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Determination of erythrocyte membrane-buffer partition coefficients by numerical analysis of multicomponent spectra

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A method suitable for the measurement of erythrocyte membrane-buffer partition coefficients by u.v.-spectrophotometry is described. This method is characterized by a proper correction for inconstant background absorption in case of bad signal to noise ratios. For this purpose the multicomponent u.v.-spectrum of a compound in the presence of absorbing materials lost by erythrocyte membranes is decomposed into its constituent spectra by means of numerical analysis. Application of this method to the determination of partition coefficients of benzhydrol, 4-bromophenol and benzyl alcohol yields satisfactory agreement with literature values for the latter two coefficients.

The determination of erythrocyte membrane-buffer partition coefficients is usually made by measurement of the solute concentration in the supernatant after centrifugation of an equilibrium mixture of membranes and solute in buffer. Tracer techniques have frequently been applied for the measurement of solute concentrations (Seeman 1969; Kwant & Seeman 1969; Colley et al 1971). They are to be highly recommended due to the absence of any necessary background correction. However, tracer techniques are costly.

The application of u.v. spectrophotometry is hampered by the presence of varying amounts of absorbing materials in the supernatant. Classical analysis of these spectra is satisfactory only when the signal to noise ratio is sufficiently high. This demands either a high partition coefficient or a high molar extinction for the material being investigated. Elferink (1977) in his study on the partition of chlorpromazine—log P = 3·20, ϵ = 31 600—corrected his spectra in the classical way by subtracting the absorbance of a proper control sample; with the background absorption an order of magnitude smaller than the solute absorption, background-fluctuations will not cause serious deteriorations in the results. These fluctuations are however inherent in the materials responsible for background absorption. These materials supposedly consist of (i): persisting residual haemoglobin; (ii): solubilized membrane proteins (Swoch & Passow 1973); (iii): enzymatic degradation products of both (i) and (ii); proteolytic enzymes, present in either the membrane preparation (Moore et al 1970) or in traces of leucocytes (Fairbanks et al 1971) are held responsible for the presence of small peptides with a molecular weight of about 5000.

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It follows that solute and background absorption are of comparable magnitude for all solutes not having the favourable characteristics mentioned. Classical correction of the u.v. spectra makes too high demands upon the reproducibility of the background spectrum. The usual fluctuations of 5–10% in the background absorption per unit weight-fraction of membranes seriously affects the final results of the measurements and led us to develop a method by which the complex u.v. spectrum provided by peptides, proteins and solute is decomposed into its constituting spectra, thus enabling us to build up a correct picture of the necessary corrections to the solute absorption.

MATERIALS AND METHODS

Reagents

All chemicals used were reagent grade. Benzhydrol and 4-bromophenol were further purified by sublimation and recrystallization.

Erythrocyte membranes

Packed cells from freshly drawn human blood were obtained from the Red Cross Blood Transfusion Laboratory of Amsterdam. The cells were centrifuged at 2500 *g* for 5 min and the remaining plasma and leucocytes were removed. Washing of the erythrocytes and preparation of ghosts was according to Kwant & Seeman (1969). The membranes were concentrated by centrifugation to 1–2% (w/w), stored at 4 °C in 10 mM phosphate buffer pH 7·0 and used within 10 days. The dry weight of membrane suspensions was determined by drying at 95 °C to constant weight. Corrections were made for the dry weight of buffer salts.

Measurement of concentrations

Partition coefficients were determined on a weight

base; solute concentrations were measured as mol kg^{-1} buffer and as mol kg^{-1} dry membranes; membrane concentrations were measured as weight-fractions: dry membrane weight/total weight of membranes and buffer.

Weighted amounts of membrane suspension and buffered solute were mixed in a 10 ml polycarbonate centrifuge tube to give a total weight of 7·5 g. The solute molality and the membrane weight-fraction chosen should provide a solute contribution to the u.v. spectrum comparable with the peptide-protein contribution. However, when equilibrium is attained, the decrease in solute molality in the aqueous phase should amount at least 10%. Two different types of control samples were prepared in an identical way, one without the solute and another without membranes.

