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MULTIVALENT INTERACTION CHROMATOGRAPHY AS EXEMPLIFIED BY THE ADSORPTION AND DESORPTION OF SKELETAL MUSCLE ENZYMES ON HYDROPHOBIC ALKYL-AGAROSES

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CONTENTS

1. INTRODUCTION

If the degree of substitution of Sepharose 4B with α-aminoalkanes (*i.e.*, alkyl-residue density) is increased, the binding capacity of the gels for proteins is a sigmoidal function of the alkyl-residue density[1]. This correlation has been shown at low ionic strength ($\mu = 0.03$–0.04)[1,2] for the enzymes phosphorylase *b*, phosphorylase kinase, phosphorylase phosphatase, glycogen synthetase, cAMP-dependent protein kinase and other skeletal muscle proteins. Similarly, a sigmoidal relationship between the binding capacity of substituted gels for phosphorylase *b* and the alkyl-residue density could be demonstrated at high ionic strength[3]. An analysis of these positive cooperative binding curves according to Hill[3–5] has led to the conclusion that the proteins described (see above) are multivalently adsorbed on at least 3–9 binding sites (multivalent interaction chromatography[6]). The minimum number of the sebinding sites appears to be temperature dependent[6].

From equilibrium binding studies of phosphorylase *b* on alkyl-Sepharoses of a specific residue density, negative cooperative ligand binding has been concluded[5]. Recently, it was reported that negative cooperativity of phosphorylase *b* binding to butyl-Sepharose might also be inferred from kinetic studies[6].

In this paper, the evidence that strongly indicates that proteins are multivalently adsorbed to alkyl-Sepharose 4B (1–4 carbon atoms) and the practical consequences for chromatographic procedures are presented. An attempt is also made to introduce a systematic order into the diverse nomenclature of chromatography on substituted carbohydrate gels on the basis of the valence involved in the adsorption of the protein ligand.

2. MATERIALS AND METHODS

The preparation of ^{14}C-labelled alkyl derivatives of Sepharose 4B has been described by Jennissen and Heilmeyer[1] and Jennissen[2]. Phosphorylase *b* (*ca.* 80 U/mg) was prepared and freed of AMP according to Fischer and Krebs[7]. All calculations are based on a subunit molecular weight of 100,000 (ref. 8). The activity was measured according to Haschke and Heilmeyer[9]. Unless otherwise stated, the capacity of the gels was determined at apparent ligand-matrix equilibrium (see the isotherms below). All experiments were performed in thermostated Plexiglas beakers (2.5 cm I.D. × 9 cm) employing a 1.5-cm magnetic stirring bar as described by Jennissen[5]. The isotherms (for a detailed description, see ref. 5) were determined by incubating increasing concentrations of phosphorylase *b* with butyl-Sepharose in buffer containing 10 m*M* tris(hydroxymethyl)aminomethane/maleate, 5 m*M* dithioerythritol, 1.1 *M* ammonium sulphate and 20% sucrose (pH 7.0) for 30 min at a stirring rate of *ca.* 700 rpm (ref. 3). The amount of phosphorylse *b* adsorbed on the gel was calculated from the difference between the values of the initial and the final free phosphorylase *b* concentration (activity measurements) after 30 min of adsorption employing unsubstituted Sepharose 4B under identical conditions as the control.

The method for the measurement of the kinetics of the desorption of phosphorylase *b* in the above buffer was described by Jennissen[6]. For the sampling procedure, see ref. 5.

3. RESULTS AND DISCUSSION

3.1. Influence of substitution procedures on the substituted matrix

3.1.1. Gel contraction and the degree of substitution

The degree of substitution is usually measured on a volume basis in micromoles of substituent per millilitre of packed Sepharose[1]. The alkyl-residue density can, however, also be expressed on a weight (moles of substituent per gram of dry agarose)[10] or molar basis (moles of substituent per mole of anhydrodisaccharide, see below). Låås[10] has shown that a drastic decrease in gel volume (50–60%) of benzylated Sepharose CL-2B may occur at substitutions of 55–110 μmole/ml gel. Therefore, large discrepancies in the value of the residue density could occur, depending on the units employed. It will be shown below (see Table 2) that under the conditions employed here[1–3] a maximal volume decrease of *ca.* 10% is found for a substituted

butyl-Sepharose containing 44 μmole/ml packed gel. This volume change has virtually no influence (see below) on the evaluation of binding data. Nevertheless, as changes in gel volume do occur, it is probably more appropriate to express the substituent density for binding site determinations on a molar (or weight) basis. However, then it is assumed that a gel contraction does not lead to a higher density of the residues on the agarose matrix.

