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THEORY OF CHROMATOGRAPHY OF RIGID ROD-LIKE MACROMOLECULES ON HYDROXYAPATITE COLUMNS

VII. INVESTIGATIONS ON THE RESOLVING POWER OF THE COLUMNS

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SUMMARY

The resolving powers of hydroxyapatite columns for several types of mixtures of rigid rod-like model molecules with different values of the adsorption energies and lengths obtained under different chromatographic conditions have been investigated theoretically. It has been shown that, when the properties of different molecules are similar, high resolutions can generally be obtained by using a small slope of the gradient of competing ions and short columns (or high loads). High resolutions can also be obtained when the activity of competing ions in the initial buffer is sufficiently small. When both the molecular length and the adsorption energy per unit molecular length are not constant, the order of elution of different molecules depends on the chromatographic conditions. When all molecules have the same length, the resolution increases with increase in molecular length.

INTRODUCTION

In Parts I–VI of this series[1–6], we developed a theory of the chromatography of rigid rod-like macromolecules, taking into account the interactions among molecules adsorbed on the surface of hydroxyapatite (HA), and we showed that the results for DNA and tropocollagen can be explained satisfactorily when repulsive interactions are assumed. In this theory, however, we assumed that an adsorbed macromolecule covers an infinite number of sites of HA. In other papers[7,8], we develop a theory which is valid for molecules of any shape and dimensions but which does not take into account interactions between adsorbed particles. Using these theories and the experimental results, several experimental parameters concerning the properties of the column and the adsorbed molecules have been estimated (see Appendix I in ref. 7 and ref. 8). It should be recalled that another hypothesis was also considered in these papers[1–7]. We assumed that molecules are adsorbed on to only a single type of crystal site and that their desorption is caused by only a single type of competing ion. This hypothesis, however, is valid for DNA that has only negatively charged adsorption groups and holds approximately even for tropocollagen[7,8]. In the present paper, using this hypothesis and taking into account both repulsive interactions among adsorbed molecules and the fact that the length of a molecule

is finite, we calculate the chromatograms for rigid rod-like model molecules and discuss the resolving power of the column under different experimental conditions obtained by changing the column length, the slope of the activity gradient of competing ions (henceforth referred to as s.o.g.) and amount of macromolecules loaded. We shall limit this study to the case when the s.o.g. is linear. In order to simplify the calculations, we also limit the maximum number of components in the mixture to two and we treat (1) the case when they have the same molecular length but different adsorption energies, (2) the case when they have different molecular lengths but the same adsorption energy per unit molecular length and (3) the case when both the molecular length and the adsorption energy per unit molecular length are not constant. We always assume that the diameter of the molecules is constant. All calculations are made only as a first approximation.

THEORETICAL

In Part II[2], we showed that the experimental parameter $B_{(\rho')}$ for a molecular species ρ' in a mixture of several species can be expressed in general by eqns. 43 and 44 in that paper. These equations can be rewritten, with slight modifications, as

$$B_{(\rho')} = 1 - \frac{\chi_{(\rho')}}{a_{(\rho')}} \tag{1}$$

$$\frac{\chi_{(\rho')}}{a_{(\rho')}} = G_{(\rho')}(\chi_{(1)}, \ldots, \chi_{(\rho)}, \Lambda_2) \tag{2}$$

and

$$G_{(\rho')}(\chi_{(1)}, \cdots, \chi_{(\rho)}, \Lambda_2) = \frac{1}{1 + \{\beta_3 \sigma_{(\rho')} p_{(\rho')}(\chi_{(1)}, \cdots, \chi_{(\rho)})\}^{-1} e^{\frac{u_{(\rho')}}{kT}\left(1 - \frac{\Xi}{w_{(\rho')}}\sqrt{\varkappa}\right)} (\Lambda_2 + 1)^{x'_{(\rho')}}} \tag{3}$$

where $\chi_{(1)}, \chi_{(2)}, \ldots, \chi_{(\rho)}$ are the "proportions" of the surface of HA occupied by species 1, 2, ..., ρ, respectively, and their sum

$$\chi = \sum_{\rho'=1}^{\rho} \chi_{(\rho')} \tag{4}$$

can vary between 0 and 1; $p_{(\rho')}(\chi_{(1)}, \chi_{(2)}, \ldots, \chi_{(\rho)})$, where ρ' indicates any one of 1, 2, ..., ρ, is the probability that, when a new molecule of species ρ' is added at random on to the crystal surface, the "proportions" $\chi_{(1)}, \chi_{(2)}, \ldots, \chi_{(\rho)}$ of which are already occupied by molecules of species 1, 2, ..., ρ, respectively, it is not superposed on the already adsorbed molecules; $a_{(1)}, a_{(2)}, \ldots, a_{(\rho)}$ are parameters proportional to the total amounts (weights) of species 1, 2, ..., ρ in a column section; Λ_2 is proportional to the activity of competing ions in that section; $u_{(1)}, u_{(2)}, \ldots, u_{(\rho)}$ are the adsorption energies of species 1, 2, ..., ρ, provided that they are adsorbed in isolated states; $w_{(1)}, w_{(2)}, \ldots, w_{(\rho)}$ are the relative values of $\xi_{(1)}, \xi_{(2)}, \ldots, \xi_{(\rho)}$ (see eqn. 45 in Part II[2]) to $\xi_{(1)}$, defined by eqn. 1 in Part V[5], respectively; $x'_{(1)}, x'_{(2)}, \ldots, x'_{(\rho)}$ are the numbers of crystal sites "covered" by a single molecule of

species 1, 2, ..., ρ, respectively; Ξ is a parameter describing the interactions among adsorbed macromolecules, its value being positive in the case of repulsions and zero when there are no interactions; $\sigma_{(1)}$, $\sigma_{(2)}$, ..., $\sigma_{(\rho)}$ are the symmetry factors of the molecules; and β_3 is a parameter related only to the properties of the column. If we use the approximation

$$p_{(\rho')}(\chi_{(1)}, \cdots, \chi_{(\rho)}) \approx p(\chi) = 1 - \chi \tag{5}$$

$G_{(\rho')}$ is a function of only χ and Λ_2. In Fig. 1, $G_{(\rho')}$ in the absence of competing ions (*i.e.*, when Λ_2 or $y = 0$) is plotted as a function of χ for ten molecular species, 1, 2, ..., 10, with the same length and with different values of $w_{(\rho')}$ (1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1, respectively). In Fig. 1, it has been assumed that $x'_{(1)} = \ldots = x'_{(10)} = 300$ and that $-u_{(1)}/kT = 20$, which are reasonable values for tropocollagen (see Appendices I and II in ref. 7). The other assumptions that have been made for the calculation of Fig. 1 are: $\Xi = 0.5$, $\beta_3 = 3 \cdot 10^{-4}$ and $\sigma_{(1)} = \ldots = \sigma_{(10)} = 1$. We took for β_3 the value of β_C in Appendix I in ref. 7, as molecules such as tropocollagen and DNA are adsorbed on to C sites (see refs. 7 and 8). The assumption that $\sigma = 1$ is reasonable because, in most actual cases, molecules are asymmetrical.

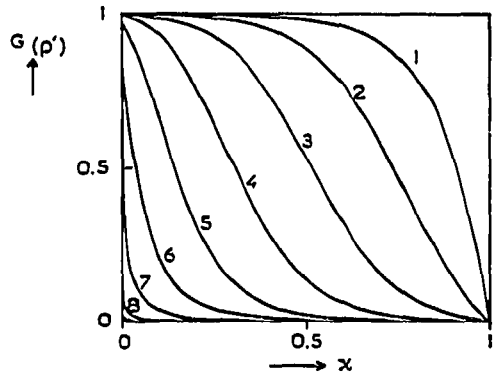
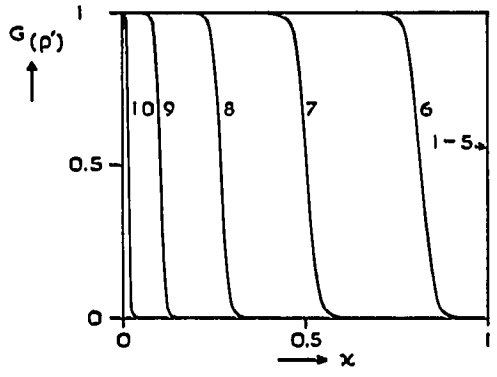


Fig. 1. $G_{(\rho')}$ in the absence of competing ions (*i.e.*, when Λ_2 or $y = 0$) as a function of χ for ten molecular species, 1, 2, ..., 10, with the same length (*i.e.*, with $x'_{(1)} = \ldots = x'_{(10)} = 300$) and different values of $w_{(\rho')}$: 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1, respectively. The numbers on the curves are the values of ρ'. When $\rho' \geqslant 9$, the curves tend essentially to $G_{(\rho')} = 0$. It has been assumed that $\beta_3 = 3 \cdot 10^{-4}$ and $\Xi = 0.5$.

Figs. 2 and 3 show how the shapes of the curves $G_{(1)}$, ..., $G_{(10)}$ change when the values of $x'_{(1)}$, ..., $x'_{(10)}$ increase and the ratios $-(u_{(1)}/kT)/x'_{(1)}$, ..., $-(u_{(10)}/kT)/x'_{(10)}$, *i.e.*, $\xi_{(1)}$, ..., $\xi_{(10)}$, are constant. In Fig. 2, the values of $x'_{(\rho')}$ (where $\rho' = 1, 2, \ldots, 10$) and $-u_{(1)}/kT$ are 3000 and 200, respectively, and in Fig. 3, both of these values are infinite. It should be noted that, owing to the approximate relationship given by eqn. 5, the curve for a particular molecular species ρ' depends uniquely on χ and is independent of the types of the other molecules in the mixture, which is evident if all molecules have the same length. (For the case when the molecules have different lengths, see below).

It should be also noted that, as the value of $\xi_{(1)}\varepsilon_3/kT$, where $-\varepsilon_3 (\varepsilon_3 > 0)$



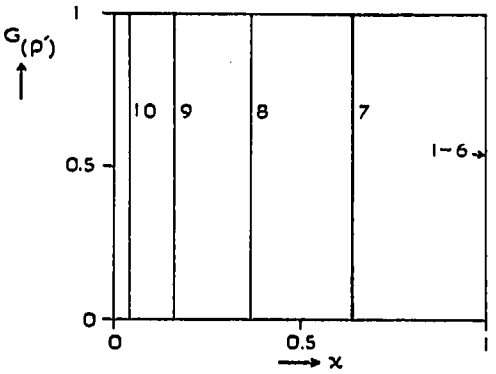


Fig. 2. As Fig. 1 when $x'=3000$ and the ratios $-(u_{(1)}/kT)/x'_{(1)}$, ..., $-(u_{(10)}/kT)/x'_{(10)}$, *i.e.*, $\xi_{(1)}$, ..., $\xi_{(10)}$, are constant.

Fig. 3. As Fig. 1 when $x'=\infty$ and $\xi_{(1)}$, ..., $\xi_{(10)}$ are constant.

is the adsorption energy of the adsorption group of a macromolecule to one of the sites of HA, is usually small, one can write

$$\Lambda_2 = y\Lambda^0_{2(1)} = y(e^{\xi_{(1)}\varepsilon_3/kT}-1) \approx y\,\frac{\xi_{(1)}\varepsilon_3}{kT}$$

where y is the relative value of the activity of competing ions defined by eqn. 58 in Part II and $\Lambda^0_{2(1)}$ is the parameter defined by eqn. 50 in Part II. When Λ_2 is not too large compared with $\Lambda^0_{2(1)}$, *i.e.*, $\xi_{(1)}\varepsilon_3/kT$, one can also write

$$(\Lambda_2+1)^{x'_{(\rho')}} \approx e^{\Lambda_2 x'_{(\rho')}} \approx e^{yx'_{(\rho')}\xi_{(1)}\varepsilon_3/kT}$$

which means that the second term of the denominator of the right-hand side of eqn. 3 can be expressed approximately as

$$\{\beta_3\sigma_{(\rho')}p(\chi)\}^{-1}\exp\left\{\frac{u_{(1)}}{kT}(w_{(\rho')}-y-\Xi\sqrt{\chi})\right\}$$

and that the curve $G_{(\rho')}$ can be considered as a function of $w_{(\rho')}-y$. Therefore, the curves for $w_{(1)}=1$, $w_{(2)}=0.9$, ..., $w_{(10)}=0.1$ in Figs. 1–3 obtained when $y=0$ are identical with those for $w_{(1)}=1$ obtained when $y=0$, $y=0.1$, ..., $y=0.9$, respectively. Curves, for instance, for $w_{(2)}=0.9$, $w_{(3)}=0.8$, ..., $w_{(10)}=0.1$ in Figs. 1–3 obtained when $y=0$ are also identical with those for $w_{(2)}=0.9$ obtained when $y=0$, $y=0.1$, ..., $y=0.8$, respectively.

In Fig. 3 or when $x'_{(1)}$, ..., $x'_{(10)}$ are infinite, $G_{(1)}$, ..., $G_{(10)}$ are step functions of χ and, if $y=0$, the values of χ at which curves $G_{(1)}(\chi)$, ..., $G_{(10)}(\chi)$ show transitions give values of $\chi'_{(1)}$, ..., $\chi'_{(10)}$ defined by eqn. 69 in Part II or the maximum possible values of χ that can be realized by species 1, by both of species 1 and 2, ..., by all of species 1, 2, ..., 10, respectively[2]. It can be seen in Fig. 3 that it is impossible for two different species to have $G_{(\rho')}$ values between 0 and 1 at the same value of χ, so that molecules with a strong affinity for HA are adsorbed in preference to those with a weak affinity for HA. If $x'_{(1)}$, ..., $x'_{(10)}$ are finite (see Figs. 1 and 2),

transitions in $G_{(1)}$, ..., $G_{(10)}$ occur gradually, and two different species can have $G_{(\rho')}$ values between 0 and 1 at the same χ value. In this case, the chromatographic separation between different molecules cannot be complete.

Fig. 4 illustrates the same plot as in Figs. 1–3 for the same model molecules as in Fig. 1 when there are no molecular interactions, *i.e.*, $\Xi=0$. We can show that when $x'_{(\rho')}$ is infinity and the ratio $-(u_{(\rho')}/kT)/x'_{(\rho')}$ is constant, then $G_{(\rho')}=0$ if $\chi=1$ or if $0\leqslant\chi<1$ and $y\geqslant w_{(\rho')}$. If $0\leqslant\chi<1$ and $y<w_{(\rho')}$, then $G_{(\rho')}=1$.

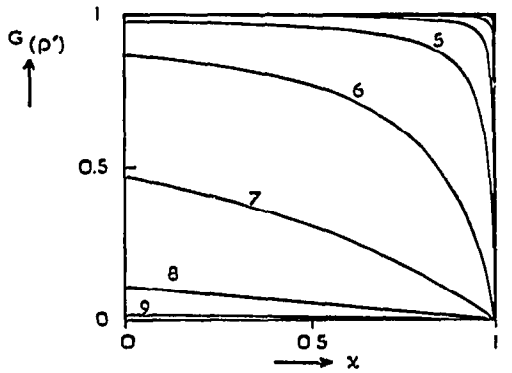


Fig. 4. As Fig. 1 when $\Xi=0$ or when there are no molecular interactions.

