CHROM. 12,881

# PROTEIN–PROTEIN INTERACTIONS STUDIED BY COUNTER-CURRENT DISTRIBUTION

## II. TEST OF THE THEORETICAL MODEL USING BOVINE SERUM ALBUMIN AND L-TRYPTOPHAN

LARS BACKMAN

*Department of Biochemistry, University of Umeå, S-901 87 Umeå (Sweden)*

(Received April 10th, 1980)

## SUMMARY

The well known association of L-tryptophan with bovine serum albumin has been studied using counter-current distribution in an aqueous–aqueous biphasic system. Analysis of the counter-current distribution patterns according to the previously developed model demonstrates the capabilities of the technique for the study of interactions between two different molecules. A general procedure for the examination of experimental results in order both to detect and to quantify a plausible interaction between two substances is described.

## INTRODUCTION

Both single-step partitions[1,2] and counter-current distribution (CCD)[3,4] in aqueous–aqueous biphasic systems have been used successfully for the study of interactions between different proteins. Theoretical models were therefore developed for the description of the CCD behaviour of interacting systems of the type $A + B \rightleftharpoons AB$[5]. The theoretical models can also describe single-step partitions, as in practice these are a special case of CCD.

In this paper, the intention is to examine and demonstrate the experimental capabilities of the CCD technique (and single-step partitions) for the study of interactions between two different molecules.

Analysis of the CCD patterns of a well known interacting system would reveal if the theoretical models developed are plausible. For this purpose, bovine serum albumin (BSA) and L-tryptophan (L-Trp) were used as the association of L-Trp with BSA has been well characterized by different methods and is known to be on a one-to-one molar basis[6–13].

A general procedure for the analysis of experimental CCD results to obtain partition coefficients (or ratios) of distributed substances, and for detecting and quantifying a conceivable interaction, is also described.

## EXPERIMENTAL

All chemicals were of analytical-reagent grade, unless specified otherwise.

Fatty acid-free bovine serum albumin, fraction V, was obtained from Miles Labs. (Slough, Great Britain), and L-tryptophan and L-[G-³H]tryptophan from Sigma (St Louis, MO, U.S.A.) and The Radiochemical Centre (Amersham, Great Britain), respectively. Picoflour³⁰ was obtained from Packard Instruments (Band-hagen, Sweden).

The polymers used for making the aqueous–aqueous biphasic system were Dextran T500 with $M_w = 5 \cdot 10^5$ (Pharmacia, Uppsala, Sweden) and poly(ethylene glycol) (PEG) with $M_n = 6000$ (Union Carbide, New York, NY, U.S.A.).

The aqueous–aqueous biphasic system contained 8% (w/w) of Dextran T500, 6% (w/w) of PEG and 25 mmol/kg of potassium phosphate buffer (pH 7.0). CCD with 29 transfers was carried out in the thin-layer CCD machine described by Alberts-son[14]. Each chamber contained 0.74 ml of both upper and lower phase. At the beginning of each experiment chamber zero contained these two phases and BSA and/or L-Trp. The shaking time after each transfer was 30 sec and the time allowed for separation between successive transfers was 5 min. All CCD experiments were carried out at 21 ± 1°C. After the experiment was completed, 1.48 ml of water were added to each chamber to give homogeneous solutions. The contents of each cham-ber were then collected and analysed.

The concentration of BSA was determined by measuring the absorbance at 280 nm, using a molar absorptivity of $4.4 \cdot 10^4$ l mol⁻¹cm⁻¹ [15]. The concentration of L-Trp was determined by measuring the absorbance at 280 nm using a molar absorptivity of $5.01 \cdot 10^3$ l mol⁻¹ cm⁻¹ when L-Trp was distributed separately.

Liquid scintillation counting was used to determine the concentration of L-Trp when BSA was present. The samples were prepared by adding 0.2 ml of the contents of each chamber to 5 ml of Picoflour³⁰.

All computations were performed on a Hewlett-Packard 9835A desk-top computer.

