. 5)*.

Both gel filtration methods indicate that RAMP interacts with BSA with the following weak association constants, as determined under the experimental conditions used: in batch experiments, $K_a = 1.21 \cdot 10^4$ l/mole and in column experiments $K_a n = 1.92 \cdot 10^4$ l/mole. Not more than one binding site ($n = 0.93$) per albumin molecule could be demonstrated in batch experiments although, on the basis of our results only, the presence of other weaker binding site(s) cannot be excluded. The K_a values obtained with the two methods are in satisfactory agreement.

Equilibrium dialysis. Some difficulties were encountered in the dialysis experiments. In effect, the high lipophilicity of the ligand molecule, in addition to favouring the adsorption of RAMP on the dialyzing membrane (5–7%), induces the antibiotic to form aggregates in aqueous solutions in the absence of proteins. For these reasons,

* The saturation phenomena in Fig. 5, which are not predicted by eqn. 3, were not investigated further. It is possible that at high protein concentrations anomalies in the interaction between BSA and RAMP may occur, resulting in a decreased binding affinity.

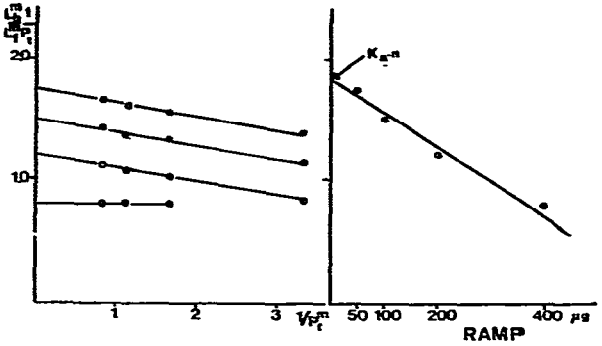


Fig. 5. Calculation of the product $K_a n$ from "large zone–small zone" elution experiments. Left: primary plot of $L_b^m/(L_f^m P_t)$, that is $([^0\sigma/\sigma] - 1)/P_t^m$, *versus* $1/P_t^m$. Right: secondary plot of the intercepts of the primary plots on the abscissa *versus* the amount of RAMP applied.

in spite of the low molecular weight of RAMP, a long dialysis time is required in order to reach equilibrium (7–8 h), and the addition of N,N-dimethylformamide (0.5%, v/v) was necessary in order to prevent aggregation, in spite of its partial inhibition of RAMP–albumin complex formation. Moreover, the range of ligand concentrations selected in the dialysis experiments was necessarily limited by the low specific activity of [^{14}C]RAMP available and the difficulties in deriving from the aforementioned phenomena. All of these limitations make the value of the association constant obtained by fitting the dialysis data reported in Fig. 6 uncertain. Nevertheless, the values obtained by dialysis ($K_a \approx 0.7 \cdot 10^4$ l/mole, $n = 0.83$) are lower but still in fair agreement with those obtained by the gel filtration methods.

