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# IMMOBILIZATION OF RESIDUES ON AGAROSE GELS: EFFECTS ON PROTEIN ADSORPTION ISOTHERMS AND CHROMATOGRAPHIC PARAMETERS

HERBERT P. JENNISSEN
*Institut für Physiologische Chemie, Ruhr-Universität Bochum, Postfach 10 21 48, D-4630 Bochum (G.F.R.)*

## SUMMARY

The concentration of immobilized residues on agarose gels largely determines their properties. If the protein has two or more available binding sites for the immobilized residue, cooperative, multivalent adsorption will be observed above a critical surface concentration of residues. A simple mathematical model for this phenomenon is suggested. Substituted agarose gels offer two possibilities for chromatographic separations: (1) so-called "irreversible" adsorption followed by eluent displacement procedures; or (2) linear zonal chromatography. In most instances the former method has been employed in affinity and hydrophobic chromatography, whereas the rarely used latter method may have been too difficult to apply on a rational basis. A mathematical model is therefore derived for the calculation of isothermal distribution coefficients and it can be shown that similar coefficients are obtainable in chromatographic runs.

## INTRODUCTION

For the monovalent adsorption of a protein from a 1–10 $\mu M$ solution on substituted agarose gels containing *ca.* 1 $\mu$mol of substituent per millilitre of packed gel (surface concentrations: $\Gamma_r^{s(Aga)} \approx 0.03$ $\mu$mol/m$^2$) apparent binding constants ($K'_a$) of *ca.* $10^4$–$10^5$ $M^{-1}$ between protein and substituent are necessary (for a review, see ref. 1).

If, on the other hand, the immobilized residue has a very low binding affinity for the protein (*e.g.*, $K'_a \approx 10$–$10^2$ $M^{-1}$) adsorption will take place only if several immobilized residues interact simultaneously with the protein (multivalent interaction chromatography[2]). Multivalent binding occurs above a critical surface concentration of *ca.* 0.03 $\mu$mol/m$^2$ (refs. 3–5) when the protein covers a surface area on the agarose containing more than one residue. Adsorption is a cooperative process[4,6,7] under these conditions and thermodynamic irreversibility in the form of adsorption hysteresis[7] may occur.

In general, substituted agarose gels have been employed for the apparent "irreversible" adsorption of proteins followed by eluent displacement, *e.g.*, in the form of gradients[2,8–10]. A much less used technique is linear zonal chromatography.

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Although theoretically both types of chromatography appear to show a similar resolution[10] they may be applied with different success to the separation of proteins. As the behaviour of adsorbed proteins is very complex, the question arises of whether the data from binding isotherms can be employed for the calculation of quantitative chromatographic parameters. Therefore, a theoretical model for the description of protein adsorption on substituted agarose gels is introduced and the differentiation between surface and bulk effects of these gels is discussed. The parameters governing linear zonal chromatography on substituted agarose gels and the possibility of obtaining this ideal are another main topic of this paper. It is shown that the constants derived from adsorption and desorption isotherms of a multivalently adsorbed protein can be employed to predict the magnitude of the distribution coefficient in a zonal chromatographic system.

## EXPERIMENTAL

The preparation, analysis and storage of $^{14}C$-labelled alkyl derivatives of Sepharose 4B (Pharmacia, Uppsala Sweden) have been described previously[3,11,12]. All methods pertaining to the preparation, determination of activity and radioactive $^{3}H$ labelling of phosphorylase *b* ([$^{3}H$]phosphorylase $b_t$) have been described[7]. The high ionic strength standard buffer (pH 7.0) employed in all experiments contained 10 m*M* tris(hydroxymethyl)aminomethane (Tris)–maleate, 5 m*M* dithioerythritol, 1.1 *M* ammonium sulphate and 20% sucrose.

Chromatographic analysis on unsubstituted and substituted (butyl) Sepharose 4B was performed as follows on a 20 × 0.62 cm I.D. column: bed height, 16.5 cm; sample volume, 0.2 ml; flow-rate, 5 ml/h; fraction volume, 1 ml; and temperature, *ca.* 5°C. The elution volume, $V_e$ was derived according to the method of Crone[13] by determining the centre of the peak at one third of the maximum height. This method leads to an apparent mean value for the elution volume in the case of asymmetric peaks. For the definition of the terms employed in this paper, see ref. 5.

