Journal of Chromatography, 260 (1983) 329–336
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,647

CHARACTERISTICS OF PROTEIN–AQUEOUS MEDIUM INTERACTIONS MEASURED BY PARTITION IN AQUEOUS FICOLL–DEXTRAN BIPHASIC SYSTEM

B. Yu. ZASLAVSKY*, N. M. MESTECHKINA and S. V. ROGOZHIN
Institute of Elementoorganic Compounds, U.S.S.R. Academy of Sciences, Moscow 117813 (U.S.S.R.)
(First received December 9th, 1982; revised manuscript received December 24th, 1982)

SUMMARY

Partitioning of a number of proteins in the aqueous Ficoll-400–Dextran-70 biphasic system was studied at pH 7.4 under varied ionic compositions. The relative hydrophobicities of the proteins have been estimated, and the contributions of the interactions of the ionogenic and non-ionic groups of a protein with an aqueous environment to the total hydrophobicity of the protein have been evaluated. Some arguments in support of the biological significance of the effect of ionic composition on the relative hydrophobicity of biological macromolecules are given. Possible applications of the partition technique to protein research are discussed.

INTRODUCTION

It has recently been shown[1–3] that the partition behaviour of solutes in aqueous Ficoll–dextran biphasic systems is governed by the hydrophobic and hydration properties of the phases, and an approach to quantify these properties was suggested[1,2]. An examination of the partitioning of a solute in aqueous Ficoll–dextran biphasic system under varied ionic compositions appears to allow an estimation of the relative hydrophobicity of the solute and the contributions of the van der Waals and hydration interactions of the ionogenic and non-ionic groups of the solute with an aqueous environment to the total hydrophobic character of the solute[3].

A number of proteins has now been studied using the aforementioned approach. The results obtained are discussed in relation to the possible applications of the partition technique to protein research.

MATERIALS AND METHODS

Materials

Ficoll-400 (Lot 11072) was obtained from Pharmacia (Sweden), Dextran-70 (Lot 310670) under the trade-name Polyglucinum from Minmedprom (U.S.S.R.).

Rat serum albumin, Cohn fraction V, was purchased from Koch-Light (Great Britain), as well as rabbit, sheep, dog, bovine, chicken, horse and porcine serum albumins (all Cohn fractions V). Two samples of crystallized fatty acid-poor bovine

0021-9673/83/$03.00

serum albumins were obtained from Calbiochem (U.S.A.) and Sigma (U.S.A.). Bovine pancreatic ribonuclease (RNase), horse heart cytochrome *c* and horse skeletal muscle myoglobin were purchased from Calbiochem. Trypsin, human oxyhaemoglobin, horse spleen ferritin, egg albumin and termolysin were purchased from Serva (G.F.R.). Two samples of human serum albumin, Cohn fraction V, were obtained from ICN Pharmaceuticals (U.S.A.) and from the Central Institute of Haematology and Blood Transfusion, Moscow, U.S.S.R. Highly purified human and bovine γ-globulins were kindly provided by Dr. R. S. Nezlin (Institute of Molecular Biology, Academy of Sciences, Moscow), toxins from *Naja naja oxiana* snake venom by Dr. E. V. Grishin (Shemyakin Institute of Bioorganic Chemistry, Academy of Sciences, Moscow) and sheep oxyhaemoglobin by Dr. Hasko Judit (Institute of Experimental Medicine, Hungarian Academy of Sciences).

Sodium salts of 2,4-dinitrophenylated (DNP) amino acids with aliphatic side-chains (glycine, L-alanine, DL-norvaline, L-norleucine and DL-2-amino-*n*-octanoic acid) were prepared as described earlier[4]. All other chemicals were of analytical reagent grade.

Biphasic systems

The aqueous polymeric biphasic systems were prepared as described[1–4] and had the following composition: 12.5% (w/w) Ficoll-400, 10.8% (w/w) Dextran-70 and the amounts of NaCl and sodium phosphate buffer, pH 7.4, according to

$$C_{\text{buffer}} = 0.11 - 0.67C_{\text{NaCl}}$$

where C_{buffer} is the concentration of buffer, varied between 0.01 and 0.11 *M*, and C_{NaCl} is the concentration of NaCl varied from zero up to 0.15 *M*.

