

## Evidence for Negative Cooperativity in the Adsorption of Phosphorylase *b* on Hydrophobic Agaroses<sup>†</sup>

Herbert P. Jennissen

**ABSTRACT:** The concept of cooperativity appears to be the key to the understanding of the complex mechanisms underlying the adsorption of proteins to agaroses substituted with hydrophobic  $\alpha$ -aminoalkanes. The adsorption of phosphorylase *b* occurs through the positive cooperative interaction of a critical number of approximately 3–5 butyl and a higher number of methyl residues with corresponding sites on the enzyme. The amount of adsorbed phosphorylase *b* per milliliter of packed gel (methyl-, butyl-Sepharose) in the absence and presence of 1.1 M ammonium sulfate at temperatures between 0 and 34 °C is a power function of the free solute equilibrium concentration (Freundlich isotherm). In contrast, the adsorption of cyanmyoglobin to phosphocellulose is described by the Langmuir equation. The surface coverage dependent isosteric heats of adsorption for phosphorylase *b* indicate an endothermic reaction only on the butyl-Sepharose in the presence

of high salt concentrations. Scatchard plots of the Freundlich isotherms of phosphorylase *b* are concave upwards, typical of negative cooperativity. Hill plots of these isotherms (5–70% saturation) yield coefficients between  $n_H = 0.39$  and 0.71. At high surface coverages, the Hill coefficients approach unity. Apparent association constants ( $K_{0.5}$ ) of  $4\text{--}39 \times 10^4 \text{ M}^{-1}$  are calculated for the adsorption of phosphorylase *b*, as compared to  $2\text{--}9 \times 10^4 \text{ M}^{-1}$  for the adsorption of cyanmyoglobin. In general, negative cooperativity of binding may be explained by changes in the affinity of the ligand for the matrix, due to the sequential, multivalent adsorption, and competition of phosphorylase *b* molecules for the critical number of alkyl residues (nonindependence of binding) on one side and to variations in the configuration of binding and entropy on the other.

**P**rotein adsorption from solution has been studied on a variety of inorganic and organic materials. McLaren (1954) concluded from adsorption isotherms of lysozyme on kaolinite that hydrogen bonding and Coulombic attraction to charges on the surface caused adsorption. Gorman et al. (1971) described the adsorption of fibrinogen to mica and Stoner et al. (1971) demonstrated that this interaction could be inhibited by heparin. Norde and Lyklema (1973) showed that nonpolar interactions play a dominant role in the isothermic adsorption of human serum albumin to negatively charged polystyrene latex.

Recently, the adsorption of proteins to agarose gels containing hydrophobic alkyl residues (Yon, 1972; Er-el et al., 1972) has gained widening interest. The protein binding capacity of such gels substituted with  $\alpha$ -aminoalkanes depends

on the alkyl residue chain length and the density of the residues on the Sepharose spheres (Jennissen and Heilmeyer, 1975). An equally important parameter in determining the critical hydrophobicity for the adsorption of proteins is the type of salt and the salt concentration employed in the buffer. Thus, hydrophobic chromatography may be divided into two subgroups: salting-in and salting-out chromatography (Jennissen, 1976a). As to the mechanism of adsorption, two theories have been put forward. Shaltiel (1974) suggested that the adsorption of a protein to a hydrophobic matrix is due to the interaction of an alkyl residue of specific length ("yard stick") with a hydrophobic pocket of the protein. Jennissen and Heilmeyer (1975) and Jennissen (1976a), on the other hand, presented evidence that the adsorption of proteins to hydrophobic agaroses occurs at a critical alkyl-group density and is a function of the hydrophobicity (i.e., the total hydrocarbon-water interface, Tanford, 1973) of the gels.

The present investigations show that the adsorption of proteins to hydrophobic agaroses does not follow a simple lock and key mechanism but is based on multivalent attachment. In general, the capacity of hydrophobic agaroses for the enzyme phosphorylase *b* characterized at different temperatures and at different salt concentrations increases as a power function of the free solute equilibrium concentration (Jen-

<sup>†</sup> From the Institut für Physiologische Chemie der Ruhr-Universität, 4630 Bochum, West Germany. Received April 23, 1976. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Je 84/1). For preliminary communications of these results see the Proceedings of the Symposium "Chromatographic Fractionation of Macromolecules", Birmingham, UK (July 1976), and the Abstract Communications of the 10th International Congress of Biochemistry, Hamburg, West Germany (July 1976).

nissen, 1976b). Analysis of the binding data indicates a non-independence of binding.

## Materials and Methods

**Agarose and Cellulose Gels.** Methylamine and butylamine (Merck, Darmstadt) were coupled to cyanogen bromide (Merck) activated Sepharose 4B (Pharmacia, Uppsala), as described (Jennissen and Heilmeyer, 1975), and modified (Jennissen, 1976a). One milliliter of packed Sepharose 4B, as defined in Jennissen and Heilmeyer (1975), contains approximately  $5 \times 10^6$  Sepharose spheres and approximately 0.0290 g of polysaccharide when taken to absolute dryness under vacuum at 80–100 °C. The density of the alkyl groups on the gel was determined by  $^{14}\text{C}$  labeling of the ligand as described (Jennissen and Heilmeyer, 1975). One batch of methyl-Sepharose (30.5  $\mu\text{mol}/\text{ml}$  of packed Sepharose activated with 30 mg of CNBr/ml of incubation mixture) and one batch of butyl-Sepharose (29  $\mu\text{mol}/\text{ml}$  of packed Sepharose activated with 27 mg of CNBr/ml of incubation mixture) were employed in this study. Only fresh, unregenerated gel was used. Volume changes of the substituted Sepharose beads as a function of the temperature were not observed. Analytical gel filtration experiments were performed on a thermostated column (1 cm i.d.  $\times$  60 cm) containing Sepharose 6B with a bed height of 52 cm. Samples were applied in a volume of 0.2 ml, the flow rate was adjusted to  $\sim 6$  ml/h, and fractions of  $\sim 1$  ml were collected. During a run, the sample was diluted approximately 100-fold on the column. Cellulose and phosphocellulose (Servacel P 23,  $\sim 0.2$  mequivalent/ml of packed gel; Serva, Heidelberg) served as adsorbents for the myoglobin isotherms. All reagents were of analytical grade. Only double-distilled water was used.

**Protein Ligands, Buffers.** Phosphorylase *b* (third crystals, 70–80 units/mg) was purified from frozen rabbit muscle according to Fischer and Krebs (1958). The enzyme was freed of AMP<sup>1</sup> on a charcoal column (Fischer and Krebs, 1958) ( $A_{260}:A_{280} \sim 0.53$ ) and the activity was measured on an AutoAnalyzer (Technicon, Tarrytown, N.Y.) according to Haschke and Heilmeyer (1972). All calculations are based on a monomer molecular weight of 100 000 (Cohen et al., 1971) for phosphorylase *b*. Phosphorylase *b* monomers were prepared as described by Shaltiel et al. (1966). The enzyme was reconstituted with the coenzyme before activity measurements. Phosphorylase protein was either determined according to the  $A_{280\text{nm}}^{1\%, 1\text{cm}^{-1}} = 13.2$  (Kastenschmidt et al., 1968), or after twofold precipitation with 5%  $\text{Cl}_3\text{CCOOH}$  (including a wash with 0.1 M NaOH and final solubilization in 0.1 M NaOH, 1% sodium dodecyl sulfate) according to Lowry (1951) on an AutoAnalyzer. The specific activity of phosphorylase *b* calculated from the protein determined by the latter method was  $\sim 10\%$  higher. Prior to adsorption, phosphorylase *b* (30–50 mg/ml) was, unless otherwise stated, extensively dialyzed against either 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 1 mM DTE (dithioerythritol), 20% sucrose, pH 7.0, *buffer A*, or 10 mM Tris-HCl, 1.1 M  $(\text{NH}_4)_2\text{SO}_4$ , 1 mM DTE, 20% sucrose, pH 7.0, *buffer B*. The ammonium sulfate concentration employed in *buffer B* does not lead to precipitation of the enzyme (Fischer and Krebs, 1958).