## RESULTS AND DISCUSSION

### *Surface area of adsorbents*

The adsorption of a protein takes place on a specifiable surface of the matrix employed for the immobilization of the residues. The specific surface area ($a_s^b$) of a gel can be calculated from the adsorption of a solute. The following assumptions (for a review, see ref. 14) underly the determination of the specific surface area by this method: 1, the whole surface can be covered by a uniform, unimolecular film of solute; 2, the achievement of complete unimolecular surface coverage can be deduced from a certain point of the adsorption isotherm ($\theta = 1, c = \infty$); 3, the area occupied by the molecule on the surface is known. As the calculated specific surface area may therefore depend on the solute employed, the latter should always be stated in connection with the determined value (see Table I). With phosphorylase *b* adsorption to butyl-Sepharose (at a saturating degree of substitution), the point of the isotherm which represents complete monolayer surface coverage is assumed to be the extrapolated saturation value of the adsorption isotherm according to the Scatchard procedure[6,7], *i.e.*, gel saturation with protein in the main hysteresis loop. With phos-

phorylase *b* a certain ambiguity arises because it is an anisometric molecule[15,16] and occupies different areas according to the way it may be placed on a surface. The enzyme can be considered either to stand up on the surface (39.7 $nm^2$/molecule) or to lie flat (73.1 $nm^2$/molecule). At identical surface coverages the latter area yields a *ca.* 2-fold higher value, *i.e.*, maximal surface area. The maximal surface area ($A_s^b$) of 1 ml of packed butyl-Sepharose 4B (20–30 $\mu$mol per millilitre of packed gel) calculated from the larger cross-sectional area of phosphorylase *b* at monolayer saturation (*i.e.*, $4.0 \cdot 10^{-7}$ mol per millilitre of packed gel at 5°C[4,6,7]) is 8.8 $m^2$. The maximal specific surface area calculated according to the equation $a_s^b = A_s^b/m$, where $m$ is the mass of the solid phase (taken here as 30 mg of dry agarose per millilitre of packed gel[6]), is 233 $m^2$/g. From this value the surface concentration of the solute, $\Gamma_b^s$, can be calculated[5]: *e.g.*, for phosphorylase *b* 1 mg per millilitre of packed gel corresponds to 11.4 nmol/$m^2$. The surface concentration of immobilized residue, $\Gamma_r^{s(Aga)}$, can be calculated[5] according to the relationship 1 $\mu$mol per millilitre of packed gel corresponds to $1.28 \cdot 10^{-2}$ $\mu$mol/$m^2$.

Up to now the specific surface area of most adsorbents for the separation of proteins has remained unknown. Therefore, a number of adsorbents in Table I are merely cited for reasons of comparison. An exception are the hydroxy methacrylate gels (Spheron; Lachema, Brno, Czechoslovakia), which appear to yield similar surface areas to Sepharose 4B.

TABLE I

SURFACE AREAS OF ADSORBENTS

For further details see text and refs. 5, 14 and 17.

| *Adsorbent** | *Adsorbate* | *Surface area* | | *Reference* |
|---|---|---|---|---|
| | | $a_s^b$ *($m^2$/g dry weight)* | $A_s^b$ *($m^2$/ml gel)* | |
| Alumina | Stearic acid | 2.3 | | 14 |
| Carbon black | Iodine | 100 | | 14 |
| Spheron-1000 | $N_2$–$H_2$ | | 5.9 | 17 |
| Spheron-500 | $N_2$–$H_2$ | | 23.0 | 17 |
| Butyl-Sepharose (max. value) | Phosphorylase | 233 | 8.8 | 5 |

* Spheron is a trade-name for hydroxyalkyl methacrylate gels; Sepharose is a trade-name for agarose gels.

## *Model for the description of protein binding on substituted agarose gels*

### *General considerations*

The binding of a protein to single independent sites on a substituted agarose gel is a function of the free protein concentration and the concentration of binding units on the agarose (*i.e.*, the available agarose surface as the sum of the binding units):

$$P + Aga \rightleftharpoons P\text{-}Aga \quad (1)$$

where P is the protein ligand, Aga a binding unit on the agarose surface and P–Aga the protein agarose complex. In addition, the amount of protein bound is a function of the immobilized substituent or residue concentration itself. Assuming a reaction between a single site on the protein and an immobilized residue, $Res_{im}$, we can write

$$P + Res_{im} \rightleftharpoons P\text{–}Res_{im} \tag{2}$$

where $P\text{–}Res_{im}$ is the monovalent protein–residue complex. In this model the surface concentration of the protein on the gel is equal to the surface concentration of the protein residue complex:

$$\{P\text{–}Aga\} = \{P\text{–}Res_{im}\} \tag{3}$$

{ } symbolizes a surface concentration by analogy with [ ] for bulk concentrations. Therefore, the concentration of the protein adsorbed on substituted agarose (measured as the protein–agarose complex) is a function of the free protein concentration, the available agarose surface area (*i.e.*, surface concentration of binding units) and the surface concentration of immobilized residues:

$$[P]\ \{Aga\}\ \{Res_{im}\} \approx \{P\text{–}Aga\} \tag{4}$$

In the absence of non-specific binding to the agarose, no protein binding will occur if either $\{Aga\} = 0$ or $\{Res_{im}\} = 0$. According to the law of mass action:

$$\frac{\{P\text{–}Aga\}}{[P]\ \{Aga\}\ \{Res_{im}\}} = K' \tag{5}$$

where $K'$ is an apparent equilibrium constant.

*Protein adsorption as a function of the immobilized residue concentration*

Eqn. 5 can be simplified by restricting the analysis to the dependence of adsorption on the surface concentration of immobilized residues and by keeping the free protein concentration at equilibrium, [P], constant:

$$\frac{\{P\text{-}Aga\}}{\{Aga\}} = K'\ [P]_{const.}\ \{Res_{im}\} \tag{6}$$

By defining $K'_1 = K'\ [P]_{const.}$:

$$\frac{\{P\text{–}Aga\}}{\{Aga\}} = K'_1\ \{Res_{im}\} \tag{6a}$$

According to mass conservation, the apparent total concentration of binding units $\{Aga\}_p$ which is a function of the constant, free protein concentration, $\{Aga\}_p = A_p\ [P]^{\alpha}_{const.}$, with $A_p$ and $\alpha$ being constants, may be defined as

$$\{Aga\}_p = \{Aga\} + \{P\text{–}Aga\} \tag{7}$$

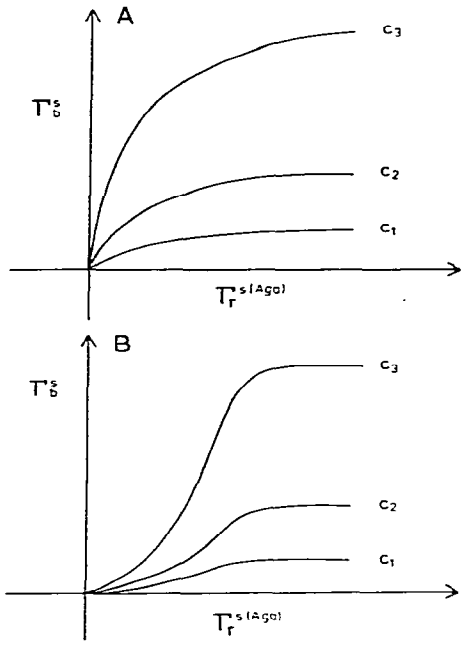


Fig. 1. Protein adsorption as a function of the immobilized residue concentration. $\Gamma_b^s$ is the surface concentration of adsorbed protein, *i.e.*, {P–Aga}, and $\Gamma_r^{s(Aga)}$ the surface concentration of immobilized residues, *i.e.*, $\{Res_{im}\}$. $C_1$–$C_3$ ($C_1 < C_2 < C_3$) indicate different values for the constant free protein concentration [P]. (A) Monovalent binding, eqn. 8 (eqn. 10, $n_{H1} = 1$); (B) multivalent binding (eqn. 10, $n_{H1} = 4$). For further details see text and ref. 5.

Substitution into eqn. 6a yields the hyperbolic equation

$$\{P\text{–}Aga\} = \frac{\{Aga\}_p \{Res_{im}\}}{\frac{1}{K'_1} + \{Res_{im}\}} \tag{8}$$

Fig. 1A shows plots of eqn. 8 at different constant values of $[P]_{const.}$ (indicated by $C_1$–$C_3$). Thus $\{Aga\}_p$ increases with increasing $[P]_{const.}$ values. By introducing fractional saturation, $\theta_1 = \{P\text{–}Aga\}/\{Aga\}_p$, eqn. 8 can be written as

$$\frac{\theta_1}{1 - \theta_1} = K'_1 \{Res_{im}\} \tag{9}$$

Because of the validity of eqn. 3, saturation (*i.e.*, $\theta = 1$) can be interpreted as the saturation of protein with immobilized residue[5].