$B_{(\rho')}$ as function of $a_{(1)}$, ..., $a_{(\rho)}$ and Λ_2

In the present paper, the theoretical chromatograms will be calculated step by step using the methods developed in the Dynamic sections in Parts I[1] and II[2]. For this purpose, we have to express the parameter $B_{(\rho')}$ as a function of $a_{(1)}$, ..., $a_{(\rho)}$ and Λ_2 (see Part II). This is possible if one can solve for χ the equation

$$\chi=\sum_{\rho'=1}^{\rho} a_{(\rho')}G_{(\rho')}(\chi,\Lambda_2) \tag{6}$$

that can be obtained from eqns. 2 and 4 and if one can estimate $G_{(1)}$, ..., $G_{(\rho)}$ for this value of χ (see eqns. 2 and 1). It should be recalled that $G_{(\rho')}$ is expressed as a function of only χ and Λ_2 by using the approximate relationship given by eqn. 5. In order to solve eqn. 6 for χ, we shall use the following approximation for $G_{(\rho')}$: it can be seen in Figs. 1–4 that $G_{(\rho')}$ decreases, in general, monotonously with increase in χ, that $0\leqslant\lim_{\chi\to 0} G_{(\rho')}\leqslant 1$ and that $\lim_{\chi\to 1} G_{(\rho')}=0$. We consider the tangent of the curve $G_{(\rho')}$ at the point P on which the ordinate value of $G_{(\rho')}$ is equal to $\frac{1}{2}G^0_{(\rho')}$, $G^0_{(\rho')}$ being the limit of G when $\chi\to 0$ (see Fig. 5). We call the corresponding abscissa value $\bar{\chi}_{(\rho')}$. If the tangent intersects with the abscissa at a value less than or equal to unity (see Fig. 5a and 5b), we call this value $\chi^B_{(\rho')}$, and if the tangent intersects with the horizontal line $G_{(\rho')}=G^0_{(\rho')}$ at zero or a positive abscissa value (see Fig. 5a), then we call this value $\chi^A_{(\rho')}$. It is evident that $0\leqslant\chi^A_{(\rho')}\leqslant\chi^B_{(\rho')}\leqslant 1$ and we can express $G_{(\rho')}(\chi)$ approximately as

$$\left.\begin{aligned} &G_{(\rho')}(\chi)\approx G^0_{(\rho')} && (\chi<\chi^A_{(\rho')})\\ &G_{(\rho')}(\chi)\approx\tfrac{1}{2}G^0_{(\rho')}+\left(\frac{dG_{(\rho')}}{d\chi}\right)_{\chi=\bar{\chi}_{(\rho')}}\cdot(\chi-\bar{\chi}_{(\rho')}) && (\chi^A_{(\rho')}\leqslant\chi<\chi^B_{(\rho')})\\ &G_{(\rho')}(\chi)\approx 0 && (\chi\geqslant\chi^B_{(\rho')}) \end{aligned}\right\} \tag{7}$$

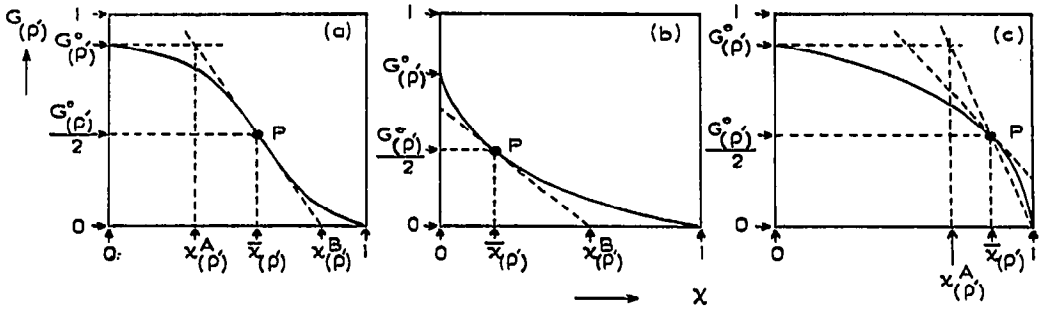


Fig. 5. Approximate representations of the curve $G_{(\rho')}(\chi)$.

in which the second equation shows the tangent of $G_{(\rho')}$ at point P (see Fig. 5a and 5b). Now, if the tangent of $G_{(\rho')}$ intersects with the abscissa at a value greater than unity (see Fig. 5c), we consider the straight line that passes through both the point P and the point (1, 0) and we define $\chi^{A}_{(\rho')}$ as the abscissa value at the intersection of this line with the horizontal line $G_{(\rho')}=G^{0}_{(\rho')}$. We can now express $G_{(\rho')}(\chi)$ approximately as

$$\left.\begin{aligned} G_{(\rho')}(\chi) &\approx G^{0}_{(\rho')} && (\chi<\chi^{A}_{(\rho')}) \\ G_{(\rho')}(\chi) &\approx -\frac{G^{0}_{(\rho')}}{2(1-\bar{\chi}_{(\rho')})}\cdot(\chi-1) && (\chi\geqslant\chi^{A}_{(\rho')}) \end{aligned}\right\} \qquad (8)$$

in which the second equation shows the straight line that passes through both the point P and the point (1, 0) (see Fig. 5c). It can be seen that, by using eqn. 7 or 8, eqn. 6 can be easily solved for χ.

RESULTS OF THE CALCULATIONS OF CHROMATOGRAMS

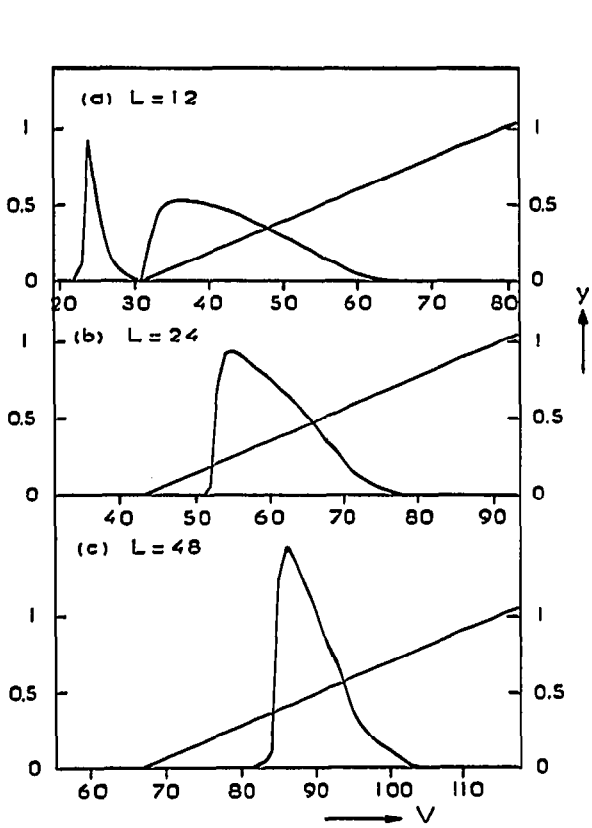
In this section, we present the results of the calculations of theoretical chromatograms in the case of the linear activity gradient. The method used is the same as that in the Dynamic sections of Parts I and II. All calculations are made using $3\cdot10^{-4}$ as the value for the parameter β_3. We assume that $\Xi=0.5$ in all instances when account is taken of the interactions among adsorbed macromolecules.

In order to simplify the discussion, we have summarized in Table I all the data corresponding to our calculated chromatograms. Each row corresponds to a different example. Column 2 refers to the corresponding figure numbers. Columns 3–8 give the characteristics of the samples and columns 9–11 give the chromatographic conditions. The last two columns are given in order to make the reading of the table simple. L^* (see column 9) is defined as the ratio of the column length to the width of the initial zone of adsorbed macromolecules, assuming saturation of the column. On each figure is given the L value which is the number of steps used for the numerical calculations. All the figures are plotted as a function of the ratio, V, of the elution volume to the interstitial volume of a hypothetical section. On the ordinate we have plotted the concentration of macromolecules, f, in arbitrary units and y values; g^* (see column 10 in Table I) is the difference in values of y at the top and the bottom of the column when $L^*=1$. It should be noted that the chromatograms as a function

of y can be characterized uniquely by L^* and g^* and we call these experimental conditions a, b, c, etc. (see column 11 in Table I).

The case of a single molecular species (Cases 1–11 in Table I)

Fig. 6 (Case 1) illustrates the simplest theoretical chromatograms for the case of a single molecular species. The amount of macromolecules loaded is equal to that which can just saturate the column of "length" $L=12$. It can be seen in Fig. 6a that some molecules are not retained on the column when $L=12$, *i.e.*, $L^*=1$, because the adsorption energy of a molecule is not large enough for the crystal surfaces to be saturated even in the absence of competing ions. Fig. 7 (Case 2) shows chromatograms for molecules which differ from Case 1 only in that they are ten times longer. We can consider that Fig. 1 in Part VI[6] (see Case 10) gives the chromatogram obtained when the length of a molecule is infinity and when the column length $L^*=1$. It can be seen that the chromatogram in Fig. 7a is almost identical with that in Fig. 1 in Part VI. Cases 3 and 4 correspond to the same molecules as in Case 1, the only



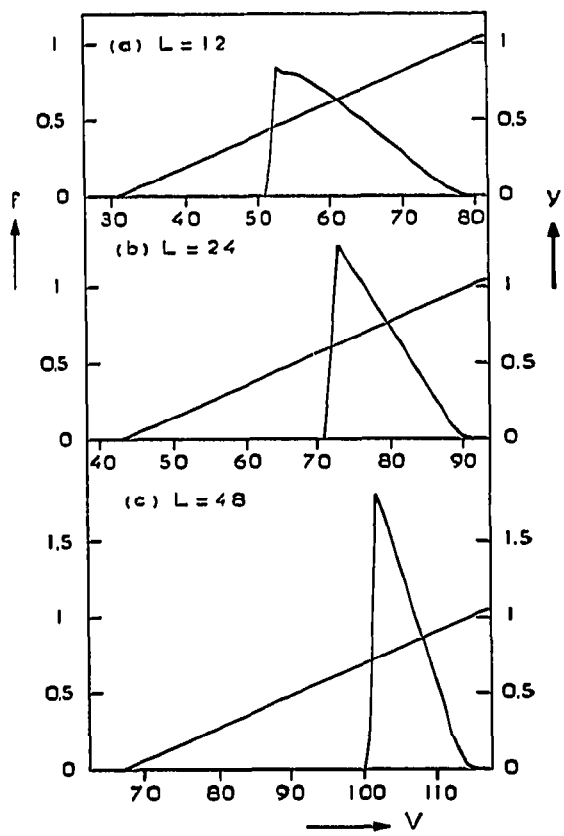


Fig. 6. Theoretical chromatograms obtained, taking into account molecular interactions, for a single type of macromolecule under the experimental conditions shown in Case 1 in Table I.

Fig. 7. As Fig. 6 but corresponds to Case 2 in Table I.

TABLE I

SUMMARY OF DATA CORRESPONDING TO CALCULATED CHROMATOGRAMS

Case	*Corresponding Figure No.*	*Species 1*			*Species 2*			*Chromatographic conditions*			*Remarks*	
		$x'_{(1)}$	$-u_{(1)}/kT$	$w_{(1)}$	$x'_{(2)}$	$-u_{(2)}/kT$	$w_{(2)}$	L^{*}	g^{**}	*Type of conditions*		
1	6	300	20	1				1,2,4	0.25	a	The case of a single molecular species.	
2	7	3 000	200	1				1,2,4	0.25	a		
3	8	300	20	1				1,2,4	0.025	b		
4	9	300	20	1				1,2,4	0.0025	c		
5	10	3 000	200	1				1,2,4	0.0025	c		
6	11	300	20	1	300	18	0.9	2,4,8	0.125	d***		
7	12§	300	20	1				1,2,4	0.25	a		
8	13§	3 000	200	1				1,2	0.25	a′		
9	14§	300	20	1	300	18	0.9	2,4,8	0.125	d***		
10	Fig. 1 in ref. 6	∞	∞	1				1	Arbitrary			
11	Fig. 4 in ref. 6	∞	∞	0.9				1	Arbitrary			
12	15	300	20	1	300	18	0.9	1,2,4	0.25	a	$w \approx 1$; $x' = 300$	
13	16§	300	20	1	300	18	0.9	1,2,4	0.25	a		
14	17	300	20	1	300	19.8	0.99	1,2,4	0.25	a		
15	18	300	20	1	300	19.8	0.99	1,2,4	0.025	b		
16	19	300	20	1	300	19.8	0.99	1,2,4	0.0025	c		
17	20	300	20	1	300	19.8	0.99	2,4,8	0.125	d***		
18	21§	300	20	1	300	19.8	0.99	1,2,4	0.0025	c		
19	22	300	40	2	300	39.8	1.99	1,2,4	0.25	a	$w \approx 2$ and 4; $x' = 300$	
20	23	300	80	4	300	79.8	3.99	1,2	0.25	a′		
21	24	300	40	2	300	39.8	1.99	1,2,4	0.025	b		
22	25	300	40	2	300	39.8	1.99	1,2,4	0.0025	c		
23	26	300	40	2	300	39.8	1.99	2,4,8	0.125	d***		The case of two molecular species with the same length.
24	27	3 000	200	1	3 000	198	0.99	1	0.25	a″	$x' = 3000$ and 30 000;	
25	28	3 000	200	1	3 000	199.8	0.999	1,2,4	0.25	a	$w \approx 1$; $g^{*} = 0.25$	
26	29(a)	30 000	2000	1	30 000	1998	0.999	1	0.25	a″		
27	29(b)	30 000	2000	1	30 000	1999.8	0.9999	1	0.25	a″		

28	30	3 000	400	2	3 000	399.8	1.999	1	0.25	a″	$x'=3000$; $w\approx 2$ and 0.5; $g^*=0.25$
29	31	3 000	100	0.5	3 000	99.8	0.499	1	0.25	a″	
30	32	3 000	200	1	3 000	199.8	0.999	1,2,4	0.0025	c	$x'=3000$; $w\approx 1$ and 0.5; $g^*=0.0025$
31	33	3 000	100	0.5	3 000	99.8	0.499	1	0.0025	c″	
32	Fig. 2 in ref. 6	∞	∞	1	∞	∞	1−ε§§	1	Arbitrary		
33	Fig. 3 in ref. 6	∞	∞	1	∞	∞	0.9	1	Arbitrary		
34	34	3 000	200	1	300	20	1	1,2,4	0.25	a	The case of two molecular species with different lengths and the same adsorption energy per unit length.
35	35	600	40	1	300	20	1	1,2,4	0.25	a	
36	36	300	20	1	297	19.8	1	1,2,4	0.25	a	
37	37	300	40	2	297	39.6	2	1,2,4	0.25	a	
38	38	30 000	2000	1	3 000	200	1	1,2,4	0.25	a	
39	39	3 000	200	1	300	20	1	1,2,4	0.0025	c	
40	40	600	40	1	300	20	1	1,2,4	0.0025	c	
41	41	300	20	1	297	19.8	1	1,2,4	0.0025	c	
42	42	300	40	2	297	39.6	2	1,2,4	0.0025	c	
43	43	30 000	2000	1	3 000	200	1	1,2	0.0025	c′	
44	Fig. 2 in ref. 6	∞	∞	1	∞	∞	1	1	Arbitrary		
45	44	3 000	200	1	300	28	1.4	1,2,4	0.25	a	The case of two molecular species with different lengths and different adsorption energies per unit length.
46	45	30 000	1940	0.97	3 000	200	1	1,2,4	0.25	a	
47	46	30 000	1920	0.96	3 000	200	1	1,2,4	0.25	a	
48	47	300	19.8	0.99	297	19.8	1	1,2,4	0.25	a	
49	48	300	19.8	0.99	297	19.8	1	1,2,4	0.0025	c	
50	49	300	19.9	0.995	297	19.8	1	1,2,4	0.25	a	
51	50	300	19.9	0.995	297	19.8	1	1,2,4	0.0025	c	
52	51(a)	300	39.6	1.98	297	39.6	2	1	0.25	a″	
53	51(b)	300	39.8	1.99	297	39.6	2	1	0.25	a″	

* L^* = column length/width of initial zone of adsorbed macromolecules on the column obtained provided that $-u_3/kT=\infty$.

** g^* = difference in the values of the parameter y at the top and the bottom of the column when $L^*=1$.

*** We can consider that this experimental condition is different from type a only in that the load is half.

§ The case of no molecular interactions.

§§ ε is a very small and positive constant.

difference being the s.o.g., which is 10 times (Fig. 8) and 100 times (Fig. 9) smaller. Case 5 (Fig. 10) corresponds to the same molecules as in Case 2 but with an s.o.g. 100 times smaller. The fluctuations in shape of chromatograms that appear particularly when the s.o.g. is small and x' is large (see Figs. 9 and 10) are artifacts due to the mathematical procedures, as in Parts II[2], III[3] and V[5]*. From these results, it appears that there is no large dependence of the shape of the chromatogram on x'. However, the chromatogram is displaced to the left if x' is small, particularly if the column is

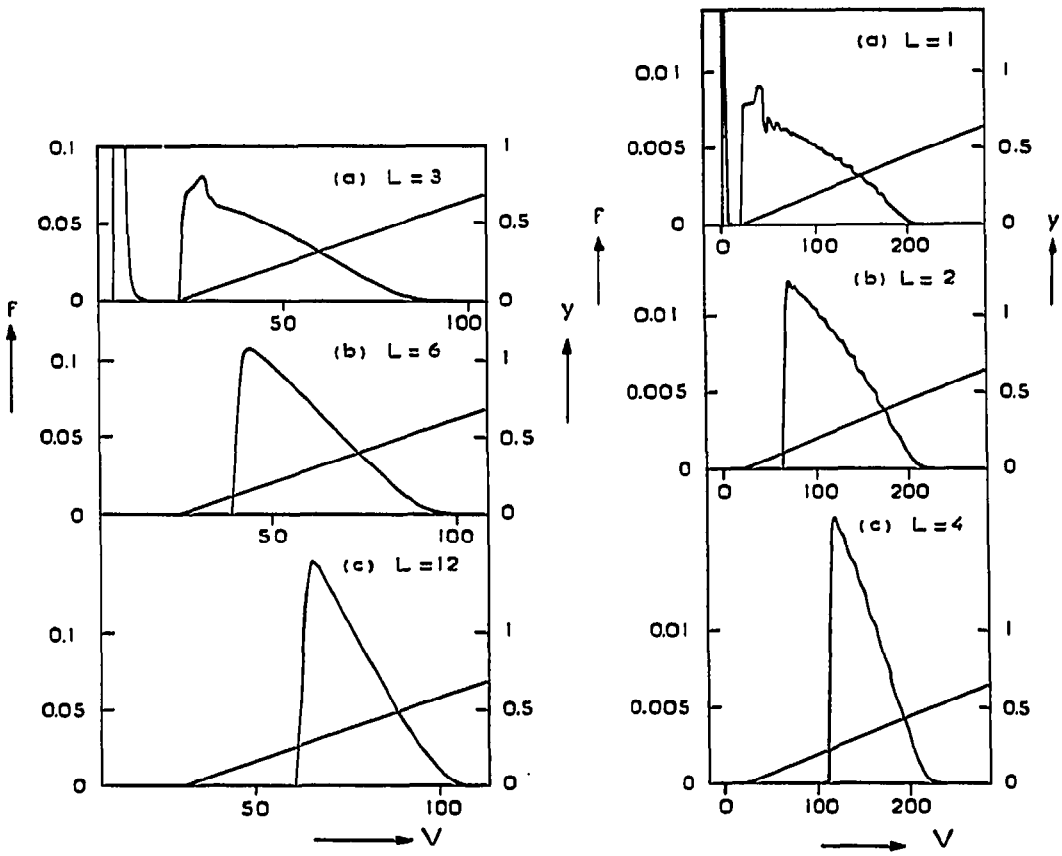


Fig. 8. As Fig. 6 but corresponds to Case 3 in Table I.