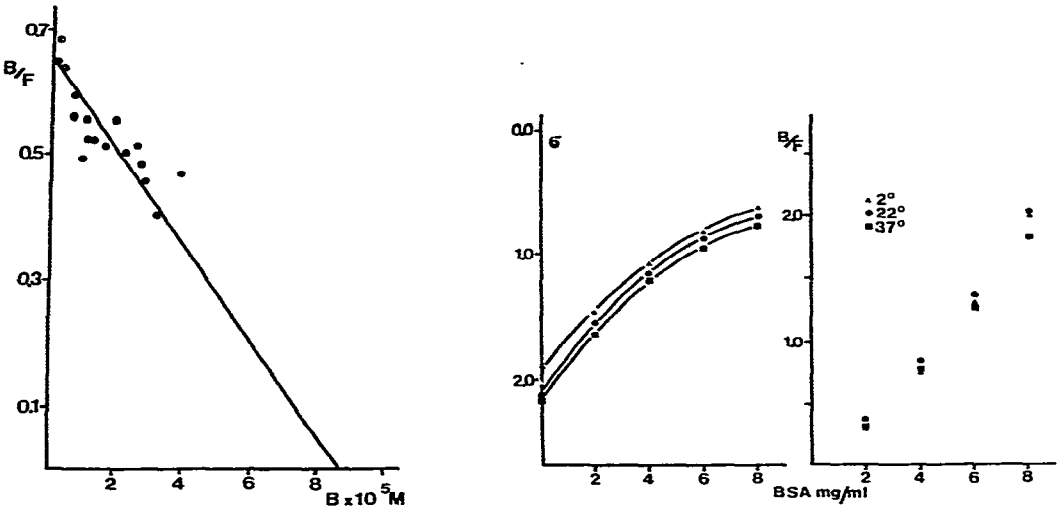


Fig. 6. Scatchard plot of the interaction between [^{14}C]RAMP and BSA as derived from equilibrium dialysis experiments. BSA concentration = 10^{-4} *M*. The plot was constructed by the least-squares method; correlation coefficient = 0.767.

Fig. 7. Variation of the RAMP chromatographic partition coefficient (σ) and of the bound/free ratio of RAMP with BSA at 2°, 22° and 37°.

Two-phase partition technique. As reported by Assandri and Moro[12], this method yielded values of the binding parameters of $K_a = 1.75 \cdot 10^4$ l/mole and $n = 0.75$ for the RAMP–BSA complex, which again are in satisfactory agreement with the methods used in this work.

Temperature dependence

Fig. 7 reports the change of σ and of the bound/free ratio by the presence of BSA at 2°, 22° and 37°; no substantial differences were observed under the different conditions.

DISCUSSION

The binding of RAMP to plasma proteins has been the subject of several papers and reviews[12–20]. Most of the data reported were qualitative or semi-quantitative, undoubtedly owing to the unfavourable physico-chemical characteristics of RAMP. This hydrophobic substance is poorly soluble in water, tends to aggregate and to adsorb at and within common dialysis membranes (*e.g.*, Visking and nitrocellulose membranes). These characteristics, however, make RAMP a useful test substance for new procedures to determine even moderately weak binding to macromolecular water-soluble ligands, such as the "large zone–small zone elution method" (this work) or the "two-phase partition technique"[12]. The reliability of these two methods has been verified for a water-soluble low-molecular-weight ligand (sodium warfarin) in comparison with equilibrium dialysis.

RAMP is adsorbed on to the gel matrix used here (Sephadex G-25), its partition coefficient in the absence of the macromolecular ligand ($^0\sigma$) being 2.136. However, the isotherms are linear (Fig. 2) and adsorption with linear isotherms was included in the derivation of eqn. 3. Hence the K_a or K_an values obtained by both the batch method and the "large zone–small zone elution method" are not affected by adsorption on to the gel. As pointed out under Results, the determinations of the binding parameters by the two gel filtration methods and by the "two-phase partition technique" were in satisfactory agreement.

The "large zone–small zone elution method" may also be useful in the fractionation of low-molecular-weight ligands on the basis of their different binding affinities to a macromolecular ligand. Fig. 4 shows that of the serum fractions tested, only fraction V (albumin) and I + III affect the chromatographic behaviour of RAMP. In addition, if the data obtained with total serum are re-plotted against the albumin concentration in the total serum, the same line is obtained as with BSA alone.

We conclude, therefore, that albumin is the major component in bovine serum capable of binding RAMP. The stoichiometry of binding is 1:1 (by the batch method as well as by the "two-phase partition technique"). The association constant is $1.2 \cdot 10^4$ or $1.9 \cdot 10^4$ l/mole (see Results) or $1.75 \cdot 10^4$ l/mole ("two-phase partition technique"). The presence of additional still weaker binding site(s) for RAMP in BSA cannot be ruled out. Little is known at present of the nature of the interaction between BSA and RAMP, except that it shows little or no temperature dependence (Fig. 7).

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