Cyanmyoglobin was prepared according to Antonini and Brunori (1971), by incubating whale myoglobin (Serva)  $\sim 40$  mg/ml in a solution containing  $\text{K}_3\text{Fe}(\text{CN})_6$ , 10% over the stoichiometric amount of myoglobin, and 0.7 mM KCN for

20 min at room temperature. The protein was dialyzed for  $\sim 12$  h against 0.2 M sodium  $\beta$ -glycerophosphate-HCl, 0.7 mM KCN, pH 7.0, and another 12 h against two changes of *buffer C* containing 10 mM maleate-Tris, 0.7 mM KCN, 20% sucrose, pH 6.5. For calculations, a molecular weight of 16 900 was employed. Cyanmyoglobin protein was either determined according to  $A_{410\text{nm}}^{1\%, 1\text{cm}^{-1}} = 41$  or after  $\text{Cl}_3\text{CCOOH}$  precipitation (see above) according to Lowry (1951).

**Adsorption Isotherms.** The adsorption rate (time curve) of the ligands was determined by adding 0.5–1.5 ml of equilibrated packed Sepharose or cellulose measured in a thermostated, graduated column (Jennissen and Heilmeyer, 1975) to 20 ml of buffer containing phosphorylase *b* or myoglobin in an initial concentration of approximately 0.01–1.5 mg/ml. The incubation mixture was stirred in a thermostated Plexiglas beaker, 2.5 cm i.d.  $\times$  9 cm, until equilibrium was reached. A homogeneous incubation mixture was obtained when the speed of the 1.5-cm stirring bar exceeded 150 rpm. Speeds over 700 rpm caused foaming of the mixture; therefore, a speed of 450–550 rpm was employed. The capacity of the gels for the ligand was independent of the stirring rate. Examination of the substituted Sepharose under a phase microscope (Leitz, Wetzlar) demonstrated that stirring at 700 rpm for 2 h did not lead to a fragmentation of the spheres. For the determination of the free solute in the mixture,  $\sim 0.2$  ml of gel-free solution was removed by suction with a disposable 2-ml syringe (B. Braun, AG Melsungen) through a small stainless-steel grid (pore diameter 20  $\mu\text{m}$ ) which excluded the Sepharose spheres (diameter ca. 40–190  $\mu\text{m}$ , Pharmacia, Uppsala) and the cellulose. After each sample, the steel grid was washed by pressing 2–4 ml of  $\text{H}_2\text{O}$ , 0.5 M NaCl,  $\text{H}_2\text{O}$ , and, finally, acetone, respectively, through the grid with the syringe. The grid was then removed and dried in a stream of pressurized air. The washing and drying procedure took about 1 min. A fresh disposable syringe was employed for each sample.

The adsorbed amount of phosphorylase *b* was calculated from the difference between the initial and equilibrium free ligand concentration obtained by incubation of the enzyme with the substituted gel (control: nonsubstituted Sepharose 4B) for  $\sim 60$  min (time curve). A new incubation mixture was employed for each time curve, i.e., each data point on the isotherm. In the case of cyanmyoglobin, equilibrium with the phosphocellulose was reached in approximately 10–15 min (time curves). After this first equilibrium had been reached, a sample was removed with the grid as described above. Then, another aliquot of myoglobin (stock solution  $\sim 60$  mg/ml) was added to the same mixture and a second sample was removed after the new equilibrium was reached. This procedure was repeated until the isotherm was completed. The difference between the free concentration of myoglobin in the phosphocellulose mixture and the control gel mixture allowed a calculation of the adsorbed amount.

In one case (Table I, column A), the gel capacity and equilibrium concentration were calculated from a column experiment. A Plexiglas column, as described by Jennissen and Heilmeyer (1975), containing 6.5 ml of packed methyl-Sepharose was loaded with phosphorylase *b* (8.5 mg/3 ml) in *buffer A* at 5 °C. The column was eluted with 50 mM  $(\text{NH}_4)_2\text{SO}_4$  in *buffer A* until constant activities were obtained in the eluted fractions. The adsorbed amount was calculated from the activity eluted with 1 M NaCl in *buffer A*.

## Results

**Kinetics.** Stirring strongly influences the rate of adsorption of phosphorylase *b*. This rate (butyl-Sepharose, initial protein

<sup>1</sup> Abbreviations used are: AMP, adenosine monophosphate; DTE, dithioerythritol; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: Adsorption of Phosphorylase *b* on Hydrophobic Agaroses at Different Ammonium Sulfate Concentrations.<sup>a</sup>

Ammonium Sulfate <sup>f</sup> (M)	Adsorbed Phosphorylase <i>b</i> mg/ml of Packed Sepharose <sup>e</sup>		
	A	B	C
	9.49 <sup>b</sup>	5.10 <sup>b</sup>	6.59 <sup>b</sup>
0.05	0.013 <sup>d</sup>		
0.11		3.84 <sup>c</sup>	
0.55			1.55 <sup>c</sup>
1.10	0.53 <sup>b</sup>	7.88 <sup>b</sup>	10.23 <sup>b</sup>

<sup>a</sup> The capacities of the gels are shown in one column of the table at constant equilibrium concentrations as calculated from <sup>b</sup> isotherms: Figure 1, 5 °C, and Figure 2, 15 °C, 18 °C; <sup>c</sup> separate time curves (15 °C); <sup>d</sup> column equilibrium experiment (5 °C). <sup>e</sup> (A) methyl-Sepharose, equilibrium concentration 0.035 mg/ml; (B) butyl-Sepharose, equilibrium concentration 0.064 mg/ml; (C) butyl-Sepharose, equilibrium concentration 0.118 mg/ml. <sup>f</sup> The experiments were performed in buffer A with the specified ammonium sulfate concentrations. For further details see legends to Figures 1 and 2, Materials and Methods, and the text.

concentration 0.75 mg/ml) is enhanced approximately sixfold if the stirring velocity is increased from 200 to 700 rpm (not shown). At 700 rpm, a first-order reaction rate ( $k = 0.11 \text{ min}^{-1}$ ,  $t_{1/2} = 6.1 \text{ min}$ ) is approximated. At stirring velocities of 450–550 rpm, equilibrium is reached in 30–60 min. No change in specific activity occurs in the presence and absence of salt at the temperatures employed for the isotherms (not shown). Unsubstituted Sepharose 4B shows no adsorptive capacity for the enzyme.

**Salt Effects.** The influence of ammonium sulfate on the capacity of hydrophobic agaroses for phosphorylase *b* is shown in Table I at constant equilibrium concentrations. Methyl- (Table I, column A) and butyl-Sepharose (Table I, columns B and C) exhibit two maxima of adsorption. A first maximum is reached in the absence of ammonium sulfate. At intermediate salt concentrations, the capacity of the gels decreases. A second maximum ensues at high salt concentrations. In comparison to the methyl derivative a minimal value of the capacity is obtained at an approximately tenfold higher concentration (i.e., 0.55 M) of ammonium sulfate on the butyl gel. In contrast to the methyl-Sepharose, the capacity of the butyl derivative at 1.1 M ammonium sulfate is 1.55-fold higher than it is in the absence of the salt.