The suspensions were left to equilibrate for 1 h at 25 °C and subsequently centrifuged at 100 000 *g* for 1 h in a MSE-75 superspeed centrifuge. During the run temperature was maintained at 25 ± 1 °C. After centrifugation 3 ml aliquots of the supernatants were added to 1 cm quartz cells. Absorption spectra were measured using a Cary 16 dual beam spectrophotometer purged with dry nitrogen. Absorbance readings were taken at 2 nm intervals in the range 230–310 nm.

Determination of partition coefficients

If the applicability of Lambert-Beer's law and the absence of chemical interactions between the constituents of a mixture of peptides, proteins and solute is assumed, the absorbance y_i at wavelength λ_i of the mixture is a linear function of the absorbancies of the 'pure' compounds (Denis & Deyrieux 1977):

$$y_i = r_M M_i + r_B B_i, \qquad (1)$$

where M_i and B_i represent the absorbancies at wavelength λ_i of control samples containing respectively: mixtures of membranes and buffer and mixtures of solute and buffer; r_B is the ratio of the solute molality in the aqueous phase at equilibrium and the corresponding solute molality in the control sample. The ratio r_M is defined analogously.

Multiple regression analysis was employed for the estimation of these ratios. The weight-fraction W of membranes and r_B were used to calculate the partition coefficient P_M by means of equation (2):

$$P_M = \frac{1}{W}\left(\frac{1}{r_B} - 1\right) + 1 \qquad (2)$$

Test of the calculated results

A constant term C and the coefficient r_M were both calculated by multiple regression analysis and submitted to tests as follows:

(i): if Lambert-Beer's law is not obeyed by the species responsible for the background absorption, then $r_M \neq 1$,

(ii): if the assumption of linearity does not hold $C \neq 0$.

The applicability of Lambert-Beer's law for a solute in the presence of materials causing background absorption was tested by adding known quantities of solute to a supernatant, emerging from mere centrifugation of membranes, and determination of the aqueous solute molality by means of equation (1).

RESULTS AND DISCUSSION

Table 1 gives the results of P_M-determinations of benzhydrol, 4-bromophenol and benzyl alcohol. Erythrocytes from 8 different donors have been used for the experiments reported in Table 1, one sample for each benzyhydrol-concentration, two for 4-bromophenol and two for benzyl alcohol.

The regression analysis of u.v. spectra of two supernatants containing 4-bromophenol at 0·17 mmol kg^{-1} is reported in Table 2. Inspection of this

Table 1. Erythrocyte membrane-buffer partition coefficients of benzhydrol, 4-bromophenol and benzyl alcohol as measured by multiple regression analysis (a) or subtraction of a background correction (b). (n = no. of determinations).

Free concn. (mol kg^{-1} buffer)	log P_M	n	Lit. value
Benzhydrol			
$0·15 \times 10^{-3}$	2·04[a]	1	—
$0·31 \times 10^{-3}$	2·02[a]	2	
$0·55 \times 10^{-3}$	1·99[a]	2	
$0·67 \times 10^{-3}$	2·01[a]	3	
4-Bromophenol			
$0·17 \times 10^{-3}$	1·99[a]	2	1·96
$1·68 \times 10^{-3}$	1·98[b]	1	
$3·53 \times 10^{-3}$	1·90[b]	1	
$5·46 \times 10^{-3}$	1·89[b]	1	
$7·39 \times 10^{-3}$	1·87[b]	1	
Benzyl alcohol			
$1·33 \times 10^{-3}$	0·48[a]	3	0·53

The standard error ranges from 0·03 to 0·07 except for benzyl alcohol where a rather high value of 0·10 was established. This is due to the small decrease—less than 10%—in aqueous benzyl alcohol molality on attaining equilibrium at the membrane concentrations used. Unfortunately, the alternative use of higher membrane concentrations is hampered by high errors originating from inhomogeneities in concentrated suspensions.

