General Aspects of Hydrophobic Chromatography. Adsorption and Elution Characteristics of Some Skeletal Muscle Enzymes†

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ABSTRACT: If the degree of substitution of Sepharose 4 B with α-alkylamines is varied gels of different hydrophobicity are produced. Proteins can be adsorbed when a critical hydrophobicity (*ca.* 10^{12} alkyl residues/Sepharose sphere) is reached. The enzymes phosphorylase kinase, phosphorylase phosphatase, 3′,5′-cAMP dependent protein kinase, glycogen synthetase, and phosphorylase *b* are successively adsorbed as the hydrophobicity of the Sepharose is increased. The capacity of the gels for these enzymes and protein in general increases exponentially and reaches plateau values as a function of the degree of substitution. There is no indication of a restriction of the hydrophobic centers for a given protein. The critical hydrophobicity needed to adsorb proteins can either be obtained in the above manner or by elongation of the employed alkylamine at a constant degree of substitution. Additionally, as the hydrophobicity of a gel is increased higher binding forces result and desorption of proteins requires an augmentation of the salt concentration in the elution buffer. Elution of proteins from a hydrophobic matrix can be described in terms of salting-in phenomena since desorption is dependent on the type of salt employed and not on the ionic strength alone. This also rules out ionic interactions as a major factor in adsorption *per se*. By rationally controlling the hydrophobicity of a Sepharose gel the adsorption and elution of a protein may be thus established that its purification or elimination can be optimally performed.

Sepharoses substituted with hydrophobic alkyl residues have been widely used for the purification of proteins. First examples were the specific adsorption of serum albumin on gels carrying aminodecyl residues (Yon, 1972) or oleyl and palmityl residues (Peters *et al.*, 1973). Shaltiel and coworkers showed that from a series of Sepharoses, substituted with homologous α-alkylamines, phosphorylase *b* could be adsorbed by the butylamine derivative or by gels carrying higher but not lower homologous amines (Er-el *et al.*, 1972; Er-el and Shaltiel, 1974). Similarly substituted Sepharoses were used to show differences in binding between negatively and positively charged proteins (Hofstee, 1973). Glycogen synthetase could be optimally adsorbed and eluted from Sepharose to which butylenediamine was linked (Shaltiel and Er-el, 1973). Agarose containing hexamethylenediamine could adsorb muscle phosphorylase kinase (Jennissen *et al.*, 1973; Jennissen and Heilmeyer, 1974b) as well as different tRNA synthetases from plants (Jakubowski and Pawelkiewicz, 1973). In all cases a minimal chain length of the substituent seemed to be required in order to obtain adsorption of these enzymes. This was interpreted as fitting a lipophilic alkyl residue into a hydrophobic pocket of the protein.

In the present study the degree of substitution of the agarose was varied thereby modifying the properties of the gel. Generally, an increase in the degree of substitution as well as an elongation of the hydrocarbon chain length of the employed amine cause an increase in the hydrophobicity of the gel, which influences the amount and the type of adsorbable protein.

Materials and Methods

Phosphorylase and phosphorylase kinase were determined on an Auto-Analyzer according to Haschke and Heilmeyer (1972) and Jennissen and Heilmeyer (1974a), respectively. Phosphorylase phosphatase was assayed according to Haschke *et al.* (1970), protein kinase according to Reimann *et al.* (1971), and glycogen synthetase according to Thomas *et al.* (1968). Protein was determined according to Lowry *et al.* (1951). Units are expressed as micromoles of substrate converted per minute except phosphorylase kinase and phosphatase (nanomoles per minute).

Phosphorylase was prepared according to Fischer and Krebs (1958) and phosphorylase kinase according to Cohen (1973). In order to remove a protein of mol wt 100,000 which contaminated the phosphorylase kinase prepared by the above method, the enzyme was further purified by chromatography on DEAE-cellulose (Serva). The gel was equilibrated with buffer containing 50 mM β-glycerophosphate, 2 mM EDTA, and 1 mM dithioerythritol at pH 7.0. A column (2.5 cm i.d., bed height 35 cm) was loaded with *ca.* 8×10^5 units and eluted by a linear NaCl gradient (0–0.5 M) of a total volume of 1.2 l. A muscle extract was prepared as described earlier (Heilmeyer *et al.*, 1970; Krebs *et al.*, 1964). An equal volume of a buffer containing 20 mM β-glycerophosphate, 40 mM mercaptoethanol, 4 mM EDTA, 40% sucrose, and 10^{-4} M phenylmethylsulfonyl fluoride (pH 7.0) was added. This solution (crude extract) contains in 1 ml *ca.* 50 units of phosphorylase, 300 units of phosphorylase kinase, 5.0 units of phosphorylase phosphatase, 0.2 unit of glycogen synthetase, $0.5–1.0 \times 10^{-3}$ unit of protein kinase, and 6 mg of protein.