Fig. 9. As Fig. 6 but corresponds to Case 4 in Table I.

* The variable χ appears in eqn. 3 in the form of $\{p(\chi)\}^{-1}\cdot\exp\{-(u_{(1)}/kT)\Xi\sqrt{\chi}\}$ where $p(\chi)=1-\chi$ (see eqn. 5). Therefore, in order to estimate the value of $\bar{\chi}_{(\rho')}$ which is defined as the value of χ giving to $G_{(\rho')}$ the value of $\frac{1}{2}G^0{}_{(\rho')}$, we have to rewrite the relationships: $\zeta=(1-\chi)^{-1}\cdot\exp\{-(u_{(1)}/kT)\Xi\sqrt{\chi}\}$ as $\chi=f(\zeta)$, which has been done approximately by expressing χ as the series of about 30 of the straight lines with slightly different slopes when the value of χ is less than some value χ^*, being chosen as about 0.95 in all calculations. When χ is larger than χ^*, we have expressed ζ approximately as $\zeta\approx(1-\chi)^{-1}\cdot\exp\{-(u_{(1)}/kT)\Xi\sqrt{\chi^*}\}$ and we have solved this equation for χ. We can show that the fluctuations in shapes of chromatograms are due to the first approximation. Therefore, these artifacts are of different types from those that appeared in refs. 2, 3 and 5.

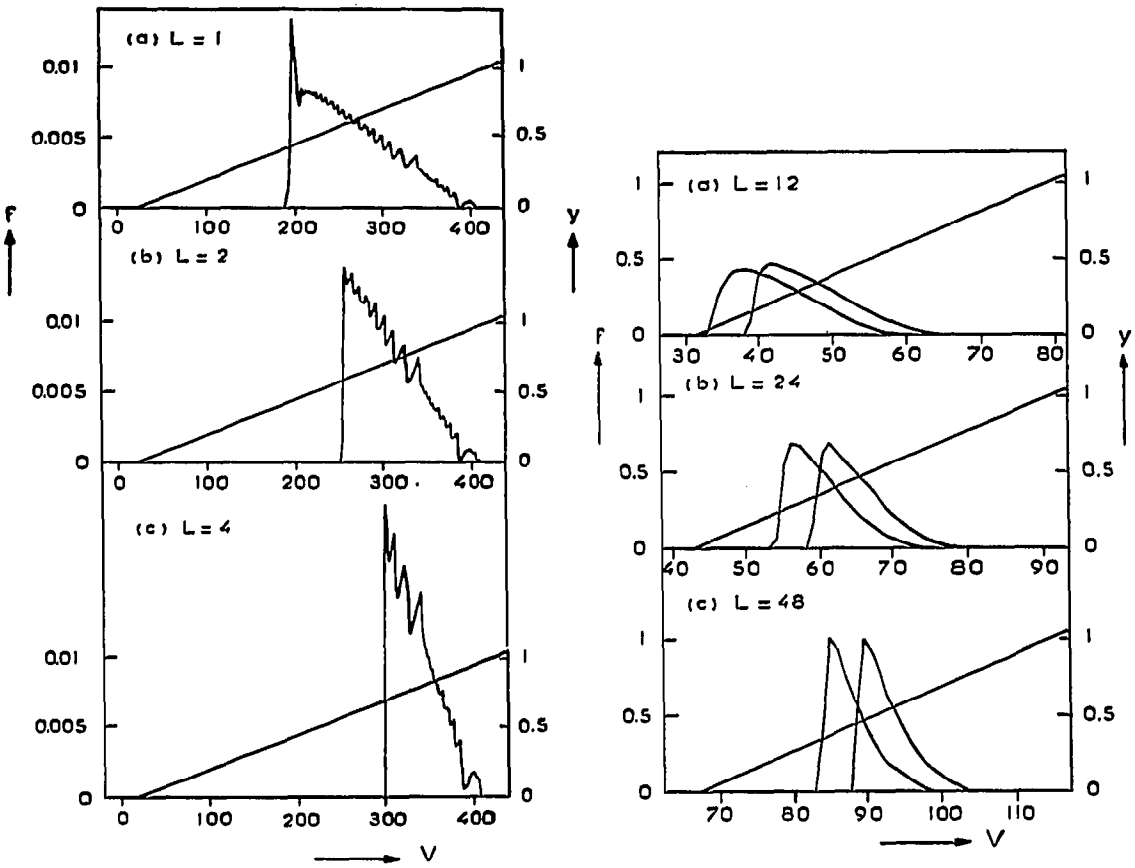
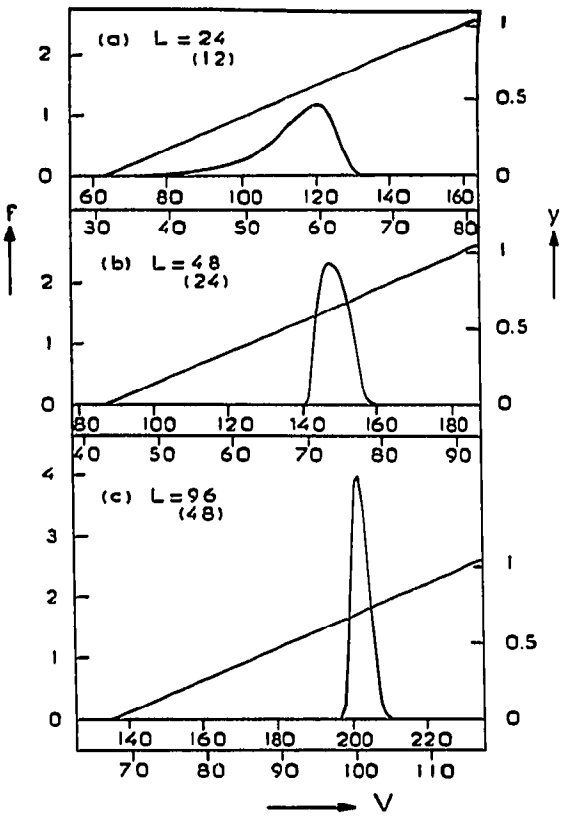


Fig. 10. As Fig. 6 but corresponds to Case 5 in Table I.

Fig. 11. As Fig. 6 but corresponds to Case 6 in Table I (right-hand and left-hand curves correspond to species 1 and 2 in Table I, respectively).

short and if the s.o.g. is small. If x' is sufficiently large (of the order of 3000), the chromatogram is almost independent of s.o.g. and coincides essentially with that obtained by assuming that $x' = \infty$. The right-hand curves of the pairs in Fig. 11a, 11b and 11c (Case 6) represent chromatograms for the same molecules as in Case 1 (Fig. 6), the only difference in the experimental conditions being that the load is half. It can be seen from Figs. 11 and 6 that when the load is low the chromatogram begins at a higher value of activity, y. It finishes, however, in the same manner as in the case when the load is high and the right-hand of the parts chromatograms obtained in cases of different loads can be exactly superimposed on one another.

Cases 7 and 8 correspond to the same molecular species and experimental conditions as in Cases 1 and 2, but the interactions have been suppressed (Figs. 12 and 13). In the case of a single molecular species and no interactions, one can calculate the experimental parameter B more precisely by using eqn. A27 in Appendix I in Part I than by using the approximations of eqns. 7 and 8. Figs. 12 and 13 are the results obtained through such calculations. It can be seen that as x' increases, the



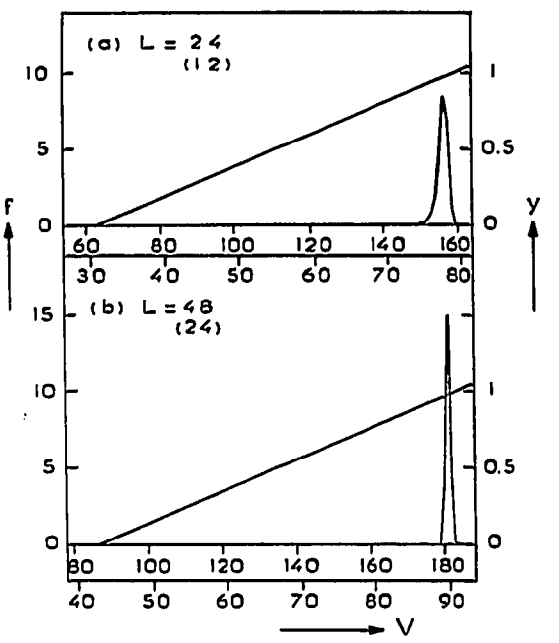
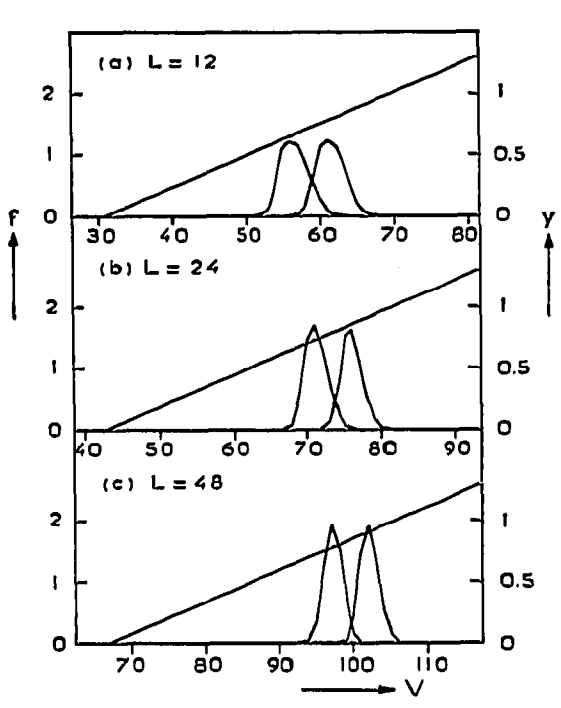


Fig. 12. As Fig. 6 in the case of no molecular interactions, corresponding to Case 7 in Table I. The values of both L and V are shown also on the same scale as in Fig. 6.

Fig. 13. As Fig. 12 but corresponds to Case 8 in Table I.

width of the chromatogram decreases rapidly (see Fig. 13), which is not observed experimentally. The general shape of the experimental chromatograms can be explained satisfactorily only when it is assumed that there are repulsive interactions among adsorbed molecules (see Figs. 6–11). However, in order to confirm this fact and also to be able to predict what will happen if the value of Ξ is different, we shall try to evaluate other chromatograms assuming that no molecular interactions occur, *i.e.*, assuming that $\Xi=0$. Fig. 14 (Case 9) shows, for the case when there are no molecular interactions, the chromatograms obtained using the same molecules and the same experimental conditions as in Case 6 (Fig. 11). The molecules represented in Fig. 14 (right-hand curves) are the same as those in Fig. 12 (Case 7) and the load relating to Fig. 14 is half of that for Fig. 12. For the calculation of Fig. 14, we used the approximations of eqns. 7 and 8. It can be seen from Figs. 12 and 14 that, even when there are no molecular interactions, the chromatogram begins at a high value of the activity if the load is low and that the right-hand part of the chromatogram is independent of the load. The slight difference in shape of the right-hand parts of



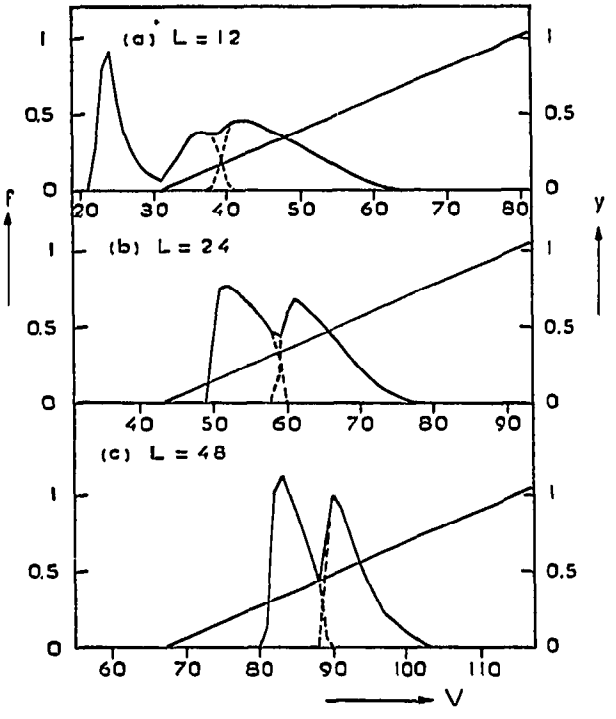


Fig. 14. As Fig. 12 but corresponds to Case 9 in Table I (right-hand and left-hand curves correspond to species 1 and 2 in Table I, respectively).

Fig. 15. Theoretical chromatograms obtained, taking into account molecular interactions, for a mixture of two types of molecules with the same length but with different adsorption energies. See Case 12 in Table I.

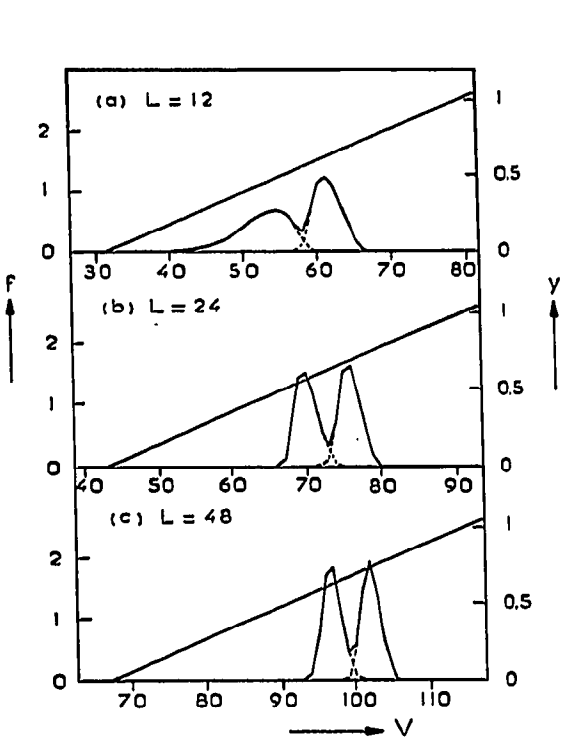
the chromatograms in Figs. 12 and 14 is due to the fact that eqns. 7 and 8 have been used only for the calculation of Fig. 14.

The case of two molecular species with the same length (Cases 12–33 in Table I)

Fig. 15 shows chromatograms, in the presence of molecular interactions, for a mixture of equal amounts of two molecular species 1 and 2 with the same value of x' (300) and different values of the adsorption energies ($-u_{(1)}/kT=20$ and $-u_{(2)}/kT=18$). Species 1 in Fig. 15 is the same as in Figs. 6, 8, 9, 11 (right-hand curves), 12 and 14 (right-hand curves) (Cases 1, 3, 4, 6, 7 and 9, respectively) and if one uses the same unit for the activity, y, as in the case of a single molecular species, one can estimate the values of $w_{(1)}$ and $w_{(2)}$ to be 1 and 0.9, respectively. In this section, unless otherwise stated, we put $w_{(1)}=1$ and we assume that $w_{(2)}<w_{(1)}$, so that $-(u_{(1)}/kT)/x'_{(1)}$, *i.e.*, $\xi_{(1)}$, is the same as that used for the species eluting at higher volumes in the cases of Figs. 11 and 14 (Cases 6 and 9). Species 2 is always on the left-hand side of species 1 in these chromatograms. The contributions of the two components are drawn as broken lines in the figures. In Fig. 15, both the load and the slope of the gradient are the same as in Fig. 6 (Case 1). It can be seen in Fig. 15

that the separation between the two molecular species is almost complete. The left-hand curves in Fig. 11 (Case 6) show the chromatograms for the same molecules as species 2 in Fig. 15 and the chromatographic conditions (except loads) are the same in the corresponding parts of Figs. 11 and 15. The loads of the two species in Fig. 11 are identical with those of the two species in Fig. 15, and Fig. 11 could represent the chromatograms obtained by re-chromatography of the two components in Fig. 15. It can be seen that the chromatograms of species 1 in Fig. 11 coincide essentially with the contributions of the same species in the corresponding parts of Fig. 15. However, in Fig. 15, the contribution of species 2 is displaced to the left by species 1.