Eqns. 8 and 9 are valid for single immobilized residues interacting with one binding site of the protein molecule on the matrix surface (Fig. 1A). For the cooperative interaction of multiple, *e.g.*, 4, immobilized residues with multiple sites on one protein molecule, an exponential term must be introduced into eqn. 8 and sigmoidal

curves (Fig. 1B) are obtained. Correspondingly, eqn. 9 becomes

$$\frac{\theta_1}{1 - \theta_1} = K_{H1} \{Res_{im}\}^{n_{H1}} \tag{10}$$

which is a form of the Hill equation[18], where $K_{H1}$ is the surface Hill constant and $n_{H1}$ the surface Hill coefficient. This equation is valid for all proteins having more than one binding site for an immobilized residue on one surface. In eqn. 9 $n_{H1} = 1$ (Fig. 1A). In Fig. 1B the sigmoidal curves show a binding experiment where $n_{H1} \approx 4$. The three curves ($C_1$–$C_3$) again show that saturation depends on the constant free ligand equilibrium concentration. For a discussion of hysteresis in connection with eqn. 10, see refs. 5 and 7.

*Protein adsorption as a function of the free protein concentration*

If the surface concentration of adsorbed protein is measured as a function of the free protein concentration under the condition that the immobilized residue concentration, $\{Res_{im}\}$, is constant, eqn. 5 becomes

$$\frac{\{P\text{–}Aga\}}{\{Aga\}} = K_2' [P] \tag{11}$$

and $K_2' = K' \{Res_{im}\}_{const.}$. For non-independent binding an exponential term must be introduced in connection with the protein concentration. Analogous to eqn. 7, we obtain $\{Aga\}_r = \{Aga\} + \{P\text{–}Aga\}$, where $\{Aga\}_r$ corresponds to the apparent total surface coverage of the matrix under the condition $\{Res_{im}\}$ = constant: $\{Aga\}_r = B_r \{Res_{im}\}^{\beta}_{const.}$, where $B_r$ and $\beta$ are constants. For single independent sites we obtain

$$\{P\text{–}Aga\} = \frac{\{Aga\}_r [P]}{\frac{1}{K_2'} + [P]} \tag{12}$$

which corresponds to a Langmuir isotherm. If the condition of binding to single independent binding units is not fulfilled, the more general equation is valid on defining $\theta_2 = \{P\text{–}Aga\}/\{Aga\}_r$:

$$\frac{\theta_2}{1 - \theta_2} = K_{H2} [P]^{n_{H2}} \tag{13}$$

where $K_{H2}$ is a bulk Hill constant and $n_{H2}$ a bulk Hill coefficient. In the absence of cooperativity (eqn. 12), $n_{H2} = 1$. In negative cooperative systems $n_{H2} < 1$ (refs. 5 and 6). For a discussion of adsorption hysteresis and the restrictions of eqn. 13, see refs. 5 and 7.

Finally, it can be concluded that both eqns. 10 and 13 are special cases of the more general equation

$$\frac{\theta}{1 - \theta} = A \{Res_{im}\}^m [P]^n \tag{14}$$

where, under the specified conditions (see text relating to eqns. 10 and 13), $A$, $m$ and $n$ are constants.

A typical isotherm for the cooperative adsorption of a protein on substituted agarose ($n_{H2} < 1$) on the basis of the formalism of eqn. 12 is shown in Fig. 2. The adsorption isotherm is not retraced by the corresponding desorption isotherm (adsorption hysteresis) so that closed adsorption–desorption loops result[7]. Within the main hysteresis loop, where the upper closure point equals the total gel surface coverage, a family of scanning curves may exist. Adsorption–desorption loops of this kind strongly suggest thermodynamic irreversibility of binding. Conformational changes of the protein may be involved. A negative cooperative mechanism of adsorption has been suggested for the binding of phosphorylase *b* on butyl-Sepharose. This appears to be substantiated by kinetic studies[5].