### Computations

If a homogeneous substance, $S_c$, is distributed by CCD, the fraction of material, $S_{i,n}$, and the total concentration, $[S_c]^{i,n}$, in the *i*th chamber after *n* transfers are given by eqns. 1 and 2, respectively, assuming that the substance is initially in-troduced only to chamber zero.

$$S_{i,n} = \binom{n}{i} \frac{G_c^i}{(1 + G_c)^n} \tag{1}$$

$$[S_c]_{cal}^{i,n} = [S_c]^{0,0} \binom{n}{i} \frac{G_c^i}{(1 + G_c)^n} \tag{2}$$

where $[S_c]^{0,0}$ is the initial concentration in the zeroth chamber and $G_c$ is the partition ratio, defined as

$$G_c = \frac{S_u}{S_l} \tag{3}$$

where $S_u$ and $S_l$ are the fraction of S material in the upper and lower phase, respectively.

The partition coefficient, $K_c$, defined as the ratio between the concentrations of the substance in the upper and lower phase, and the partition ratio are related by

$$G_c = K_c \cdot \frac{V_u}{V_l} \qquad (4)$$

in which $V_u$ and $V_l$ are the volumes of the upper and lower phase, respectively.

All subsequent equations have been derived for the case when the volume of the upper phase equals that of the lower phase, i.e., when $G_c = K_c$.

When a substance is distributed alone, its partition coefficient can be determined, either by comparing the concentration in two consecutive chambers, $i$ and $i + 1$, since

$$K_c = \frac{[S_c]_{exp}^{i+1,n}}{[S_c]_{exp}^{i,n}} \cdot \frac{i+1}{n-i} \qquad (5)$$

or by minimizing

$$U_1 = \sum_{i=0}^{n} ([S_c]_{exp}^{i,n} - [S_c]_{cal}^{i,n})^2 \, \omega_{c,i} \qquad (6)$$

where $[S_c]_{exp}^{i,n}$ and $[S_c]_{cal}^{i,n}$ are the experimental and calculated (according to eqn. 2) concentrations of $S_c$ in the $i$th chamber, respectively.

Examination of several CCD curves showed that in areas form the peak maximum all experimental curves differed from the model (eqn. 2). This discrepancy was interpreted as being due to both imperfections in the model and to low precision of the concentration measurements in these areas. To compensate for this non-ideal behaviour a weighted least-squares criterion was used, with the weights defined by

$$\omega_{c,i} = \frac{[S_c]_{exp}^{i,n}}{[S_c]_{exp}^{max}} + 1 \qquad (7)$$

in which $[S_c]_{exp}^{max}$ is the highest experimentally observed concentration of $S_c$. Hence, the weights have values from ca. 1 far from a peak maximum to 2 at the peak maximum.

If eqn. 2 is approximated by Laplace's formula[17], defined as

$$[S_c]_{cal}^{i,n} = [S_c]_{cal}^{0,0} \cdot \frac{K_c}{\sqrt{2\pi n K_c}} \cdot e^{\frac{-(i+iK_c-nK_c)^2}{2nK_c}} \qquad (8)$$

then non-linear regression analysis can be used to find the best fitting parameters, $K_c$ and $[S_c]_{cal}^{0,0}$, i.e., to minimize eqn. 6.

For 1:1 interacting systems, pairs of theoretical CCD curves can then be calculated according to the models described[5] by using the known initial concentrations and the partition coefficients of both components, and assigning values to the partition coefficient of the complex, $K_{complex}$, and to the association constant in the

lower phase, $K_1$. By comparing such theoretical pairs of curves with experimental ones, the best fitting $K_{complex}$ and $K_1$ can be obtained.

As the concentrations of the two components may differ significantly in magnitude, summation of the ordinary least-squares criterion (eqn. 9) was found to be inadequate.

$$U_2 = \sum_{c=1}^{2} \sum_{i=0}^{n} ([S_c]_{exp}^{i,n} - [S_c]_{tot}^{i,n})^2 \omega_{c,i}$$  (9)

where $[S_c]_{tot}^{i,n}$ is the total concentration in the $i$th chamber calculated according to model $1^5$. Therefore, the criterion was transformed to a normalized form:

$$U_3 = \sum_{c=1}^{2} \sum_{i=0}^{n} \frac{v_c}{v_1 + v_2} \left( \frac{[S_c]_{exp}^{i,n} - [S_c]_{tot}^{i,n}}{[S_c]_{exp}^{max}} \right)^2 \omega_{c,i}$$  (10)

The distribution factors, $v_1$ and $v_2$, i.e., the inverted values of the relative errors, were included to correlate the separate CCD behaviour of both components; $v_1$ and $v_2$ are calculated from the minimum of eqn. 6 using

$$\frac{1}{\sqrt{v_c}} = \frac{\sqrt{U_1}}{\sum_{i=0}^{n} [S_c]_{exp}^{i,n}} \qquad c = 1, 2$$  (11)

Hence, the more ideal the distribution behaviour the higher is the value of $v$, and if both components behave equally ideally (or non-ideally) their distribution factors will be identical.