Cohen et al. (1971) reported that phosphorylase *b* dissociates to monomers in the presence of 2 M NaCl. This was examined (see Table II) by analytical gel filtration of the native dimeric and the monomeric forms of phosphorylase *b* in buffer B containing 1.1 M ammonium sulfate. At 5 and 35 °C, native phosphorylase *b* (lower  $V_e/V_0$  ratio) elutes before the monomer. Furthermore, the  $V_e/V_0$  ratio of the native enzyme is independent of the effluent protein concentration over 2 magnitudes. However, gel filtration in the presence of 1.1 M ammonium sulfate is problematical, as can be seen by a decrease of the  $V_e/V_0$  ratio at 35 °C in comparison to the value at 5 °C. This observation is illustrated by the large decrease of this ratio for bromphenol blue when the temperature is raised to 35 °C. At high protein concentrations of the native enzyme (e.g., 30 mg/ml in the sample) and during all runs with the monomer of phosphorylase *b*, asymmetric peaks with backward tailing are found (not shown).

**Isotherms of Phosphorylase *b*.** If the amount of adsorbed phosphorylase *b* on alkyl-Sepharose is plotted as a function of the free solute equilibrium concentration at different tem-

TABLE II: Analytical Gel Filtration of Phosphorylase *b* in the Native and Monomeric Form on Sepharose 6B in the Presence of 1.1 M Ammonium Sulfate.<sup>a</sup>

Compound	Temp (°C)	$V_e/V_0$	Effluent Protein Conc'n (mg/ml)
Phosphorylase <i>b</i> (native)	5	2.21	0.0024
		2.23	0.0490
Phosphorylase <i>b</i> (monomer)		2.40	0.0250
Bromphenol blue		6.01	
Phosphorylase <i>b</i> (native)	35	2.15	0.0012
		2.15	0.0240
Phosphorylase <i>b</i> (monomer)		2.12	0.2700
Bromphenol blue		2.30	0.1000
		4.71	

<sup>a</sup> All runs were performed on one column as described under Methods. The void volume ( $V_0$ ), determined with dextran blue, was 12.3 ml at both temperatures. For further details see Materials and Methods and the text. For definition of  $V_e$  see Crone (1974). The  $V_e/V_0$  ratio is characterized by Determann and Michel (1966).

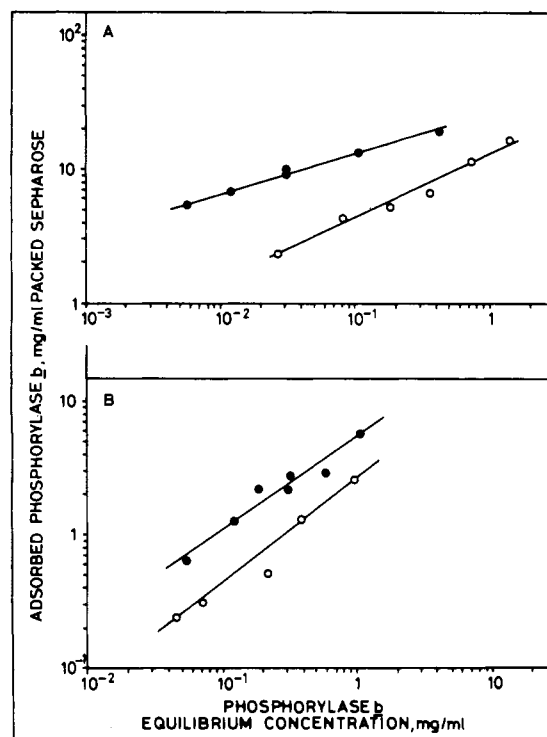


FIGURE 1: Adsorption isotherms of phosphorylase *b* on methyl-Sepharose at 5 and 34 °C in the absence (A) and in the presence (B) of 1.1 M ammonium sulfate (buffer A and B, respectively). The amount of phosphorylase *b* adsorbed by the substituted agarose (0.5–1.5 ml of packed gel) added to the incubation mixture (20 ml) was calculated from the difference between the initial and the 60-min (equilibrium) value of the free-ligand concentration (phosphorylase *b* activity) of the time curves run for each point. The activity measurements were controlled by protein determinations. For further details see Materials and Methods and the text. (●), 5 °C; (○), 34 °C.

peratures in double-logarithmic coordinates, linear isotherms result (Figures 1 and 2). Linearity is observed in an equilibrium concentration range of up to 2.5 magnitudes. Saturation of the gels is not obtained. This binding data corresponds to the

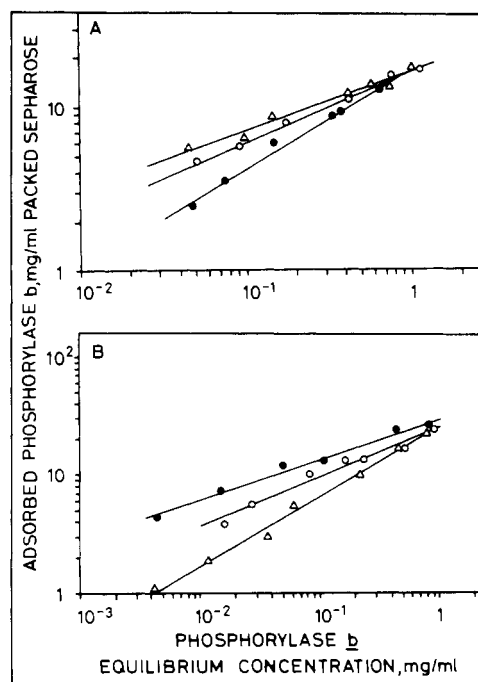


FIGURE 2: Adsorption isotherms of phosphorylase *b* on butyl-Sepharose at different temperatures in the absence (A) and in the presence (B) of 1.1 M ammonium sulfate (buffer A and B, respectively). For further details, see legend to Figure 1, Materials and Methods, and the text. (A) ( $\Delta$ ), 8; ( $\circ$ ), 18; ( $\bullet$ ), 34 °C. (B) ( $\Delta$ ), 0; ( $\circ$ ), 15; ( $\bullet$ ), 34 °C.

Freundlich type of isotherm (Freundlich, 1922), which is a power function:

$$a = \alpha c^{1/n} \quad (1)$$

Logarithmation leads to:

$$\log a = \log \alpha + (1/n \log c) \quad (1a)$$

where  $a$  is the amount of adsorbed ligand (in mg/ml of packed gel),  $c$  is the equilibrium concentration (in mg/ml),  $\alpha$  is the adsorption constant (in mg/ml of packed gel), and the slope ( $1/n$ ) is the adsorption exponent. The data of the isotherms (Figures 1 and 2) were fitted to the above power function by the least-squares method and the constants are presented in Table III. On methyl-Sepharose (Figure 1) an increase in temperature from 5 to 34 °C leads to a decline in the capacity in the absence (Figure 1A) and in the presence (Figure 1B) of 1.1 M ammonium sulfate; for example, at an equilibrium concentration of 0.04 mg/ml only ~15 and ~38% of the amount at 5 °C is adsorbed in the absence (Figure 1A) and in the presence (Figure 1B) of salt, respectively. This is reflected, in part, by a decreased adsorption constant,  $\alpha$ , at 34 °C (Table III). As the temperature is increased to 34 °C, the slope ( $1/n$ ) is enhanced (Table III) by a factor of 1.63 (Figure 1A) and 1.12 (Figure 1B) in the absence and presence of salt, respectively. The addition of 1.1 M ammonium sulfate (Figure 1B) decreases the capacity of the gel (equilibrium concentration 0.05 mg/ml) by ~1 magnitude (compare Figure 1A and Table I); the slope increases by a factor of ~2.

