# Gels Mimicking Antibodies in Their Selective Recognition of Proteins

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## Key Words

Affinity chromatography
Entrapment
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## Summary

In a previous paper we presented preliminary experiments aimed at the preparation of gel particles with the property to recognize selectively some particular protein (hemoglobin, cytochrome C, transferrin) [1]. Using the same method we show in this article that human growth hormone, ribonuclease and myoglobin from horse can also be adsorbed specifically, indicating that the method may be universal or at least applicable to a great number of proteins. A gel with specific adsorption of three model proteins was synthesized in order to demonstrate that the beds can be employed to remove (traces of) several proteins contaminating a sample ("negative purification"). The degree of selective recognition is high, to judge from the fact that myoglobin from horse, but not that from whale, was adsorbed onto a column designed to bind specifically the former protein. This selectivity is noteworthy, since these two proteins have similar amino acid sequences and 3-D structures. The method for the synthesis of the specific gels involves polymerization of appropriate monomers (for instance acrylamide and its derivatives) in the presence of the protein to be adsorbed specifically, granulation of the gel formed, packing a column with the gel particles, washing the column to remove the protein and finally application of the sample for selective adsorption of the protein. The approach resembles that used for entrapment (immobilization) of proteins for affinity chromatography and that for molecular imprinting, with the distinct difference that the monomer composition is quite different and thereby the binding mechanism. This mechanism is discussed, for instance, in terms of (1) a new classification system for chromatographic beds based on the number of bonds between the solute and the matrix and the strength of each bond and (2) "non-specific bonds" (these bonds are often harmful in conventional chromatography, but we have used them to advantage). In this classification system the selective recognition is characterized by a large number of weak bonds. Therefore, so-called functional monomers are not used for the preparation of the gels because they often are charged and, accordingly, give rise to strong electrostatic interactions, i.e. the beds behave to some extent as ion-exchangers. In most experiments we have used a polyacrylamide gel with large pores to facilitate diffusion of proteins into and out of the gel granules. When used in chromatography these soft gels (which can be used repeatedly) allow only rather low flow rates. This problem can be overcome by a new approach to prepare the granules. Potential applications of the selective beds are discussed, as well as future improvements.

## Introduction

Recently, we described briefly a technique for selective adsorption of a protein (1). The method is based on the preparation of a gel (for instance crosslinked polyacrylamide) in the presence of the protein of interest, granulation of the gel, packing a chromatographic column with the granules and, finally, removal of the protein. Upon application of a sample containing this protein and other proteins only the same protein will become adsorbed. The preparation of the selective bed is thus similar both to (1) that employed in affinity chromatography for immobilization of a protein (for instance an antibody) by entrapment of the protein in a polyacrylamide gel [2, 3], although the composition of the entrapping gel must differ from that of "our" selec-



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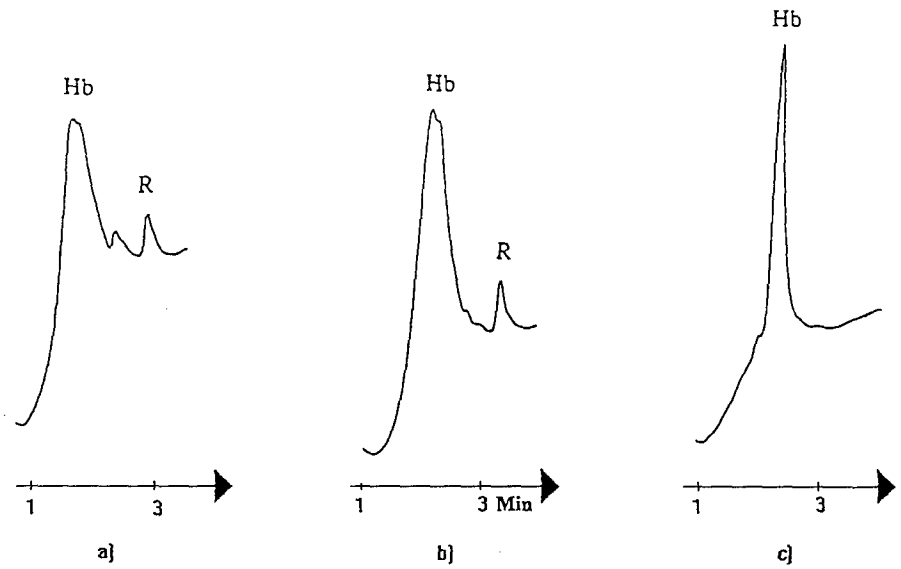