Thus, the best fitting $K_1$ and $K_{complex}$ are obtained by minimizing eqn. 10. (For further explanation, see Fig. 1.)

For comparison between association constants obtained with different phase systems or other methods, neither the association constant in the lower phase nor that in the upper phase is adequate. A more comparable quantity is the overall association constant, $K_T$, defined by

$$K_T = \frac{[\text{Complex}]}{[S_1]_{free} [S_2]_{free}}$$  (12)

where the concentration terms denote the total concentration of each component in any one chamber. By the same formal procedure as before[5], the overall association constant can be derived in terms of the individual equilibria:

$$K_T = K_1 \cdot \frac{q + pK_{complex}}{[(q + pK_{c_1}) (q + pK_{c_2})}$$  (13)

where $p = V_u/(V_u + V_l)$ and $q = V_l(V_u + V_l)$. If the volume of the upper phase equals that of the lower phase, eqn. 13 becomes

$$K_T = 2K_1 \cdot \frac{1 + K_{complex}}{(1 + K_{c_1}) (1 + K_{c_2})}$$  (14)
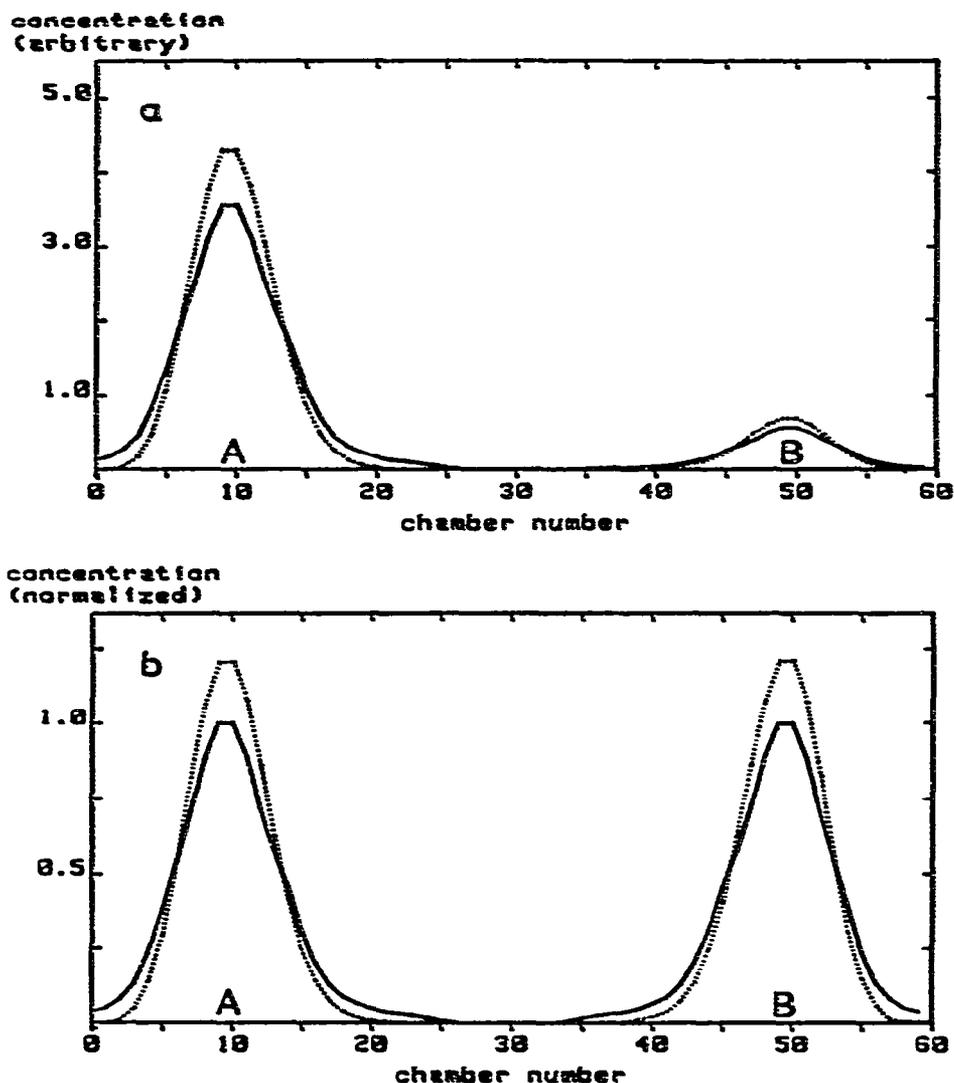
Fig. 1. Experimental (full lines) and theoretical (dotted lines) pairs of CCD curves of A and B for a hypothetical case when $[A]_{exp}^{t,o} \gg [B]_{exp}^{t,o}$ and $v_A = v_B$. (a) The ordinary least-squares criterion (eqn. 9) is mainly dependent on the contribution from A as $\sum_{i=0}^{n} ([A]_{exp}^{i,n} - [A]_{tot}^{i,n})^2 \omega_{A,i} \gg \Sigma([B]_{exp}^{i,n} - [B]_{tot}^{i,n})^2 \omega_{B,i}$. Hence, the best fitting $K_1$ and $K_{complex}$ would be very sensitive to the initial concentrations and mainly dependent on the fit of the component in excess. (b) This inadequacy is avoided by first transforming the experimental and theoretical obtained concentrations to normalized values, i.e., $[A]_{exp}^{i,n}/[A]_{exp}^{max}$, $[A]_{tot}^{i,n}/[A]_{exp}^{max}$, $[B]_{exp}^{i,n}/[B]_{exp}^{max}$ and $[B]_{tot}^{i,n}/[B]_{exp}^{max}$, hence making their magnitudes equal and insensitive to the initial concentrations and then minimizing the differences between these normalized values. As the magnitude of the normalized values are equal, also, under the assumptions made, the sum of the squares of the differences must be near equal. Thus, the best fitting $K_1$ and $K_{complex}$ would be equally dependent on the fit of both A and B and insensitive to the initial concentrations.