On butyl-Sepharose (Figure 2A) an elevation of the temperature from 8 to 34 °C in the absence of salt produces a decrease in the amount of enzyme bound and a 1.7-fold increase of the slope (Table III). This change of slope leads to a convergence of the isotherms. The adsorption constant is practically independent of the temperature (Table III). After addition of 1.1 M ammonium sulfate (Figure 2B), the capacity

TABLE III: Constants of the Freundlich Equation Calculated from the Isotherms of Phosphorylase *b* on Methyl- and Butyl-Sepharose.<sup>a</sup>

Gel	Buffer	Temp (°C)	$\alpha$	$1/n$	$r^2$
Methyl-Sepharose	A	5	25.66	0.30	0.99
		34	13.16	0.49	0.97
	B	5	5.15	0.68	0.93
		34	2.22	0.76	0.95
Butyl-Sepharose	A	8	15.78	0.35	0.94
		18	116.26	0.42	0.99
		34	17.12	0.61	0.98
	B	0	25.97	0.58	0.99
		15	25.45	0.43	0.95
		34	29.15	0.34	0.98

<sup>a</sup> The adsorption constants ( $\alpha$ ), expressed in mg/ml of packed Sepharose, the adsorption exponents ( $1/n$ ), and the coefficients of determination ( $r^2$ ) were calculated from the data of Figures 1 and 2 on a Hewlett-Packard HP 65, employing the power-curve fit program STAT 1-24 A. For further details, see the legends to Figures 1 and 2, and the text.

of the gel is of similar magnitude, as in the absence of salt (see Table I). In contrast to the previous isotherms (Figure 2A), an increase in temperature from 0 to 34 °C enhances the capacity ~3.7-fold (equilibrium concentration 0.04 mg/ml). Concomitantly, the slope decreases 1.7-fold (Table III) and the isotherms converge in one area. Phosphorylase *b* is not cold inactivated (Graves et al., 1965) under these conditions at 0 °C.

**Cooperativity.** Figure 3 depicts Scatchard plots (Scatchard, 1949) of the isotherms carried out on the two Sepharose derivatives. The solid lines curved concave upwards were calculated according to eq 1 employing the constants of Table III. From the intersects with the abscissas extrapolated from the experimental points of the curves, an estimate of the stoichiometry, i.e., the total coverage of available surface, may be derived. In the absence of ammonium sulfate, saturation apparently occurs at ~29 mg/ml of packed methyl-Sepharose (Figure 3A), at ~30 mg/ml of packed butyl-Sepharose (Figure 3B) and in the presence of salt at ~36 mg/ml of packed butyl-Sepharose (Figure 3C). In these plots, the binding of phosphorylase *b* is observed in a range between approximately 5–70% saturation. The concave Scatchard plots of phosphorylase *b* adsorption on methyl-Sepharose in the presence of 1.1 M ammonium sulfate (not shown) were not evaluated, since the isotherms lie below ~40% saturation.

Hill plots (Hill, 1910; Koshland, 1970) of the data on butyl-Sepharose are shown in Figure 4. The solid lines were calculated according to the Freundlich equation (eq 1) employing the data of Table III. At low surface coverage, each plot rises linearly with a slope  $n_H < 1$  (see Table IV). As high coverage (saturation) is approached, the plots curve upwards to a slope of ~1. In the absence of ammonium sulfate, the Hill coefficients (Table IV) of the adsorption of phosphorylase *b* on methyl- and butyl-Sepharose lie between 0.39 and 0.71, increasing with temperature. However, in the presence of 1.1 M ammonium sulfate on the butyl derivative, the Hill coefficients decrease from 0.63 to 0.40 as the temperature is raised. The value of the Hill coefficient is between 1.1 and 1.4 times higher than that of the adsorption exponent (Table IV). An increase in temperature from 5 to 34 °C decreases the apparent association constant of half-maximal saturation ( $K_{0.5}$ ) of

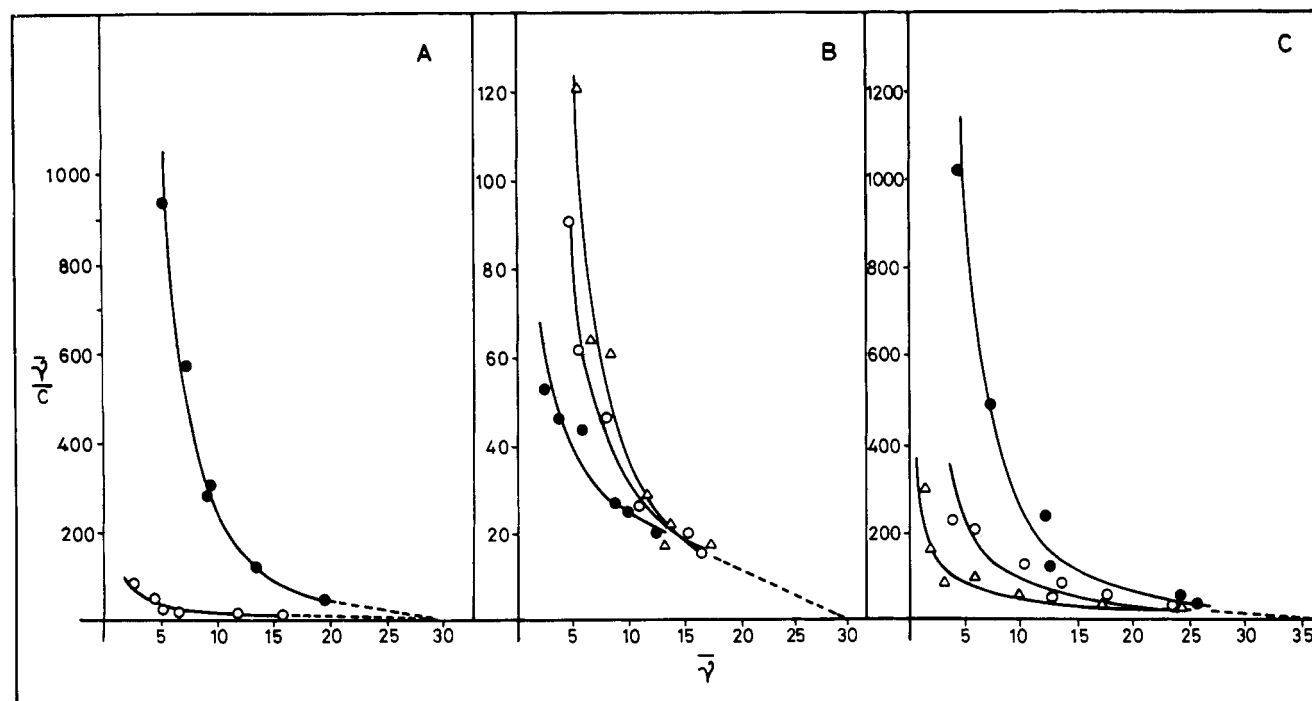


FIGURE 3: Scatchard plots of the isotherms of phosphorylase *b* on methyl- (A) (Figure 1) and butyl-Sepharose (B and C) (Figure 2). The solid lines were calculated according to eq 1 and the constants of Table III. The measured values are included as data points. The dotted lines denote the mode of extrapolation to the abscissa.  $\bar{\gamma}$  symbolizes the amount of adsorbed phosphorylase *b* in mg/ml of packed gel and *C* denotes the equilibrium concentration of free ligand in mg/ml. For further details, see the legends to Figures 1 and 2, and the text. (A) Methyl-Sepharose, buffer A, 5 °C (●), 34 °C (○); (B) butyl-Sepharose, buffer A, 8 °C (▲), 18 °C (○), 34 °C (●); (C) butyl-Sepharose, buffer B, 0 °C (▲), 15 °C (○), 34 °C (●).