The difference in the hydrophobic characters of the phases employed was estimated by partitioning of DNP-amino acids with aliphatic side-chains as described[1,4]. It was found to be 17.4 cal/mole of CH_2, expressed in terms of the free energy of transfer of a CH_2 group from the Ficoll-rich phase to the dextran-rich phase of the system. The difference in the hydration properties of the phases was estimated as described earlier[2,3] by the *C* value corresponding to the logarithm of the partition coefficient of DNP-glycine.

Partition experiments

The partition experiments were carried out as described before[1,3,4]. The phases of the biphasic system were allowed to settle at 25°C for 21–24 h. Then aliquots of both phases were pipetted from the system and each was diluted in an appropriate amount of buffer and used for the solute concentration measurements.

The concentrations of the proteins studied were determined either by absorbance measurements or by the Coomassie G-250[5] or fluorescamine[6] technique. In the separate experiments it was verified that the two latter techniques give essentially agreeing results when expressed in terms of the partition coefficient.

The partition coefficient of a protein, *K*, is defined as the ratio of the protein concentration in the Ficoll-rich phase to the protein concentration in the dextran-rich phase. The *K* values were measured for each solute over approximately ten-fold concentration ranges and were found to be independent of the solute concentration

under all the conditions employed. Each partition coefficient was determined as the mean from two measurements on three dilutions of each partition experiment carried out three or four times at a given ionic composition. Deviation from the mean K value did not exceed 3% for all the proteins examined.

RESULTS

It has been shown previously[7] that estimates of the hydrophobic character of solutes depend on the particular biphasic system used. As the system containing 12.5% (w/w) Ficoll-400 (Lot 11069, Pharmacia) and 10.8% (w/w) Dextran-70 (lot 580870, Minmedprom) has been chosen as the reference system[7], the results obtained in this work were transformed to the reference system according to

$$\ln K_i = a_i \cdot \ln K_0 + b_i \quad (1)$$

where K_i is the partition coefficient of a solute in the system employed, K_0 is the partition coefficient of the solute in the reference system and a_i and b_i are constants.

In order to determine a_i and b_i, partitioning of several solutes (see Fig. 1) was studied in the phase system employed. The results were compared to those reported

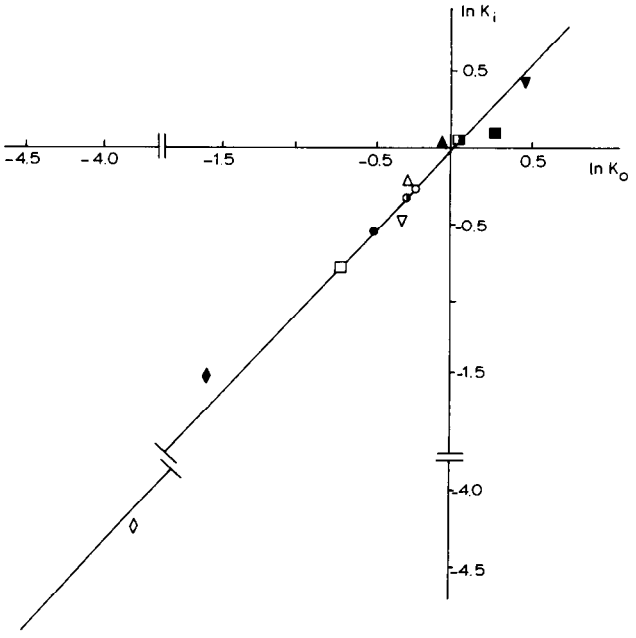

Fig. 1. Relationship between the partition coefficients of different solutes in two aqueous Ficoll–dextran biphasic systems of various polymer compositions. K_0 = Partition coefficient of a solute in the reference biphasic system; K_i = partition coefficient of the solute in the biphasic system used in this work —for explanations see text. Solutes: ○, cytochrome *c*; □, human serum albumin; ◇, polyuridylic acid; △, AMP; ▽, human plasma proteins; ◨, thymine; ◑, myoglobin. (Shaded symbols denote the partition coefficient of the solutes in the presence of 0.11 *M* sodium phosphate buffer, pH 7.4; unshaded symbols those in the presence of 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer, pH 7.4.

earlier[1,7–9] in the reference system and the relationship established according to eqn. 1 is shown in Fig. 1. The a_i and b_i values are found to be independent of the ionic composition of the compared systems under the conditions employed and amount to 1.083 and -0.010, respectively.