## RESULTS AND DISCUSSION

In this test case it was assumed that neither BSA nor L-Trp polymerizes, that no higher aggregates of the complex are formed, and that the polymers do not interfere with the interaction. Under these assumptions the interaction between BSA and L-Trp occuring in a biphasic system can be described completely by

$$
\begin{array}{ccc}
& K_u & \\
BSA_u + L\text{-}Trp_u & \rightleftharpoons & BSA\text{-}Trp_u \\
\big\Vert K_{BSA} \quad \big\Vert K_{Trp} & & \big\Vert K_{complex} \\
BSA_l + L\text{-}Trp_l & \rightleftharpoons & BSA\text{-}Trp_l \\
& K_l &
\end{array}
$$
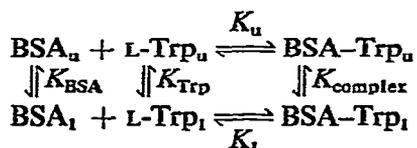
where the subscripts u and l refer to the upper and lower phase, respectively.

To compute $K_l$ (or $K_u$) and $K_{complex}$ it is necessary first to determine $K_{BSA}$ and $K_{Trp}$. The partition coefficients of the individual components can be obtained from the separate CCDs of BSA and L-Trp by means of eqn. 5 or, preferably, by non-linear regression analysis.

In Fig. 2 the distribution curve of L-Trp separately is shown. As can be seen, L-Trp behaved like a homogeneous substance with a well defined partition coefficient, $K_{Trp}$, of 0.89.
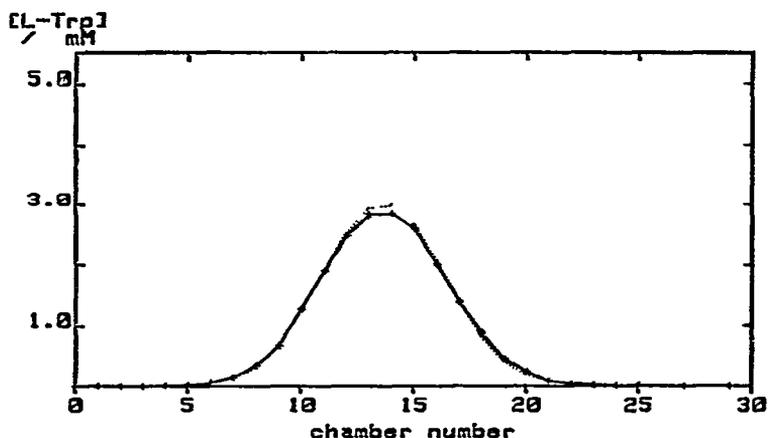