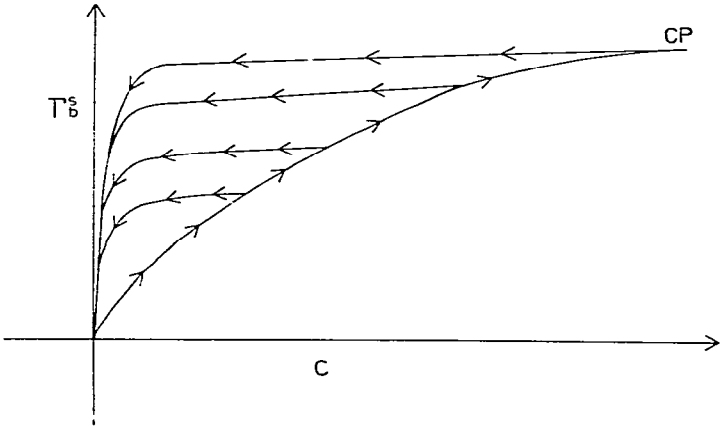


Fig. 2. Protein adsorption as a function of the free protein concentration and adsorption hysteresis. $\Gamma_b^s$ is the surface concentration of adsorbed protein, *i.e.*, {P–Aga}, and $c$ the apparent free equilibrium concentration of solute, *i.e.*, [P], at a constant surface concentration of immobilized residues. The arrows indicate the direction of the closed adsorption–desorption loops. The curves are based on the cooperative, multivalent protein adsorption (see eqns. 12 and 13). CP indicates the upper closure point of the hysteresis loop. For further details, see text and refs. 5–7.

## *Dependence of the chromatographic behaviour of proteins on constants derived from binding isotherms*

### *Theoretical considerations*

The chromatographic separation of proteins depends on the differential accumulation of molecules at certain sites within a chromatographic system. In general, two possibilities for the accumulation of proteins in chromatographic systems exist: (1) accumulation in the volume of a liquid (*i.e.*, a three-dimensional system) giving rise to such purification procedures as a*b*sorption[14] or partition chromatography and countercurrent distribution[19]; (2) accumulation on a surface (*i.e.*, two-dimensional structure), providing the basis for a*d*sorption chromatography.

It has been pointed out, however (for a review, see ref. 14), that there appears to be no clear boundary between partition and adsorption. Extensively hydrated hydrophilic gels whose bulk may take part in the adsorption reaction may also be

described in terms of volume. Thus, in a dualistic, complementary sense it should be possible to describe some phenomena of protein adsorption on substituted agarose gels by a surface model (see the section *Model for the description of protein binding on substituted agarose gels*) and others by a bulk model (see below).

*The isothermal distribution coefficient.* Initially adsorption was described as a surface phenomenon[20]. Therefore, in the following discussion the symbols of surface chemistry[21] will be employed. Solute adsorption to a surface from dilute solutions is classically described by the Langmuir adsorption isotherm[20] (see also eqn. 12), which can be derived from the Gibbs adsorption isotherm (for a review, see ref. 22). The following fundamental postules for this type of isotherm were set up by Langmuir[23]: (1) the adsorption sites (*i.e.*, surface cells) are all identical; (2) each surface cell can hold only one molecule; (3) the potential energy of a molecule in a surface cell is independent of the presence of molecules in other cells. If true equilibria are obtained:

$$\Gamma^s = \frac{\Gamma_0^s \, c}{\frac{1}{K_a} + c} \tag{15}$$

$\Gamma^s$ corresponds to the surface concentration (surface excess) of solute, $\Gamma_0^s$ to the saturating surface concentration of solute, $c$ to the equilibrium concentration of free solute in the bulk and $K_a$ to an association constant. Cross-multiplication and rearrangement lead to:

$$\frac{\Gamma^s}{c} = K_a \, (\Gamma_0^s - \Gamma^s) \tag{16}$$

which is equivalent to the Scatchard equation[24] for the adsorption of ligands on surfaces. For $\Gamma^s \ll \Gamma_0^s$, eqn. 16 reduces to

$$\frac{\Gamma^s}{c} = K_a \, \Gamma_0^s = D_a^i \tag{17}$$

where $D_a^i$ corresponds to the isothermal adsorption distribution coefficient[19] employed for ideal linear zonal chromatographic systems. Thus, at very low fractional saturation of a gel the ratio of the surface concentration of the solute to the equilibrium concentration of the free bulk solute is a constant. In general, the separation of solutes in zonal chromatography is possible if the condition $1 < D_a^i < 100$ is fulfilled. Although the problem of so-called irreversible adsorption ($D_a^i > 100$) in affinity and hydrophobic chromatography can often be overcome by gradient elution techniques, linear zonal chromatography should nevertheless be one aim in the optimization of chromatographic systems. If the factors governing the magnitude of the distribution coefficient can be controlled, a more rational approach to affinity and hydrophobic chromatography could be possible. The main obstacle to meaningful isothermal distribution coefficients has been the fact that $\Gamma_0^s$ is a surface concentration, in general eluding exact determinations.