The method employed for the activation of the Sepharose (Pharmacia) is based on the procedures described by Cuatrecasas (1970) and Porath *et al.* (1973). Cyanogen bromide was dissolved in absolute dioxane (30–50% w/v). In

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Table I: Covalent Substitution of Sepharose with Alkylamines.[a]

BrCN (mg/ml)	μmol of Methylamine/ml of Packed Sepharose	BrCN (mg/ml)	μmol of Ethylamine/ml of Packed Sepharose	BrCN (mg/ml)	μmol of Butylamine/ml of Packed Sepharose
3.2	5.7	0.43	3.3	0.29	2.9
9.7	12.1	1.4	7.2	1.4	8.5
19.5	20.7	3.6	9.6	3.6	15.6
70.0	32.5	10.7	19.2	14.3	24.0
		21.4	25.1	21.0	36.8
		71.0	32.6	75.0	51.0

[a] The moles of amine linked to 1 ml of each gel depending on the cyanogen bromide concentration in the incubation mixture were determined in duplicate as described in the text. For other conditions see text.

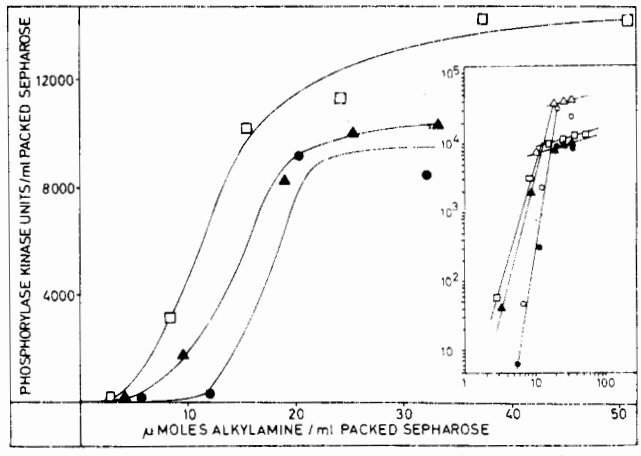

FIGURE 1: Adsorption of phosphorylase kinase from a crude extract on alkylamine–Sepharose derivatives. The amount of adsorbed enzyme activity per milliliter of packed Sepharose was calculated from the difference between the total amount of applied units and the amount excluded from the gel. Each column contained *ca.* 10 ml of packed Sepharose. Insert: Double logarithmic plots of adsorbed phosphorylase kinase as a function of the degree of substitution. Purified phosphorylase kinase (8.2–9.6 × 10^3 units/mg at pH 8.6) was applied in a concentration of 1.3 mg/ml to *ca.* 10 ml of packed agarose derivative as described under Materials and Methods. Unity of ordinates and abscissas is identical in both figures. The definition of 1 ml of packed Sepharose and the determination of the amount of bound alkylamine are described in Materials and Methods: methylamine–Sepharose, (●) crude extract, (○) homogeneous enzyme; ethylamine–Sepharose, (▲) crude extract, (△) homogeneous enzyme; butylamine–Sepharose, (□) crude extract.

order to use reproducible amounts of cyanogen bromide, the concentration was determined iodometrically before each activation of Sepharose. A typical activation mixture contained 25 g wet weight Sepharose (= Sepharose from which the outer liquid phase has been removed by suction on a Büchner funnel), 12 ml of 5 M phosphate (pH 12.0), 12 ml of H_2O, and 0.1–5 g of BrCN dissolved in 20 ml of dioxane. The reaction was allowed to proceed for 12 min at 20°. When necessary ground ice prepared from deionized water was added to control the temperature and 5 M NaOH was added to keep the pH above 11. The reaction was terminated by washing this gel with 750 ml of 0.1 M β-glycerophosphate buffer (pH 7.0, 0°) in a Büchner funnel. The dry Sepharose was then added to 50 ml of 2 M alkylamine solution (pH 10.0) which was labeled with the corresponding [^{14}C]alkylamine (New England Nuclear), 0.25 μCi/ml. The butylamine was labeled with [^{14}C]ethylamine. The coupling reaction was allowed to proceed for 16 hr at 5°. The gel was then successively washed with 10–20 vol each of H_2O, 0.01 M HCl, 0.01 M NaOH, and finally again H_2O. The material was stored at 5°. In order to determine the amount of covalently linked alkylamine on the Sepharose a standardized amount of the gel was hydrolyzed in 32% HCl. An aliquot, *ca.* 1 ml of the agarose suspension, was applied to a small column custom made from a 5-ml glass pipet. The Sepharose was allowed to settle and subsequently the column was allowed to run dry by gravity. The volume of the *packed* Sepharose was determined from the bed height in the graduated column. One milliliter of packed Sepharose contains *ca.* 5 × 10^6 agarose spheres as determined by counting the spheres suspended in 1 mg/ml of bovine serum albumin (Serva) in a Neubauer cell. The bottom adapter of the column was then removed and the total contents blown into a test tube. An equal volume of 32% HCl was added and the mixture hydrolyzed for 45 min at 50°. After addition of 0.1 ml of 20 N NaOH to the hydrolysate it was transferred to 18 ml of a scintillation fluid as described by Feldmann *et al.* (1972). The results are shown in Table I. Even at the highest concentration of cyanogen bromide used only *ca.* 0.3% of the added amine was covalently linked to the agarose. Since it is known that by the reaction of cyanogen bromide activated agarose with α-alkylamines positively charged groups are introduced into the matrix (Porath, 1968), the product was tested in this respect. At a concentration of *ca.* 7, 23, and 70 mg of cyanogen bromide per ml of reaction *ca.* 1, 3.7, and 8.3 μequiv of basic groups were titratable per milliliter of packed Sepharose, respectively. Before adsorption of enzymes the alkylamine–Sepharose was equilibrated with buffer composed of 10 mM β-glycerophosphate, 20 mM mercaptoethanol, 2 mM EDTA, 20% sucrose, and 5 × 10^{-5} M phenylmethylsulfonyl fluoride (pH 7.0). Plexiglas columns of the dimensions 2 cm i.d. × 15 cm were filled with 5–15 ml of the gel. Purified phosphorylase kinase, phosphorylase *b*, or crude extract was added to the columns until no more enzyme was adsorbed. The columns were washed (10- to 50-fold volume) until enzymatic activity could no longer be detected in the eluate.