TABLE IV: Hill Coefficients ( $n_H$ ) and Apparent Association Constants ( $K_{0.5}$ ) of the Adsorption of Phosphorylase *b* and Cyanmyoglobin on the Respective Matrices.<sup>a</sup>

Gel	Ligand	Buffer	Temp (°C)	Hill Coefficient ( $n_H$ )	Hill Coefficient: Adsorption Exponent	$K_{0.5} \times 10^{-4} \text{ M}^{-1}$
Methyl-Sepharose	Phosphorylase <i>b</i>	A	5	0.39	1.30	38.5
			34	0.56	1.14	3.8
		B	5	Nd <sup>b</sup>	Nd <sup>b</sup>	Nd <sup>b</sup>
			34	Nd <sup>b</sup>	Nd <sup>b</sup>	Nd <sup>b</sup>
Butyl-Sepharose	Phosphorylase <i>b</i>	A	8	0.48	1.37	8.7
			18	0.53	1.26	8.0
			34	0.71	1.16	7.9
		B	0	0.62	1.07	9.5
			15	0.48	1.12	10.6
			34	0.40	1.18	21.3
Phosphocellulose	Myoglobin	C	4.5	1.0		9.4 <sup>c</sup>
			34	1.0		2.3 <sup>c</sup>

<sup>a</sup> The data were calculated for the linear portion of the Hill plots (Figure 4) derived from the fitted data of the Freundlich equation (eq 1) and the Langmuir equation (see legend to Figure 5). For further details and definition of  $K_{0.5}$ , see legend to Figure 4 and the text. <sup>b</sup> Nd: Could not be determined accurately. <sup>c</sup> Identical to the reciprocal of the dissociation constants (*k*) determined from the intersects with the abscissa in Figure 5.

phosphorylase *b* in the absence of the salt on methyl-Sepharose by a factor of  $\sim 10$  and on the butyl gel by  $\sim 10\%$ ; a  $\sim 2.5$ -fold increase of this constant is found on the butyl gel in the presence of 1.1 M ammonium sulfate.

**Isotherms of Myoglobin.** As an example for a qualitatively different type of adsorption, the binding of cyanmyoglobin to phosphocellulose was studied. The adsorption of cyanmyoglobin to this gel is a hyperbolic function of the free myoglobin equilibrium concentration corresponding to a Langmuir isotherm (Langmuir, 1916; Barrow, 1966). These isotherms can

be linearized in double-reciprocal coordinates (Figure 5). From Figure 5, the adsorption maximum (saturation)  $a_0 = 10.53$  mg of cyanmyoglobin/ml of packed cellulose can be calculated from the ordinate intersect. It is identical for the two temperatures. However, a rise in temperature from 4.5 to 32 °C decreases the association constant by a factor of 4 (Table IV). The insert in Figure 5 demonstrates that these isotherms are nonlinear functions in double-logarithmic plots. The Scatchard plots of myoglobin adsorption on phosphocellulose are linear and Hill coefficients of 1 are obtained (Table IV).

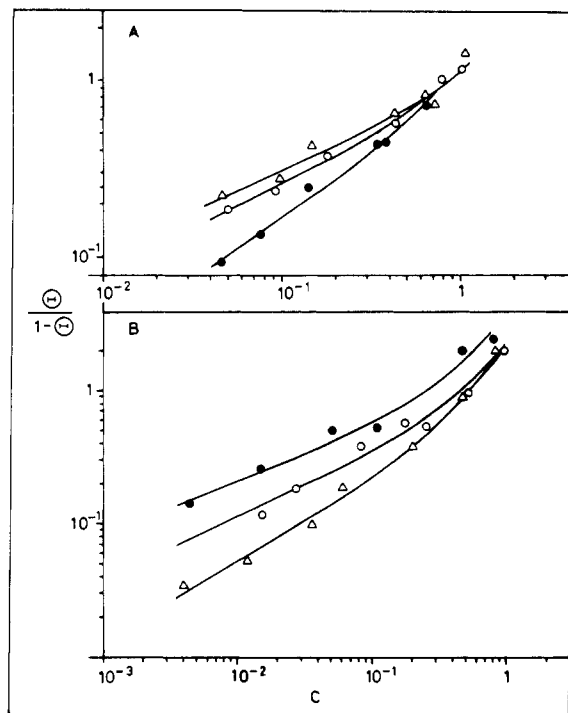


FIGURE 4: Hill plots of the isotherms of phosphorylase *b* on butyl-Sepharose (Figure 2) in buffer A (A) and in buffer B (B) at different temperatures. The solid lines were calculated according to eq 1 and the constants of Table III. From the logarithmic form of the Hill equation,  $\log(\theta/(1-\theta)) = \log K_H + n_H \log c$ , where  $\theta$  denotes fractional saturation and  $c$  is the equilibrium concentration, the apparent association constant ( $K_{0.5}$ ) and the Hill coefficient ( $n_H$ ) were determined (see Table IV). The Hill constant ( $K_H$ ) can be calculated from the apparent association constant of half-maximal saturation according to:  $K_H = (K_{0.5})^{n_H}$ . The values for the saturation of the gel with ligand were extrapolated from the Scatchard plots (see Figure 3). For further details see the legend to Figure 2 and the text. (A) ( $\Delta$ ), 8; ( $\circ$ ), 18; ( $\bullet$ ), 34 °C. (B) ( $\Delta$ ), 0; ( $\circ$ ), 15; ( $\bullet$ ), 34 °C.

**Heats of Adsorption.** Under the condition that the adsorption reaction is a reversible process, the heat of adsorption can be derived from the isotherms according to the Clausius-Clapeyron equation (Patat et al., 1964; Barrow, 1966). From the corresponding isosteres (e.g., Figure 6), the heats of adsorption were calculated and are presented in Table V. The negative enthalpies of adsorption of phosphorylase *b* on methyl-Sepharose (presence and absence of salt) and on the butyl gel (absence of salt, Figure 6A) indicate an exothermic reaction. In the presence of 1.1 M ammonium sulfate, the adsorption of phosphorylase *b* on butyl-Sepharose is endothermic (Figure 6B). The reaction enthalpies are dependent on the surface coverage and may approach zero at high values of fractional saturation.

The adsorption of cyanmyoglobin on phosphocellulose is an exothermic reaction with a negative enthalpy change of ca.  $-8.5$  kcal/mol ( $\Delta G' \sim -6$  kcal/mol; see Table IV). In contrast to the isotherms of phosphorylase *b* on hydrophobic agaroses, there is practically no dependence of the heat of adsorption on the surface coverage.