We can consider that Fig. 3 of Part VI[6] (Case 33) represents the chromatogram of a mixture of the homologues of species 1 and 2 in Fig. 15 but with infinite lengths. We can also consider that the chromatographic conditions in Fig. 3 in Part VI are the same as those in Fig. 15a. The right-hand areas of the chromatograms in Figs. 1 and 4 in Part VI (Cases 10 and 11) could also represent chromatograms obtained following the re-chromatography of species 1 and 2 in Fig. 3 in Part VI (Case 33).



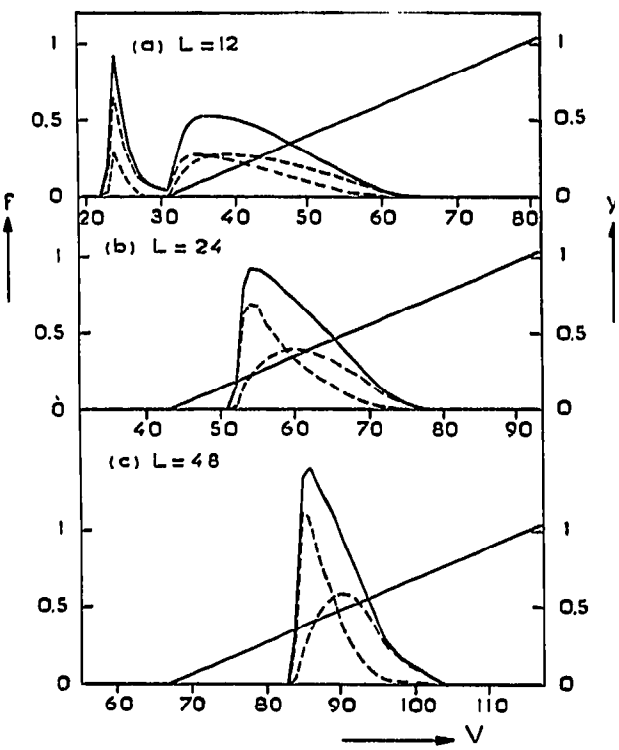


Fig. 16. As Fig. 15 in the case of no molecular interactions, corresponding to Case 13 in Table I.

Fig. 17. As Fig. 15 but corresponds to Case 14 in Table I and to Case 1 in Table II.

Fig. 16 (Case 13) shows what happens when interactions are suppressed. The chromatograms correspond exactly to the exper mental conditions of Fig. 15. Species 2, appearing at lower V values in Fig. 16, is the same as in the chromatograms in Fig. 14 (Case 9). The total load relating to Fig. 14 is half that relating to Fig. 16; the chromatographic conditions (except load) are also the same for the corresponding parts of Figs. 16 and 14. It can be seen in Figs. 16 and 14 that the chromatogram for the component with a high w value is generally not influenced by the presence of the component with a low w value. The chromatogram for the component with the low w value is displaced to a low value of the activity y in the presence of the component with a high w value when the column is very short. If it is sufficiently long, the chromatogram for any component is independent of the presence of the other components.

Fig. 17 (Case 14) shows chromatograms, when there are molecular interactions, for a mixture of molecules with the same length as those represented in Fig. 15, *i.e.*, assuming $x'=300$ but with very similar adsorption energies. We have assumed that $-u_{(1)}/kT=20$ and $w_{(1)}=1$ and that $-u_{(2)}/kT=19.8$ and $w_{(2)}=0.99$. All chromatographic conditions in Fig. 17 are the same as those in Fig. 15. It can be seen

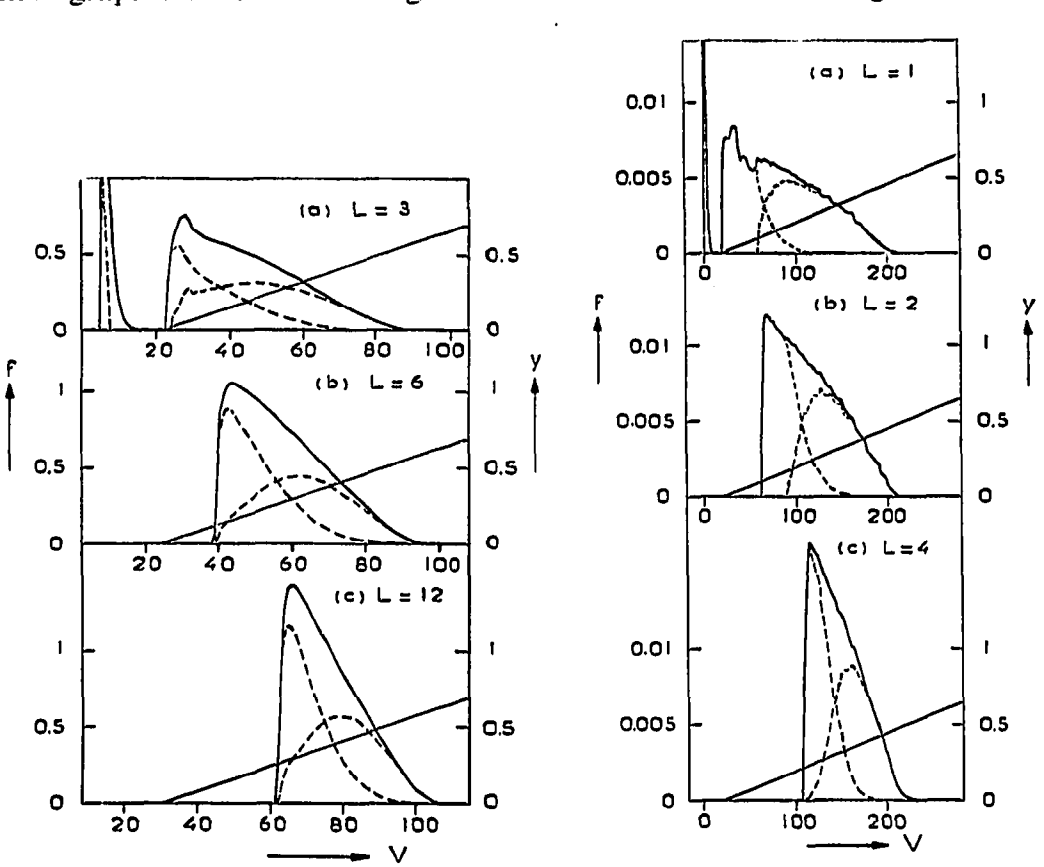


Fig. 18. As Fig. 15 but corresponds to Case 15 in Table I.

Fig. 19. As Fig. 15 but corresponds to Case 16 in Table I and to Case 1 in Table II.

in Fig. 17 that only poor separations of molecules can be achieved when the adsorption energies are so similar. In this case, the chromatograms following the rechromatography of any components will give identical shapes represented by the right-hand curves of Fig. 11 (Case 6) and the shape of the total chromatogram is almost identical with that obtained in the case of a single component with $w=1$ (compare Fig. 17 with Fig. 6 (Case 1)). It should be noted that the shape of the contribution to the total chromatogram of species 1 which has the highest energy is no longer identical with that obtained when the chromatography of species 1 is carried out independently (see the right-hand curves of Fig. 11 (Case 6)). Figs. 18 and 19 (Cases 15 and 16) show the effect of s.o.g., using values 10 and 100 times smaller than those used in Fig. 17, all the other conditions remaining the same. In these cases also the shapes of the total chromatograms are almost identical with those obtained when there is only a single species with $w=1$ (compare Figs. 18 and 19 with Figs. 8 and 9 (Cases 3 and 4), respectively). It can be seen clearly, however, in Figs. 17a, 18a and 19a that, when the column is short, the separation between different molecules increases with a decrease in s.o.g. If the column length increases, the s.o.g. no longer has an effect on the resolution of the column (see parts (b) and (c) of Figs. 17–19) and the best separation is obtained when the column is short and the s.o.g. is small. It should be noted that both of the components of the mixture are always involved in the chromatographic peak that appears before the gradient

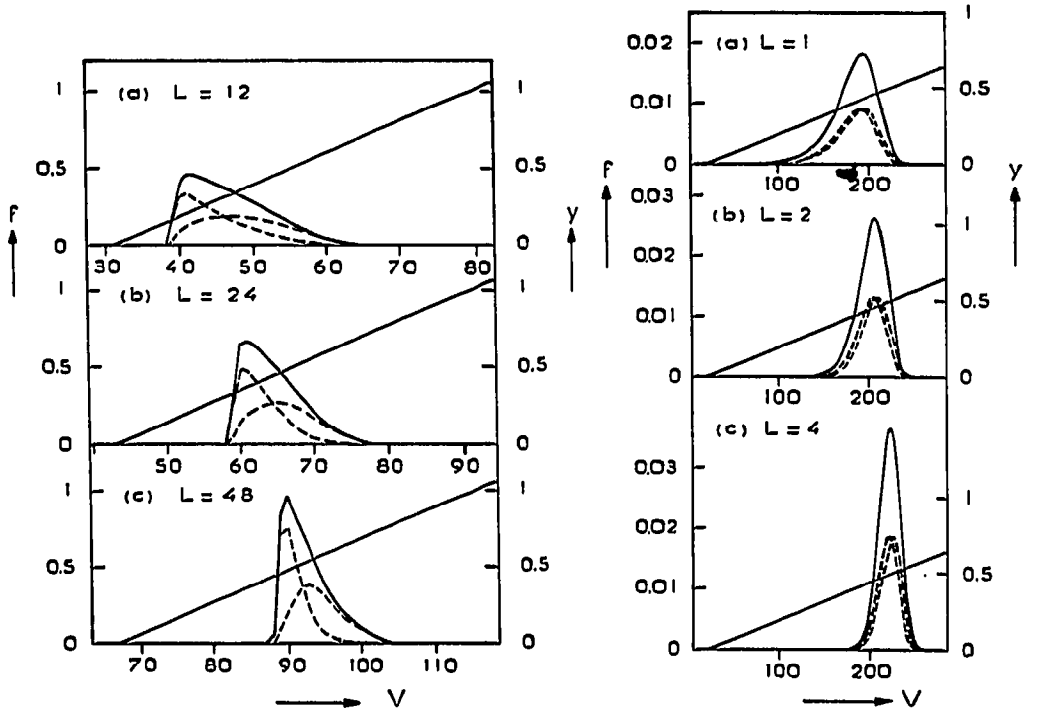


Fig. 20. As Fig. 15 but corresponds to Case 17 in Table I.

Fig. 21. As Fig. 15 in the case of no molecular interactions, corresponding to Case 18 in Table I

begins when the column is short (see parts (a) of Figs. 17–19) and that this peak is independent of s.o.g.

Fig. 20 (Case 17) shows the chromatograms for the same mixture as in Figs. 17–19 obtained when the load is half and when the s.o.g. is the same as in Fig. 17. In this case, the shapes of the total chromatograms are almost identical with those of the right-hand curves in Fig. 11 (Case 6).

Fig. 21 (Case 18) shows chromatograms obtained, assuming that no molecular interactions occur, for the same mixture as in Figs. 17–20 (Cases 14–17) and for the same experimental conditions as in Fig. 19 (Case 16). It can be seen from Figs. 21 and 19 that the chromatographic separation between molecules with very similar adsorption energies is due mainly to the repulsive interaction between them.

Up to now we have investigated the resolution of the columns under several experimental conditions for mixtures of macromolecules assuming always that $x' = 300$ and that the mean value of $-u_3/kT$ is about 20, which may represent the case of tropocollagen. It should be recalled that this value of $-u_3/kT$ is not sufficiently large enough for the surface of HA to be initially saturated by molecules. Now, the value of x' for tropocollagen could also be applied to some types of DNA. However, the absolute value of the adsorption energy of DNA molecules with the same dimensions as those of tropocollagen must be more than twice the value for tropocollagen, as the elution molarity of DNA is usually more than twice that for tropocollagen. Figs. 22 and 23 (Cases 19 and 20) show the chromatograms when there are molecular interactions for mixtures of equal amounts of two molecular

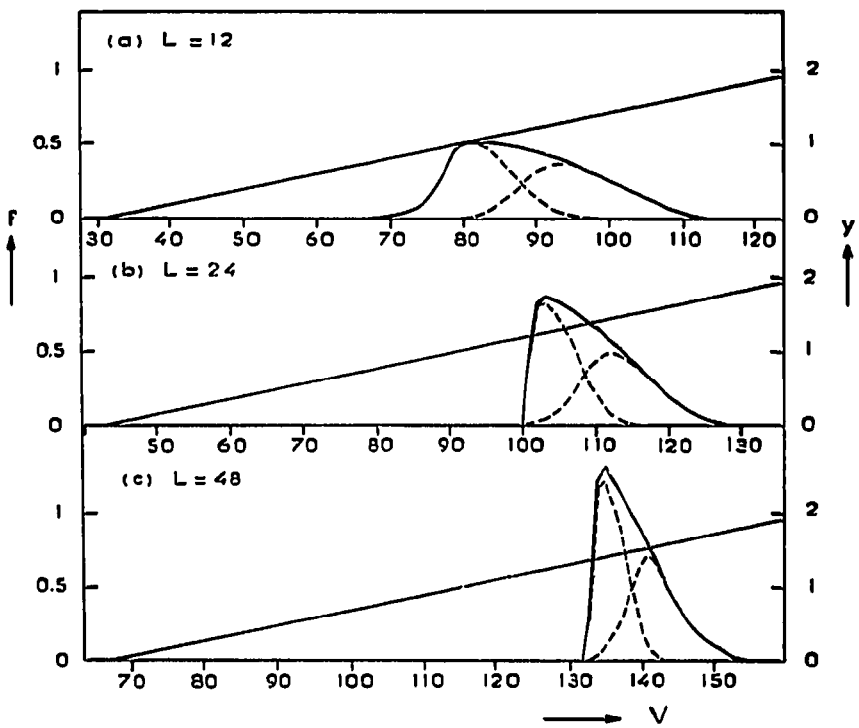


Fig 22. As Fig. 15 but corresponds to Case 19 in Table I.