3.1.2. Uniform distribution of residues and substitution procedures

In the described coupling procedure (see Section 2) the reactive sites are introduced by varying the concentration of cyanogen bromide (see Table 2) in the reaction mixture. The coupling to the alkylamine[11] occurs at a constant (maximal concentration change 0.3%)[1] and very high amine concentration (2 *M*). It is therefore unlikely and can be excluded[1] (see Table 2) that the introduction of alkyl residues is a sigmoidal (see the implications below) function of the initial cyanogen bromide concentration. A uniform substitution of Sepharose by the cyanogen bromide method is also indicated by the electron microscopy of ferritin coupled to Sepharose 6B[12].

In contrast, if an alkyl halide is reacted directly with non-activated Sepharose CL-6B[13] the degree of substitution increases as a sigmoidal function of the dilute and limiting initial concentration of the halide in the mixture. This behavior has been interpreted as indicating an uneven (cooperative) distribution of alkyl residues on the matrix[13].

3.2. Positive cooperative binding as a function of the alkyl-residue density

3.2.1. Low ionic strength (e.g., $\mu = 0.03$–0.04)

In Table 1, the data so far reported on the positive cooperative adsorption of enzymes on alkyl-Sepharoses at low ionic strength are summarized.

In a similar manner to Freundlich isotherms (see negative cooperativity below), the sigmoidal curves resulting from a plot of the adsorbed amount of ligand per unit of packed Sepharose against the degree of gel substitution with alkyl residues can easily be analysed according to the power function of Freundlich[4,5,15]:

$$\log a = \log \alpha + 1/n \log c \tag{1}$$

where a (also called binding capacity[1]) is the amount of ligand adsorbed (*e.g.*, in milligrams per millilitre of packed gel), α is the adsorption constant (*e.g.*, in milligrams per millilitre of packed gel), $1/n$ is the adsorption exponent and c is either the alkyl-residue density (*e.g.*, in micromoles per millilitre of packed gel, see Table 1) or the free ligand equilibrium concentration (*e.g.*, in milligrams per millilitre, see Table 3). The adsorption exponent (c = alkyl-residue density) corresponds to the initial increment of the sigmoidal, positive cooperative binding curves when these are plotted in double logarithmic coordinates.

If saturation is approximated, analysis according to Scatchard[16] is feasible. With the extrapolated saturation value of the Scatchard plot, the corresponding Hill plot[5,17] can be obtained for analysing the positive cooperativity. The calculated Hill coefficient (n_H), which is larger than unity, is an indication of the minimum number of binding sites involved in adsorption[5,18]. This type of ligand adsorption, which ideally

TABLE 1

CONSTANTS OF THE FREUNDLICH AND THE HILL EQUATION OF THE BINDING OF SKELETAL MUSCLE ENZYMES TO ALKYL-SEPHAROSES AS A FUNCTION OF THE DEGREE OF SUBSTITUTION AT LOW IONIC STRENGTH

The original experimental data employed in the calculations are given in the corresponding reference. For definition of $1/n$, n_H, $K_{0.5}$ and packed Sepharose, see ref. 5 and the text. The enzymes were adsorbed from crude muscle extracts[1] from either mixed, white or red muscle[14]. Saturation of the gels with phosphorylase kinase was extrapolated from Scatchard plots to be: mixed muscle 1.0 (methyl), 1.4 (ethyl), 1.8 (butyl); white muscle 0.5 (methyl); and red muscle 0.16 (methyl) mg/ml packed gel, respectively, assuming a specific activity of the pure enzyme to be 8500 units/mg. For further details see the text, Tables 3 and 5 and the references.

Enzyme	*Alkyl residue*	$1/n$	n_H	$K_{0.5} \cdot 10^{-1}$ (*l packed Sepharose/mole*)	*Reference*
Phosphorylase Kinase					
mixed muscle	Methyl	5.8	6.7	5.5	1
	Ethyl	3.6	3.8	6.8	
	Butyl	3.5	3.7	8.2	
Fast twitch muscle (white)	Methyl	7.5	9.2	6.8	
Slow twitch muscle (red)	Methyl	7.8	8.7	7.9	2
Phosphorylase Phosphatase					
Fast twitch muscle (white)	Methyl	2.9			2
Slow twitch muscle (red)	Methyl	3.6			
Phosphorylase *b*					
mixed muscle	Ethyl	8.5			1
	Butyl	4.8			

corresponds to an "all-or-none" model, has been called imperative multivalent binding[3].