It has recently been shown[3] that the partition coefficient of a solute can be described by

$$\ln K = n_0^{CH_2}E + mC \tag{2}$$

where E characterizes the difference in the hydrophobic characters of the two phases of the system (according to $\Delta G_{tr}^{CH_2} = RTE$, where $\Delta G_{tr}^{CH_2}$ is the free energy of transfer of a CH_2 group between the phases[1]), C represents the difference in the hydration properties of the two phases in relation to the α-carboxyl group of DNP-amino acid[2]; m characterizes the strength of the hydration interactions of all the ionogenic groups of the solute relative to that of the α-carboxyl group of DNP-amino acid[3] and $n_0^{CH_2}$ characterizes the strength of all the interactions of the solute with an aqueous environment (except for processes of ionic hydration) relative to that of a CH_2 group[1–3].

Parameter E is independent of the ionic composition of the Ficoll–dextran biphasic system under the conditions employed[1]. Parameter C depends on the ionic composition of the system as shown in Fig. 2. It is seen that all the relationships established are in agreement with eqn. 2. Treatment of the data obtained according to eqn. 2 led to the $n_0^{CH_2}$ and m values listed in Table I.

It has been shown previously[3,7,9] that the relative hydrophobicity of a solute under the given conditions can be estimated from

$$n^{CH_2} = (\ln K)/E \tag{3}$$

where n^{CH_2} is the equivalent number of CH_2 groups. Estimates of the relative hydrophobicities of the proteins examined are also given in Table I.

DISCUSSION

It should be emphasized, first, that the characteristics of the two samples of fatty acid-free bovine albumin from different manufacturers are completely identical. This is important as it indicates that the partition technique, which is highly sensitive to the individual features of a given solute, can be used to estimate the quality of a biochemical preparation.

The difference observed between the two different samples of human serum albumin is of the same order of magnitude as that reported earlier[10]. No difference between the samples could be detected by paper and free-boundary electrophoresis, polyacrylamide gel electrophoresis, immunoelectrophoresis, electrophoresis in sodium dodecyl sulphate (SDS)-containing polyacrylamide gel and analytical electrofocusing in polyacrylamide gel[10]. It seems that the difference in the solvent interactions of the proteins is due to the different natures and amounts of lipids present in the samples, and is in accord with the difference observed (Table I) in the characteristics of bovine serum albumin containing lipids and fatty acid-free bovine albumin. It is seen that the presence of lipids in serum albumin in both cases alters the parameter