Elution was accomplished by salt (see figure legends) in a stepwise or gradient fashion. Fractions of 5 ml were collected. After use gels could be regenerated by washing with 10–20 vol of 1 M NaCl, H_2O, 10 mM NaOH, 10 mM HCl, and H_2O, respectively.

Results

The capacity of the gels for phosphorylase kinase, *i.e.* the amount of enzyme per milliliter of Sepharose adsorbed from a crude extract, increases exponentially as a function of the degree of substitution and reaches plateau values with the methyl-, ethyl-, and butylamine (Figure 1). In comparison to the methylamine substituted agarose the exponential increase is obtained with ethylamine at a lower degree of substitution and this curve is shifted to a still lower level by employing butylamine. Similarly, sigmoidal curves are obtained if the capacities of the gels are determined with purified phosphorylase kinase yielding, how-

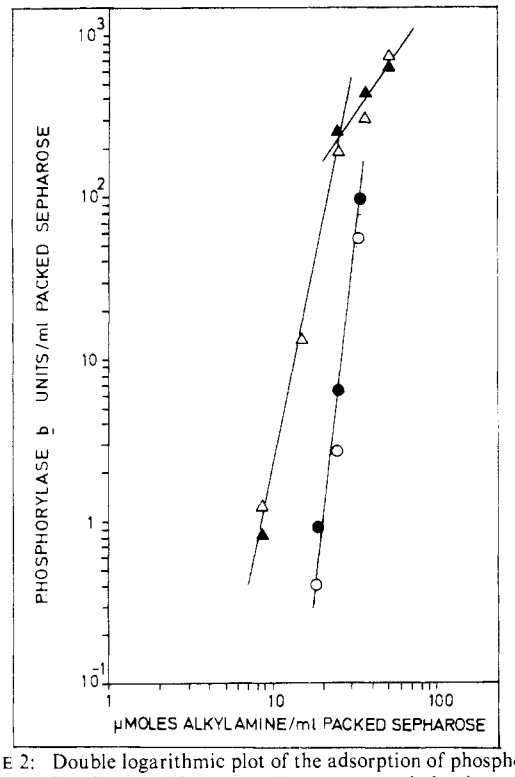

FIGURE 2: Double logarithmic plot of the adsorption of phosphorylase *b* in the purified form and from a crude extract on ethylamine- and butylamine-Sepharose. Purified phosphorylase (76 units/mg) was applied in a concentration of 1 mg/ml. Adsorption of phosphorylase *b* from a crude extract is described under Materials and Methods. The amount of adsorbed enzyme was calculated as described in the legend of Figure 1. For the definition of 1 ml of packed Sepharose and the determination of the amount of bound amine see Materials and Methods: ethylamine-Sepharose, (●) crude extract, (○) purified phosphorylase; butylamine-Sepharose, (▲) crude extract, (△) purified phosphorylase.