As agarose gels are highly porous and hydrophilic, analysis could be simpli-

fied[5] by assuming that a Sepharose 4B sphere is a superstructured macromolecule containing $n$ binding sites, *i.e.*, binding units. A molar concentration of beads in 1 ml of packed gel can be calculated from the number ($5 \cdot 10^6$) of spheres[3] per millilitre of packed gel to be $c_{0,\mathrm{Aga}} = 8.3$ f$M$. From the saturation value with phosphorylase $b$ of $4.0 \cdot 10^{-4}$ mol per litre of packed gel, the concentration of binding units can be calculated for packed gel to be $[\mathrm{Aga}] = n\, c_{0,\mathrm{Aga}} = 4 \cdot 10^{-4}$ $M$. The number of binding units for phosphorylase $b$ on one agarose sphere at saturation is $n = 4.8 \cdot 10^{10}$. Eqn. 17 can now be written as

$$\frac{\tilde{v}}{c} = K'_a \tilde{v}_0 = D'^{\mathrm{i}}_a \tag{18}$$

where $\tilde{v}$ corresponds to moles of adsorbed protein ligand per litre of packed gel, $\tilde{v}_0$ to the saturation value, $K'_a$ to the corresponding apparent association constant and $D'^{\mathrm{i}}_a$ to the isothermal distribution coefficient on a volume basis.

*The chromatographic distribution coefficient.* Experimentally, the chromatographic distribution coefficient can be determined according to the equation[19,25]

$$V_e = V_\mathrm{m} + D^{\mathrm{c}}_a V_\mathrm{s} \tag{19}$$

where $V_e$ is the elution volume of the adsorbed solute, $V_\mathrm{m}$ the dead retention volume, $V_\mathrm{s}$ the volume of the stationary phase and $D^{\mathrm{c}}_a$ the chromatographic distribution constant. Rearrangement leads to

$$D^{\mathrm{c}}_a = \frac{V_e - V_\mathrm{m}}{V_\mathrm{s}} \tag{19a}$$

For butyl-Sepharose 4B, which additionally has gel filtration properties, $V_\mathrm{m}$ corresponds to the elution volume of the protein on unsubstituted Sepharose 4B: $D^{\mathrm{c}}_a = 0$ and $V_e = V_\mathrm{m}$. For agarose gels the total stationary phase volume ($V_\mathrm{av}$) (see also refs. 10 and 26), *i.e.*, the sum of the internal gel volume ($V_\mathrm{i}$) and the volume occupied by the gel matrix ($V_\mathrm{g}$), can be defined as:

$$V_\mathrm{av} = V_\mathrm{t} - V_0 \tag{20}$$

where $V_\mathrm{t}$ is the total gel volume (*i.e.*, packed gel) and $V_0$ the void volume (determined with blue dextran). Therefore, the chromatographic distribution coefficient on substituted Sepharose 4B would be

$$D^{\mathrm{c}}_\mathrm{av} = \frac{V_e - V_\mathrm{m}}{V_\mathrm{t} - V_0} \tag{21}$$

It is evident from eqn. 21 that the calculation of $D^{\mathrm{c}}_\mathrm{av}$ is based on the total stationary phase volume ($V_\mathrm{t} - V_0$) and not on $V_\mathrm{t}$ (total gel volume, *i.e.*, packed gel) as was employed for the derivation of $D'^{\mathrm{i}}_a$ (eqn. 18). As the isothermal and the chromato-

graphic distribution coefficients should be based on the same parameter, eqn. 18 can be rewritten as

$$D'^{i}_{av} = K'_a \bar{v}^{av}_0 \quad (22)$$

where $\bar{v}^{av}_0$ corresponds to moles of adsorbed ligand per litre of total stationary phase (eqn. 20) at saturation.