With phosphorylase kinase (Table 1), where both the adsorption exponent and the Hill coefficient have been calculated, the value of the Hill coefficient is consistently 1.1–1.2-fold higher than the adsorption exponent. A similar relationship has been shown for negative cooperative binding[5] (see below). One can therefore conclude that, in those instances when a Hill coefficient cannot be calculated (*e.g.*, phosphorylase phosphatase and phosphorylase *b*) because saturation is not obtained, the adsorption exponent gives a good approximation of a minimum value of the Hill coefficient. For the adsorption of phosphorylase kinase on methyl-agarose, the Hill coefficients indicate that at least 7–9 binding sites are necessary. There appears to be no difference in the number of binding sites essential for the two isoenzymes[14] from fast and slow twitch muscle. This may not be true for phosphorylase phosphatase from these two types of muscle.

If the chain length of the alkyl residue is increased from methyl to ethyl and butyl, the adsorption exponents and the corresponding Hill coefficients (n_H) decrease from 6.7 to 3.8 and 3.7, respectively. A similar decrease in the adsorption exponent is observed for phosphorylase *b* adsorption on ethyl- ($1/n = 8.5$) and butyl-Sepharose ($1/n = 4.8$). It has therefore been concluded[4,5] that the number of binding sites necessary for adsorption of a ligand molecule decreases as a function of the alkyl-

residue chain elongation. Therefore, if the chain length is sufficiently great and the interaction of a single alkyl residue with the protein yields the free energy necessary for adsorption, the Hill coefficient should approach unity and a non-cooperative relationship between the gel capacity and the alkyl-residue density is to be expected. This, however, does not exclude multiple contacts between a single residue and the protein ligand. Such a binding behaviour of low residue cooperativity appears to have been observed on uncharged alkyl derivatives of Sepharose[13,20] (see Section 3.1.2.).

The apparent association constant of half-maximal saturation ($K_{0.5}$; Tables 1 and 5) corresponds to the reciprocal of the alkyl-residue density at half-maximal saturation with ligand. With phosphorylase kinase it can be calculated from these constants (see Table 1) that the apparent alkyl-residue density essential for half-maximal saturation decreases from 18.2 to 14.7 and 12.2 μmole/ml packed Sepharose for the methyl, ethyl and butyl derivative, respectively (see Table 5).

3.2.2. High ionic strength ($\mu > 1$)

Various studies[21,22] have indicated that ionic groups can be involved in the adsorption of proteins on alkyl derivatives of cyanogen bromide-activated Sepharoses. These interactions should be reduced or eliminated at high ionic strength. Recently, it was shown[5] that the adsorption of phosphorylase *b* on butyl-Sepharose in the presence of 1.1 *M* ammonium sulphate is in fact an endothermic reaction. Therefore, it was pertinent to show that the multivalent interactions observed at low ionic strength (Table 1) can also be detected in the case of truely hydrophobic (*i.e.*, endothermic) adsorption of proteins on alkyl-Sepharoses.

Therefore, a new series of Sepharose derivatives of increasing degree of substitution was synthesized (Table 2). From the data, it can be calculated that the volume of the gel substituted with 44 μmole/ml packed gel has decreased by *ca.* 10%. The parameters calculated from binding studies are not significantly different whether the butyl-residue density is expressed on a volume or a molar basis (compare the results of Jennissen[6] with Fig. 1). This also demonstrates that the previous evaluations (Table 1) are soundly based.

TABLE 2

SUBSTITUTION OF CYANOGEN BROMIDE-ACTIVATED SEPHAROSE 4B WITH BUTYL RESIDUES

The absolute dry weight of the substituted agarose gels[5] was employed for the calculation of moles of anhydrodisaccharide (molecular weight 306). For the butyl derivative (CH_3–CH_2–CH_2–CH_2–N(H)–C(=O)–) a molecular weight of 83 was employed. For further details see the text, Section 2 and ref. 1.

BrCN (mg/ml)	*Degree of substitution*	
	μMoles/ml packed gel	*Moles butyl residue per mole anhydrodisaccharide*
1	3.8	0.038
2	6.2	0.062
4	12.1	0.121
8	20.6	0.204
15	29.9	0.308
30	44.0	0.401

In the data presented previously for low ionic strength (Table 1), the capacity of the gels was measured by a column technique[1,2] under the assumption of high binding constants (see $K_{0.5}$ in the paper by Jennissen[5] and below) and low ligand leakage during the column wash. These assumptions can be neglected if the gel capacity is measured at apparent ligand-matrix equilibrium and if the gel capacity of the various gels is compared at identical equilibrium, free ligand concentrations[3,19]. These conditions are most easily met if adsorption isotherms are performed for each gel under study. The adsorption constants (α) and the adsorption exponents ($1/n$) of the Freundlich isotherms are listed in Table 3. The adsorption constants characteristically increase and reach a plateau while the adsorption exponents decrease as a function of the butyl-residue density.