Table II: Enzymes of Glycogen Metabolism Adsorbed from a Crude Extract by Methylamine-Sepharoses of Increasing Hydrophobicity.[a]

	Units/ml of Packed Sepharose				
μmol of Methylamine/ml of Packed Sepharose	Phosphorylase *b*	Phosphorylase Kinase	Phosphorylase Phosphatase	Protein Kinase	Glycogen Synthetase
5.7	—	6.5	—	—	—
12.1	0.8	312	0.12	0.147	21.7
20.0	0.7	9603	9.1	2.74	216
32.5	61	8324	18.7	4.25	228

[a] The amount of covalently linked amine and the adsorbed proteins were determined as described under Materials and Methods and in the legend of Figure 1. Dashes indicate that enzyme activity was not detectable in the eluate.

ever, a *ca.* 4 times higher value in the plateau region (compare insert of Figure 1).

If the data are plotted in a double logarithmic form (see insert, Figure 1), the sigmoidal curves can be linearized. The first part of the sigmoidal curves corresponds to the steep slope whereas the plateau region yields low increments. As shown with agaroses carrying methyl or ethyl residues the slopes are the same whether the enzyme is adsorbed from a mixture of proteins or from a solution containing the pure enzyme.

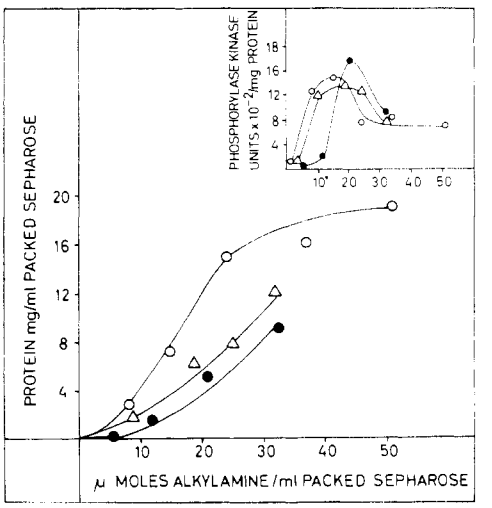

FIGURE 3: Adsorption of protein from a crude extract on alkylamine-Sepharose derivatives. The amount of protein adsorbed was calculated analogous to the determination of adsorbed enzyme activity as described in the legend of Figure 1. Saturation of the gel was taken as the amount of crude extract applied when the enzyme activities tested were excluded. Insert: Specific activity of phosphorylase kinase adsorbed under the conditions described above. Unity of the abscissas is identical in both figures. For the definition of 1 ml of packed Sepharose and the determination of bound amine see Materials and Methods: (●) methylamine-Sepharose; (△) ethylamine-Sepharose; (○) butylamine-Sepharose.

Phosphorylase *b*, an enzyme of very different structure and function, shows the same behavior. Again, linear relationships are found if the capacity of the gel is plotted as a function of the degree of substitution in the double logarithmic form (Figure 2). There is again no difference in the slope whether the crystalline enzyme or the enzyme present in a crude extract is employed.

In addition, several other enzymes, phosphorylase phosphatase, 3′,5′-cAMP-dependent protein kinase, and glycogen synthetase can be adsorbed on these Sepharose derivatives. Table II shows that the capacity of the methylamine-substituted agarose for these enzymes rises as a function of the hydrophobicity of the gel in the same manner as observed with phosphorylase kinase and phosphorylase *b*. Finally protein in general follows the same pattern of adsorption when a crude extract is applied to these three types of agarose (Figure 3). These curves can also be linearized by double logarithmic plotting (not shown). From the amount of enzymatic activity adsorbed on an agarose gel (32.5 μmol of methylamine/ml, Table II) and the known specific activities of the purified enzymes it can be calculated that in this case they comprise *ca.* 23% of the total protein. The specific activity of phosphorylase kinase (calculated from the data above) in the adsorbed state shows distinct maxima for each type of substituted Sepharose (insert Figure 3). Furthermore, a change of the alkylamine from methyl- to butylamine shifts this maximum from 20 to 15 μmol of bound amine/ml of packed Sepharose.

The amount of covalently linked alkylamine per milliliter of packed Sepharose capable of adsorbing 1 mg of protein can be taken as a characteristic value of the binding property of the gel. Thus, this standard amount of either crude extract protein, purified phosphorylase kinase, or crystallized

Table III: Stepwise Elution of Enzymes of Glycogen Metabolism from Methylamine-Sepharoses of Increasing Hydrophobicity.[a]

Elution Step	μmol of of Methyl-amine/ml of Packed Sepharose	Units/ml of Packed Sepharose				
		Phospho-rylase *b*	Phospho-rylase Kinase	Phosphorylase Phosphatase	Protein Kinase	Glycogen Synthetase
50 mM	5.7	—	6.5	—	—	—
NaCl	12.1	0.8	301	0.12	0.147	21.7
	20.0	0.65	6554	2.0	1.4	146.
	32.5	32.0	353	0.3	0.014	32.0
120 mM	5.7	—	—	—	—	—
NaCl	12.1	—	7.3	—	—	—
	20.0	—	2839	1.6	1.2	58.0
	32.5	16.0	1304	1.8	0.04	35.0
1 M	5.7	—	—	—	—	—
NaCl	12.1	—	3.6	—	—	—
	20.0	—	210	1.2	0.09	12.0
	32.5	14.0	6666	16.6	4.2	161

[a] The gels used for the determinations in Table II were eluted with buffer containing salt as shown. Dashes indicate that enzyme activity was not detectable in the eluate. The amount of covalently linked amine and the adsorbed proteins were determined as described under Materials and Methods and in the legend of Figure 1.