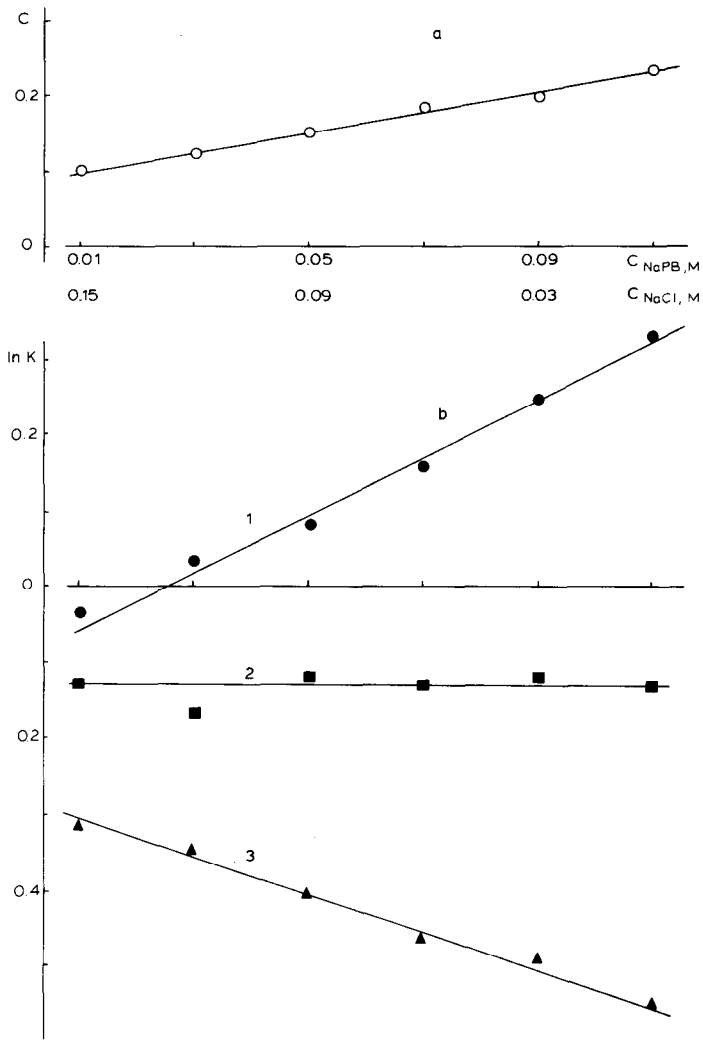

Fig. 2. Parameter C and partition coefficients of proteins as a function of the ionic composition of the aqueous Ficoll–dextran biphasic system. Curves: 1 = trypsin; 2 = ribonuclease; 3 = cytochrome *c*. NaPB = Sodium phosphate buffer.

$n_0^{CH_2}$ but not m. This implies that the binding of lipids to albumin is accompanied by some changes in the macromolecular surface which affect the nature of the non-ionic residues exposed to the aqueous environment of the macromolecule. It is of interest in this context that the immunologic properties of human serum albumin are unaltered by removal of the fatty acids, as are the circular dichroism spectra and other features of the tertiary structure of the protein[11–13].

The differences in the characteristics of the solvent interactions with the albumins examined are of interest as it is generally supposed[13] that the folding of these serum albumins is very similar. However, this conclusion[13] was made on the basis of detergent binding studies. The data in Table I seem to indicate that the solvent interactions of macromolecules can differ even when the binding sites of the macromolecules are similar, and support the view[14] that the hydrophobic character of a

TABLE I

CHARACTERISTICS OF PROTEIN INTERACTIONS WITH AQUEOUS MEDIA AND THE RELATIVE HYDROPHOBICITIES OF THE PROTEINS

In the presence of sodium chloride and sodium phosphate buffer, pH 7.4. The relative hydrophobicity is expressed in terms of the equivalent number of CH_2 groups (for explanation see text).

Protein	$n_0^{CH_2}$	m	n^{CH_2*}	n^{CH_2**}
Albumins:				
Sheep	−32.0 ± 1.5	2.67 ± 0.11	−4.6	−22.6
Bovine	−39.7 ± 1.8	3.68 ± 0.12	−1.9	−26.7
Bovine***	−33.6 ± 0.9	3.37 ± 0.05	1.1	−21.6
Rabbit	−40.1 ± 1.0	4.26 ± 0.08	0.3	−28.4
Rat	−33.8 ± 0.9	3.84 ± 0.06	5.6	−20.3
Horse	−37.7 ± 1.0	3.74 ± 0.07	0.7	−24.5
Porcine	−37.5 ± 1.0	3.95 ± 0.06	2.1	−24.5
Dog	−44.5 ± 0.9	4.06 ± 0.05	−2.9	−30.2
Human-I §	−46.1 ± 1.0	5.42 ± 0.06	9.6	−27.0
Human-II §	−40.5 ± 1.0	5.03 ± 0.15	11.2	−22.8
Chicken	−60.1 ± 1.0	6.53 ± 0.08	6.9	−37.1
Egg	−18.8 ± 0.6	2.38 ± 0.04	5.6	−10.4
γ-Globulins:				
Bovine	31.6 ± 1.0	1.19 ± 0.06	43.9	35.9
Human	16.2 ± 1.5	0	16.2	16.2
Oxyhaemoglobins:				
Sheep	10.3 ± 1.3	0.90 ± 0.09	−1.0	−7.1
Human	15.1 ± 0.9	0.53 ± 0.05	20.7	17.1
Insulin	−0.04 ± 1.0	1.52 ± 0.06	14.3	4.0
Myoglobin	−10.1 ± 1.0	0	−10.1	−10.1
Ribonuclease	−4.2 ± 0.9	0	−4.2	−4.2
Termolysin	−5.0 ± 1.5	0.92 ± 0.11	4.5	−1.7
Trypsin	−9.8 ± 1.0	2.10 ± 0.06	11.8	−2.3
Ferritin	−59.0 ± 2.0	12.30 ± 0.20	67.6	−15.3
Cytochrome *c*	−3.6 ± 0.6	−1.62 ± 0.04	−20.3	−9.4
Toxins from snake venom:				
Neurotoxin-I	3.3 ± 0.9	0.42 ± 0.05	7.6	4.8
Neurotoxin-II	4.1 ± 1.0	−1.08 ± 0.06	−7.0	0.3
Cytotoxin-I	11.6 ± 1.0	−1.48 ± 0.06	−3.6	6.4
Cytotoxin-II	16.5 ± 0.9	−1.96 ± 0.07	−3.5	9.6

* In the presence of 0.11 *M* sodium phosphate buffer, pH 7.4.