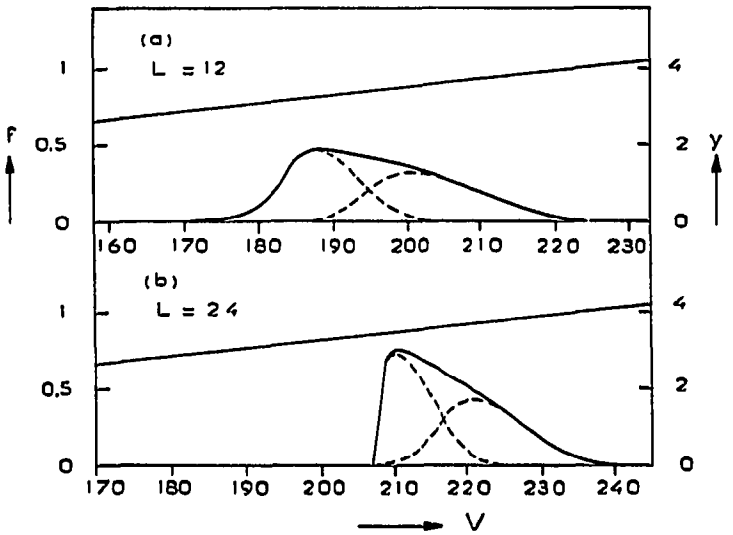


Fig. 23. As Fig. 15 but corresponds to Case 20 in Table I.

species with $x' = 300$ and with mean values of $-u_3/kT$ of about 40 and 80, respectively. The chromatographic conditions in Figs. 22 and 23 are the same as those in Fig. 17 (Case 14) and the differences in the adsorption energies between the two molecular species in Figs. 22 and 23 are the same as those in Figs. 17–21 (Cases 14–18). It can be seen in Figs. 22 and 23 that all of the molecules loaded are retained on the column

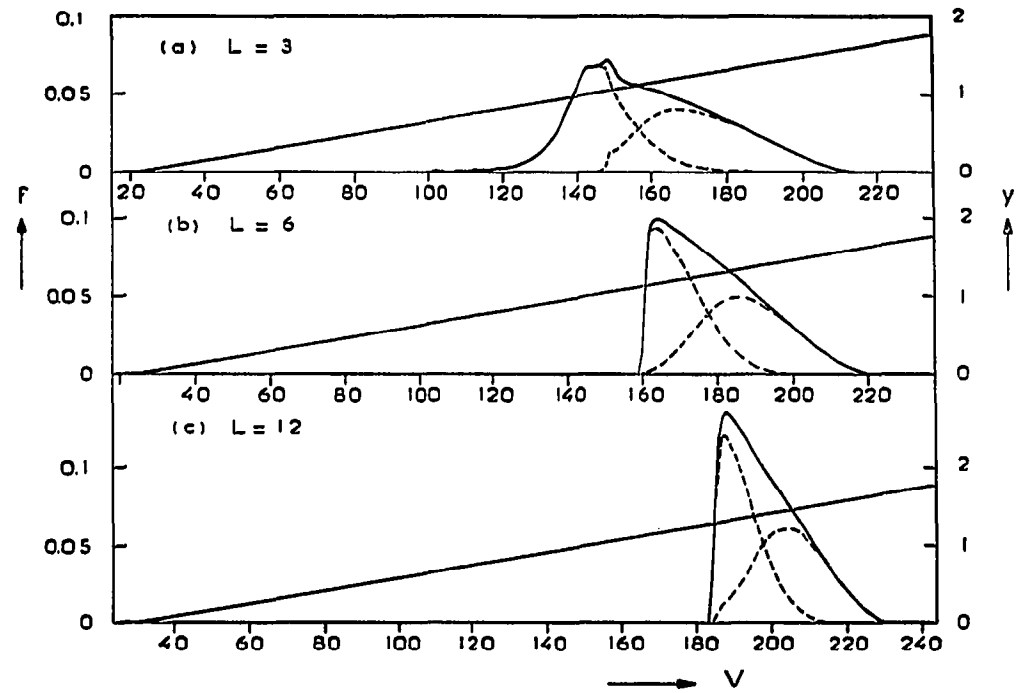


Fig. 24. As Fig. 15 but corresponds to Case 21 in Table I.

of length $L = 12$, which means that the adsorption energies of molecules are sufficiently large and that the surface of HA is initially saturated by them. It can also be seen that the shapes of the total chromatograms as well as those of the contributions of the two components are almost identical between corresponding parts of Figs. 22 and 23, and that better chromatographic separations can be achieved in Figs. 22 and 23 than in Fig. 17, which is the case when the mean absolute value of the adsorption energies of molecules is too small for saturation to occur initially. Figs. 24 and 25 (Cases 21 and 22) illustrate the chromatograms for the same mixture as in Fig. 22 (Case 19) but with the s.o.g. decreased by factors 10 and 100, respectively. It can be seen, on comparing Figs. 22, 24 and 25, that the resolution of the column increases slightly with a decrease in the column length. When the column is short, the resolution seems to increase very slightly with a decrease in the s.o.g. (compare Fig. 22a with Fig. 25a). At the smallest s.o.g. (see Fig. 25), the separation between different molecules is similar to that obtained in the case of molecules with small adsorption energies (see Fig. 19 (Case 16)). Fig. 26 (Case 23) shows chromatograms for the same mixture as in Figs. 22, 24 and 25 (Cases 19, 21 and 22) when the load is half. The other chromatographic conditions are the same as those in Fig. 22 (Case 19). It can be seen in Figs. 26 and 22 that, especially when the column is short (see parts (a) of Figs. 26 and 22), a better resolution can be achieved by increasing the load.

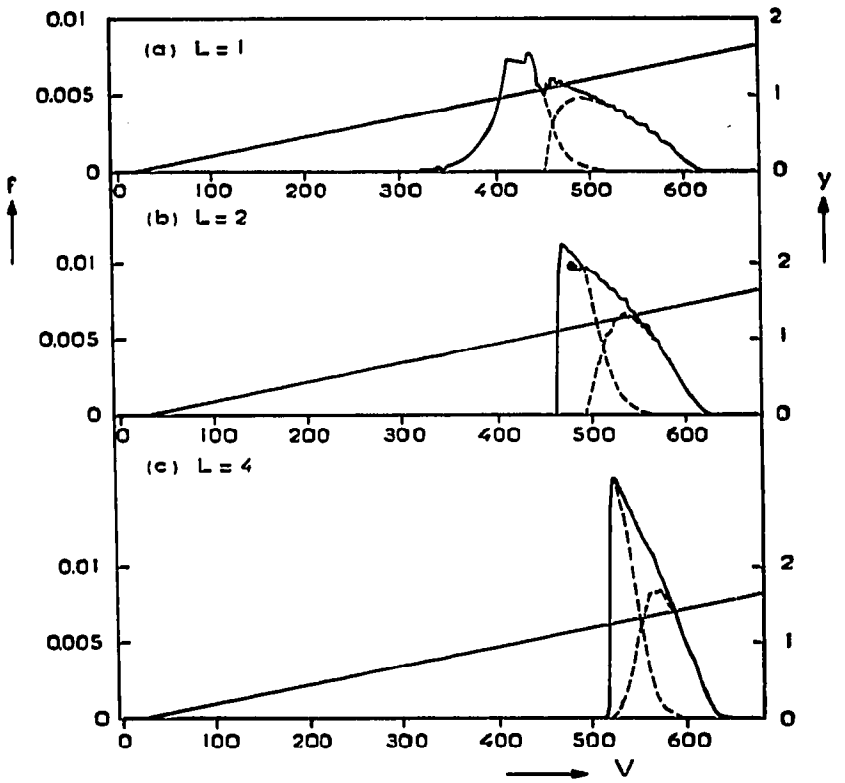


Fig. 25. As Fig. 15 but corresponds to Case 22 in Table I.

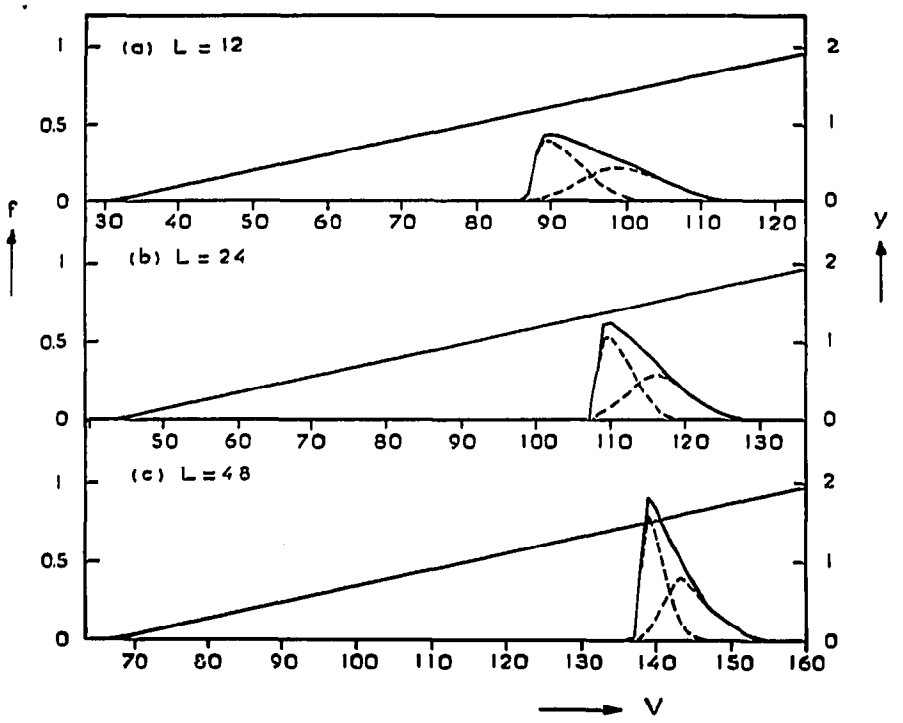


Fig. 26. As Fig. 15 but corresponds to Case 23 in Table I.

All of the results mentioned above are for mixtures of molecules with a constant value of x' (300). In many instances, *e.g.* in the case of DNA, x' could be much larger. Fig. 27 (Case 24) shows the chromatogram for a mixture of equal amounts of molecular species 1 and 2 with a value of x' ten times larger than that for the molecules represented in Figs. 15–26 (Cases 12–23), *i.e.*, 3000, and with the same values of the adsorption energies per unit molecular length as those of species 1 and 2 in Figs. 17–21 (Cases 14–18), respectively. The chromatographic conditions in Fig. 27 are the same as in parts (a) of Figs. 15–17, 22 and 23 (Cases 12–14, 19 and 20). Fig. 28 (Case 25) shows the chromatograms obtained when the adsorption energy of species 2 is ten times closer to the adsorption energy of species 1. than in the case of Fig. 27. The chromatographic conditions in Fig. 28 are the same

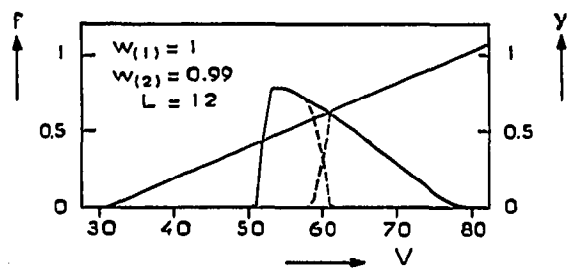
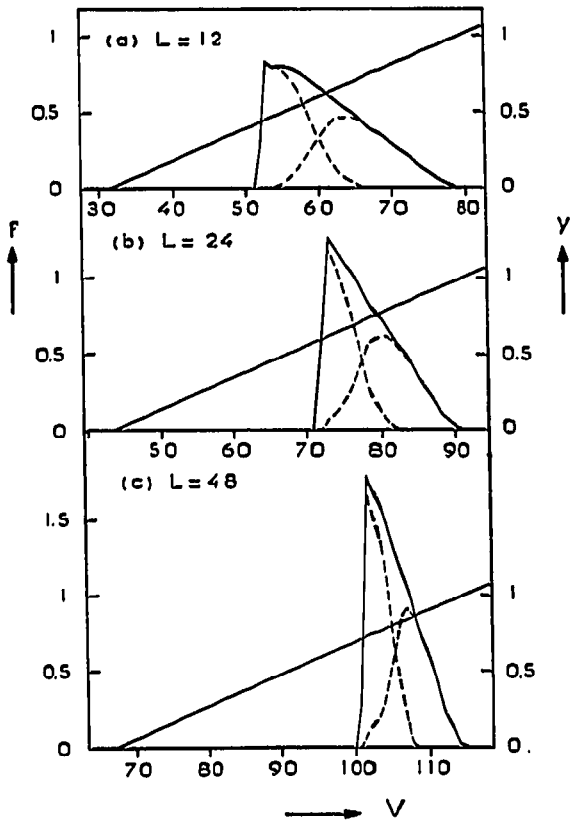


Fig. 27. As Fig. 15 but corresponds to Case 24 in Table I.

as those in the corresponding parts of Figs. 15–17, 22 and 23. It can be seen in Figs. 27 and 28 that, if the difference in the values of w between two molecular species is 0.01, the chromatographic separation between them is almost complete (see Fig. 27 (Case 24)). The separation is no longer complete if the difference in w values is 0.001 (see Fig. 28 (Case 25)). It should be recalled that when molecules are ten times smaller or when $x'=300$, the chromatographic separation is almost complete if the difference in the w values of the molecules is 0.1 (see Fig. 15 (Case 12)). If the difference in w values is 0.01, however, the separation between two components can no longer be complete (see Figs. 17, 22 and 23 (Cases 14, 19 and 20)). Fig. 29 (Cases 26 and 27) shows what happens if the dimensions are increased by a factor of ten ($x'=30{,}000$). The chromatographic conditions in Fig. 29 are the same as those in Fig. 27 (Case 24) and in parts (a) of Figs. 28, 15, 17, 22 and 23 (Cases 25, 12, 14, 19 and 20). It can be seen in Fig. 29 that the separation between two molecular species is complete if the difference in w values is 0.001 (part (a)) and is no longer complete if the difference in w values is 0.0001 (part (b)). We can consider that Fig. 2 in Part VI (Case 32) represents the chromatogram for a mixture of molecular species 1 and 2 with infinite lengths and with a very small difference in the adsorption energies per unit molecular length. We can also consider that the mean value of w of species 1 and 2 and the chro-



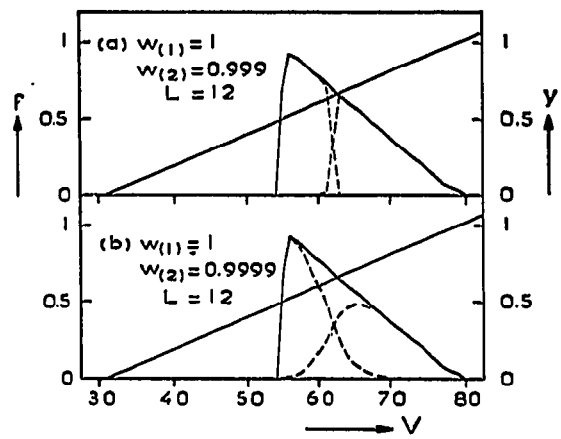


Fig. 28. As Fig. 15 but corresponds to Case 25 in Table I.

Fig. 29. As Fig. 15 but parts (a) and (b) correspond to Cases 26 and 27 in Table I, respectively.

matographic conditions in Fig. 2 in Part VI are almost identical with those in Figs. 15a, 17a, 27, 28a and 29 (Cases 12, 14, 24, 25 and 26–27), respectively. It can be concluded from these figures that when $x' \geqq 3000$, if the difference in w values between two components is small (see Figs. 27, 28a and 29), the total chromatogram is almost identical with that obtained assuming that $x' = \infty$ (see Fig. 2 in Part VI). If the difference in w values is small, but not very small, the contributions of the two components to the total chromatogram are also similar to those realized in practice, provided that the lengths of the molecules are infinite (compare Figs. 27 and 29a with Fig. 2 in Part VI). It should be noted that the adsorption energy per unit molecular length or the parameter w reflects the compositions or the sequences of amino acids and nucleotides in the cases of protein and DNA, respectively (see Discussion).

Up to now, we have discussed the chromatography of two-component systems when x' changes and when the mean values of $w_{(1)}$ and $w_{(2)}$ are almost unity. If $x' \geqq 3000$, the latter values are large enough for the surfaces of HA in the column to be initially saturated with macromolecules. Figs. 30 and 31 (Cases 28 and 29)

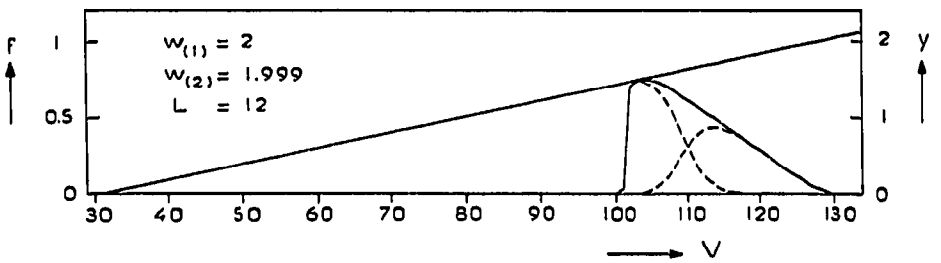


Fig. 30. As Fig. 15 but corresponds to Case 28 in Table I.

show the chromatograms for mixtures of equal amounts of molecular species 1 and 2 with $x' = 3000$ and with mean values of w of about 2 and 0.5, respectively. In both Figs. 30 and 31, the differences in w values between the two molecular species are the same as in Fig. 28 (Case 25), *i.e.*, 0.001. The chromatographic conditions in Figs. 30 and 31 are the same as those in Figs. 15a, 17a, 22a, 23a, 27, 28a and 29 (Cases 12, 14, 19, 20, 24, 25, 26 and 27). It can be seen that when $w \approx 2$, both the shape of the total chromatogram and the shapes of the contributions of the two components are almost identical with those in the case when the mean value of w is about unity (compare Fig. 30 with Fig. 28a (Case 25)). When the mean value

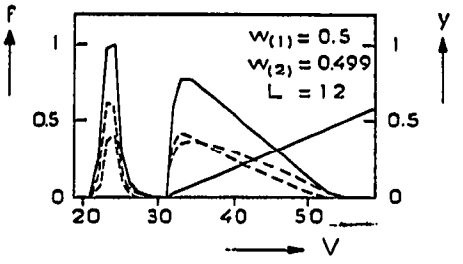
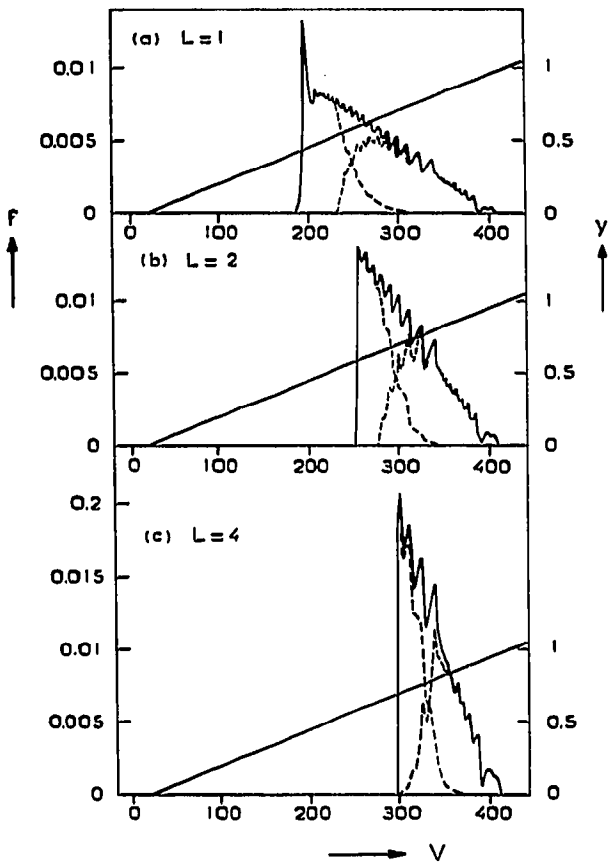


Fig. 31. As Fig. 15 but corresponds to Case 29 in Table I.

of w is about 0.5 (see Fig. 31), some molecules cannot be retained on the column and good resolution cannot be obtained. This situation is similar to the case when the value of x' is ten times smaller or when $x'=300$. In this case, however, a better separation has been obtained when the mean value of w is equal to or greater than 2 (see Figs. 22 and 23 (Cases 19 and 20)) and when it is unity, the resolution of the column decreasing together with a decrease in its adsorption capacity (see Fig. 17 (Case 14)).