## Discussion

Control experiments demonstrated that the experimental conditions, especially stirring, do not lead to denaturation of phosphorylase *b* in solution nor to a mechanical disruption of the adsorbed ligand layer on the gel. Desorption experiments (Jennissen and Heilmeyer, 1975; Jennissen, 1976a) have also

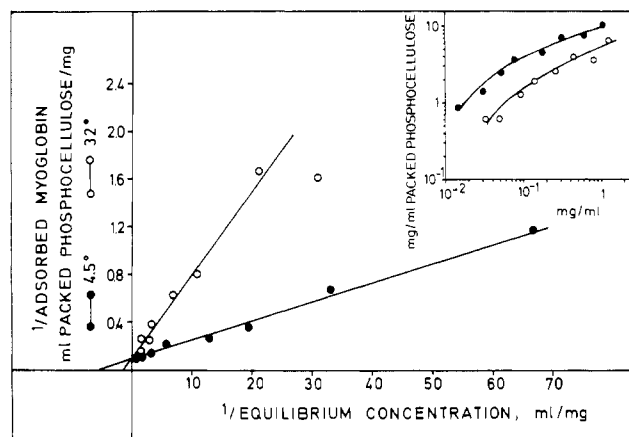


FIGURE 5: Adsorption isotherms of cyanmyoglobin on phosphocellulose in double-reciprocal coordinates at 4.5 and 32 °C. The adsorption was performed in buffer C and the myoglobin concentration was determined optically at 410 nm. Langmuir isotherms correspond to a hyperbolic function:  $a = a_0 c / (k' + c)$ , where  $a$  corresponds to the adsorbed amount of ligand/ml of packed cellulose,  $a_0$  to the amount of ligand adsorbed at saturation (ordinate intersect of double-reciprocal plot),  $c$  to the free-solute equilibrium concentration, and  $k'$  to a dissociation constant which is equal to the equilibrium concentration of half-maximal saturation (intersect with abscissa in double-reciprocal plot). Insert: plot of the adsorption of cyanmyoglobin on phosphocellulose in double-logarithmic coordinates. The logarithm of the adsorbed amount of myoglobin (mg/ml of packed phosphocellulose) is plotted vs. the logarithm of the equilibrium concentration (mg/ml). For further details, see Table IV, Materials and Methods, and the text.

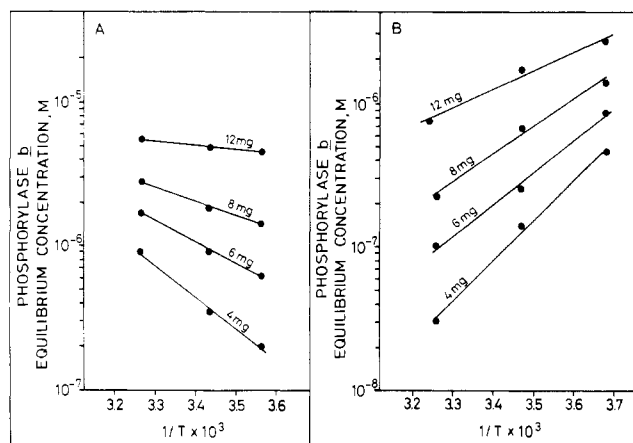


FIGURE 6: Isosteres of the adsorption of phosphorylase *b* on butyl-Sepharose in buffer A (A) and buffer B (B); depicted as semilogarithmic plots of the molar equilibrium concentration vs. the reciprocal of the absolute temperature. In the Clausius-Clapeyron equation,  $\Delta H / 2.3 R = d(\log c) / d(1/T)$ ,  $\Delta H$  is the difference of the partial molal enthalpies (cal/mol) of the ligand on the surface and in the liquid,  $R$  is the gas constant (1.987 cal/deg  $\times$  mol),  $c$  is the equilibrium concentration (mol/l.), and  $T$  is the absolute temperature (K). The number on each isostere denotes the constant amount of phosphorylase *b* adsorbed in mg/ml of packed Sepharose. The isosteres were calculated from the data of Figure 2 and Table III. For further details, see the legend to Figure 2, Table V, and the text.

shown that the native and intact enzyme can be regained. It was also excluded (see Methods) that significant fragmentation of the gel occurs, e.g., by stirring at 700 rpm for 2 h. Kinetic experiments indicate a pseudo-first-order reaction at stirring velocities over 700 rpm. Deviations from this behavior are probably due to interference by convection and diffusion. Empirically, the adsorption of phosphorylase *b* can be correlated to an exponential time function (not shown).

TABLE V: Isosteric Heats of Adsorption of Phosphorylase *b* and Cyanmyoglobin on Different Adsorbents.<sup>a</sup>

Gel	Ligand	Buffer	Adsorbed Ligand mg/ml of Packed Gel	$\Delta H$ , kcal/mol
Methyl-Sepharose	Phosphorylase <i>b</i>	A	4.0	-22.0
			6.0	-18.8
			8.0	-16.8
		B	0.6	-8.4
			1.0	-7.9
			3.0	-6.9
Butyl-Sepharose	Phosphorylase <i>b</i>	A	4.0	-9.9
			6.0	-7.3
			8.0	-4.6
			12.0	-1.3
		B	4.0	13.4
			6.0	10.4
			8.0	8.9
			12.0	5.9
Phosphocellulose	Myoglobin	C	0.8	-8.7
			2.0	-8.5
			5.0	-8.6

<sup>a</sup> For further details see the legend to Figure 6 and the text.

The effect of salt on the adsorption of phosphorylase *b* to agaroses containing hydrophobic groups is probably a sum of multiple influences exerted on the protein, as well as on the matrix. At high concentrations, ammonium sulfate adversely affects the solubility of proteins by decreasing the availability of water molecules in the bulk; furthermore, the extent of hydrophobic associations increases as a result of enhanced interfacial tension (Lewin, 1974). In contrast, at concentrations below 0.5 M most electrolytes reduce the surface tension of water (Lewin, 1974). Therefore, hydrophobic associations are promoted at relative maxima of the surface tension, i.e., at very low and at very high ammonium sulfate concentrations. Under these conditions, the capacity of the alkyl-Sepharoses reach relative adsorption maxima (Table I). At intermediate ammonium sulfate concentrations, the salting-in effect (increase in solubility, decrease of surface tension) outweighs the salting-out influence so that the capacity of the gel for the ligand falls to low values. Similarly, the decrease in the capacity of the alkyl-Sepharoses for phosphorylase *b* when the temperature is raised (see Figures 3 and 4) may also be due to increased solvation and decreased surface tension. On butyl-Sepharose in the presence of salt, the hydrophobic nature of the interaction probably dominates over secondary influences on solubility and surface tension. The quantitative difference in the capacity of the methyl- and butyl-Sepharose in the presence of 1.1 M ammonium sulfate (Tables I and III) could be accounted for by differences in the net hydrophobicity of the gels. The decrease in capacity of the methyl-Sepharose after addition of salt may not only be a result of solubility and surface tension effects but could result from a rupture of electrostatic interactions (ratio of alkyl residues to positive charges ca. 4-8:1, Jennissen and Heilmeyer, 1975) cooperating with the alkyl groups in adsorption in the absence of the salt. The latter effects on the adsorption of phosphorylase *b* appear minimal in  $\beta$ -glycerophosphate buffer (see Table II and Figure 2 in Jennissen and Heilmeyer, 1975), where the capacity ratio of the methyl derivative (0.78 mg/ml of packed gel) to the butyl gel (5.4 mg/ml of packed gel) at a density of  $\sim 33 \mu\text{mol/ml}$  of packed gel more readily reflects the capacity ratio found in the

presence of 1.1 M ammonium sulfate (Table I, columns A and B). Below a density of  $\sim 30 \mu\text{mol}$  of methylamine/ml of packed gel, practically no adsorption of phosphorylase *b* occurs in the  $\beta$ -glycerophosphate buffer (Jennissen, 1976a).