Fig. 2. CCD of L-Trp separately. $K_{Tpr} = 0.89$ was determined by non-linear regression analysis. Best fitting theoretical curve (dotted line) calculated according to eqn. 2.

Preparations of BSA are composed of two types of albumin, one devoid of sulphydryl group and the other with a single sulphydryl group. They also contain small amounts of dimers and polymers of albumin[17,18]. Owing to this heterogeneity it was not surprising that the CCD curve of BSA became broader than a single theoretical one, as shown in Fig. 3. However, if the experimental conditions are chosen such that the initial concentration of BSA is well in excess of the initial concentration of L-Trp when they are distributed together, the change in the CCD curve of BSA due to interaction with L-Trp should be negligible[5]. Therefore, in order
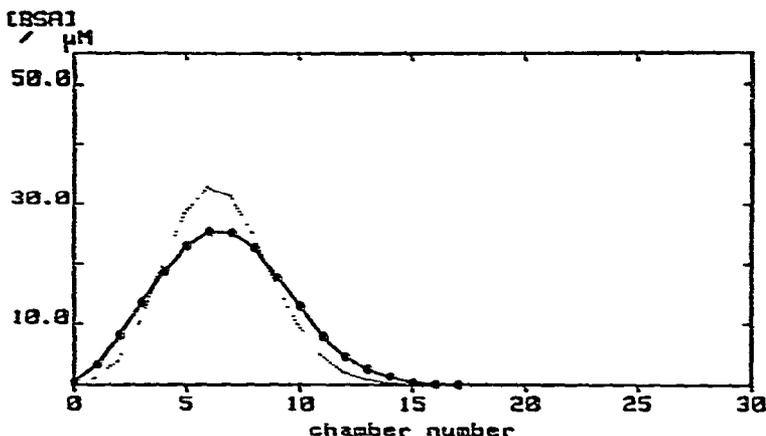
Fig. 3. CCD of BSA separately. Best fitting theoretical curve (dotted line) calculated according to eqn. 2 with $K_{BSA} = 0.29$.

to simplify the forthcoming computations it seems reasonable to assume that BSA distributes as a single homogeneous substance. Further, such an experimental arrangement would also make the measurements of the concentration of BSA more reliable in that L-Trp should give an exceedingly small contribution to the absorbance at 280 nm.

Thus, from CCDs of BSA separately a best fitting partition coefficient, $K_{BSA}$, of 0.29 was obtained. The distribution factors, $\nu_{BSA}$ and $\nu_{Trp}$, were calculated to be 103 and 5055, respectively. Fig. 4 shows experimental curves when BSA and L-Trp are distributed together. As the initial concentration of L-Trp was increased while that of BSA was held constant and well in excess, the distribution of L-Trp became increasingly distorted and retarded.

Under the assumptions made, the distortion of the distribution behaviour of the component in deficit is sufficient evidence of an interaction[5]. However, if the initial concentrations are of the same magnitude an interaction would be indicated by a mutual influence of the CCD behaviours of both components.

$K_1$ and $K_{complex}$ were computed from the known initial concentrations of BSA and L-Trp using eqn. 10, and the separately determined partition coefficients and distribution factors. The resulting best matching theoretical pairs of curves are also shown in Fig. 4. The results are summarized in Table I. The computations yielded unequivocal values of both the association constant in the lower phase and of the partition coefficient of the complex. Hence, the described model fits the experimental results very well.

