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Hydrophobic interaction chromatography of proteins on Separon HEMA

III. Selection of suitable gradient conditions for the separation of proteins by hydrophobic interaction chromatography

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ABSTRACT

The aim of this work was to verify the possibility of the application of the simple approach to the prediction of the peak maximum position and peak width. The system studied was based on the sorbent Separon HEMA 1000 H benzoyl and on several proteins which were eluted with a decreasing concentration of ammonium sulphate. It was established that the relationships based on the approach according to Yamamoto *et al.* required correction, because a "limit" concentration of salt exists. A protein leaves the column at this "limit" concentration always if the initial concentration of salt in the gradient is higher than this "limit" value. A knowledge of the "limit" values of salt concentrations together with a knowledge of mutual ln k' *vs.* $[(NH_4)_2SO_4]$ dependences of the mixture of proteins (determined from isocratic experiments) is necessary in order to choose the elution conditions, *e.g.*, the gradient time, initial and final salt concentrations and in some instances even the length of the column.

INTRODUCTION

In previous work [1] we studied hydrophobic interaction chromatography (HIC) on unmodified Separons HEMA and determined the influence of the initial concentration of salt (φ_0) on gradient separations and of the mutual position of the isocratic dependences ln $k' \approx \varphi$ on the separation of a mixture of standard proteins. In subsequent work [2], we achieved a substantial improvement in separation efficiency by chemical modification of Separon HEMA 1000 H. In this work, we investigated the possibility of utilizing isocratic ln $k' = f(\varphi)$ data for the selection of the optimum separation conditions.

The theory of gradient elution was developed by various groups [3–11]. A number of Snyder and co-workers' papers [4–9] are devoted to the application of this theory in the chromatography of proteins, especially in the area of reversed-phase

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chromatography (RPC). Considering the calculation of retention time, t_R, and retention volume, V_R, the method is based on a simple integral equation balancing the volume of mobile phase passing through the centre of a peak during its movement along the column. The analytical solution of this equation has frequently been published with respect to the linear dependence of the logarithm of the capacity factor on the concentration of salt and with respect to the linear course of a gradient [5]. The calculation of the number of plates is based on Knox's equation, the coefficients of which are determined from empirical equations [6]. On the basis of the relationships obtained it is possible to establish an optimization strategy for gradient parameters.

Katti *et al.* [12] tried to predict the retention times of protein peaks in hydrophobic interaction chromatography (HIC) with the help of a mathematical model. In our own work we applied the ideas published by Yamamoto *et al.* [13] on ion-exchange chromatography (IEC) in the field of HIC. Our aim was to determine not only the retention time but also the peak width so as to follow the optimization of the separation of a model pair of proteins by HIC.

THEORY

Concerning the calculation of t_R, Yamamoto *et al.*'s [13] model began with an equation expressing the velocity of the peak movement along a column which is valid for equilibrium chromatography:

$$\frac{dz_p}{dt} = \frac{u}{1 + k'} \tag{1}$$

where z_p is the coordinate of the peak maximum ($z_p \in \langle 0,L \rangle$, where L is the column length), t is time, u is the linear velocity of eluent, k' is the capacity factor [for gradient conditions $k' = f(\varphi)$, where φ is the concentration of salt]. The steepness of the linear gradient of salt concentration is defined by

$$g = \frac{\varphi_0 - \varphi_t}{t_g} \tag{2}$$

where φ_t is the terminal concentration of salt and t_g is the duration of the gradient. Considering a gradient mixer delay t_D and zero salt retention, the course of salt concentration behind the column is expressed by

$$\varphi(t) = \varphi_0 - g(t - t_0 - t_D) \tag{3}$$

where t_0 is the dead time of the column, $t_0 = L/u$.

The salt concentration (φ) at the peak maximum during its movement along the column is described by the function

$$\varphi(t,z_p) = \varphi_0 - g\left(t - \frac{z_p}{u} - t_D\right) \tag{4}$$

and the following relationship describes its change:

$$\frac{d\varphi}{dz_p} = -g\left(\frac{dt}{dz_p} - \frac{1}{u}\right) \tag{5}$$

Combining eqns. 1 and 5, we obtain

$$\frac{d\varphi}{dz_p} = -g\left[\frac{k'(\varphi)}{u} + \frac{1}{u} - \frac{1}{u}\right] = -g \cdot \frac{k'(\varphi)}{u} \tag{6}$$