**Figure 1**

Selective recognition of ribonuclease. Analysis on a cation exchanger of a) the sample itself (consisting of hemoglobin (Hb) and ribonuclease (R)) prior to passage through any column; b) the fraction collected from a blank column (prepared in the absence of ribonuclease); c) the fraction collected from the column prepared in the presence of ribonuclease. The ribonuclease peak is present in Figures 1a and 1b, but absent in Figure 1c, indicating that ribonuclease had been specifically adsorbed only onto the column prepared in the presence of ribonuclease.

tive gel in that the protein (antibody) should not be desorbed in any step and (2) that used for molecular imprinting [4–12], with the important difference that the degree of crosslinking is relatively low and that we do not include special, so-called functional monomers in the monomer solution since they may decrease drastically the possibility to get a high specificity toward macromolecules (see Discussion).

In this paper we present further studies of the selective beds and discuss the mechanism of the selective recognition.

## Experimental

### Materials

Ribonuclease, lysozyme, myoglobin from horse and whale were obtained from Sigma (St. Louis, MD, USA). Growth hormone was a gift from Professor Paul Roos, this department, and human serum albumin from Dr Lars-Olov Andersson, KABI (Stockholm, Sweden). Hemoglobin was prepared from normal human serum. Acrylamide, N,N′-methylenebisacrylamide, ammonium persulfate, N, N, N′,N′-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), and a cation-exchanger (CB-S, i.d. 7 mm, length 40 mm) were from Bio-Rad Laboratories (Hercules, CA, USA). An anion-exchanger (Poros Q/H, i.d. 4 mm, length 50 mm) was purchased from PerSeptive Biosystems (Cambridge, MA, USA).

### Preparation of a Ribonuclease-Specific Gel

Acrylamide (0.0582 g), N,N′-methylenebisacrylamide (0.0018 g) and ribonuclease (0.003 g) were dissolved in 1 mL of 0.01 M sodium phosphate, pH 7.0. Following addition of 20 µLof a 10 % (w/v) solution of ammonium persulfate and deaeration, 20 µL of a 5 % (v/v) TEMED solution was added. The polymerization proceeded for thirty minutes, producing a gel with the total concentration (T) = 6 % (w/v) and the crosslinking concentration (C) = 3 % (w/w); for definition of T and C, see. Ref. [13]. The formed gel was then pressed through a 60-mesh net to produce granules, which were packed into a Pasteur pipette with glass wool in the constriction as support for the gel particles. The Pasteur pipette had i.d. = 5 mm and the bed height was 4.5 cm. The granules were washed with 0.8 mL of a solution of Savinase (a proteinase obtained from Novo Nordisk A/S, Denmark) to remove ribonuclease and equilibrated with 3 mL of 0.01 M sodium phosphate, pH 7.0. About 50 µL of a sample solution of hemoglobin (10 mg $mL^{-1}$) and ribonuclease (3 mg $mL^{-1}$) was applied. The column was then washed with 0.01 M sodium phosphate, pH 7.0, and a 500-µL fraction was collected and analyzed by cation-exchange chromatography at 220 nm. The results are shown in the chromatogram traces of Figure 1. Figure 1a is a chromatogram of the sample itself prior to passage through any column ("Hb" designating hemoglobin and "R" designating ribonuclease); Figure 1b is a chromatogram of the fraction collected from a blank column (prepared in the absence of ribonuclease); and Figure 1c is a chromatogram of the fraction collected from the column prepared in the presence of ribonuclease. The ribo-