Table 2. Decomposition of the u.v.-spectrum of a supernatant containing 4-bromophenol at 0·17 mmoles kg^{-1}. buffer into a solute- and a background-spectrum. Absorption readings were taken at 38 wavelengths between 240 and 314 nm. Absorptions of two 4-bromophenol-containing supernatants are referred to as Y_i and Y'_i; absorptions of control samples are referred to as B_i, B'_i, M_i and M'_i.

Regression	R	S	F	DW	r_M	$t(r_M)$	r_B	$t(r_B)$	$\log P_M$
Y_i vs M_i and B_i	0·9998	0·003	48600	2·40	0·983	125	0·609	69·7	1·995
Y_i vs M_i and B'_i	0·9999	0·003	62100	2·08	0·993	145	0·607	78·8	1·998
Y_i vs M'_i and B_i	0·9998	0·003	42200	1·65	0·953	117	0·630	68·3	1·956
Y_i vs M'_i and B'_i	0·9998	0·003	43800	1·47	0·963	122	0·629	69·6	1·959
Y'_i vs M_i and B_i	0·9997	0·004	31900	2·44	1·001	103	0·595	55·0	2·019
Y'_i vs M_i and B'_i	0·9998	0·003	41600	2·29	1·010	120	0·594	62·9	2·021
Y'_i vs M'_i and B_i	0·9997	0·004	34800	1·97	0·970	107	0·617	60·5	1·980
Y'_i vs M'_i and B'_i	0·9998	0·003	40400	1·80	0·980	118	0·616	65·2	1·983

Meaning of the symbols used:
R = correlation coefficient adjusted for degrees of freedom
S = standard deviation in extinction units
F = F-test for significance of fit
DW = Durbin-Watson test,
$t(r_M)$ = Student's t-test for r_M
$t(r_B)$ = Student's t-test for r_B

Table shows that the reproducibility of the background-spectrum was not better than 5%. For the experiments with benzyl alcohol rather high membrane concentrations were needed to obtain a measurable decrease in aqueous benzyl alcohol concentration on attaining equilibrium. It was found that the reproducibility in this case was not better than 10%. Generally, the reproducibility is supposed to depend on the amount of membranes involved and on the presence of inhomogeneities unavoidable in membrane suspensions prepared by hypotonic haemolysis.

These considerations led us to the conclusion that acceptable regressions should result in an r_M-value between 0·9 and 1·1. Unacceptable low r_M-values were systematically found when the signal to noise ratio was very small. These findings are in accordance with Legget's statement (1977) that multiple regression analysis will fail in those cases where the numerical contribution to the absorption of one or more components of a multicomponent system approaches zero. It seems evident that these spectra can be decomposed by means of non-negative least squares- or simplex optimalization algorithms as pointed out by Legget.

Table 3 gives the result of a control experiment designed to test the validity of Lambert-Beer's law for benzhydrol in the presence of materials causing background absorption. Generally, unknown concentrations can be estimated with errors less than 3%.

Addition of $HClO_4$ to a final concentration of 5% to the supernatants seems to result in complete denaturation of residual haemoglobin, as indicated by the disappearance of the Soret band. The remaining absorption band at 270 nm showed virtually the same characteristics as the original band, apparently because the same amino acids are responsible for the absorption in both cases. Further support for this conclusion comes from the observation that inclusion of a haemoglobin u.v. spectrum in the regression analysis did not result in significant alterations of the results.

Table 3. Determination of benzhydrol in the presence of materials causing background absorption.

Concn. (mol kg^{-1} buffer)	Estimated concn	s.e.
$0·68 \times 10^{-3}$	$0·66 \times 10^{-3}$	$0·03 \times 10^{-3}$
$0·69 \times 10^{-3}$	$0·67 \times 10^{-3}$	$0·03 \times 10^{-3}$
$0·78 \times 10^{-3}$	$0·79 \times 10^{-3}$	$0·03 \times 10^{-3}$
$0·77 \times 10^{-3}$	$0·77 \times 10^{-3}$	$0·03 \times 10^{-3}$

Inclusion of a Durbin-Watson test in the multiple regression program as proposed by Madsen et al (1974) gave ambiguous results; the test was sensitive towards minor baseline fluctuations but, on the other hand, did not detect the occurrence of stray light in the cells caused by spontaneous precipitation of haemoglobin after overnight storage of supernatants at 4 °C. For this reason, preference was given to the above-mentioned physically based tests.