TABLE 3

CONSTANTS OF THE FREUNDLICH ISOTHERMS OF THE BINDING OF PHOSPHORYLASE *b* TO BUTYL-SEPHAROSES AS A FUNCTION OF THE FREE PHOSPHORYLASE *b* EQUILIBRIUM CONCENTRATION IN THE PRESENCE OF 1.1 *M* AMMONIUM SULPHATE

The adsorption constant (α, mg/ml packed Sepharose) and the adsorption exponent ($1/n$) were calculated by the least-squares method (eqn. 1) from the isotherms. For further details, see the legend to Fig. 1, Table 1, methods, the text and ref. 5.

Temperature (°C)	*μMoles/ml packed gel*	α	$1/n$
5	3.8	1.1	0.95
	6.2	3.7	0.72
	12.1	12.2	0.55
	20.6	22.6	0.54
	29.9	21.4	0.46
	44.0	24.7	0.52
34	3.8	2.5	0.95
	6.2	4.6	0.68
	12.1	18.9	0.57
	20.6	34.7	0.52
	29.9	34.6	0.43
	44.0	38.2	0.42

From the isotherms (Table 3) the isosteric heats of adsorption (ΔH) at a capacity of $a = 1.5$ mg of phosphorylase *b* per millilitre of packed gel can be calculated to lie between 2 and 13 kcal/mole (for method, see ref. 5). This demonstrates the endothermic nature of phosphorylase binding on all butyl-Sepharoses (Table 2) independent of the alkyl-residue density. In Fig. 1A, the capacity of the butyl-agaroses for the ligand phosphorylase *b* (calculated from the isotherms in Table 3 at an apparent equilibrium concentration of 0.07 mg/ml) is plotted as a function of the butyl-residue density. Sigmoidal curves result which, when re-plotted according to Scatchard, are concave downwards (see insert in Fig. 1A). An increase in temperature from 5° to 34° leads to an increase in the capacity of the gel (see ΔH above). Fig. 1B shows the corresponding Hill plots (*ca.* 1–90% saturation). An increase in temperature from 5° to 34° decrease the Hill coefficient from 3.6 to 3.0. The apparent association constant of half-maximal saturation is reduced from 7.3 to 6.0 mole of anhydrodisaccharide

per mole of butyl residue (see also ref. 6). It can therefore be concluded that an increase in temperature from 5° to 34° leads to a decrease in the minimum number of binding sites (from 4 to 3, *i.e.*, nearest integers of the Hill coefficients) necessary for the adsorption of a phosphorylase *b* molecule. Correspondingly, the slight decrease in the $K_{0.5}$ value indicates a decrease in affinity as a result of the temperature enhancement.