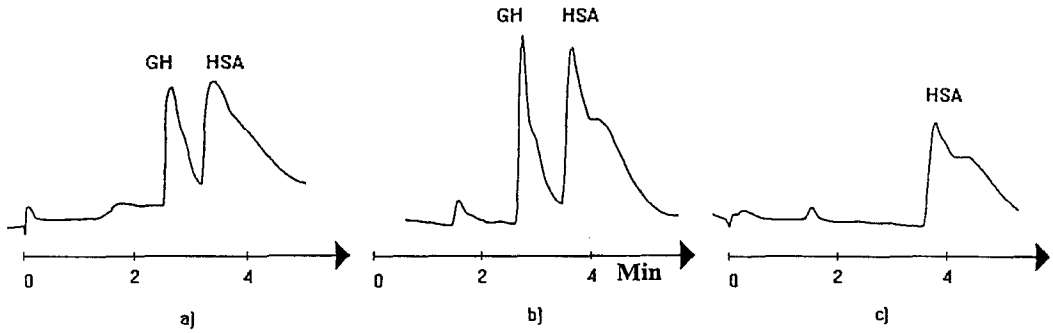


**Figure 2**

Selective recognition of human growth hormone. The sample, consisting of human growth hormone (GH) and human serum albumin (HSA), was applied (1) directly on an anion exchanger for analysis (chromatogram a); (2) on a blank column prepared in the absence of growth hormone and the eluated protein fraction was analyzed on the same ion-exchanger (chromatogram b); (3) on a column prepared in the presence of growth hormone and the eluated protein fraction was analyzed on the ion-exchanger (chromatogram c). A comparison of the three chromatograms shows that the growth hormone was adsorbed only onto the gel synthesized for specific recognition of this protein (i.e., the gel prepared in the presence of the growth hormone).

nuclease peak is present in Figures 1a and 1b, but absent in Figure 1c, indicating that ribonuclease was adsorbed only by the column prepared in the presence of ribonuclease.

## Preparation of a Gel for Specific Adsorption of Human Growth Hormone

Human growth hormone (3 mg) was dissolved in 1 mL of 0.1 M sodium phosphate, pH 6.2. Acrylamide (0.0576 g), N,N′-methylenebisacrylamide (0.0024 g) and ammonium persulfate (10 µL of a 10 % (w/v) aqueous solution) were added. The deaerated solution was supplemented with 10 µL of a 5 % (v/v) aqueous solution of TEMED. About thirty min following polymerization, the gel (which had the composition T6, C4) was granulated by pressing it through a 100-mesh net and packed into a Pasteur pipette. To desorb the growth hormone two column volumes of sodium phosphate (0.1 M, pH 6.2) were passed through the column followed by five column volumes of 10 % (v/v) acetic acid containing 10 % (w/v) SDS. The column was washed with the buffer until the pH of the effluent reached 6.2 and no precipitate of dodecyl sulfate could be detected upon addition of potassium chloride (potassium dodecyl sulfate is only slightly soluble in water). A 10-µL volume of the sample, a mixture of growth hormone (5 mg $mL^{-1}$) and human serum albumin (2 mg $mL^{-1}$) in the phosphate buffer, was added. The eluate was analyzed on an anion exchanger using a 5 min linear concentration gradient created by the equilibration buffer and the same buffer containing 0.5 M sodium chloride (Figure 2c). The eluate from a blank column (prepared in the absence of growth hormone) was studied on the same ion exchanger (Figure 2b), as was the original sample (Figure 2a). The chromatograms show that the growth hormone, but not albumin, had adsorbed to the column prepared in the presence of the hormone.