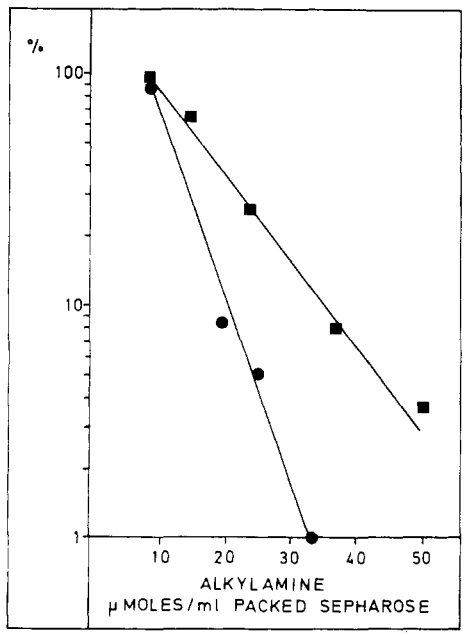

FIGURE 4: Semilogarithmic plot of the fraction of enzyme liable to elution by a buffer containing 50 mM NaCl: (■) butylamine-Sepharose, purified phosphorylase *b*; (●) ethylamine-Sepharose, purified phosphorylase kinase. One-hundred per cent is that amount of enzyme adsorbed to the alkylamine-Sepharose derivatives under the conditions described under Materials and Methods and in the legend to Figures 1 and 2.

phosphorylase *b* is adsorbed by ethylamine substituted agarose containing 7, 19, and 34 μmol/ml, respectively.

The fraction of purified phosphorylase kinase, which can be eluted from Sepharose substituted with ethylamine by a buffer containing 50 mM NaCl, decreases exponentially with increasing hydrophobicity of the gel (Figure 4). The residual amount of adsorbed enzyme can be eluted if the salt concentration is raised to 1 M. In this way even from the gel of the highest degree of substitution (32 μmol of amine/ml) up to 80% of the adsorbed enzyme can be recovered (not shown). The same relationship between the exponential decrease of desorbable phosphorylase *b* and the hydrophobicity of the gel is observed on Sepharoses carrying butylamine residues (Figure 4). Only *ca.* 60% of this adsorbed enzyme on a gel with the highest hydrophobicity (51 μmol of amine/ml) can be desorbed by buffer containing 1 M NaCl (not shown). Similar patterns of elution are found for phosphorylase phosphatase, glycogen synthetase, and 3′,5′-cAMP-dependent protein kinase. The amount of activity of these enzymes which can be eluted with different salt concentrations from methylamine substituted Sepharoses is shown in Table III. Generally, that fraction of enzyme activity eluted with buffer containing 50 mM NaCl decreases, whereas the fraction subsequently eluted with buffer containing 1 M NaCl increases as a function of the hydrophobicity of the gel. The type of salt employed to elute phosphorylase kinase determines the concentration which is needed to obtain desorption (Figure 5). Sepharose substituted with ethylamine (25 μmol/ml) was saturated with this enzyme and gradients employing salts chosen from the Hofmeister series (von Hippel and Schleich, 1969) were used. As can be seen the ionic strength of the peak fraction increases parallel with the salting out effect of the employed cations and anions; concurrently the sharpness of the peaks declines.

Uniquely, phosphorylase *b* can be eluted by adjusting the buffer to slightly acidic pH values as shown by Er-el and Shaltiel (1974). Employing a pH gradient from 7.0 to 5.6 the peak fraction has a pH value of 6.2. Using this latter technique, phosphorylase *b* can be enriched *ca.* eightfold (Table IV) from a crude extract with a yield of *ca.* 80%. Following ammonium sulfate precipitation (42% saturation) the enzyme can be crystallized, total purification being *ca.* tenfold. Since phosphorylase kinase is not eluted at the low pH value, subsequent elution with buffer containing 1 M NaCl leads to a *ca.* 13-fold purified enzyme (Table IV). Optimally, *ca.* 20-fold enrichment of this latter enzyme is obtained with a methylamine-substituted Sepharose (20 μmol/ml) employing 50 mM NaCl in the elution buffer. From an agarose containing 32 μmol of methylamine/ml phosphorylase phosphatase was enriched *ca.* 25-fold (yield