** In the presence of 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer, pH 7.4.

*** Bovine albumin containing less than 0.05% of fatty acids. The data are given for two different samples (Sigma and Calbiochem).

§ Human albumins-I and -II are from different manufacturers, ICN Pharmaceuticals and Central Institute of Haematology and Blood Transfusion, Moscow, respectively.

protein is essentially a surface property of the macromolecule which is not directly affected by the residues buried in its interior or by those forming a binding pocket. The data also indicate the dominant rôle of hydrophilic residues exposed to an aqueous environment in the total relative affinity of the albumins for the aqueous medium under the conditions employed. Only human oxyhaemoglobin and bovine and human γ-globulins appear to differ in this respect from the other mammalian proteins studied so far. The reasons for this are not clear at present.

The data appear to indicate that the molecular weight of a protein is not of great importance in protein–solvent interaction, rather that the nature of the residues exposed is of much greater significance. This conclusion is confirmed by the

results (Table I) for the snake venom toxins. Both cytotoxins examined contain 60 amino acid residues and the amino acid composition of the proteins is distinguished by ten residues, the sequence of the proteins differing at thirteen locations[15]. The difference observed in the $n_0^{CH_2}$ values of the cytotoxins is significant and seems to support the assumption that the partition technique is highly sensitive to features of the protein folding.

The difference in the effect of ionic composition upon the relative hydrophobicity of different human or bovine plasma proteins appears to confirm the hypothesis[10] that the ionic composition of the blood stream can affect the hydrophobic properties of the blood components and thus influence their functions. Additional support for this assumption follows from a comparison of the effects of ionic composition on the hydrophobic properties of serum albumins and red blood cells from different mammalian species[16]. It has been shown previously[1–4,7,9,10,16,17] that the ionic composition can be represented by the corresponding ionic strength, I, whereupon the following relationship between the partition coefficient of a solute (or particle) and the ionic composition of the phase system used can be derived

$$\ln K = A + BI \tag{4}$$

where A and B are constants. It is obvious that parameter B represents the effect of the ionic composition of the medium on the relative hydrophobicity of the solute under the conditions employed.

Values of B found for the albumins considered are listed in Table II together with those reported earlier[16] for red blood cells from the same species. It can be seen that the B values are related by

$$B_{cell} = 26.63 + 2.28B_{alb} \tag{5}$$

where B_{cell} and B_{alb} are the values for erythrocytes and serum albumin from the same species. The correlation coefficient of this relationship is 0.943, but the number of species examined is small and therefore eqn. 5 cannot be claimed to be statistically significant. The dog albumin and red cells do not fit eqn. 5, probably owing to the difference in breed of dogs from which the protein and the cells were obtained. However, although statistically unreliable, the above relationship does seem to support the assumption[9,10,17] that the effect of the composition of an aqueous medium upon the hydrophobic properties of biological solutes and particles is important for regulation of their biological functions.

TABLE II

PARAMETER B FOR SERUM ALBUMINS, B_{alb}, AND RED BLOOD CELLS, B_{cell}, FROM DIFFERENT SPECIES

For explanation see text. It should be noted that the B_{alb} and B_{cell} values were obtained in different aqueous Ficoll–dextran biphasic systems.

Species	B_{alb}	B_{cell}
Dog	6.60 ± 0.41	33.9 ± 1.4
Man	8.81 ± 0.38	45.8 ± 1.9
Rabbit	6.91 ± 0.49	42.4 ± 1.2
Rat	6.23 ± 0.56	42.9 ± 1.5
Sheep	3.91 ± 0.49	35.3 ± 1.3

The results obtained indicate the advantages of the partition technique over other methods used in the study of the hydrophobic character of biological macromolecules. Approaches based on the frequency of non-polar side-chains in proteins[18] or on the features of the amino acid sequence of the protein[19] appear to ignore the fact that the hydrophobic character of a protein is governed not by the total amino acid composition but by the residues exposed to an aqueous environment. Attempts to calculate the non-polar surface area of a macromolecule[20] are of obvious interest but do not take into account the specific features of the non-polar residues located at the macromolecular surface and the fact that the relative hydrophobicity of the residues depends on their surroundings[17] and on the composition of the aqueous medium. The approaches based on a study of the protein–ligand interactions[21–23] provide important information about the features of the binding sites in the proteins but not about the macromolecule–solvent interactions.