and in an integral form

$$\int_{\varphi_0}^{\varphi_e} \frac{d\varphi}{k'(\varphi)} = -g \cdot \frac{1}{u} \int_0^L dz_p \tag{7}$$

$$\int_{\varphi_e}^{\varphi_0} \frac{d\varphi}{k'(\varphi)} = gt_0 \tag{8}$$

The value of φ_e results from the numerical solution of eqn. 8; then, t_R can easily be calculated:

$$t_R = \frac{\varphi_0 - \varphi_e}{g} + t_0 + t_D \tag{9}$$

Yamamoto *et al.* [13] assumed that so-called "steady state" is reached, *i.e.*, the state when the peak broadening caused by its movement along the column is in equilibrium with its sharpening which is a consequence of the faster movement of the rear edge of peak in comparison with the front edge. If we accept this assumption, the solution of the fundamental mathematical model based on the Plate theory [13] gives the relationships for the number of plates, N_p, and σ^2:

$$N_p = \frac{L}{\frac{2D_L}{u} + \frac{d_p^2\, u\, k'_e}{30\bar{D}_e(1 + k'_e)^2}} \tag{10}$$

$$\sigma_\theta^2\, N_p = -\frac{1}{2} \cdot \frac{(1 + k'_e)^3}{gt_0\left(\frac{dk'}{d\varphi}\right)_e} \tag{11}$$

where D_L is the coefficient of axial dispersion, $\bar{D}$ is the coefficient of diffusion inside the particle, the subscript e indicates the values of the salt concentration equal to that at which a protein leaves the column and θ is non-dimensional time or volume ($\theta = t/t_0$ or V/V_0).

EXPERIMENTAL

Ribonuclease A (RIB), lysozyme (LYS), chymotrypsin (CHYM), soybean trypsin inhibitor (STI) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, U.S.A.). Proteins were dissolved in 0.1 *M* phosphate buffer (pH 7.0) containing the initial concentration of ammonium sulphate chosen for a given gradient. A volume of 20–60 μl of each protein solution was injected onto the column. The stainless-steel columns (50 × 4 mm I.D., void volume 0.5 ml) were packed with Separon HEMA 1000 H (10 μm) (Tessek, Prague, Czechoslovakia) modified by esterification with benzoyl chloride [2].

All experiments were performed at room temperature using an LKB (Bromma, Sweden) high-performance liquid chromatographic system consisting of a pump (Model 2150), a diode-array detector (Model 2140), a controller (Model 2152) and an AT computer with LKB evaluation software.

RESULTS

To utilize the relationships given above, a knowledge of the dependence k' *vs.* φ is necessary. Hence it is necessary to carry out and to evaluate isocratic experiments. The relationships between ln k' and φ in the isocratic HIC of proteins are known frequently to be linear. Analogous relationships were used to describe the dependence σ^2 *vs.* φ:

$$\ln k' = A + B\varphi \quad (12)$$

$$\ln \sigma^2 = a + b\varphi \quad (13)$$

The parameters A, B, a and b were calculated from experimental data by linear regression. The results are summarized in Table I, including correlation coefficients. The experimental data for ln $k' \approx \varphi$ for the proteins studied are shown in Fig. 1.

Several gradient experiments were carried out with STI and the concentration of salt, φ_e (concentration of salt at which protein left the column), was found to be almost constant over a wide range of g (g = 0.02–0.1 mol dm^{-3} min^{-1}). The value of φ_e calculated according to eqn. 8 was also nearly constant (the parameters from Table I were used); however this value was significantly lower than that obtained from the experimental results. Similar results were obtained with the other proteins. To achieve

TABLE I

THE PARAMETERS OF EQNS. 12 AND 13

Protein	Ln $k' = A + B\varphi$			Ln $\sigma^2 = a + b\varphi$		
	A	B	Correlation coefficient	a	b	Correlation coefficient
STI	−8.3464	8.4988	0.9952	−6.419	12.66	0.9967
LYS	−7.4092	5.8509	0.9930	−3.1929	7.1571	0.9838
CHYT	−10.0397	8.2847	0.9983	−6.0483	10.5500	0.9995
RIB	−9.1737	5.8085	0.9978	−7.9846	8.5036	0.9577
BSA	−16.7388	1.4250	0.9995	−21.508	19.5000	0.9863