*Determination of the distribution coefficient of [$^3$H]phosphorylase $b$, on butyl-Sepharose*

Table II shows the application of eqn. 22 to previously published data for adsorption and desorption isotherms under the assumption that $K'_{0.5} \approx K'_a$ (see refs. 5–7). Owing to adsorption hysteresis, a definite isothermal distribution coefficient for the adsorption of [$^3$H]phosphorylase $b_r$ on butyl-Sepharose cannot be calculated. Instead, one coefficient can be calculated for the adsorption branch of the hysteresis loop and another for each desorption isotherm. Nevertheless, the isothermal distribution coefficients of $1.2 \cdot 10^2$ to $6.7 \cdot 10^5$ (see Table II) calculated for the highly substituted gel (21 μmol per millilitre of packed gel) clearly indicate that zonal chromatography will not be feasible. In contrast, at a low degree of substitution (5 μmol per millilitre of packed gel) finite isothermal distribution coefficients between 2.0 and 5.5 are obtained. In this instance the determination of a chromatographic distribution coefficient appeared possible.

TABLE II

DISTRIBUTION COEFFICIENTS OF [$^3$H]PHOSPHORYLASE $b_r$ ON BUTYL-SEPHAROSE

The isothermal distribution coefficient, $D'^{i}_{av}$ (see eqn. 22), was calculated from apparent association constants, $K'_{0.5}$ (ref. 7), and gel saturation, $\bar{v}^{av}_0$, which was derived from the apparent saturation, $\bar{v}_{sat} = 6.8$ mg/ml of packed gel, and the virtual saturation, $\bar{v}'_{sat} = 1.5$ mg/ml of packed gel for the adsorption and desorption isotherms, respectively[7], on the gel substituted with 5 μmol/ml of packed gel. At 21 μmol/ml of packed gel the respective values are $\bar{v}_{sat} = 45$ mg/ml of packed gel and $\bar{v}'_{sat} = 9.6$ mg/ml of packed gel[7]. In the table, 1 ml packed gel contains 0.6 ml of total stationary phase. The chromatographic distribution coefficient ($D^{c}_{av}$, see eqn. 21) was derived from the experiment in Fig. 3B. For further details, see ref. 7, Fig. 3B and the text.

| *Adsorbent* | *Constants of sorption isotherms* | | | *Distribution coefficients* | |
|---|---|---|---|---|---|
| | $K'_{0.5}$ ($M^{-1}$) | *Gel saturation,* $\bar{v}^{av}_0$ | | $D'^{i}_{av}$ *(calculated)* | $D^{c}_{av}$ *(experimental)* |
| | | *mg/ml stationary phase* | *M* | | |
| *Butyl-Sepharose:* | | | | | |
| 5 μmol/ml of packed gel | | | | | |
| Adsorption isotherm | $1.8 \cdot 10^4$ | 11.3 | $1.1 \cdot 10^{-4}$ | 2.0 | 3.7 |
| Desorption isotherm | $2.2 \cdot 10^5$ | 2.5 | $2.5 \cdot 10^{-5}$ | 5.5 | |
| 21 μmol/ml of packed gel | | | | | |
| Adsorption isotherm | $1.6 \cdot 10^5$ | 75 | $7.5 \cdot 10^{-4}$ | $1.2 \cdot 10^2$ | |
| Desorption isotherm | $4.2 \cdot 10^9$ | 16 | $1.6 \cdot 10^{-4}$ | $6.7 \cdot 10^5$ | |