Table IV: Differential Elution and Purification of Phosphorylase *b* and Phosphorylase Kinase on Butylamine-Sepharose.[a]

Enzyme	Crude Extract		Elution at pH 5.6		Elution with 1 M NaCl	
	Units Adsorbed/ml of Sepharose	Units/mg	Units Eluted/ml of Sepharose	Units/mg	Units Eluted/ml of Sepharose	Units/mg
Phosphorylase *b*	259	8	202	60	10	1.8
Phosphorylase kinase	11,155	105	3	1.4	7651	1362

[a] The butylamine-Sepharose employed contained 24 μmol of amine/ml of matrix. The gel volume indicates packed Sepharose. Adsorption of enzymes from a crude extract is described under Materials and Methods. Conditions for the elution are described in the text.

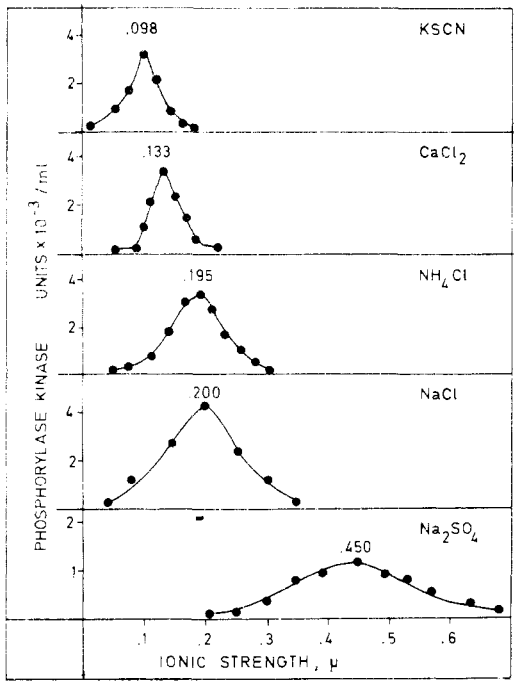

FIGURE 5: Desorption of purified phosphorylase kinase from ethylamine-Sepharose (25 μmol/ml) with salt gradients of different ionic composition. Each column with 5 ml of the above gel was loaded with *ca.* 11 mg of purified phosphorylase kinase (see legend of Figure 1). The linear gradients were produced from 100 ml of adsorption buffer and 100 ml of salt containing buffer. The ionic strength of these salts in the latter buffer was for KSCN 0.5, $CaCl_2$ 0.48, NH_4Cl 0.75, NaCl 1.5, and Na_2SO_4 0.75. The gradients were controlled by measurements of the conductivity. The number at the maximum of the elution profiles indicates the ionic strength of the peak fraction.

ca. 110%) and 3′,5′-cAMP-dependent protein kinase, *ca.* 19-fold (yield *ca.* 111%), after first washing with 120 mM NaCl and then employing 1 M NaCl to elute these enzymes. Optimal enrichment of glycogen synthetase (*ca.* threefold, yield *ca.* 90%) was obtained on ethylamine-substituted Sepharose (32 μmol/mol) in the same manner.

Discussion

By variation of the concentration of cyanogen bromide in the activation mixture the amount of hydrophobic residues on the gel, *i.e.* its hydrophobicity, could be varied. The adsorbed amount of phosphorylase kinase, phosphorylase *b*, phosphorylase phosphatase, glycogen synthetase, and 3′,5′-cAMP dependent protein kinase, as well as protein in general, increases exponentially if the degree of substitution of the gel is enhanced. As shown exemplarily for phosphorylase kinase and phosphorylase *b* this exponential increase occurs in a very narrow range of hydrophobicity change. Augmentation of ethylamine residues from 0.36×10^{12} to 1.2×10^{12}/sphere (calculated from a number of 5×10^6 spheres/ml of packed Sepharose; see Materials and Methods) causes an enhancement of adsorbed phosphorylase kinase molecules from 4.9×10^5 to 4.9×10^8/sphere. Concomitant with the enhancement of ethylamine residues on a Sepharose sphere the amount of positively charged groups increases from 0.42×10^{11} to 1.10×10^{11}. Since the same number of charged groups is introduced into methylamine-Sepharose, which does not adsorb phosphorylase kinase in this range of substitution (compare Figure 1), the charge cannot be the cause for the observed adsorption of this enzyme on the ethylamine-substituted gels.