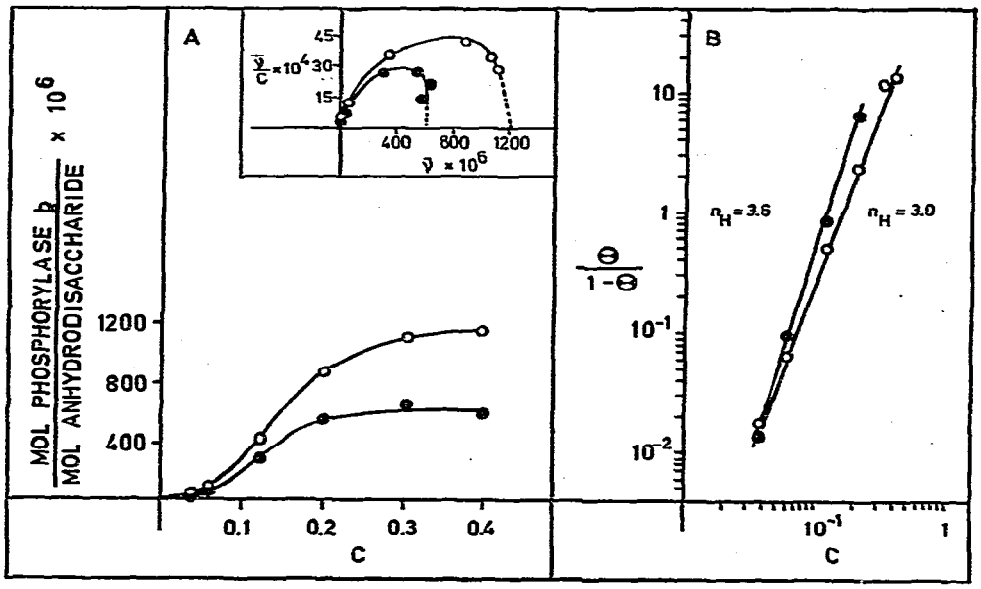

Fig. 1. (A) Adsorption of phosphorylase *b* in the presence of 1.1 *M* ammonium sulphate at 5° (●) and at 34° (○) as a function of the butyl-residue density (equilibrium concentration *ca.* 0.07 mg/ml). Insert: Scatchard plots of the sigmoidal binding curves. $\bar{\nu}$ denotes the gel capacity for phosphorylase *b* in moles of enzyme per mole of anhydrodisaccharide. *C* is the alkyl-residue density in moles of residue per mole of anhydrodisaccharide. The broken lines indicate the mode of extrapolation. (B) Hill plot of the adsorption of phosphorylase *b* as a function of the butyl-residue density under the conditions described in (A). The fractional saturation of the gel (θ) was calculated from the extrapolated value of 610 and 1220 μmole of phosphorylase *b* per mole of anhydrodisaccharide at 5° and 34°, respectively, as shown in the Scatchard plot in (A). n_H is the Hill coefficient. The apparent association constants of half-maximal saturation ($K_{0.5}$) are 7.3 and 6.0 mole of anhydrodisaccharide per mole of butyl residue at 5° and 34°, respectively. For further details, see the text and ref. 5.

The following simplified model may explain these results. For example, at 5° twelve alkyl residues corresponding to twelve *binding sites* are capable of binding three molecules of phosphorylase *b*. Each molecule of the enzyme covers the minimum number of four binding sites, which are called a *binding unit*. As one binding unit contains four binding sites, twelve binding sites are capable of binding three molecules of ligand. If the temperature is increased to 34°, one binding unit now contains only three binding sites. Thus twelve binding sites are now capable of binding four rearranged molecules of the enzyme. The temperature increase apparently leads to an enhancement of the free energy of a single hydrophobic interaction. Therefore, at the higher temperature apparently only three binding sites are now necessary for the adsorption of one phosphorylase *b* molecule. However, the affinity of the binding unit

containing three binding sites appears nevertheless to be lower than that of the binding unit containing four binding sites. One would therefore expect that the described temperature rise would result in a capacity ratio of 4:3 for the gel capacities at 34° and 5°, respectively. Experimentally, the gels show a capacity ratio of *ca.* 4:2.4, which is higher than the model predicts. This difference may be due to the simplifications involved.

3.3. Negative cooperativity as a function of fractional ligand saturation of the gel

3.3.1. Low ionic strength

Equilibrium binding studies of the exothermic adsorption of phosphorylase *b* on methyl- and butyl-agaroses (Freundlich isotherms) at low ionic strength yield non-linear Scatchard plots (curved concave upwards) and Hill coefficients below unity[5]. This behaviour has been interpreted as a decrease in ligand affinity as a function of fractional saturation (negative cooperativity) on a qualitatively heterogeneous (*e.g.*, ionic and hydrophobic binding sites) lattice of binding sites[5].

3.3.2. High ionic strength

Similar isotherms have been obtained for phosphorylase *b* in the presence of 1.1 *M* ammonium sulphate[5] (see Table 3) and have led to the same interpretation as indicated above. From the positive heats of adsorption (see ΔH above) it has been concluded that this reaction is entropy driven and therefore may occur on a qualitatively homogeneous (hydrophobic binding sites) lattice of binding sites[5]. The observed decrease in affinity (negative cooperativity) is probably due to a number of factors[5], *e.g.*, sequential adsorption, competition of ligands, binding-unit overlap (together often termed non-independent binding) and steric factors such as configurational entropy. These factors are linked to the basic multivalent interactions between matrix and ligand which underly the above effects.