Finally, we discuss the influence of the s.o.g. on the resolution of the column when $x'=3000$. Fig. 32 (Case 30) shows the chromatograms for the same mixture as in Fig. 28 (Case 25) obtained by using an s.o.g. 100 times smaller than that in Fig. 28. The fluctuations in Fig. 32 are artifacts that have been mentioned before. It can be seen in Figs. 28 and 32 that the resolution of the column, in general, increases slightly with a decrease in the column length and that when the column is the shortest (see parts (a) of Figs. 28 and 32), the resolution of the column also increases slightly with a decrease in the s.o.g., which is the same result as that obtained in the case of smaller molecules with $x'=300$ and with adsorption energies sufficiently large for



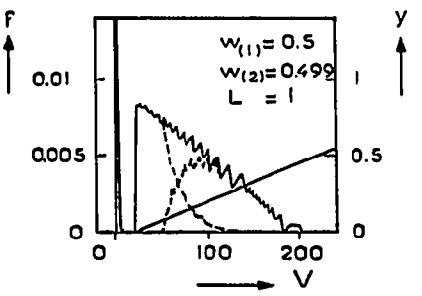


Fig. 32. As Fig. 15 but corresponds to Case 30 in Table I.

Fig. 33. As Fig. 15 but corresponds to Case 31 in Table I.

the crystal surfaces of the column to be initially saturated by them (see Figs. 22, 24 and 25 (Cases 19, 21 and 22)). Fig. 33 (Case 31) shows the chromatogram obtained under the same experimental conditions as those in Fig. 32a (Case 30) for the same mixture as in Fig. 31 (Case 29), which is the case when some molecules cannot be retained on the column. The fluctuations in the chromatograms in Fig. 33 are the same type of artifacts as those in Fig. 32. It can be seen in Figs. 33 and 31 that the resolution of the column increases significantly with a decrease in s.o.g. and that, at the smallest s.o.g., both the shape of the total chromatogram and the separation between different molecular species are very similar to the case when the mean adsorption energy of the molecules is sufficiently large for all of them to be initially retained on the column (compare Fig. 33 with Fig. 32a). It should be noted, however, that in the first chromatographic peak which appears before the gradient begins in Fig. 33, both components in the mixture are involved and that this peak is identical with the corresponding peak in Fig. 31. The conclusions thus obtained from Figs. 31, 33 and 32a (Cases 29, 31 and 30) are parallel to those for smaller molecules with $x' = 300$ obtained from Figs. 17a, 19a and 25a (Cases 14, 16 and 22).

The case of two molecular species with different lengths and with the same adsorption energy per unit length (Cases 34–44 in Table I)

In the following sections, all calculations of chromatograms are carried out assuming the existence of repulsive interactions among adsorbed macromolecules,

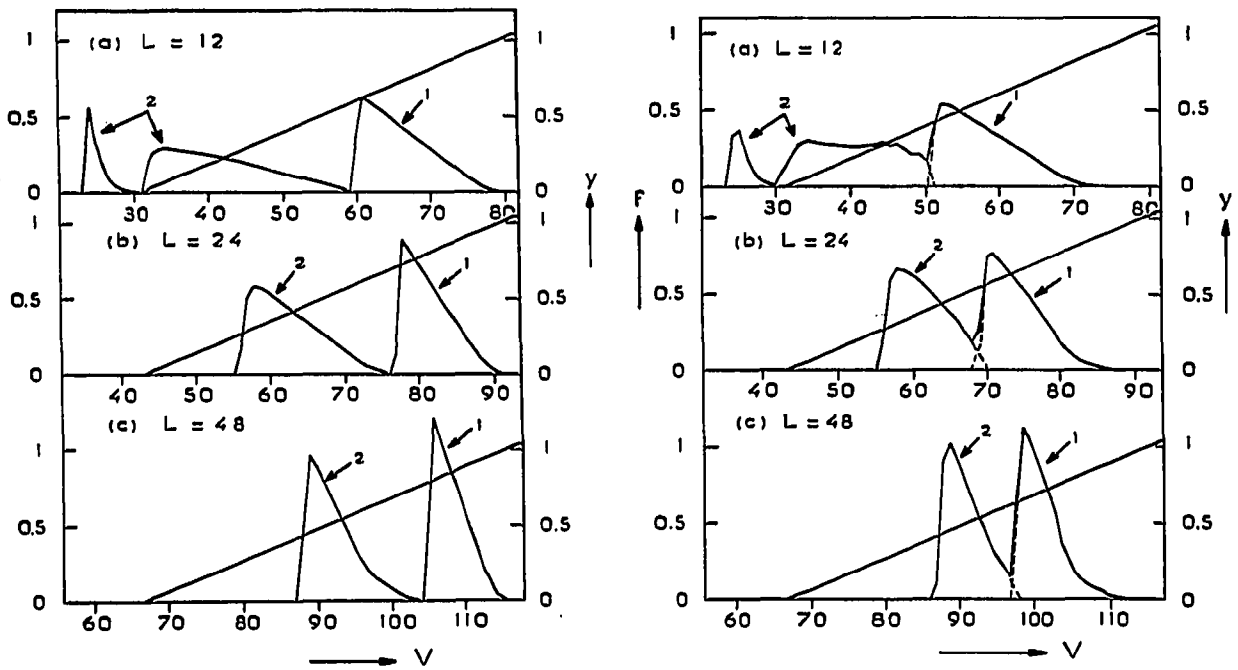


Fig. 34. Theoretical chromatograms obtained, taking into account molecular interactions, for a mixture of two types of molecules with different lengths but with the same adsorption energy per unit length. See Case 34 in Table I.

Fig. 35. As Fig. 34 but corresponds to Case 35 in Table I.

and subscripts (1 and 2) are used to represent the larger and the smaller molecules in the mixture, respectively. We always use the approximate relationship given by eqn. 5. It should be noted that the actual values of the probabilities $p_{(1)}$, ..., $p_{(\rho)}$ are, in general, smaller than the values estimated by eqn. 5 and that the error is more important when there are large molecules in the mixture. It can be shown, however, from Part II that if molecules are sufficiently large, their contribution to the total chromatogram is independent of $p_{(\rho')}$.

Figs. 34–38 (Cases 34–38) show the chromatograms for mixtures of equal amounts of molecular species 1 and 2 with different lengths and with the same values of w. In Fig. 34, we have assumed that the sizes of the molecules are very different ($x'_{(1)}=3000$ and $x'_{(2)}=300$), in Fig. 35 their sizes are not so different ($x'_{(1)}=600$ and $x'_{(2)}=300$), while in Fig. 36 their sizes are almost identical ($x'_{(1)}=300$ and $x'_{(2)}=297$). It should be noted that in Figs. 34–36 the column is not initially saturated with molecules, whereas in Figs. 37 and 38 (Cases 37 and 38) we have assumed intial saturation. The molecular dimensions of both species in Fig. 37 are identical with those in Fig. 36. In Fig. 37, however, the mean value of the adsorption energies of molecules is about twice the value in Fig. 36. Fig. 38 shows chromatograms for values of x' that are ten times larger than those in Fig. 34 ($x'_{(1)}=30{,}000$ and $x'_{(2)}=3000$). Figs. 39–43 (Cases 39–43) are chromatograms for the same mixtures as in Figs. 34–38 but with an s.o.g. 100 times smaller. Fluctuations in the chromatograms

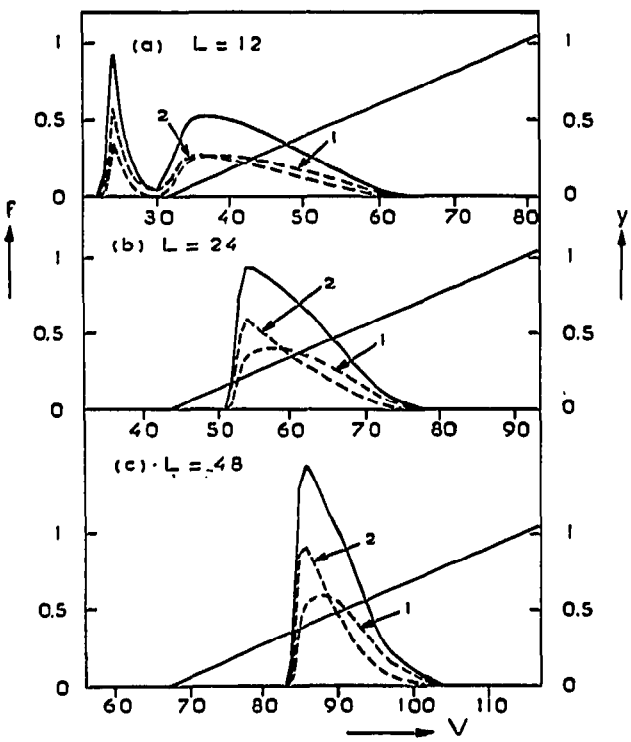


Fig. 36. As Fig. 34 but corresponds to Case 36 in Table I and to Case 2 in Table II.

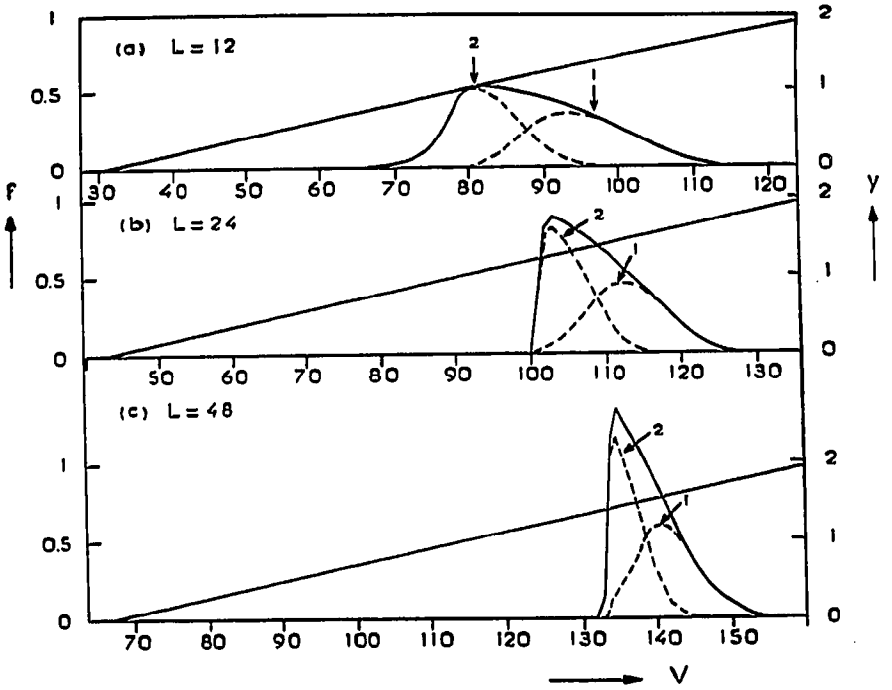
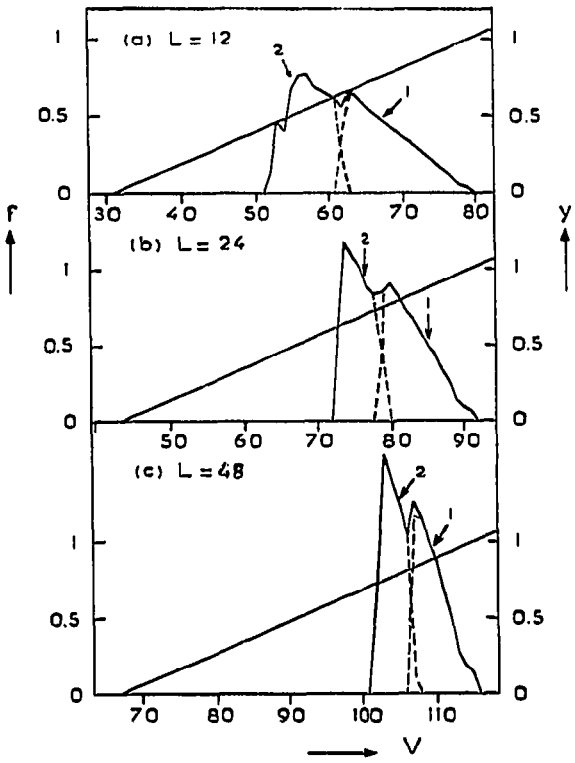


Fig. 37. As Fig. 34 but corresponds to Case 37 in Table I.

in Figs. 39–43 are the artifacts mentioned before. It should be noted that in Figs. 34–43 (Cases 34–43) the larger component 1 appears at higher activities, y, than the smaller component 2. We can consider that Fig. 2 in Part II (see Case 44) represents the chromatogram for a mixture of two molecular species with different infinite lengths (the ratio of which has a finite value, not equal to unity). It should be recalled that Fig. 2 in Part II also represents the chromatogram for a mixture of molecules with the same infinite length and with a small difference in adsorption energies per unit length (see Case 32). As in the preceding case, there is only a small difference between $x' \geqq 3000$ and $x' = \infty$ (compare Figs. 38a and 43a with Fig. 2 in Part II). It can be also seen in Figs. 34–43 that the best separation is generally obtained by using a small value of s.o.g. Especially if the adsorption energies of two molecular species are not sufficiently large for the surface of HA to be initially saturated by them and if the values of x' are similar, a decrease in the s.o.g. has a striking effect on the resolution of the column (see Figs. 36 and 41). When both the s.o.g. and the difference in molecular lengths are small, the best resolution can be obtained by using short columns (see Figs. 41a and 42a).