It could be assumed that the covalently linked alkyl molecules cannot be reached by a protein at a low degree of substitution. Additional fixation of alkyl residues to the matrix could enhance only that fraction of hydrophobic centers which is accessible to the enzyme, thus leading to the observed exponential increase. However, this explanation is unlikely. When the amount of adsorbed phosphorylase *b* is at the lowest limit of detection an ethylamine, substituted Sepharose contains *ca.* 2.3×10^{12} residues/sphere. Since phosphorylase kinase of mol wt 1.3×10^6 can be adsorbed on an ethylamine-substituted agarose which contains *ca.* 6 times less alkyl residues/sphere than is needed to adsorb phosphorylase *b* of mol wt 2×10^5 (see above), it is very improbable that only the former enzyme has access to alkyl residues at a low degree of substitution. Furthermore, the ratio of alkyl residues to enzyme molecules adsorbed decreases during the exponential increase of adsorbed amount of enzyme as a function of the hydrophobicity of the gel from 7.4×10^5 to 2.9×10^3 for phosphorylase kinase and from 7.6×10^4 to 8.7×10^3 for phosphorylase *b*.

Therefore, it can be concluded that the availability of alkyl residues is not restricted, but that apparently a critical hydrophobicity of the gel is needed in order to obtain adsorption of a certain protein. This critical hydrophobicity can be produced either by an increase in the amount of residues per sphere as demonstrated here or by elongation of the employed alkylamine. For example, 1 ml of gel containing 20 μmol of ethylamine adsorbs only *ca.* 0.6 unit of phosphorylase *b* and the equivalently substituted butylamine Sepharose absorbs *ca.* 100 units (compare Figure 2). Approximately the same conditions as above were employed by Er-el *et al.* (1972) and Shaltiel and Er-el (1973) who in agree-

ment with these results found that phosphorylase *b* can be adsorbed by the butylamine derivative whereas matrices carrying lower homologous amines excluded the enzyme. When gels containing 6 μmol of amine/ml are compared in respect to their adsorption capacity for phosphorylase kinase (crude extract) 1 ml of methylamine derivative binds *ca.* 6 units, whereas the ethyl and butyl derivatives bind *ca.* 800 and 1000 units, respectively (Figure 1).

Saturation characteristics seen as a general phenomenon with all enzymes tested might be due to total coverage of the available, effective surface of the sphere. Therefore, an additional increase in the number of covalently linked amine molecules/sphere results mainly in a higher force of binding of the protein (see below) and not in an increase in the amount of adsorbed protein. Alternatively, saturation characteristics could result from intermolecular interactions of the hydrophobic groups which may form metamers at high densities on the Sepharose sphere.

Proteins may, however, also compete for hydrophobic centers. If to a series of gels of different hydrophobicity a crude extract containing hydrophobically differing proteins is applied, the enzyme of the highest hydrophobicity will be adsorbed by the gel of the lowest degree of substitution. As the number of alkyl residues on the matrix increases, proteins of lower hydrophobicity are additionally adsorbed. A comparison of Figures 1 and 2 shows that at the degree of substitution with ethylamine where adsorption of phosphorylase kinase (an enzyme of high hydrophobicity) levels off into a plateau region phosphorylase *b* adsorption begins (see also Table II). As the hydrophobicity of the gel is further enhanced, adsorption of proteins of low hydrophobicity, *e.g.*, phosphorylase *b*, increases exponentially. Therefore, the specific activity of the enzyme adsorbed first must go through a maximum (shown for phosphorylase kinase, Figure 3) as a function of the hydrophobicity of the gel.

Even though an increase of the titratable basic groups is found on the substituted gels, ionic interactions cannot be responsible for the adsorption of these enzymes. For example, phosphorylase *b* is not adsorbed on DEAE-cellulose carrying 90 μequiv of basic groups/ml packed matrix under the conditions employed here (compare Mayer and Krebs, 1970). A methylamine-substituted Sepharose which contains *ca.* 8 μequiv of titratable basic groups, however, binds 61 units (0.75 mg) of phosphorylase *b*/ml of packed gel (see Table II and Materials and Methods). As shown here and by Er-el *et al.* (1972) and Er-el and Shaltiel (1974), employing an agarose with a constant degree of substitution adsorption of phosphorylase *b* can be accomplished by increasing the chain length alone, which does not alter the amount of titratable groups. It may be that the hydrophobicity of the gel is a function of both the amount of alkyl residues linked to the matrix and the number of ionic and hydroxyl groups present. However, it can be concluded from the data that hydrophobic interactions are the primary factor for the adsorption of proteins.

The increased hydrophobicity causes a tighter binding of the adsorbed protein to the matrix as shown by the exponential decrease of the fraction of enzyme elutable with buffer containing 50 mM salt. The same phenomenon can be observed if the chain of the employed amine is elongated (Er-el *et al.*, 1972).

As demonstrated the elution power of the anions corresponds directly to the order of the Hofmeister series (von Hippel and Schleich, 1969; Lewin, 1974) and that of the cations to the reverse order in respect to the salting-out or - in effect. The salt might directly penetrate into the interphase between the protein and the hydrophobic surface of the Sepharose sphere leading to desorption. If ionic interactions were responsible for the adsorption, protein elution would depend on the ionic strength and not on the salt species employed.