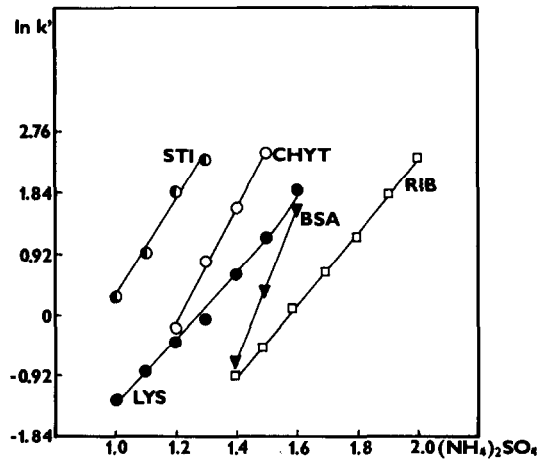

Fig. 1. Experimental dependences of ln k' on concentration of ammonium sulphate, φ. Sorbent: Separon HEMA 1000 H benzoyl. Concentration in mol dm^{-3}.

agreement between the two sets of values we introduced a correction parameter k'' into eqn. 8:

$$\int_{\varphi_e}^{\varphi_0} \frac{d\varphi}{k' - k''} = gt_0 \tag{14}$$

It would have been advantageous if one could have put instead of k'' the value of k' for φ corresponding to the experimentally determined constant φ mentioned above. Considering the course of the exponential functions on the left-hand sides of eqns. 8 and 14, it is clear that this simplification will not produce great differences. The values of φ_e for the proteins studied are given in Table II.

The concentration of salt at which a protein leaves the column was almost constant if the starting concentration of salt was chosen to be higher than the value $(\varphi_e)_{lim}$. Then the solution of eqn. 10 indicates that the number of theoretical plates, N_p, is also constant provided that the column length and the mobile phase velocity are also

TABLE II

THE VALUES OF k'' AND φ_e FOR THE STUDIED PROTEINS

Protein	k''	φ_e (mol dm^{-3})
STI	6.0	1.19
LYS	4.5	1.52
CHYT	5.0	1.41
RIB	6.0	1.89
BSA	6.0	1.62

constant. In addition, the relationship for σ_θ^2 at two different gradients follows from eqn. 11:

$$\frac{(\sigma_\theta^2)_1}{(\sigma_\theta^2)_2} = \frac{g_2}{g_1} \tag{15}$$

The applicability of eqn. 15 in HIC systems has been shown by Miller and Karger [10], as peak height is inversely proportional to peak area.

Using parameters a and b we could determine σ^2 for $\varphi = (\varphi_e)_{lim}$ under isocratic conditions. In addition, we assumed this value of σ^2 to be the same even for a very gentle gradient with $\varphi_0 > (\varphi_e)_{lim}$, *i.e.*, for very small g. However, if g reaches a certain value (critical value), σ^2 will fall according to eqn. 15. The values of these g_{crit} for individual proteins were determined from eqn. 15 utilizing the value of σ^2 for $(\varphi_e)_{lim}$ and the results of gradient experiments for $\varphi_0 > (\varphi_e)_{lim}$. These calculations resulted in average values of g_{crit} for the proteins studied (see Table III). Utilizing the parameters A, B, a, b, k'' and g_{crit}, the calculations of t_R and $W_{1/2}$ ($W_{1/2} = 2.35\sigma$) were carried out for gradient experiments. Calculated t_R and $W_{1/2}$ values for LYS and RIB are given in Tables IV and V as examples, together with the experimental results.

The other results were obtained when LSS theory [5] was applied to the calculations of some retention times and the values of φ_e are given in the Tables VI–VIII. The experimental isocratic data were employed (see Fig. 1). The following equations were used in t_R calculations [5]:

$$t_R = \frac{t_0}{b} \cdot \log\left(2.3\, k'_0 b \cdot \frac{t_{sec}}{t_0} + 1\right) + t_{sec} + t_D \tag{16}$$

where $k'_0 = k'$ for φ_0 (see eqn. 10), $b = (B/2.303)\Delta\varphi t_0/t_g$ ($\Delta\varphi = \varphi_0 - \varphi_{final}$), $t_0 = V_0/F = 0.515/0.5 = 1.03$ min, t_{sec} is the elution time of the excluded proteins and $t_D = V_D/F = 4$ min.