During the adsorption of phosphorylase *b* on hydrophobic agaroses, the heat of adsorption changes characteristically as a function of the surface coverage (see Table V) and may approach zero. In contrast, the enthalpy change for myoglobin on phosphocellulose is practically independent of the surface coverage in the range determined. However, as can be derived from Figure 5, in this case, the heat of adsorption is zero at saturation, i.e., at an infinitely high equilibrium concentration. In the absence of ammonium sulfate, the adsorption of phosphorylase *b* on the methyl and butyl derivative is an exothermic reaction. In comparison, negative enthalpy changes are also observed with detergents. For example, micelle formation of sodium dodecyl sulfate and other detergents in water (Brandts, 1969) is an exothermic process at room temperatures. If the adsorption of proteins to hydrophobic matrices is considered to be a process of limited micelle formation, a negative enthalpy change would, therefore, not preclude the hydrophobic nature of binding (see also: Jost et al., 1974). As is also indicated by the exothermic adsorption of phosphorylase *b* on methyl-agarose in the presence of 1.1 M salt (Table V), a negative enthalpy change does not prove the presence of ionic interactions (see discussion on salt effects). On the butyl gel in the presence of 1.1 M ammonium sulfate, the positive enthalpy change in phosphorylase *b* adsorption indicates an entropy-driven reaction.

The Freundlich type of isotherm (Figures 1 and 2) appears to be independent of the hydrophobicity of the gel and, from experiments with phosphorylase kinase (unpublished results), also independent of the protein employed. The utilization of the Freundlich equation is limited by the fact that it predicts an infinite capacity at infinite equilibrium concentrations. However, Freundlich (1922) pointed out that the equation is only valid in dilute solutions of ligand. Secondly, the equation is limited by predicting an infinite heat of adsorption at infinitely low surface coverages (Adamson, 1967; see Table V).

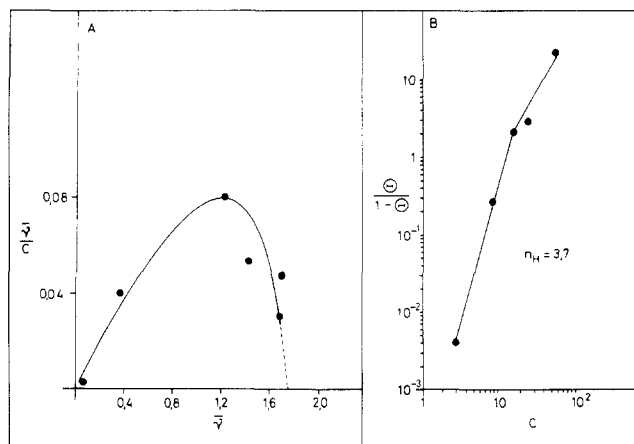


FIGURE 7: Scatchard plot (A) and Hill plot (B) of the adsorption of phosphorylase kinase on butyl-Sepharoses of increasing alkyl group density. The data were taken from Figure 1 in Jennissen and Heilmeyer (1975). In the plots,  $\bar{\nu}$  denotes the amount of phosphorylase kinase adsorbed in mg/ml of packed Sepharose (calculated with a sp act. of  $8.5 \times 10^3$  units/mg),  $\theta$  symbolizes the fractional surface coverage (saturation being extrapolated as 1.75 mg of enzyme/ml of packed Sepharose, dotted line), and C corresponds to the density of butyl groups in  $\mu\text{mol/ml}$  of packed gel. For further details see Jennissen and Heilmeyer (1975), the legend to Figure 4, and Discussion.

The drawbacks may be overcome if the isotherms are analyzed with the tools employed in the analysis of regulatory proteins (Figures 3 and 4). In contrast to the binding of myoglobin to phosphocellulose, the Scatchard plots of the binding of phosphorylase *b* result in curves that are concave upwards. According to Levitzki and Koshland (1969), binding curves of this type indicate negative cooperativity. By extrapolation to the abscissa, a crude estimate of the total available surface coverage (i.e., stoichiometry) may be obtained (Koshland, 1970). The Hill coefficients ( $n_H$ ) (Table IV) obtained from plots, as shown in Figure 4, are smaller than unity, also indicating negative cooperativity (Levitzki and Koshland, 1969). The errors inherent in the extrapolation of saturation from the Scatchard plots (Figure 3) do not exclude this interpretation. For example, in the case of phosphorylase *b* adsorption on butyl-Sepharose in the presence of 1.1 M salt at 34 °C (Figure 3C;  $n_H = 0.40$ , Table IV), a saturation value of 36 mg/ml of packed gel was extrapolated. If the final experimental point of the isotherm (Figure 2B), ~26 mg/ml of packed gel, is employed for saturation (lowest saturation possible) in the Hill plot a coefficient  $n_H = 0.44$  is calculated. If, on the other hand, saturation is assumed at a far higher capacity, e.g., 50 mg/ml of packed gel, a lower Hill coefficient of  $n_H = 0.38$  is obtained. In summary, an error of ca.  $\pm 30\%$  in the extrapolation (see above) leads to an error of  $\pm 5\%$  in the minimal Hill coefficient; the error in the apparent association constant  $K_{0.5}$  (Table IV) is, however,  $\pm 40\%$  under these conditions.

Electrostatic repulsion cannot be the cause for the negative cooperative behavior of the phosphorylase *b* molecules at increasing surface coverage, since no qualitative change in the form of the Scatchard or the Hill plots is observed by adding 1.1 M ammonium sulfate to the buffer. Under these conditions, all charge effects must be eliminated.

Polymorphic forms of the enzyme are also improbable. Crystallized, native, AMP-free rabbit phosphorylase *b* in solution is a dimer of two identical (Fischer et al., 1970) subunits with a monomer molecular weight of 100 000 (Cohen et al., 1971). At low and intermediate salt concentrations and at temperatures between 4 and 35 °C, no change in molecular

weight has been reported, as determined by gel filtration (DeVincenzi and Hedrick, 1967), ultracentrifugal studies (Seery et al., 1967), and electrophoresis on polyacrylamide (Pfeuffer et al., 1972). From the analytical gel filtration experiments shown here (see Table II), it can be concluded that in the presence of 1.1 M ammonium sulfate neither a dissociation to monomers nor association to tetramers occurs.