Undoubtedly, the presence of high concentrations of polymers in the phase system alters the solvation properties of the solvent. Owing to the excluded volume effect in polymer solutions[19,20], the effective concentrations of added macromolecules, i.e., BSA and BSA–Trp complex, can be expected to be higher than the calculated values. On the other hand, a decrease in the effective concentration of L-Trp can be expected, at least, in the presence of PEG[20]. Thus, the measured association constant for the binding of L-Trp to BSA is probably higher in the presence than in the absence of polymers. Therefore, as shown in Table II, it was not surprising
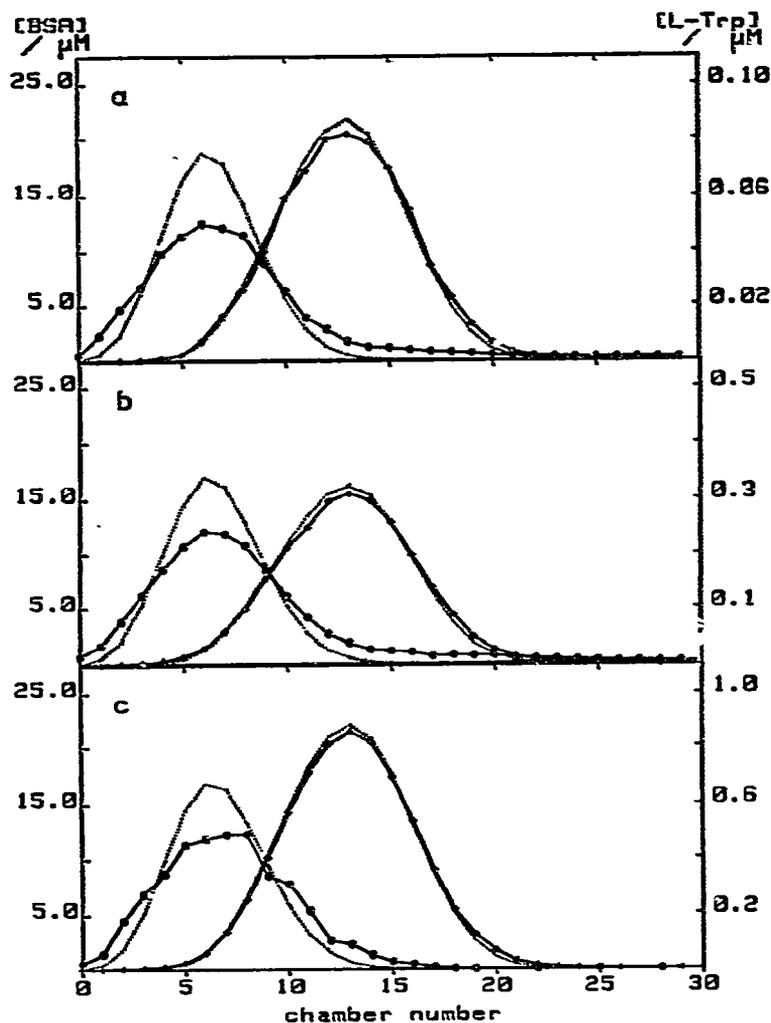
Fig. 4. CCD of BSA (○) and L-Trp (×) together. Initial concentrations of BSA and L-Trp were as follows: (a) $[BSA]^{0.0}_{exp} = 0.11$ m$M$ and $[L\text{-}Trp]^{0.01}_{exp} = 0.67$ $\mu M$; (b) $[BSA]^{0.0}_{exp} = 0.10$ m$M$ and $[L\text{-}Trp]^{0.0}_{exp} = 2.5$ $\mu M$; (c) $[BSA]^{0.0}_{exp} = 0.10$ m$M$ and $[L\text{-}Trp]^{0.01}_{exp} = 6.7$ $\mu M$. Best fitting theoretical pairs of curves (dotted lines) obtained using $K_{BSA} = 0.29$, $K_{Trp} = 0.89$, $\nu_{BSA} = 103$ $\nu_{Trp} = 5055$, and the initial concentrations of BSA and L-Trp.