It should be noted also that the results obtained in this study suggest that the partition technique can be used for studying features of the protein tertiary structure, conformational changes accompanying protein–ligand binding and similar processes. The high sensitivity of the technique to the quality of the protein preparations provides possible applications to the analysis of purity of different protein samples. Finally, the technique is not limited by the nature of the solutes examined and could be as promising in the field of nucleic acids and other solutes of biological interest.

REFERENCES

1 B. Yu. Zaslavsky, L. M. Miheeva, N. M. Mestechkina and S. V. Rogozhin, *J. Chromatogr.*, 253 (1982) 139–148.
2 B. Yu. Zaslavsky, L. M. Miheeva, N. M. Mestechkina and S. V. Rogozhin, *J. Chromatogr.*, 253 (1982) 149–158.
3 B. Yu. Zaslavsky, N. M. Mestechkina, L. M. Miheeva and S. V. Rogozhin, *J. Chromatogr.*, 256 (1983) 49–59.
4 B. Yu. Zaslavsky, N. M. Mestechkina, L. M. Miheeva and S. V. Rogozhin, *J. Chromatogr.*, 240 (1982) 21–28.
5 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
6 P. Böhlen, S. Stein, W. Dairman and S. Udenfriend, *Arch. Biochem. Biophys.*, 155 (1973) 213–219.
7 B. Yu. Zaslavsky, L. M. Miheeva, N. M. Mestechkina, L. G. Shchyukina, M. A. Chlenov, L. I. Kudrjashov and S. V. Rogozhin, *J. Chromatogr.*, 202 (1980) 63–73.
8 B. Yu. Zaslavsky, L. M. Miheeva and S. V. Rogozhin, *J. Chromatogr.*, 216 (1981) 103–113.
9 B. Yu. Zaslavsky, L. G. Shchyukina and S. V. Rogozhin, *Mol. Biolog. (USSR)*, 15 (1981) 1315–1320.
10 B. Yu. Zaslavsky, N. M. Mestechkina and S. V. Rogozhin, *Biochim. Biophys. Acta*, 579 (1979) 463–465.
11 K. Zakrewski and H. Goch, *Biochemistry*, 7 (1968) 1835–1842.
12 R. F. Chen, *J. Biol. Chem.*, 242 (1967) 173–181.
13 W. W. Sukow, J. Healey and P. Witkewicz, *Physiol. Chem. Phys.*, 10 (1978) 133–138.
14 W. Melander and Cs. Horváth, *Arch. Biochem. Biophys.*, 183 (1977) 200–215.
15 E. V. Grishin, A. P. Sukhikh, T. B. Adamovich and Yu. A. Ovchinnikov, *Bioorg. Khim.*, 2 (1976) 1018–1034.
16 B. Yu. Zaslavsky, L. M. Miheeva and S. V. Rogozhin, *Biochim. Biophys. Acta*, 588 (1979) 89–101.
17 B. Yu. Zaslavsky, N. M. Mestechkina, L. M. Miheeva, S. V. Rogozhin, G. Ya. Bakalkin, G. G. Rjazhsky, E. V. Chetverina, A. A. Asmuko, J. D. Bespalova, N. V. Korobov and O. N. Chichenkov, *Biochem. Pharmacol.*, 31 (1982) 3757–3762.
18 R. A. Capaldi and G. Vanderkooi, *Proc. Nat. Acad. Sci. U.S.*, 69 (1972) 930–932.
19 J. P. Segrest and R. J. Feldman, *J. Mol. Biol.*, 87 (1974) 853–858.
20 C. Chothia and J. Janin, *Nature (London)*, 256 (1975) 705–708.
21 A. Helenius and K. Simons, *J. Biol. Chem.*, 247 (1972) 3656–3661.
22 S. Clarke, *Biochem. Biophys. Res. Commun.*, 79 (1977) 46–52.
23 J.-L. Ochoa, *Biochimie*, 60 (1978) 1–15.