DISCUSSION

It is difficult to explain the existence of $(\varphi_e)_{lim}$ and consequently the necessity for the correction of eqn. 8 with the parameter k''. Applying the isocratic data in eqn. 8, one would have to obtain values of t_R close to the experimental values. We did not

TABLE III

THE VALUES OF g_{crit} FOR STUDIED PROTEINS

Protein	g_{crit} (mol dm^{-3} min^{-1})
STI	0.02
LYS	0.02
CHYT	0.012
RIB	0.013
BSA	0.0022

TABLE IV

CALCULATED AND EXPERIMENTAL DATA FOR LYS

t_0 (min)	t_g (min)	φ (mol dm^{-3})	g (mol dm^{-3} min^{-1})	t_R (min)		φ_e (mol dm^{-3})		$W_{1/2}$ (ml)	
				Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
1.03	30	2.0	0.0667	11.77	11.75	1.55	1.552	0.62	0.56
1.03	20	2.0	0.100	9.50	9.69	1.55	1.534	0.40	0.46
1.03	20	1.8	0.090	7.82	7.96	1.55	1.536	0.52	0.48
1.03	30	1.8	0.060	9.17	9.15	1.55	1.553	0.65	0.59
1.03	30	1.6	0.053	6.12	6.17	1.54	1.539	0.62	0.63
1.03	20	1.6	0.080	5.62	5.89	1.55	1.531	0.46	0.51
1.03	20	1.4	0.070	3.23	3.28	1.40	1.40	0.54	0.59
1.03	30	1.4	0.0467	3.23	3.28	1.40	1.40	0.55	0.59
1.03	20	1.2	0.060	1.77	1.73	1.20	1.20	0.24	0.29
1.03	30	1.2	0.040	1.73	1.73	1.20	1.20	0.39	0.29
2.06	20	2.0	0.10	11.70	10.82	1.44	1.524	0.40	0.46

succeed even in the application of the model based on the rate eqn. 1 [1]. We always obtained values higher then experimental values [*i.e.*, $\varphi_e < (\varphi_e)_{lim}$]. Analogous results were obtained using eqn. 16 for $t_{sec}/t_0 = 1$ (see Tables VI–VIII), although the exclusion limit of the sorbent used is 1000 kilodaton and the relative molecular weights of the proteins examined are much smaller. On the other hand, excellent agreement between the calculated and experimental data was achieved for $t_{sec}/t_0 = 0.5$. This value is too small, in our opinion, because the column volume accessible to proteins would be only 20%. Nevertheless, it follows from these calculations that the concentration of salt at which a protein leaves the column is also nearly constant. In the work of Yamamoto *et al.* [13], the coefficient k'' has the real meaning of being the capacity factor of a salt, *e.g.*, sodium chloride, which is not equal to zero in IEC. On the other hand, its physical interpretation is unclear in HIC because the capacity factor of ammonium sulphate is

TABLE V

CALCULATED AND EXPERIMENTAL DATA FOR RIB

t_0 (min)	t_g (min)	φ_0 (mol dm^{-3})	g (mol dm^{-3} min^{-1})	t_R (min)		φ_e (mol dm^{-3})		$W_{1/2}$ (ml)	
				Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
1.03	30	2.0	0.0667	7.12	6.59	1.86	1.846	0.56	0.52
1.03	20	2.0	0.100	6.25	6.13	1.88	1.890	0.39	0.42
1.03	20	1.8	0.090	4.25	4.79	1.80	1.80	0.43	0.76
1.03	30	1.8	0.060	4.37	4.74	1.80	1.80	0.62	0.76
1.03	30	1.6	0.053	2.32	2.19	1.60	1.60	0.38	0.32
1.03	20	1.6	0.080	2.25	2.19	1.60	1.60	0.34	0.32
1.03	30	2.2	0.073	9.33	9.13	1.88	1.899	0.56	0.49
1.03	20	2.2	0.110	7.80	7.84	1.89	1.891	0.37	0.40
1.03	20	2.5	0.125	9.67	9.91	1.92	1.890	0.34	0.37
1.03	30	2.5	0.083	12.17	12.27	1.905	1.897	0.44	0.46
2.06	20	2.2	0.110	9.88	8.90	1.78	1.888	0.28	0.40