## Can a Bed Be Designed with Specificity to More Than One Protein?

Acrylamide (0.171 g), N,N′-methylenebisacrylamide (0.009 g), hemoglobin (60 µL of a 1 % (w/v) aqueous solution), cytochrome C (5 mg) and lysozyme (5 mg) were dissolved in 2.94 mL of water. Following addition of 30 µL of a 10 % (w/v) aqueous solution of ammonium persulfate and deaeration by aspiration the mixture was supplemented with 30 µL of a 5 % (v/v) aqueous solution of TEMED. The polymerization was allowed to proceed for three hours. The gel, which had the composition T6, C5 was granulated by pressing it through a 100-mesh net and packed into a 5-mL plastic Eppendorf-syringe (the piston was removed), that had been fitted at the outlet with a piece of a cut nylon stocking. The column was washed for 3 h with 10 mL of a 10 % (w/v) SDS solution in 10 % (v/v) acetic acid to desorb the three proteins, but was still somewhat colored, indicating that hemoglobin and cytochrome C (and therefore probably also lysozyme) could not be released completely. The column was equilibrated with 12 mL of 0.1 M sodium phosphate, pH 6.2, for 1 h and was then stored for two days before it was tested for adsorption of hemoglobin, cytochrome C and lysozyme by applying 35 µL of these proteins dissolved in 0.1 M sodium phosphate, pH 6.2, at a final concentration of 0.020, 0.085 and 0.085 % (w/v), respectively. The column was washed with 0.1 M sodium phosphate, pH 6.2, and the eluate was analyzed at 220 nm by cation-exchange chromatography (Figure 3b). Evidently, all three proteins had adsorbed. A similar experiment was performed on a blank column, i.e., a column prepared in the absence of the three proteins (Figure 3a). These Figures show that more than one protein can be adsorbed specifically on the same column.

In a pre-experiment on the same adsorbent we used glass wool to support the bed. Because glass wool ap-

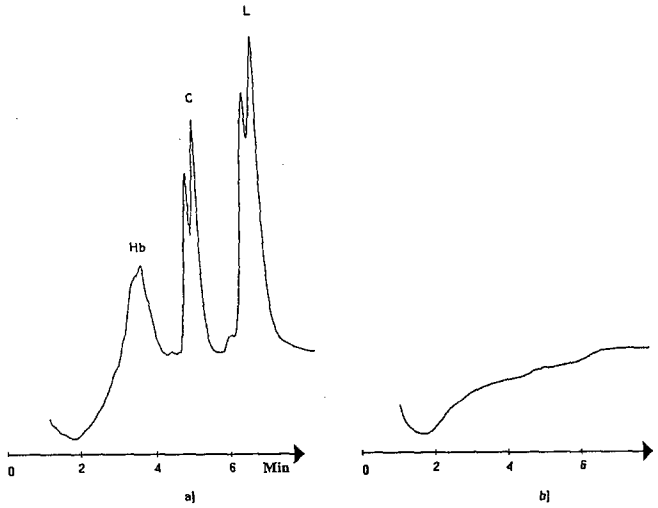


**Figure 3**
Selective recognition of three proteins. Sample: hemoglobin (Hb), cytochrome C (C) and lysozyme (L). Analysis on a cation exchanger of a) the fraction collected from a blank column (prepared in the absence of any protein) and b) the fraction collected from the column prepared in the presence of the sample. A comparison of the two chromatograms indicates that hemoglobin, cytochrome C and lysozyme were adsorbed onto the column prepared in the presence of these three proteins.