3.3.3. Kinetics of desorption

The rate of [^{3}H]phosphorylase *b* desorption during incubation of butyl-Sepharose (20.6 μmole/ml packed gel) pre-loaded with reduced [^{3}H]phosphorylase *b*

TABLE 4

DESORPTION KINETICS OF TRITIUM-LABELLED PHOSPHORYLASE *b* ON BUTYL-SEPHAROSE

The desorption experiments were performed after 20-fold dilution of the gel in the presence of 1.1 *M* ammonium sulphate at 5°. The apparent, initial desorption rates in the presence and absence of unlabelled, reduced phosphorylase *b* (Cold phos) was calculated between 0 and 2 min of desorption. The specific radioactivity of the labelled enzyme was *ca.* 10^5 cpm/mg. For a description of the experimental procedure, see Section 2, the text and refs. 6 and 23.

Gel load (mg [^{3}H]phosphorylase/ml packed gel)	*Initial rate of desorption (μg [^{3}H]phosphorylase/ml packed gel × min)*		*Stimulation (n-fold)*
	Control	*+Cold phos*	
2.0	16	87	5.44
4.5	48	118	2.46
16.5	393	345	0.88

in buffer without and with *ca.* 1 mg/ml of non-labelled, reduced enzyme, respectively, is shown in Table 4. In the presence of non-labelled phosphorylase *b* the initial desorption rate is stimulated up to 5-fold (Table 4). This enhancement of the desorption rate decreases as a function of fractional saturation of the gel and is absent if the gel is pre-loaded with *ca.* 16 mg of [^{3}H]phosphorylase *b*/ml packed gel (*ca.* 50% fractional saturation).

These preliminary results may be explained by the tentative model[23] that the binding of a non-labelled phosphorylase *b* molecule to a matrix containing adsorbed ^{3}H-labelled enzyme leads to a decrease in the affinity of the matrix for the ^{3}H-labelled enzyme molecule. This decrease in affinity would be manifested by an enhanced initial desorption rate. Hence these kinetic results would be in agreement with negative cooperativity of ligand binding. Similar observations have been reported for the binding properties of insulin receptors[31]. The kinetic data are also in agreement with the proposed model of multivalent effector–receptor interactions[5,6,23].

3.4. Practical consequences of multivalent interactions in the optimization of chromatographic procedures on alkyl-agaroses

3.4.1. Optimal alkyl-residue density

The binding affinity of the matrix is a result of the simultaneous interaction of a critical number of multiple sites (depending, for example, on the alkyl-residue density) and the affinity of a single binding site (depending, for example, on the number of carbon atoms per residue). To purify a protein, one must therefore determine the optimal density and the optimal chain length of an alkyl residue for the adsorption and elution of the ligand. How this can be done is illustrated for the enzyme phosphorylase kinase which is adsorbed from a crude muscle extract at low salt concentrations on alkyl-Sepharoses[1] containing a heterogeneous lattice of binding sites (Table 5).

The chromatographic optimum of the listed gels for phosphorylase kinase at low ionic strength (Table 5) has been reported by Jennissen and Heilmeyer[1]. If the specific activity of this enzyme in the adsorbed state (calculated from the total enzyme activity and the total protein adsorbed per millilitre of packed gel) is plotted against the alkyl-residue density, a maximal specific activity is obtained at the optimal alkyl-residue density. These gels therefore exhibit a certain binding specificity as the maximal specific activity of the enzyme adsorbed to the gel is *ca.* 15-fold higher than in the crude extract (see Table 5). It is evident from Table 5 that this optimal density compares quite well with the reciprocal of the apparent association constant of half-maximal saturation ($K_{0.5}$; compare the values given in Tables 1 and 5). The optimum may in fact be identical with this value, but gels containing this degree of substitution, $(K_{0.5})^{-1}$, were not obtained. As the maximal specific activity of the enzyme on the gel correlates with the purification factor obtained (12–18-fold from a crude extract, yield 80–98%; see Table 5), it can be concluded that irrespective of the residue chain length the three gels are equally efficient for the purification of phosphorylase kinase provided that an alkyl-residue density of half-maximal saturation is employed and the salt concentration for elution is increased.

Optimization of a gel for the purification of a new protein would therefore commence by testing one or two gels of intermediate hydrophobicity (3–6 carbon

TABLE 5

OPTIMIZATION OF THE MULTIVALENT INTERACTION CHROMATOGRAPHY OF PHOSPHORYLASE KINASE ON ALKYL-SEPHAROSES

The specific activity is calculated for the enzyme in the adsorbed state. Recovery is expressed as that percentage of total enzyme units adsorbed (100%) which can be eluted by a given salt concentration. The specific activity of phosphorylase kinase in the crude extract prior to adsorption was 113, 86 and 105 units/mg for the experiment employing the methyl, ethyl and butyl derivative, respectively. For further details and definitions, see Table 1, the text and ref. 1.