TABLE VI

THE EXPERIMENTAL AND CALCULATED t_R AND φ_e VALUES (USING LSS THEORY) FOR LYS

t_g (min)	φ_0 (mol dm^{-3})	t_R (min)			φ_e (mol dm^{-3})		
		Exp.	Calc.		Exp.	Calc.	
			A[a]	B[b]		A[a]	B[b]
30	2.0	11.77	11.56	13.80	1.55	1.56	1.42
20	2.0	9.50	9.86	11.54	1.55	1.52	1.35
20	1.8	7.82	7.74	9.94	1.55	1.56	1.36
30	1.8	9.17	9.14	10.33	1.54	1.55	1.38
30	1.6	6.12	6.93	8.83	1.54	1.50	1.40
20	1.6	5.62	6.62	8.14	1.55	1.47	1.35

[a] $t_{sec}/t_0 = 0.5$.
[b] $t_{sec}/t_0 = 1.0$.

really zero, as was verified experimentally. Nevertheless, this correction is necessary. The reason probably lies in changes in protein conformation during separation, *i.e.*, different conformations for isocratic *vs.* gradient elution, as postulated by Hodder *et al.* [14].

A comparison of the experimentally obtained and calculated values of t_R and $W_{1/2}$ (see Tables IV and V) gives very good agreement. For $\varphi_0 < (\varphi_e)_{lim}$ the calculation is carried out as for isocratic conditions for the whole length of the column if the retention volume is smaller then 2.5 cm^3 $[(t_0 + t_D)F]$. If V_R is higher, the calculation is

TABLE VII

THE EXPERIMENTAL AND CALCULATED t_R AND φ_e VALUES (USING LSS THEORY) FOR CHYM

t_g (min)	φ_0 (mol dm^{-3})	t_R (min)			φ_e (mol dm^{-3})		
		Exp.	Calc.		Exp.	Calc.	
			A[a]	B[b]		A[a]	B[b]
30	2.0	14.15	14.05	14.83	1.39	1.40	1.35
20	2.0	10.85	11.36	12.72	1.42	1.37	1.23
20	1.8	9.33	9.77	11.22	1.41	1.37	1.24
30	1.8	11.82	11.62	13.52	1.39	1.40	1.29
30	1.6	8.98	8.81	10.72	1.39	1.40	1.30
20	1.6	7.43	7.90	9.39	1.41	1.37	1.25
30	2.2	15.50	16.11	17.21	1.43	1.39	1.27
20	2.2	11.90	12.65	13.41	1.44	1.36	1.22
20	2.5	13.27	14.20	14.87	1.47	1.35	1.20
30	2.5	17.55	18.51	19.47	1.46	1.38	1.25

[a,b] See Table VI.

TABLE VIII

THE EXPERIMENTAL AND CALCULATED t_R AND φ_e VALUES (USING LSS THEORY) FOR RIB

t_g (min)	φ_0 (mol dm^{-3})	t_R (min)			φ_e (mol dm^{-3})		
		Exp.	Calc.		Exp.	Calc.	
			A[a]	B[b]		A[a]	B[b]
30	2.0	7.12	7.58	8.95	1.86	1.83	1.70
20	2.0	6.25	7.07	8.06	1.88	1.80	1.64
30	2.2	9.33	9.69	11.57	1.88	1.86	1.71
20	2.2	7.80	8.53	9.55	1.89	1.82	1.64
20	2.5	9.67	10.52	11.47	1.92	1.75	1.63
30	2.5	12.17	12.74	14.13	1.91	1.86	1.70

[a,b] See Table VI.

carried out as for gradient conditions from the point in the column when the peak maximum is "matched" by the descending concentration of salt (the beginning of the gradient). Eqns. 10 and 11 were used for the calculation of σ^2 for connection of two identical columns (50 × 2 mm I.D.). In this instance the experimental values of t_R were always nearly 1 min higher. This difference was found repeatedly even for a 100-mm column. This disagreement could be removed applying another value of k''.

From Yamamoto *et al.*'s [13] conclusions on the influence of the gradient conditions selected on the resolution of a pair of proteins (R_s) it follows that (i) resolution increases with depression of the gradient slope, $R_s \approx [(t_g F)/\Delta\varphi]^{1/2}$, (ii) if $(\Delta\varphi/t_g F)$ is constant, R_s is independent of the length of column and (iii) if $(V_0 \Delta\varphi/t_g F)$ is constant, R_s increases with $L^{1/2}$.

To obtain a better experimental verification of the given conclusions, the gradient conditions and a pair of proteins were chosen for which separation is more efficient. The results, including calculated data, are given in Table IX and confirm that the conclusions given above are valid for the system studied.