The case of two molecular species with different lengths and with different adsorption energies per unit length (Cases 45–53 in Table I)

It is evident that a good separation between large molecules with large absolute values of the adsorption energies per unit length (*i.e.*, large values of w) and small molecules with small adsorption energies per unit length (*i.e.*, small values of w)



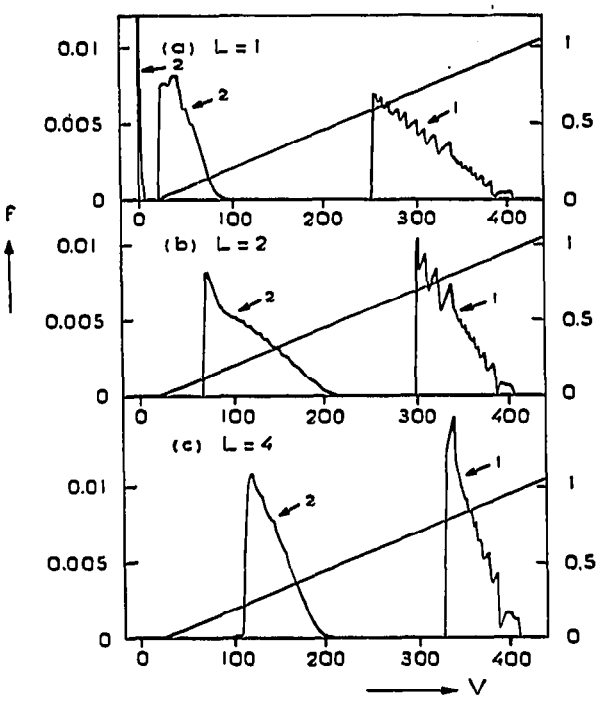
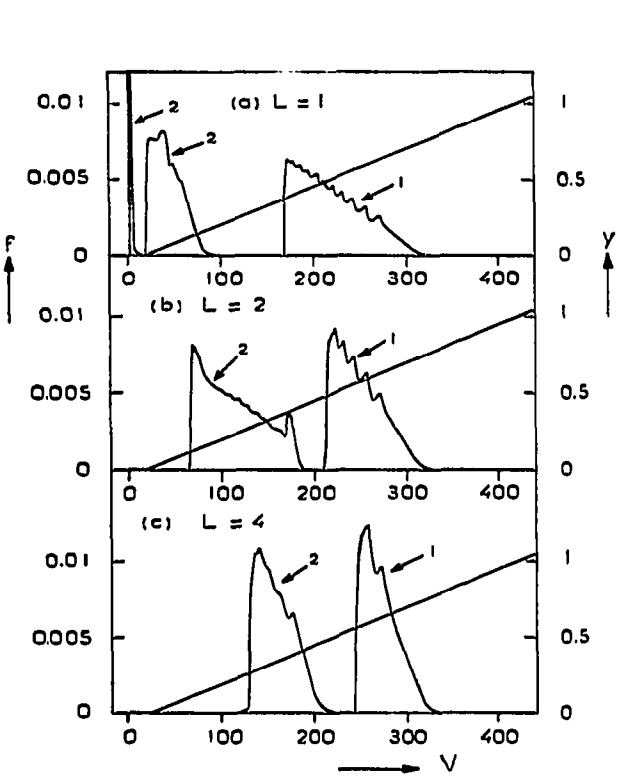


Fig. 38. As Fig. 34 but corresponds to Case 38 in Table I.

Fig. 39. As Fig. 34 but corresponds to Case 39 in Table I.

can be achieved by using short columns and a small value of the s.o.g., as the value of the activity, y, at which the chromatographic peak of the small component appears decreases more rapidly with a decrease in both the column length and the s.o.g. than in the case of large molecules (see Figs. 6–10 (Cases 1–5)). If large molecules have small w and small molecules have large w and if the difference in w values between the two components is sufficiently large, then a good chromatographic separation could be obtained by using long columns and large values of the s.o.g., provided that the effect of the axial diffusion is negligible (see Discussion). We shall now present some results for large and small molecules that have small and large values of w, the difference between these values being small.

Fig. 44 corresponds to a mixture of equal amounts of species 1 and 2 with x' values of 3000 and 300 and with w values of 1 and 1.4, respectively (Case 45). The chromatographic conditions are the same as those for Figs. 34–38 (Cases 34–38). It should be noted that species 1 in Fig. 44 is identical with species 1 in Fig. 34 (Case 34) and that the values of $x'_{(2)}$ for species 2 in both Figs. 44 and 34 are also the same. It can be seen in Fig. 44 that when the column is short the peak of species 2 with small length appears before the peak of the larger component 1 (see Fig. 44a), which is the same as in Fig. 34. When the column is long, however, the order of the



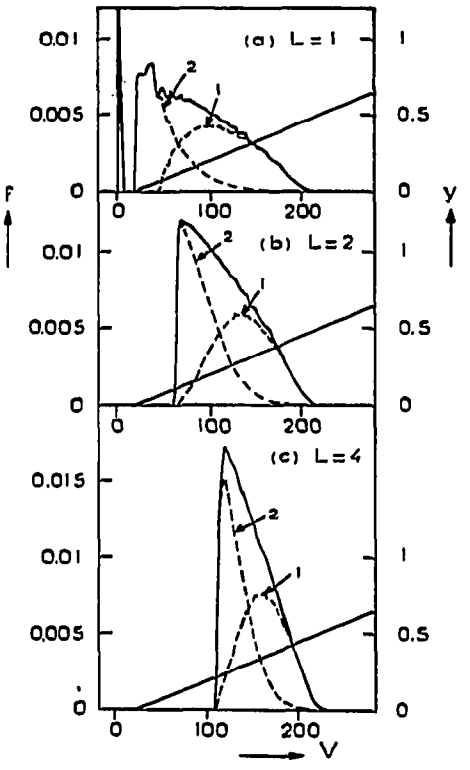


Fig. 40. As Fig. 34 but corresponds to Case 40 in Table I.

Fig. 41. As Fig. 34 but corresponds to Case 41 in Table I and to Case 2 in Table II.

appearance of the two molecular species is reversed (see Fig. 44b and c) and the best separations of the different molecules can be obtained by using either a very short or a very long column.

In Cases 46 and 47 (Figs. 45 and 46), the experimental conditions are the same as in Case 45 (Fig. 44) but the dimensions of the molecules are ten times larger, *i.e.*, $x'_{(1)}=30{,}000$ and $x'_{(2)}=3000$. There are also slight differences in the adsorption energies per unit length. Species 2 in both Figs. 45 and 46 is identical with species 2 in Fig. 38 (Case 38) and also with species 1 in Fig. 44 (Case 45). The molecular length of species 1 in both Figs. 45 and 46 is the same as the molecular length of species 1 in Fig. 38. It can be seen that if $w_{(1)}=0.97$ and $w_{(2)}=1$, the chromatogram is almost identical with that obtained when both $w_{(1)}$ and $w_{(2)}$ are unity (compare Fig. 45 with Fig. 38). If the value of $w_{(1)}$ is slightly smaller or if $w_{(1)}=0.96$, it is only when the column is short that species 2 appears before species 1 in the chromatogram (see Fig. 46a) and the order of elution between the two molecular species is reversed when the column is long (see Fig. 46c). It should be noted, however, that the shapes of the total chromatograms are very similar in Figs. 38, 45 and 46.

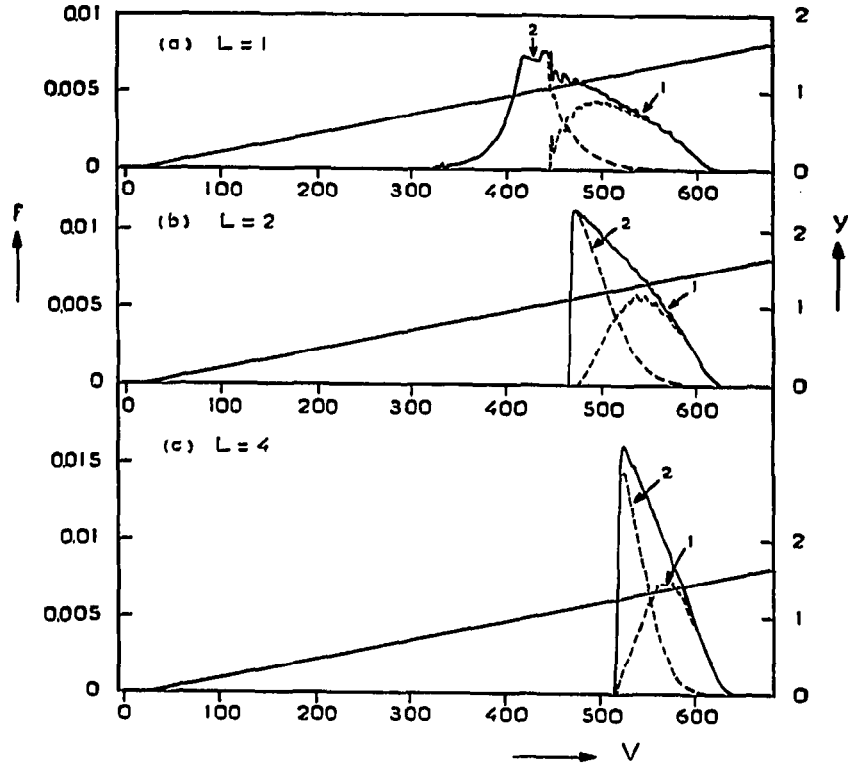


Fig. 42. As Fig. 34 but corresponds to Case 42 in Table I.

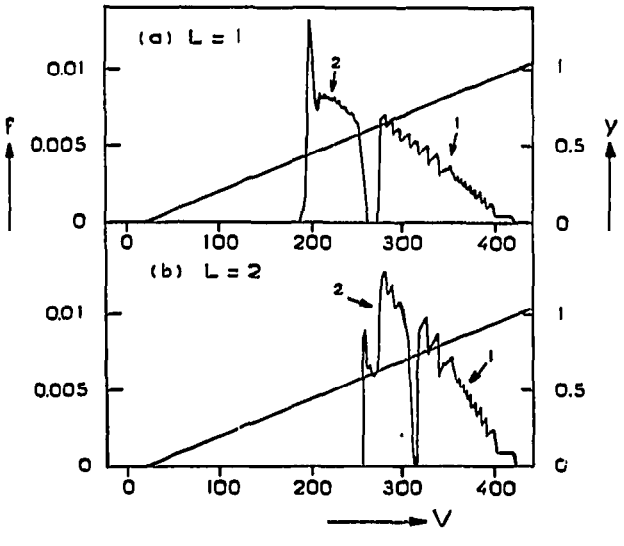
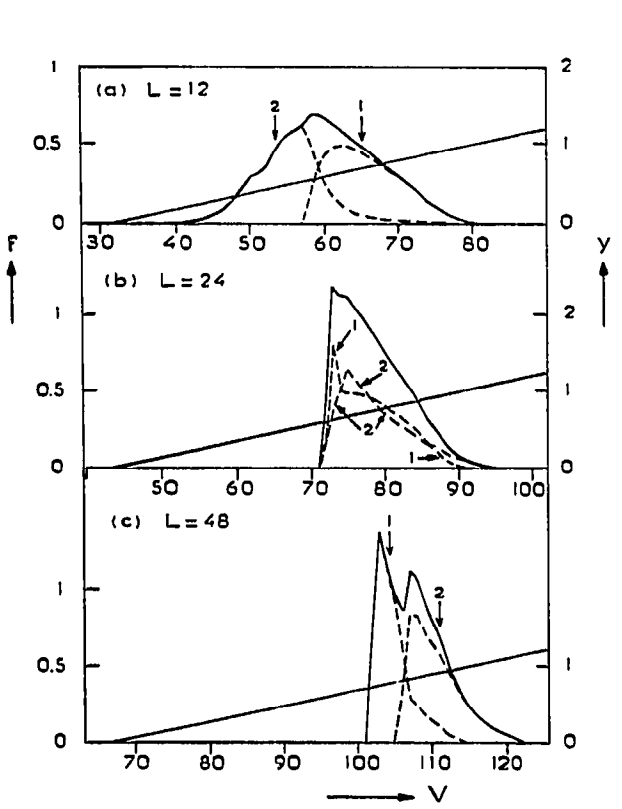


Fig. 43. As Fig. 34 but corresponds to Case 43 in Table I.



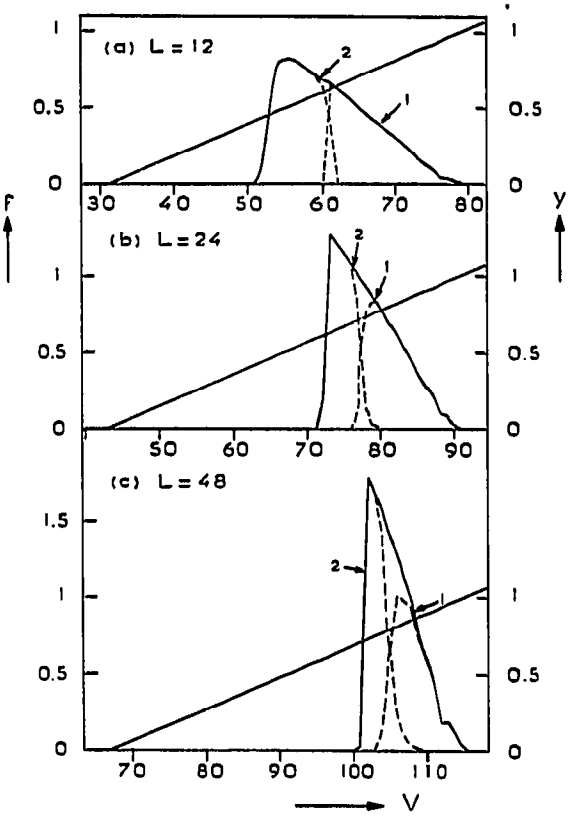


Fig. 44. Theoretical chromatograms obtained, taking into account molecular interactions, for a mixture of two types of molecules with different lengths and different adsorption energies per unit length. See Case 45 in Table I.

Fig. 45. As Fig. 44 but corresponds to Case 46 in Table I.

Fig. 47 (Case 48) shows the chromatograms for equal amounts of molecular species 1 and 2 with values of x' and $-u_3/kT$ very close to 300 and 20 and therefore with w very close to unity. The chromatographic conditions in Fig. 47 are the same as those in Figs. 44–46 (Cases 45–47) and also those in Fig. 17 ($x'_{(1)}=x'_{(2)}=300$ and both $w_{(1)}$ and $w_{(2)}$ are very close to unity (see Case 14)) and Fig. 36 (the case when both $x'_{(1)}$ and $x'_{(2)}$ are very close to 300 and $w_{(1)}=w_{(2)}=1$ (see Case 36)). Fig. 48 (Case 49) shows the chromatograms for the same mixture as those in Fig. 47 obtained when the s.o.g. is 100 times smaller. Chromatograms obtained under the same experimental conditions as those in Fig. 48 for mixtures with constant molecular length and with a constant value of w were shown in Figs. 19 and 41 (Cases 16 and 41), respectively. It can be seen in Figs. 47 and 48 that (1) the reversal of the order of the elution does not occur under these experimental conditions, (2) the resolution of the column increases strikingly with a decrease in the s.o.g. and (3) if the s.o.g. is small, the best resolution can be achieved when the column is short (see Fig. 48a). This is the same result as in the two cases when the molecular length and

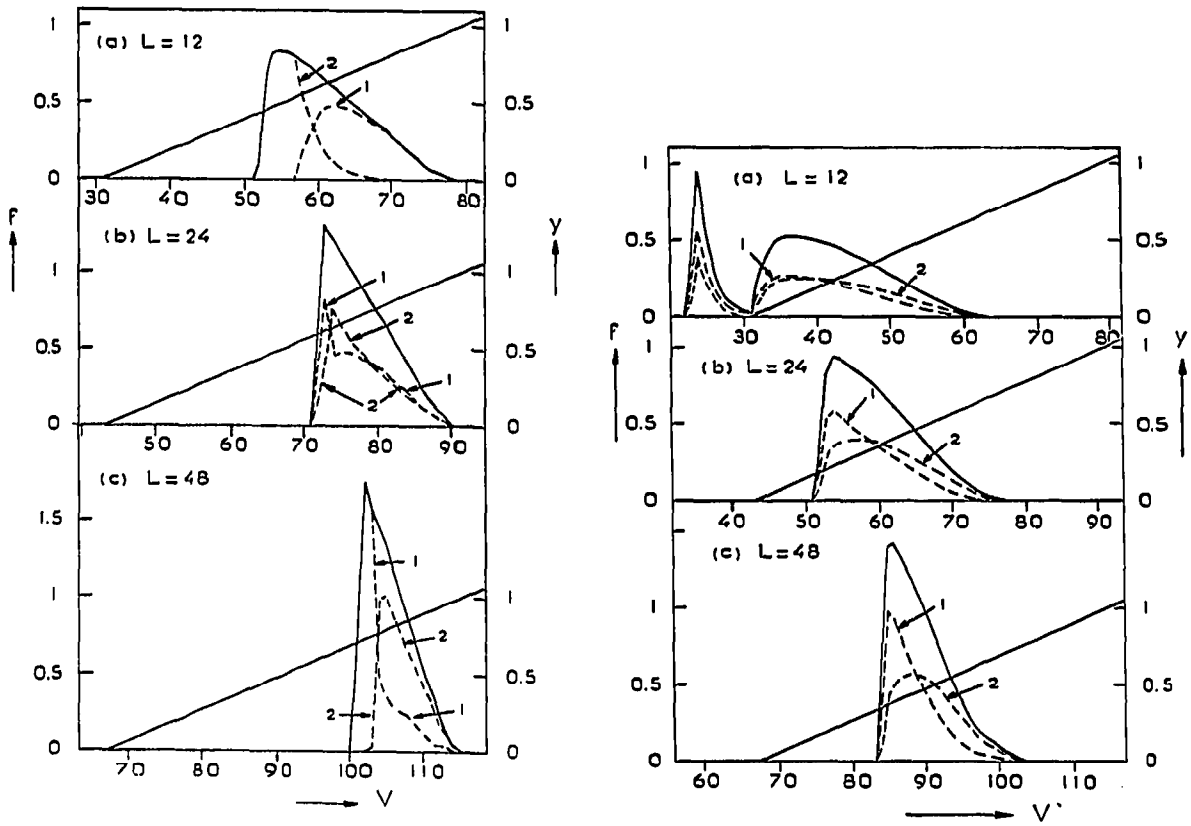


Fig. 46. As Fig. 44 but corresponds to Case 47 in Table I.