Sepharose derivative	$(K_{0.5})^{-1}$ *(μmole/ml packed gel)*	*Chromatographic optimum*			
		Residue density (μmole/ml packed gel)	*Specific activity (units/mg)*	*Elution with NaCl*	
				[NaCl] (M)	*Recovery (%)*
Methyl	18.2	20.7	1790	0.05	68
				0.12	98
Ethyl	14.7	19.2	1320	0.12	47
				1.0	77
Butyl	12.2	15.6	1460	1.0	84

atoms) from a homologous series (series I) of alkyl-Sepharoses to elucidate which alkyl derivative is capable of adsorbing the protein at all. Then a second series (series II) of alkyl-Sepharoses of increasing alkyl-residue density is synthesized. A sufficiently long hydrocarbon chain (series I) should be employed so that a saturation of the binding capacity of the gel as a function of the residue density can be expected. (For examples of enzymes where saturation is not yet obtained at short alkyl-residue chain lengths, see phosphorylase *b* and phosphorylase phosphatase in Table 1.) The Hill plot of the binding data then yields the minimum number of binding sites (n_H) and the optimal alkyl-residue density (calculated from $K_{0.5}$).

3.4.2. Fractional saturation of gel with protein ligand

As has been shown above, the affinity of an alkyl-Sepharose matrix decreases as a function of the fractional saturation with ligand. Therefore, high equilibrium concentrations of ligand do not lead to the expected, proportional increase in capacity. Furthermore, enzyme leakage from the gel will be observed at high fractional saturation. Efficient adsorption is therefore obtained at relatively low enzyme concentrations (crude extracts) where the apparent, high affinity sites are preferentially employed. From Table 4 and the conclusion of binding unit overlap (see Section 3.3.3), one ligand molecule can displace another on alkyl-Sepharoses. This effect at high concentrations of displacing proteins is highest at very low (*e.g.*, 1–5%) fractional saturation (Table 4). In general, therefore *ca.* 15–20% fractional saturation of a gel with protein for a chromatographic procedure appears optimal (for the role of hysteresis see ref. 23). A decrease in affinity with fractional saturation and competitive displacement of ligand by contaminating proteins also make it understandable that the maximal purification of a protein from a crude extract by multivalent interaction chromatography is in general no higher than 10–30-fold (*e.g.*, ref. 1).

3.4.3. Elution

The desorption of a multivalently bound protein from an alkyl-Sepharose at

the optimal residue density is best obtained by a procedure that decreases the affinity and capacity of the gel by one or two orders of magnitude. In general, this is most easily achieved by increasing salt gradients in salting-in chromatography[2] and by decreasing salt gradients when salting-out chromatography is employed. The type of salt should be chosen from the Hofmeister series[1].

Temperature gradients alone are not likely candidates for an efficient elution procedure. As can be concluded from Fig. 1, a temperature change of *ca.* 30° leads to a relatively small change in affinity (compare the $K_{0.5}$ values) and to only a 30–40% change in gel capacity. Therefore, the above conditions for efficient elution are not fulfilled.

3.5. Nomenclature

A problem in all newly emerging and rapidly developing fields is the definition of terms. In Fig. 2 an attempt has been made to enumerate in a systematic manner some of the terms so far suggested for the different types of adsorption chromatography on substituted gels. The term *univalent interaction chromatography* is taken to classify adsorption described by Langmuir-type isotherms which indicate single, independent binding sites. Multiple contacts occurring in single, non-overlapping binding units may, however, not be differentiated by such isotherms. It appears that