TABLE IX

THE CALCULATED AND EXPERIMENTAL DATA INCLUDING RESOLUTION FOR RIB AND BSA

L (mm)	t_g (min)	φ_0 (mol dm^{-3})	RIB				BSA				R_s	
			t_R (min)		$W_{1/2}$ (ml)		t_R (min)		$W_{1/2}$ (ml)		Exp.	Calc
			Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.		
50	60	2.0	8.22	7.58	0.89	0.78	16.87	16.10	0.99	0.87	2.70	3.05
50	60	2.4	16.45	16.73	0.66	0.76	24.53	24.36	0.85	0.76	2.91	2.94
100	60	2.0	11.83	9.18	0.71	0.72	19.85	17.37	1.09	0.80	2.62	3.17
100	60	2.4	20.10	18.63	0.69	0.66	27.03	25.51	0.93	0.73	2.51	2.93
50	30	2.0	6.83	6.59	0.49	0.51	10.94	10.68	0.68	0.57	2.07	0.24

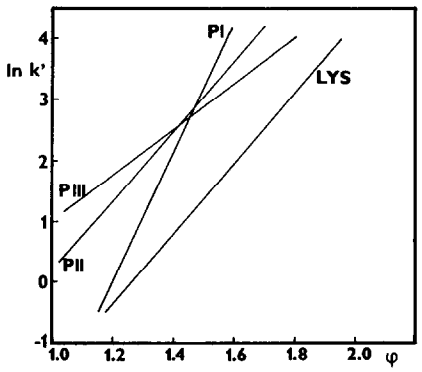

Fig. 2. Dependences of ln k' on φ (mol dm^{-3}) for hypothetical proteins and LYS.

In addition, we investigated the relationships between R_s and φ_0, t_g and L for different mutual positions of dependences ln $k' \approx \varphi$. We chose three pairs of hypothetical proteins, PI, PII and PIII, with lysozyme. The courses of ln $k' \approx \varphi$ are demonstrated in Fig. 2. Parameters a and b describing the relationship $\sigma^2 \approx \varphi$ were chosen to be identical with those for LYS.

The value of k'' [or $(\varphi_e)_{lim}$] and g_{crit} were chosen in the same way. The results of calculations are shown in Fig. 3. If $\varphi_0 > (\varphi_e)_{lim}$ (1.52 mol dm^{-3}), the suggested relationships correspond with the conclusions of Yamamoto *et al.* [13] and others [3–9]. It is also clear that a knowledge of $(\varphi_e)_{lim}$ is very important. With the pair PI–LYS and PII–LYS it is always advantageous to choose φ_0 near to $(\varphi_e)_{lim}$, where an optimum (not too significant) exists. For the pair PIII–LYS it is more advantageous to start the gradient below this limit value or to chose isocratac conditions and to profit from the influence of a longer column. This information was verified experimentally with the pair LYS–BSA, where the mutual position of the dependence ln $k' \approx \varphi$ is analogous to that of LYS–PIII (see Fig. 1). An increase in the column length (50, 100 and 150 mm) resulted in an increase in resolution (R_s = 0.56, 0.82 and 0.99, respectively) under isocratic elution conditions (φ = 1.4 mol dm^{-3}). On the other hand, this pair of proteins could not be separated with any gradient elution if $\varphi_0 \geqslant (\varphi_e)_{lim}$.

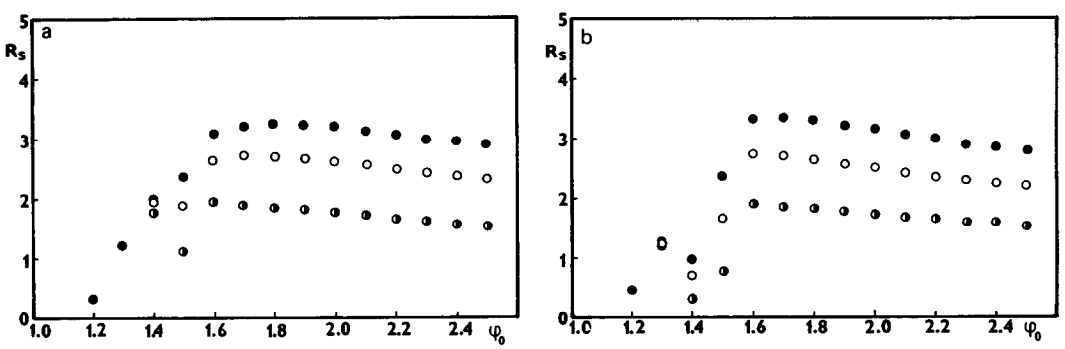

Fig. 3.