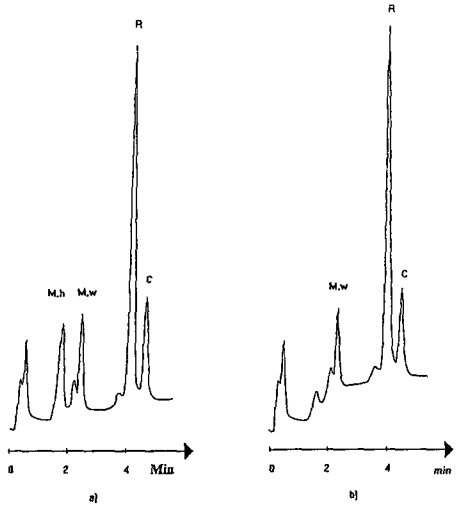


**Figure 4**
Demonstration of the high degree of selective recognition of the adsorbent. Sample: myoglobin from horse (M,h), myoglobin from whale (M,w), ribonuclease (R) and cytochrome C (C). Analysis on a cation exchanger of a) the fraction collected from a blank column prepared in the absence of any protein and b) the fraction collected from a column prepared in the presence of myoglobin from horse. A comparison of the two chromatogram traces shows that myoglobin from horse, but not that from whale, was adsorbed onto the column prepared in the presence of the former protein, indicating a high degree of selective recognition.

peared to interact with hemoglobin we exchanged it for a piece of a nylon stocking in the runs described.

## The Degree of Specificity of the Adsorption

Horse myoglobin (3 mg) was dissolved in 1 mL of 0.01 M sodium phosphate, pH 6.2. Acrylamide (57 μg), N,N′-methylenebisacrylamide (2.5 μg) and 10 μL of a 10 % aqueous solution of ammonium persulfate were added. Following deaeration for one min the solution was supplemented with 5 μL of a 5 % aqueous solution of TEMED. The polymerization proceeded overnight. The gel formed was pressed through a 60-mesh net and packed into a Pasteur pipette (i.d., 5 mm; bed height, 4 cm) with glass wool at the outlet. The column was washed overnight with a 10 % (v/v) solution of acetic acid containing 10 % (w/v) sodium dodecyl sulfate to remove the horse myoglobin and was then equilibrated with 0.01 M sodium phosphate, pH 6.2. This column is denoted "horse myoglobin column". Another column was prepared in the same way, but in the absence of horse myoglobin ("blank column"). Horse myoglobin, whale myoglobin, ribonuclease and cytochrome C were dissolved in 0.01 M sodium phosphate, pH 6.2. The final concentration of each protein was 1 mg $mL^{-1}$. A 10-μL volume of this sample was applied on each of the two columns. Non-adsorbed proteins were eluted with the phosphate buffer used for equilibration of the columns and analyzed on a cation exchanger. Detection was done at 220 nm. The chromatogram in Figure 4a was obtained from the "blank column" and that in Figure 4b from the "horse myoglobin column". Horse myoglobin was adsorbed only on the latter column, and whale myoglobin on neither of the two columns. Figure 5 shows that the 3-D structures of these proteins are similar.

## Selective Bed with Higher Flow Rate

The polyacrylamide beds described above had the composition T = 6 % (w/w) and C = 3–5 % (w/w). The beds described previously had similar composition [1]. From molecular-sieve chromatography experiments it is known that such gels give a relatively low flow rate [13]. Since this can be improved by using denser, i.e., more rigid gels [14], we prepared a bed with the composition T 20, C3. However, the selective adsorption of proteins was low, which could be due to the smaller pore size of these gels with attendant difficulty to remove the protein to be specifically adsorbed. Therefore, we studied another alternative to increase the flow rate: to embed grains of a soft gel with specific adsorption of a certain protein into grains of a rigid gel. The detailed procedure was as follows. 100-mesh granules of a T6, C5 gel specific for adsorption of hemoglobin, were prepared in 1 mL of 0.01 M sodium phosphate pH 7.0, essentially as described for the above "growth hormone column" (the hemoglobin concentration in the monomer solution was 1.2 mg $mL^{-1}$). The column was emptied and the gel particles were pressed three times through a 100-mesh net and mixed with a monomer solution of the composition T20, C3 in the volume ratio 1:2. The same catalyst was used as in the above experiment with the "ribonuclease column" although the volumes of the ammonium per-