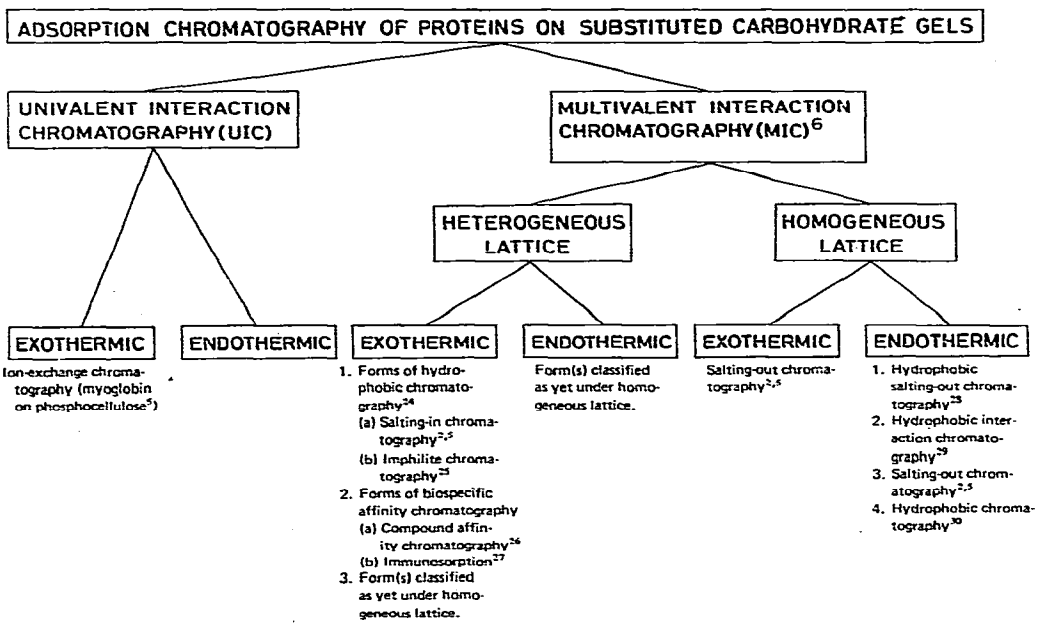

Fig. 2. Tentative classification of the nomenclature frequently employed for the various types of adsorption chromatography on carbohydrate gels. Heterogeneity and homogeneity are defined here on a qualitative basis, *e.g.*, a heterogeneous lattice may contain effective binding sites of both ionic and hydrophobic character. On a homogeneous lattice it is assumed that the effective binding sites are qualitatively homogeneous, *e.g.*, exclusively ionic or hydrophobic in nature. For further details, see the text and the references cited.

virtually all forms of chromatography defined by the listed terms employing substituted agarose gels are based on multivalent matrix-ligand interactions. Therefore, the general term *multivalent interaction chromatography* has been proposed[6]. The subgroup to which the listed forms belong has in most instances still to be determined by thermodynamic and binding experimental methods.

In the group, exothermic multivalent interaction chromatography on an effective, qualitatively heterogeneous lattice of binding sites, those chromatographic types which are performed at initially low ionic strength are listed and to which "detergent chromatography"[32] may be added. All forms of chromatography at high ionic strength ($\mu > 1.0$) to which "phosphate-induced chromatography"[33] may be added are tentatively listed under multivalent interactions on an effective, qualitatively homogeneous lattice under the assumption that interactions depending primarily on water structure (*e.g.*, surface tension effects and hydrophobic interactions) are the basis for adsorption.

An unambiguous classification of ion-exchange chromatography (*e.g.*, myoglobin on phosphocellulose[5]) and salting-in chromatography (*e.g.*, phosphorylase *b* on methyl- and butyl-Sepharose, at low ionic strength[5]) is possible. In exothermic and endothermic salting-out chromatography (phosphorylase *b* on methyl- and butyl-Sepharose, respectively, at high ionic strength[5]), an effective, homogeneous lattice is proposed as ionic interactions are improbable. However, especially in exothermic salting-out chromatography, the exact adsorption mechanism has still to be elucidated.

From the large number of different terms often employed for identical or similar procedures, it appears that the time is now ripe for the IUPAC Nomenclature Commission to decide on clear definitions.

4. ACKNOWLEDGEMENT

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5. SUMMARY

Cyanogen bromide-activated Sepharose 4B substituted with α-aminoalkanes (1–4 carbon atoms) has been shown to adsorb proteins by multivalent interactions (multivalent interaction chromatography) at low and at high salt concentrations (salting-in and salting-out chromatography, respectively). Equilibrium binding studies and desorption kinetics indicate that the affinity of alkyl-Sepharoses decreases as a function of fractional saturation (negative cooperativity). Temperature-induced changes in gel capacity appear to be linked to the minimum valence of adsorption.

In spite of these very complex ligand–matrix interactions, such gels can easily be optimized for a 10–20-fold chromatographic purification of proteins from crude extracts. The degree of substitution (alkyl-residue density) at the chromatographic optimum corresponds closely to the alkyl-residue density of half-maximal saturation, $(K_{0.5})^{-1}$. This value can be determined from a Hill plot of the binding capacity of the gel *versus* the alkyl-residue density. The same plot simultaneously yields the minimum number of binding sites involved in the adsorption of the protein.

An attempt is made to classify the confusing nomenclature employed in adsorption chromatography in terms of univalent and multivalent interactions.

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