TABLE I

BEST FITTING $K_{complex}$ AND $K_i$ VALUES

Best fitting $K_i$ and $K_{complex}$ obtained with $K_{BSA} = 0.29$, $K_{Trp} = 0.89$, $\nu_{BSA} = 103$, and $\nu_{Trp} = 5055$.

| Initial concentrations | | | | |
|---|---|---|---|---|
| [BSA] (mM) | [L-Trp] (µM) | $K_{complex}$ | $K_I \cdot 10^{-4}$ (l mol$^{-1}$) | $K_T \cdot 10^{-4}$ (l mol$^{-1}$) |
| 0.11 | 0.67 | 0.63 | 5.2 | 6.8 |
| 0.10 | 2.5 | 0.62 | 5.0 | 6.6 |
| 0.10 | 6.7 | 0.66 | 6.4 | 8.8 |
| | | Mean: 0.64 ± 0.02 | | Mean: 7.4 ± 1.0 |

TABLE II

ASSOCIATION CONSTANTS ($K_{ass}$) FOR THE INTERACTION BETWEEN BSA AND L-TRP OBTAINED WITH VARIOUS METHODS

As the binding is pH dependent[6] the pH of the buffers used are given.

| $K_{ass} \cdot 10^{-4}$ ($l\ mol^{-1}$) | pH | Temperature (°C) | Method | Reference |
|---|---|---|---|---|
| 7.4 | 7.0 | 21 | CCD | This work |
| 1.1 | 7.4 | 20 | Dialysis rate | 10 |
| 1.4 | 7.4 | 22 | Equilibrium dialysis | 8 |
| 2.1 | 7.95 | 24 | Equilibrium dialysis | 9 |
| 2.3 | 7.4 | 20 | Ultrafiltration | 12 |
| 19.1 | 9.0 | 25 | Gel filtration | 7 |

that the overall association constant obtained with the CCD technique was higher than those determined with other methods. Another reason for the disparity probably lies in the assumption that BSA behaves like a homogeneous substance. Naturally, it should be possible to interpret the results in terms of interactions between two or more forms of BSA and L-Trp, but the number of unknowns would increase, making the computations much more difficult and unreliable.

This test case has shown clearly that (1) the interaction patterns actually follow the described model, (2) the computations yield unequivocal and reproducible results and (3) the overall association constant obtained agrees well with association constants obtained with other methods. In other words, the CCD technique is applicable to the study of molecular interactions.

Although the method has been developed primarily for the study of protein–protein interactions, for which other methods, e.g., gel filtration and equilibrium dialysis, are not useful, it should have a very wide field of application.

REFERENCES

1 L. C. Petersen, FEBS Lett., 94 (1978) 105–108.
2 G. Fex, P.-Å. Albertsson and B. Hansson, Eur. J. Biochem., 99 (1979) 353–360.
3 L. Backman and G. Johansson, FEBS Lett., 65 (1976) 39–43.
4 D. N. Silverman, L. Backman and C. Tu, J. Biol. Chem., 254 (1979) 2588–2591.
5 L. Backman and V. P. Shanbhag, J. Chromatogr., 171 (1979) 1–13.
6 R. H. McMenamy and J. L. Oncley, J. Biol. Chem., 233 (1958) 1436–1447.
7 G. F. Fairclough, Jr., and J. S. Fruton, Biochemistry, 5 (1966) 673–683.
8 J. N. McArthur and P. D. Dawkins, J. Pharm. Pharmacol., 21 (1969) 744–750.
9 T. P. King and M. Spencer, J. Biol. Chem., 245 (1970) 6134–6148.
10 R. W. Fuller and B. W. Roush, Res. Commun. Chem. Pathol. Pharmacol., 8 (1974) 563–566.
11 C. S. Pande and R. H. McMenamy, Biochim. Biophys. Acta, 342 (1974) 60–68.
12 V. J. Cunningham, L. Hay and H. B. Stoner, Biochem. J., 146 (1975) 653–658.
13 C. Corbion and P. Lazzlo, Nouv. J. Chim., 2 (1978) 309–316.

14 P.-Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Almqvist and Wiksell, Stock-holm, and Wiley, New York, 2nd ed., 1971.

15 R. C. Feldhoff and T. Peters, Jr., *Biochem. J.*, 159 (1976) 529–533.

16 B. M. Shchigolev, in H. Eagle (Editor), *Mathematical Analysis of Observations*, Iliffe Books, London, and American Elsevier, New York, 1965, pp. 123–127.

17 J. Janatova, J. K. Fuller and M. J. Hunter, *J. Biol. Chem.*, 243 (1968) 3612–3622.

18 E. M. Spencer and T. P. King, *J. Biol. Chem.*, 246 (1971) 201–208.

19 C. Tanford, *Physical Chemistry of Macromolecules*, Wiley, New York, London, Sidney, 1965.

20 K. C. Ingham, *Arch. Biochem. Biophys.*, 184 (1977) 59–68.