Fig. 47. As Fig. 44 but corresponds to Case 48 in Table I and to Case 3 in Table II.

the adsorption energy per unit molecular length, respectively, are constant (see Figs. 17 and 19 and Figs. 36 and 41). Table II lists the values of the parameters that characterize the model molecules in Figs. 17, 19, 36, 41, 47, 48, 49 and 50 (for the last two figures, see below). It should be recalled that the preference of the adsorption is governed either by the factor w or by molecular length x' (see Cases 1 and 2 in Table II). Figs. 47 and 48 (Case 3 in Table II) show cases where the adsorption is governed by w in spite of the fact that the total molecular length is not constant. Figs. 49 and 50 (see Case 4 in Table II) show the chromatograms when $w_{(1)}$ is intermediate between the corresponding values in Figs. 36 and 41 (Case 2 in Table II) and in Figs. 47 and 48 (Case 3 in Table II). In this case, the chromatographic separation between species 1 and 2 cannot be achieved (see Figs. 49 and 50). If the value of $w_{(1)}$ increases slightly and approaches the value of $w_{(2)}$, the order of preference of the adsorption of molecules will be governed by the molecular length.

Parts (a) and (b) in Fig. 51 (Cases 52 and 53 in Table I) show chromatograms when the mean values of x' and w are almost 300 and 2 obtained under the same experimental conditions as in parts (a) of Figs. 47 and 49 (Cases 48 and 50) and also

TABLE II

VALUES OF PARAMETERS THAT CHARACTERIZE THE MODEL MOLECULES IN FIGS. 17, 19, 36, 41, 47–50

Case	*Corresponding Figure No. (corresponding Case in Table I)*		*Species 1*			*Species 2*			*Order of elution*
	$g^*=0.25$	$g^*=0.0025$	$x'_{(1)}$	$-u_{(1)}/kT$	$w_{(1)}$	$x'_{(2)}$	$-u_{(2)}/kT$	$w_{(2)}$	
1	17 (14)	19 (16)	300	20	1	300	19.8	0.99	2, 1
2	36 (36)	41 (41)	300	20	1	297	19.8	1	2, 1
3	47 (48)	48 (49)	300	19.8	0.99	297	19.8	1	1, 2
4	49 (50)	50 (51)	300	19.9	0.995	297	19.8	1	Species 1 and 2 both elute at the same time

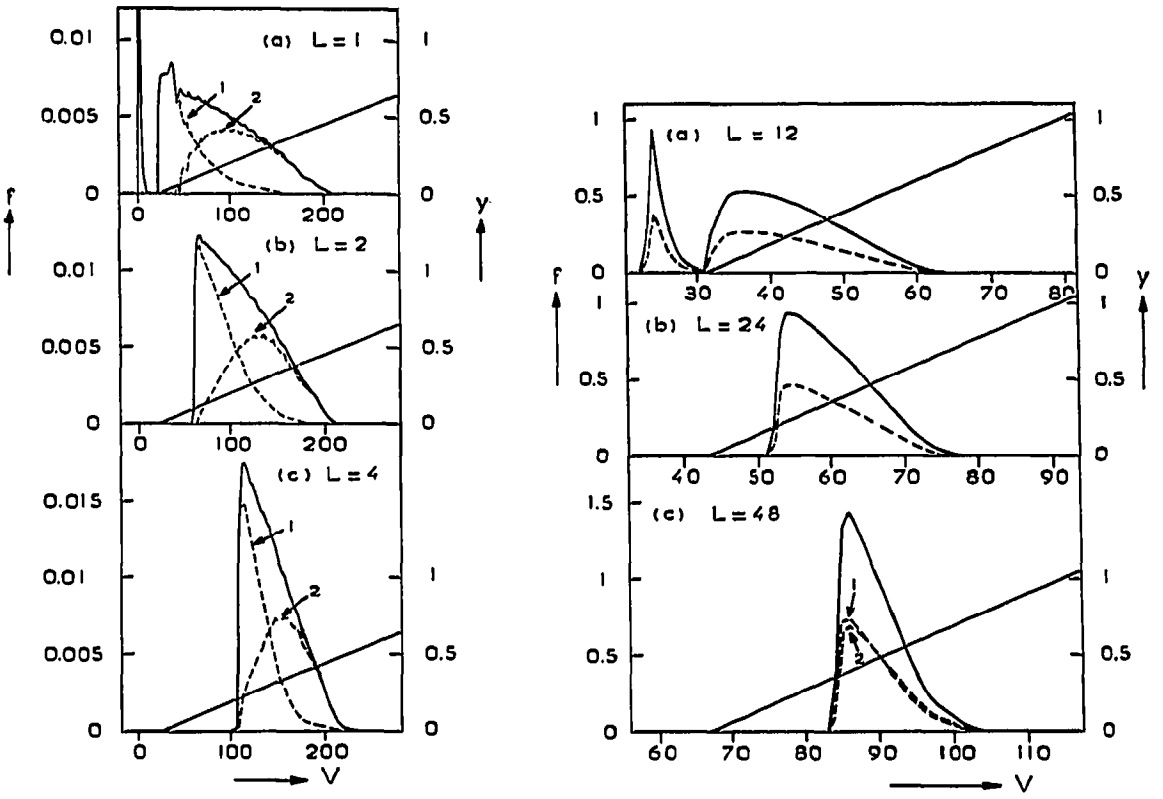
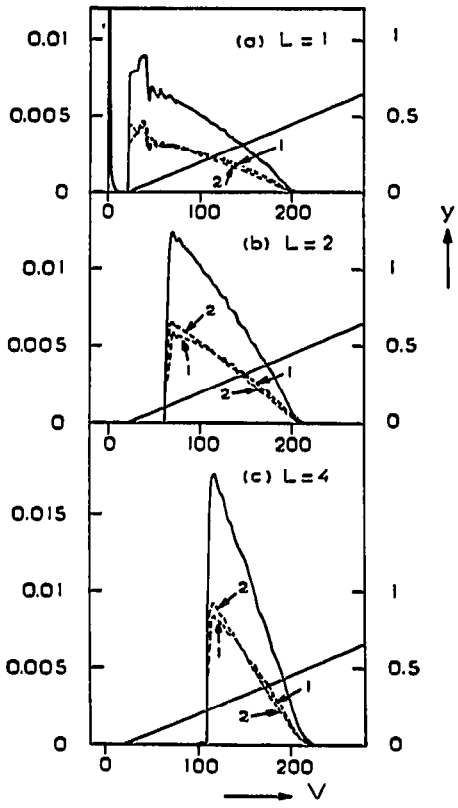


Fig. 48. As Fig. 44 but corresponds to Case 49 in Table I and to Case 3 in Table II.

Fig. 49. As Fig. 44 but corresponds to Case 50 in Table I and to Case 4 in Table II.

as in Fig. 22 (the case when $x'_{(1)}=x'_{(2)}=300$ and both $w_{(1)}$ and $w_{(2)}$ are very close to 2 (see Case 19)) and Fig. 37 (the case when both $x'_{(1)}$ and $x'_{(2)}$ are very close to 300 and $w_{(1)}=w_{(2)}=2$ (see Case 37)). It can be seen in Fig. 51 that the reversal of the order of the elution between the two molecular species with a 1% difference in molecular length occurs when w for the larger component is between 99 and 99.5% of its value for the smaller component.

Finally, it should be recalled that, in Part V[5], the order of the preferential adsorption on to HA of molecules with both different lengths and w values was discussed, assuming that molecules have infinite lengths. It has been shown that the elution of molecules always follows the order of w and that it depends on the molecular length or the value of x' only when all molecules have the same value of w. The elution is independent of the order of the preferential adsorption (which is a function of both w and x') of molecules on to the crystal surfaces in the initial step of the chromatography when the value of the activity, y, of the competing ions is too small for desorption of macromolecules to occur. We have shown in this paper that, in some actual cases when molecules have finite lengths, the order of the elution of molecules depends on the value of x' if the column is short (see parts (a) of Figs. 44



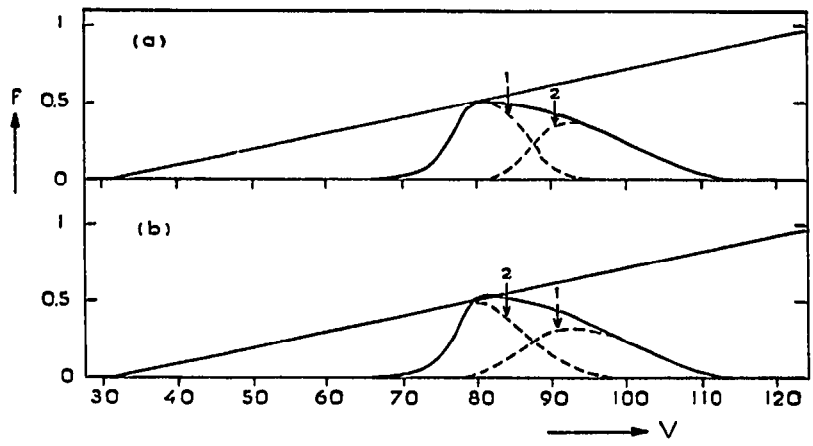


Fig. 50. As Fig. 44 but corresponds to Case 51 in Table I and to Case 4 in Table II.

Fig. 51. As Fig. 44 but parts (a) and (b) correspond to Cases 52 and 53 in Table I, respectively.

and 46). If the column is sufficiently long, however, it will depend on the order of the values of w, as the elution of molecules depends mainly on the critical value Λ^0_2 (see Parts I and II), which is proportional to w (see also parts (c) of Figs. 44 and 46).

DISCUSSION

We have shown that the shape of the total chromatogram for a mixture of different molecules is essentially identical with that obtained in the case of a single component, if both the adsorption energies per unit molecular length and the total lengths of the different molecules are similar (compare Figs. 17–19, 27–29, 32, 36, 41 and 47–50 with Figs. 6–10) or if the adsorption energies per unit length, w, are similar and all molecules have extremely large dimensions (compare Figs. 38, 43, 45 and 46 with Figs. 7 and 10, and Fig. 2 in Part VI with Fig. 1 in Part VI). In these cases, the separation is always improved by using a small value of the s.o.g. and a "short column" (see below) (see Figs. 19a, 32a, 41a, 43a and 48a, and Figs. 25a, 33 and 42a). It should be noted that in this paper, the column length, the s.o.g.

and the load are all expressed as relative values and that the chromatogram as a function of y can be characterized only in terms of the ratio among these three quantities, which means that one can replace the term "short column" above by the term "high load". Now, when all molecules have the same length x' and similar values of w, the resolution of the column for molecules with the same difference in the values of w increases with the increase in the length of each molecule. It can be seen in Figs. 15a, 17a, 22a, 23a, 27, 28a and 29 that almost complete separations between molecules with values of x' of 300, 3000 and 30,000 can be obtained when the differences in the values of w between them are 0.1, 0.01 and 0.001, respectively*. When the differences in the latter values are ten times smaller, *i.e.*, 0.01, 0.001 and 0.0001, good separations can no longer be achieved under the same experimental conditions, which means that the separation depends essentially on the difference in the values of the total adsorption energies per molecule, *i.e.*, the product of w and x'. It should be noted that the value of w reflects the compositions and the sequences of amino acids and nucleotides in the cases of protein and DNA, which means that, when the molecules are large, those with very similar chemical and physical properties can be separated on the column. It is probable that sometimes different molecules that have been fractionated on the column cannot be distinguished by the usual chemical and physical methods. Now, let us assume that we have obtained a chromatogram with a single peak and that the re-chromatography of any part of the original chromatogram has given essentially the same patterns and the chemical and physical properties of the molecules in any part of the chromatogram are also identical with one another, within the limit of the experimental error. We then have to consider the following three possibilities: (1) the molecules are homogeneous; (2) the molecules are heterogeneous but sufficient chromatographic separations among different molecules do not occur; and (3) the chromatographic separations among different molecules do occur but the differences in their chemical and physical properties cannot be detected by the usual methods. It is possible, however, to detect the last possibility by introducing markers into some chromatographic fraction, by mixing all the chromatographic fractions and by chromatographing the mixture again. The shape of the total chromatogram in the second chromatography will be the same as in the initial chromatography but, if there are chromatographic separations among different molecules, the markers will be concentrated in the position where there have been the same molecules in the initial chromatogram.

A column generally gives a good resolution for a mixture of molecules with similar properties when the mean value of the parameter w among them is sufficiently large for the surfaces of HA in the column to be initially saturated with them (compare Fig. 17 with Figs. 22 and 23, Fig. 31 with Figs. 28a and 30, and Fig. 36 with Fig. 37). It has been mentioned, on the other hand, that the contribution of the component ρ' to the total chromatogram involves the variables y and $w_{(\rho')}$ approximately in the form of $w_{(\rho')}-y$, so that the chromatogram obtained by using the initial buffer that contains competing ions of activity y is identical with the chromatogram for a mixture of molecules with values of $w_{(1)}$, ..., $w_{(\rho)}$ that are smaller by a factor y,

* It should be noted that when one of the components in the mixture has $w=1$ (Figs. 15, 17 and 27–29), the difference in the values of w between two components could represent the ratio of the deviation in values of the adsorption energies to the mean value. When one of the components has $w=2$ (Figs. 22 and 23), for instance, the difference in w is half of the above ratio.

obtained by using the initial solution with no competing ions. Therefore, we can consider, for instance, that Fig. 17 can represent the chromatograms for the same mixtures as in Figs. 22 and 23 obtained in the cases when the values of the activity y in the initial solutions are 1 and 2 instead of zero, 1 and 2 being the differences in the mean values of w between Figs. 22 and 17 and between Figs. 23 and 17, respectively. Similarly, Fig. 31 can represent the chromatograms for the same mixtures as in Figs. 28a and 30 obtained by using the initial solutions that contain the competing ions with activities of 0.5 and 1.5, respectively, and Fig. 36 the chromatograms for the same mixtures as in Fig. 37 obtained by using the initial solution with an activity of unity. It should be recalled[9] that we sometimes use, in the case of the chromatography of DNA, the initial buffer that contains about 0.1 mole/l of phosphate ions (a type of competing ion (see Appendix I in Part III[3])), which, according to the above conclusion, generally cannot be the best chromatographic conditions. However, if the s.o.g. is sufficiently small and if the column is short (or the load is high), a good resolution can be achieved even when the initial solution contains a considerable amount of the competing ions (see Figs. 19a, 33 and 41a).

We have mentioned above that the best resolution of the column can be obtained, in general, by using a small value of the s.o.g. and a short column (or a high load) when there are no large differences between the properties of the molecules in the mixture. In Figs. 1, 2 and 6 in an earlier paper[10], it was shown experimentally that the resolution of the column increases strikingly with a decrease in the s.o.g. in the case of tropocollagen. In Figs. 1, 2 and 6 in ref. 10, the reduced values of the s.o.g. relating to a column diameter of 1 cm are about 10^{-3}, $4\cdot10^{-4}$ and $4\cdot10^{-5}$ M/ml, respectively. In Appendix II in ref. 7, the critical value of the elution phosphate molarity, $m^0_{(P)}$, for the molecular species of tropocollagen that appears in the highest part of the chromatogram has been estimated as about 0.12 M. Using this estimate and a value of 0.8 for the ratio of the interstitial volume to the packed crystal volume in the column[9], one can estimate, for instance, for a column of length 50 cm, that the differences in the values of y at the top and the bottom of the column are about 0.3, 0.1 and 0.01 when the slopes of the phosphate gradients are 10^{-3}, $4\cdot10^{-4}$ and $4\cdot10^{-5}$ M/ml, respectively. On the other hand, one can also estimate, by using the values of the experimental parameters in Table I in Appendix I in ref. 7, that the difference in the values of $-u_3/kT$ between the neighbouring chromatographic peaks of tropocollagen obtained in the case of the smallest slope of the phosphate gradient (see Fig. 6 in ref. 10) is about 2, or about 0.1 in units of w. In Fig. 15, we have shown that the chromatographic separation of molecules with $x'=300$, which could be applied to tropocollagen, and with a difference of 0.1 in the values of w, is almost complete even when the s.o.g. is large, *i.e.*, when the difference in the values of y at the top and the bottom of a column of length $L=48$ is unity. It should be recalled that the theory is based on the assumption that there is no longitudinal diffusion of molecules on the column. The increase in the resolution of the column with a decrease in the slope of the phosphate gradient observed in the experimental chromatography of tropocollagen could be at least partially explained by the fact that the effect of the longitudinal diffusion of molecules decreases with an increase in the width of the chromatographic peak of each component which occurs when the s.o.g. decreases. The conclusion that a good resolution of the column can be obtained by using a small value of the s.o.g. and a short column (or a high

load) is valid even taking into account the longitudinal diffusion of macromolecules on the column, as this effect also decreases for short columns.

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