Heterogeneity of independent sites on the gel is also an unlikely explanation for the observed isotherms. For example, the adsorption of phosphorylase *b* and phosphorylase kinase on hydrophobic agarose increases in a sigmoidal fashion as a function of the alkyl-residue density on the Agarose spheres (Figures 1 and 2 in Jennissen and Heilmeyer, 1975). This adsorption of phosphorylase kinase on butyl-Sepharose at different alkyl-residue densities plotted according to Scatchard and Hill is shown in Figure 7. Both the Scatchard (concave downwards) and the Hill plot (maximal  $n_H = 3.7$ ,  $K_{0.5}^2 = 8.2 \times 10^4 \text{ M}_s^{-1}$ ) are typical for positive cooperative binding. Similarly, Hill coefficients ( $n_H$ ) of 3.8 ( $K_{0.5}^2 = 6.8 \times 10^4 \text{ M}_s^{-1}$ ) and 6.7 (mean value) ( $K_{0.5}^2 = 5.5 \times 10^4 \text{ M}_s^{-1}$ ) are calculated for the ethyl and methyl derivatives, respectively (not shown). It may be concluded that the adsorption of phosphorylase kinase and phosphorylase *b* (see below) depends on the positive cooperative interaction of the substituted alkyl residues. A single alkyl residue is not capable of binding a molecule of these enzymes. The power function of Freundlich (eq 1) can also be applied to the double-logarithmic plots of the adsorption of these proteins to Sepharoses with increasing density of alkyl groups (see inserts to Figures 1 and 2 in Jennissen and Heilmeyer, 1975). For the adsorption of phosphorylase kinase, exponents ( $1/n$ ) of 5.8, 3.6, and 3.5 can be calculated for the 1, 2, and 4 carbon-atom derivatives, respectively. The interconversion factor between these values and the Hill coefficients (see above) is  $\sim 1.1$ . For phosphorylase *b* adsorption in  $\beta$ -glycerophosphate buffer on ethyl- and butyl-Sepharose (Figure 2 in Jennissen and Heilmeyer, 1975), exponents ( $1/n$ ) of 8.5 and 4.8 are obtained, respectively. If the interconversion factor of 1.1 (see above) is employed for phosphorylase *b*, apparent Hill coefficients ( $n_H$ ) of 9.4 and 5.3 can be calculated for the 2 and 4 carbon-atom derivatives, respectively. In the presence of 1.1 M ammonium sulfate the capacity of butyl agaroses for phosphorylase *b* at a constant equilibrium concentration is also a sigmoidal function of the butyl residue density (not shown). Maximal Hill coefficients ( $n_H$ ) of 3.8 ( $K_{0.5}^2 = 8 \times 10^4 \text{ M}_s^{-1}$ ) and 3.1 ( $K_{0.5}^2 = 6 \times 10^4 \text{ M}_s^{-1}$ ) are calculated from the corresponding Hill plots at 5 and 34 °C, respectively. The discussed Hill coefficients may be interpreted (Koshland, 1970) as an indication of the minimum number of binding sites (e.g., alkyl residues) necessary for the adsorption of one ligand molecule. Furthermore, it may be concluded from the changes in the Hill coefficient as a function of the length of the alkyl residue that the minimum number of residues necessary for the binding of the protein ligand increases as the chain length is reduced. Klotz (1970) concluded from the x-ray diffraction data of four proteins that up to 50% and more of the apolar residues may be fully or partially exposed on the surface to the aqueous solvent. Protein ligands should, therefore, be capable of multivalent reactions with alkyl-agaroses. Thus, on the basis of multiple contact sites, an argument of heterogeneous, single, independent sites does

<sup>2</sup>  $K_{0.5}^2$  denotes the reciprocal of the alkyl residue density, i.e., concentration in (moles of alkyl residue/1 packed Sepharose) $^{-1}$  symbolized by  $\text{M}_s^{-1}$  at half-maximal saturation with ligand.



not invalidate the above interpretation of negative cooperativity.

That the interpretation of multivalent cooperative binding is compatible with the data can be shown in the following way. From a molecular weight of 306 for the anhydrosaccharide unit of agarose, it can be calculated that 1 ml of packed gel (0.029 g of polysaccharide) contains  $\sim 95 \mu\text{mol}$  of this unit. At a density of  $30 \mu\text{mol}$  of alkyl residues/ml of packed gel,  $\sim 1$  residue is linked/hexasaccharide unit. Presuming an unaltered agarose structure in the substituted Sepharose, it can be calculated from the pitch (1.9 nm) of the agarose helix (Arnott et al., 1974) and 6 monosaccharide residues/turn that the alkyl residues are  $\sim 1.9$  nm apart on the axis of a polysaccharide strand. The two strands in a double helix are separated by 0.45–0.6 nm. Assuming a uniform distribution (Lasch et al., 1975), a mean spacing of 0.55 nm between single strands (diameter ca. 0.7 nm), and double helices in the stacked, double-helical structure of the agarose, an average density of  $\sim 1$  alkyl residue/ $2.3 \text{ nm}^2$  may be calculated. From the molecular dimensions of the phosphorylase *b* dimer (rectangular prism,  $6.3 \times 5.5 \times 10.9$  nm, Puchwein et al., 1970) on the basis of area, one side of the enzyme is capable of maximally covering an area of agarose containing ca. 15–30 alkyl residues. The simultaneous contact between 3–5 or more alkyl residues and the protein ligand is therefore quite possible. Due to the higher molecular weight ( $1.3 \times 10^6$ , Cohen, 1973; Hayakawa et al., 1973), phosphorylase kinase can be expected to cover an even larger area.

The aberrant adsorption isotherms of phosphorylase *b* on alkyl-agaroses may, therefore, be explained on the basis of multiple contact sites. In the buffer employed and in the absence of ammonium sulfate, ionic interactions may cooperate in binding; this is excluded at high salt concentrations. Therefore, an interpretation of the data must encompass these two possibilities. The adsorption of phosphorylase *b* may be viewed as occurring on a heterogeneous or homogeneous planar lattice of binding sites. If, for example, a homogeneous lattice on the adsorbent (butyl-Sepharose) and ligand (phosphorylase *b*) in the presence of high salt concentrations is assumed, the following model could be envisioned. The Sepharose spheres are rigid bodies containing an external and internal surface (effective pore radius for Sepharose 4B larger than  $\sim 40$  nm, Lasch et al., 1975) from which alkyl residues protrude. The rigid ligand, phosphorylase *b* (rectangular prism, Puchwein et al., 1970), has surface sites capable of reacting with the alkyl groups. Binding occurs when a critical number of residues (critical hydrophobicity) comes into contact with corresponding sites on the surface of the ligand. The more residues that make contact with the protein the stronger is the binding affinity (Jennissen and Heilmeyer, 1975). In a binding experiment (e.g., Figure 2), the most alkyl residues are available to the first molecules adsorbed; each oncoming ligand finds a smaller number of available residues for multivalent binding until the critical value is reached and adsorption ceases. This may be termed *sequential adsorption*. Sequential adsorption may be modified by the competition of the ligand molecules for the residues. Sequential adsorption and competition would thus lead to a nonindependent binding of the ligands. Furthermore, variations in the number of binding residues per ligand probably are responsible for differences in the configuration of binding and in the degrees of freedom of the adsorbed ligand. This would produce differences in configurational entropy. Thus, negative cooperativity of binding could be due to changes in the affinity of the ligand for the matrix produced by sequential adsorption and competition for alkyl groups

(nonindependence of binding), on one hand, and variations in the configurations of binding and entropy, on the other. From the data, it appears that a very similar mechanism can be postulated for the adsorption of phosphorylase *b* on a heterogeneous lattice of multiple binding sites (e.g., in the absence of ammonium sulfate). The noncooperative adsorption of myoglobin on phosphocellulose (Figure 5, Table IV) leads to the conclusion that this protein reacts with single, independent, and identical binding sites on the gel.

One of the first statistical mechanical theories (for review, see Patat, 1964; Roe, 1974) of the adsorption of flexible homopolymers was developed by Simha et al. (1953) (SFE isotherm) and Frisch and Simha (1954). Their equation (not shown), which is similar to Hill equation (see legend to Figure 4), states that the reciprocal of the slope deduced from a plot of  $\log(\theta/1-\theta)$  vs.  $\log c$  is an indication of the average number of contact sites (see also Krauss and Dugone, 1955; Binford and Gessler, 1959). If an analogy to the Hill equation is accepted, this would mean that the reciprocal of a Hill coefficient below unity is an indication of the number of involved contact sites. The increase of the Hill coefficient from below unity to 1 at high surface coverage (Figure 4) may thus reflect the decrease in contact sites due to sequential adsorption. Laiken and Némethy (1970a,b, 1971) have shown in a statistical mechanical model that a negative cooperative mechanism of binding (nonlinear Scatchard plot) describes the adsorption of flexible, small-molecular-weight ligands to a two-dimensional lattice of multiple binding sites on a protein. Nonindependence of ligand binding on a one-dimensional lattice has been treated on a theoretical basis by McGhee and von Hippel (1974), who also report binding curves concave upwards in Scatchard plots in the absence of interligand interactions.

Multivalent, nonindependent adsorption of ligands may play an important role in cellular effector–receptor interactions (manuscript in preparation).

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