The ability to devise agarose gels of a specific hydrophobicity should enable one to find the critical value for the adsorption of the protein under study. Often a gel of low hydrophobicity can be employed to eliminate the proteins of higher hydrophobicity than the protein to be purified. Finally the type of salt best suited to elute the protein must be determined. It should then be possible to employ hydrophobic chromatography as a standard procedure (*e.g.*, gel filtration, ion exchange chromatography, etc.) for the purification of proteins in general.

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Hybridization of Ribosomal RNA Labeled to High Specific Radioactivity with Dimethyl Sulfate†

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ABSTRACT: RNA radioactively methylated with dimethyl sulfate has the advantage of relatively low background noise level when utilized in DNA saturation hybridization employing the membrane filter technique. In addition, the RNA can be methylated with either ^{3}H- or ^{14}C-labeled methyl groups. However, the low specific radioactivity usually obtained with dimethyl sulfate has limited the use of this labeling technique. We describe a detailed characterization of the methylation of rRNA with dimethyl sulfate giving specific radioactivities on the order of 10^5 cpm/μg. Kinetics and optimum conditions for the methylation reaction of rRNA were studied. The methylation did not cause excessive degradation of RNA in neutral aqueous solution, and the methyl derivative of RNA was stable under normal hybridization conditions. Specific radioactivity of the methylated RNA was found to be a linear function of the product of RNA concentration and specific radioactivity of the dimethyl sulfate in the reaction mixture at a constant incubation time. The methylated bases of the RNA lowered the thermal stability of the DNA–RNA hybrids by 1° in T_m per 1.6% methylated RNA bases. rRNA gene dosage values using high specific radioactive methylated RNA were determined for mouse and human liver tissues and were found to be 81 and 180 genes/haploid genome, respectively. Dissociation constants of the hybridization reaction ranged from 0.90×10^{-10} to 2.37×10^{-10} M.

A problem frequently encountered in designing DNA–RNA hybridization experiments is to obtain either DNA or RNA at sufficiently high specific radioactivities. These high specific radioactivities are particularly difficult to achieve in *in vivo* experiments using whole animals because of the low efficiency of label incorporation. A rapid, simple, and economical means of introducing sufficient amounts of radioactive label into nucleic acids would therefore be of considerable advantage. The radioactive labeling of purified nucleic acids *in vitro* has several important advantages. In addition to being able to obtain nucleic acids at a high specific radioactivity more economically, different nucleic acid species can be radioactively labeled at a uniform specific radioactivity independent of their *in vivo* nucleotide precursor pool size or synthesis rate.

Radioisotopes may be introduced into nucleic acids *in vitro* by several methods. The *in vitro* enzymatic synthesis of RNA by DNA-dependent RNA polymerase (Melli and Pemberton, 1972) and the enzymatic synthesis of DNA by RNA-dependent DNA polymerase (Kacian *et al.,* 1972) in the presence of labeled nucleotides have been used to obtain nucleic acids with high specific radioactivities. The disadvantages of these techniques are the requirements for a suitable enzyme and a highly purified template and the resultant low fidelity and nonuniformity of copying. Another method is to label nucleic acids directly by chemical modification. This has been achieved by halogenation (Commerford, 1971), photoreduction (Lee and Gordon, 1971), and alkylation (Smith *et al.,* 1968). While iodination affords a means of labeling DNA and RNA to as much as 10^7 cpm/μg (Tereba and McCarthy, 1973; Scherberg and Refetoff, 1973), the isotope is not randomly introduced into the preformed polynucleotide but reacts exclusively with cytosine (Commerford, 1971). In addition, there are difficulties encountered with the stability of the label (Holmes and Bonner, 1974; Getz *et al.,* 1972; Scherberg and Refetoff, 1973), excessively high nonspecific background binding to membrane filters (Scherberg and Refetoff, 1973; Tereba and McCarthy, 1973), and loss of the ability to renature when extensively iodinated (Commerford, 1971). The *in vitro* introduction of label into nucleic acids by photoreduction of uracil and thymine with [^{3}H]borohydride circumvents some of the problems of iodination (Lee and Gordon, 1971), but rather low specific radioactivities are obtained, and the isotope is not randomly introduced (Cerutti *et al.,* 1965).

Dimethyl sulfate has been used to introduce label into RNA (Smith *et al.,* 1968) and DNA (Akiyoshi and Yamamoto, 1970). The background noise level using the membrane filter technique for methylated RNA ranges from 0.003 to 0.01% of total input counts, whereas the level normally obtained for iodinated RNA is tenfold greater—which seriously limits sensitivity. Polynucleotides were found to be randomly labeled with no apparent degradation (Smith *et al.,* 1968; Lawley and Shah, 1972) and nucleic acids could be labeled with either ^{3}H or ^{14}C-labeled di-

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