Comparison with single-wavelength analysis

In the classical analysis of u.v. spectra the use of the absorbance at one wavelength λ_a is assumed to be sufficient for the calculation of r_B in equation 1. Furthermore, the contribution to the absorbance at λ_a of materials responsible for background absorp-

tion is supposed to be equal to the absorption at λ_a of a control sample. These assumptions convert equation 1 into:

$$r_B = \frac{Y_a - r_M M_a}{B_a} \qquad (3)$$

where r_M is supposed to be equal to 1. This assumption, however, is not in accordance with the reproducibility of the background spectrum as discussed in the foregoing section. It will follow that the use of equation 3 with $r_M = 1$ for the calculation of r_B only gives reliable results if the signal to noise ratio is high ($Y_a \gg M_a$). This is illustrated in Fig. 1.

If solute- and background-absorption are of comparable magnitude, the use of equation 3 with $r_M = 1$ will give rise to inaccuracies as illustrated in Fig. 2. The improvement achieved by multiple regression consists of estimating a more realistic value for r_M. Insertion of this r_M-value in equation 3 would result in an r_B-value identical to the r_B-value established by the regression analysis itself.

Conclusion

Two compounds for which accurately measured values were available from literature have been included in our reporting as they provide an external check of the accuracy of the proposed method. Colley et al (1971) reported a value of 0·53 for log P_M of benzyl alcohol at 5 mM, Machleidt et al (1972) a value of 1·96 for log P_M of 4-bromophenol. From the agreement between these and our log P_M values we conclude that the multicomponent approach, which circumvents the use of labelled molecules, can serve as a budget-saving alternative for existing methods.

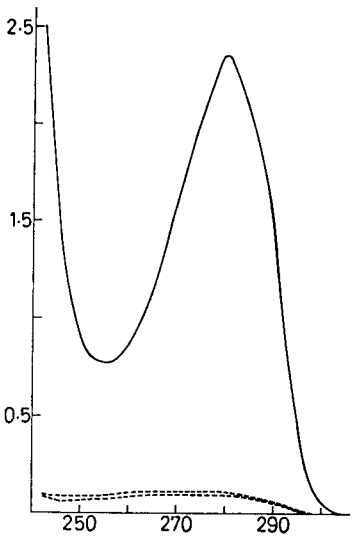

FIG. 1. U.v. absorption (ordinate) of supernatants emerging from centrifugation of erythrocyte membranes in the presence (——) and absence (– – –) of 4-bromophenol at 1·68 mmol kg^{-1}. Abscissa: wavelength (nm).

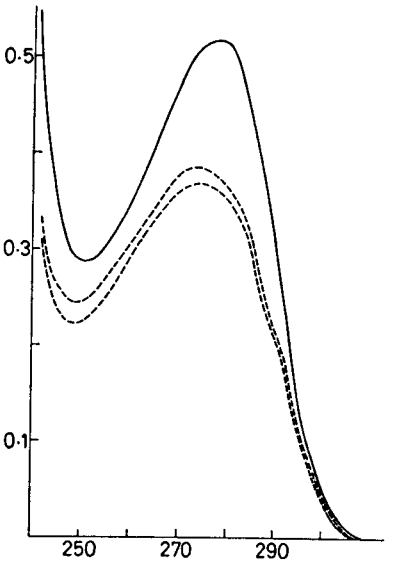

FIG. 2. U.v. absorption (ordinate) of supernatants emerging from centrifugation of erythrocyte membranes in the presence (——) and absence (– – –) of 4-bromophenol at 0·17 mmol kg^{-1}. Abscissa: wavelength (nm).

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