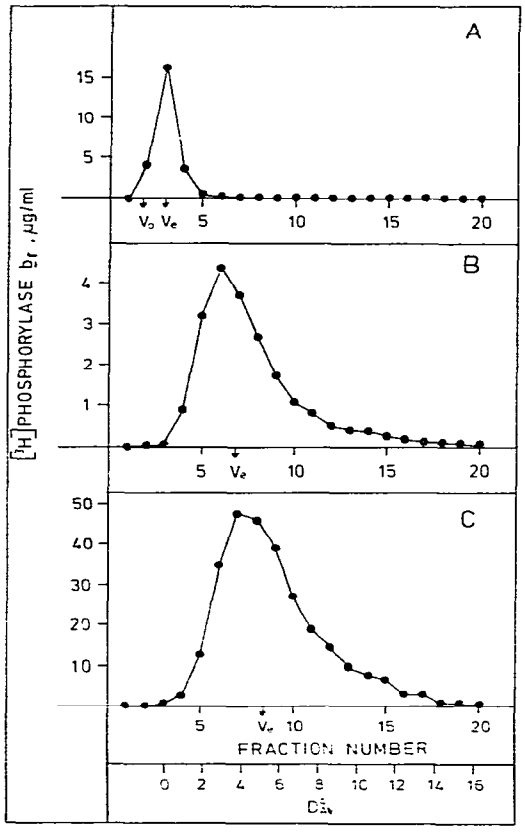


Fig. 3. Determination of the chromatographic distribution coefficient ($D^s_{av}$) for [$^3$H]phosphorylase $b_r$ on butyl-Sepharose (5 μmol per millilitre of packed gel). Chromatography was performed at 5°C on a 20 × 0.62 cm I.D. column: bed height, 16.5 cm; flow-rate, 5 ml/h; fraction volume, 1 ml. No corrections were made in the fraction number for the dead volume of the system. The specific activity of the enzyme was 46 units/mg and the specific radioactivity was $2.8 \cdot 10^5$ cpm/mg. (A) Sepharose 4B; sample 0.2 ml, protein 0.39 mg/ml; (B) butyl-Sepharose; sample 0.2 ml, protein 0.39 mg/ml; (C) butyl-Sepharose; sample 0.2 ml, protein 3.6 mg/ml. For further details, see text, eqn. 21 and Table II.

Therefore, [$^3$H]phosphorylase $b_r$ was applied to a small column of butyl-Sepharose 4B substituted with 5 μmol per millitre of packed gel (Fig. 3). Fig. 3A shows a control run for the enzyme on unsubstituted Sepharose 4B in standard buffer. After correction for the dead volume of the chromatographic system, $V_0$ was determined with blue dextran to be 1.98 ml ($V_t$ = 5.0 ml) and $V_e$ (= $V_m$) to be 5.2 ml with a total protein yield of 92%. On butyl-Sepharose (Fig. 3B), [$^3$H]phosphorylase $b_r$ is eluted with $V_e$ = 14.8 ml and a yield of 77%. The ratio of applied protein (*ca.* 80 μg) to the value of gel saturation with protein (*ca.* 34 mg per 5 ml of packed gel) is 1:425. If the amount of protein applied is increased to 720 μg (Fig. 3C) this ratio increases to 1:47 and $V_e$ = 21.7 ml (yield of protein *ca.* 100%).

The chromatographic distribution constant calculated from eqn. 21 for the run in Fig. 3B is 3.7 and for the run in Fig. 3C it is 5.5. For the condition $\Gamma^s \ll \Gamma^s_0$ (eqn. 16), *i.e.*, $\tilde{v}^{av} \ll \tilde{v}^{av}_0$ (eqn. 22), the value of 3.7 (Fig. 3B) appears most plausible (Table II).

TABLE III

PROPERTIES OF SUBSTITUTED AGAROSE GELS

| *Property* | *High-affinity residue–protein interaction, e.g., affinity chromatography* | *Low-affinity residue–protein interaction, e.g., hydrophobic chromatography* |
|---|---|---|
| Approximate minimal binding constant of single-site interaction | $10^4$–$10^5$ $M^{-1}$ | 10–$10^2$ $M^{-1}$ |
| Specificity | Through specific binding site on protein | Through steric arrangement of immobilized residues and protein surface sites |
| Linear zonal chromatography | Possible | Can only be approximated |
| Valence of interaction | Univalent (at low degree of substitution) | Multivalent |

Even at the low sample loading and the low degree of substitution a symmetrical peak is not obtained. This is probably due to adsorption hysteresis (non-equilibrium states) and negative cooperativity of binding (see fundamental postulate No. 3 in the section *The isothermal distribution coefficient*).

From the experimental data in Fig. 3 and Table II the following preliminary conclusions (see also Table III) are possible: (1) the data from protein binding isotherms on substituted agarose gels can be employed for the calculation of isothermal distribution coefficients; (2) the chromatographic distribution coefficients are in reasonable agreement with the values predicted from the sorption isotherms; (3) in an affinity chromatographic system in which a relatively high binding constant between the affinity residue and the protein exist (*e.g.*, $10^4$–$10^5$ $M^{-1}$), linear zonal chromatography should be possible at a low degree of substitution (monovalent interaction); (4) in hydrophobic chromatography where the binding constant between alkyl residue (4–6 carbon atoms) and a single binding site, *e.g.*, on phosphorylase *b*, appears to be very low (*ca.* 10–$10^2$ $M^{-1}$) since no high-affinity alkyl binding sites or pockets can be detected[27] and multivalent interactions are necessary for binding. Interference from adsorption hysteresis and negative cooperativity can be minimized at low degrees of substitution, making zonal chromatography possible.

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