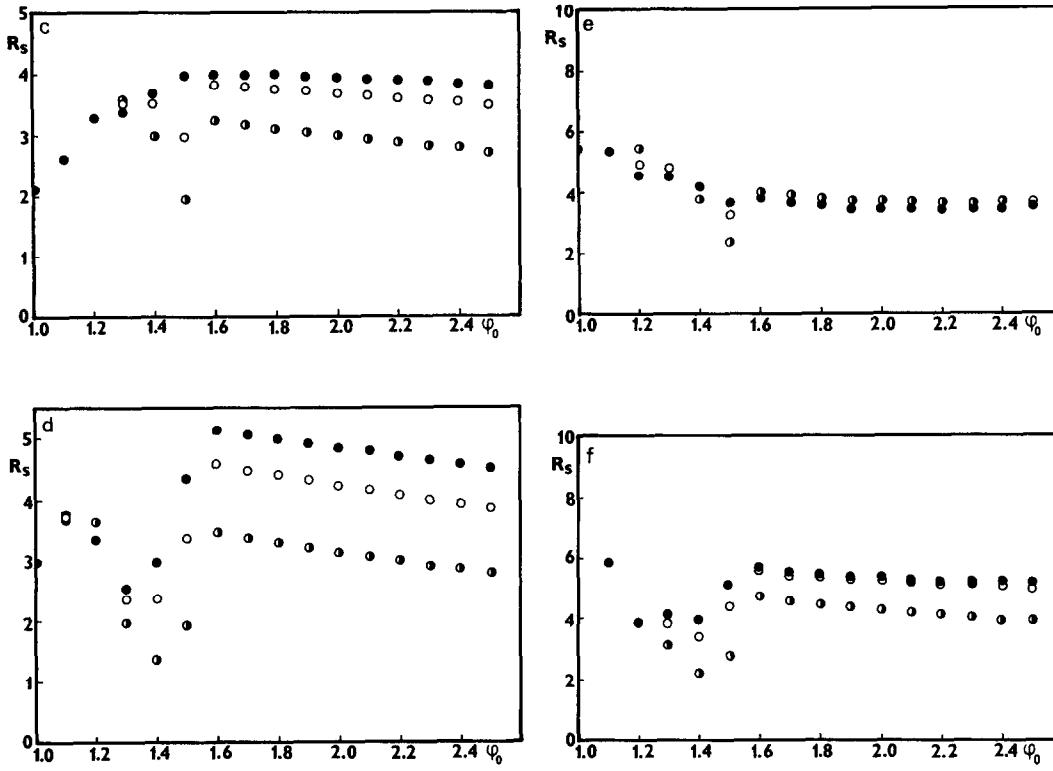

Fig. 3. Influence of φ_0 (mol dm^{-3}), t_g and L on the resolution of hypothetical proteins and LYS. (a) LYS–PI, L = 5 cm; (b) LYS–PI, L = 10 cm; (c) LYS–PII, L = 5 cm; (d) LYS–PII, L = 10 cm; (e) LYS–PIII, L = 5 cm; (f) LYS–PIII, L = 10 cm. In all instances: ●, 60 min; ○, 40 min; ◑, 20 min.

CONCLUSION

The strategy of the selection of gradient elution conditions, from the point of view of the resolution of proteins, following from the model used here does not differ in principle from those proposed by other workers [3–9,13]. From the experiments and calculations in this work it follows that in the HIC of proteins there can exist very gentle dependence of the salt concentration at which a protein leaves the column at $(\varphi)_e$ on the gradient steepness (g) and on the value of the product gt_0, so that this value of φ_e will be almost constant, $(\varphi_e)_{lim}$, for a given protein. For an unknown mixture of proteins it is not difficult to determine the values of $(\varphi_e)_{lim}$ for individual proteins if the quality of the separation allows the identification of individual peaks. One has only to carry out two or three gradient experiments with a high starting concentration of salt (φ_0), then φ_0 should be chosen to be close to the highest $(\varphi_e)_{lim}$. A knowledge of isocratic relationships ($\ln k' \approx \varphi$) even gives the chance to decide on isocratic elution in some special cases.

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