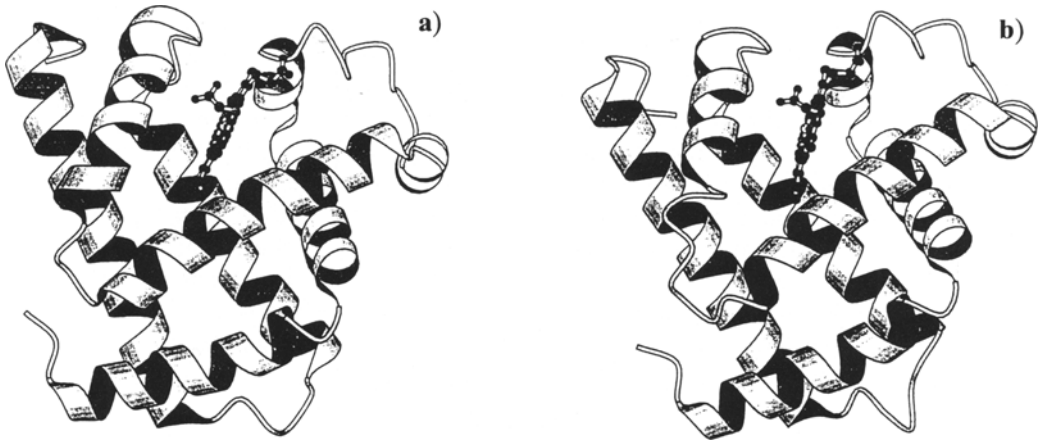


**Figure 5**
The 3-D structure of horse myoglobin (a) and whale myoglobin (b). Despite the great structural similarities only horse myoglobin, but not whale myoglobin, was adsorbed onto the column prepared to be specific for horse myoglobin (Figure 4). The column was thus highly selective.

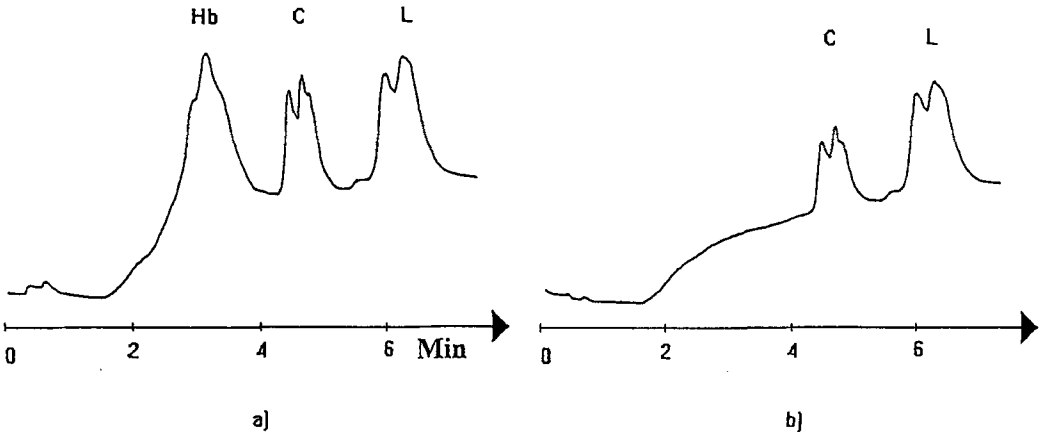


**Figure 6**
Preparation of a selective bed with higher flow rate. A polyacrylamide column of the composition T6 C5 and specific for hemoglobin was prepared essentially as described for the "ribonuclease column" (Figure 1). The column was emptied and the granules were suspended in a monomer solution of the composition T20, C3. Following polymerization the gel was granulated by pressing it through a 100-mesh net and packed in a Pasteur pipette. A blank column was prepared in the same way but in the absence of hemoglobin. A standard solution consisting of hemoglobin (Hb), cytochrome C (C ) and lysozyme (L) was (a) passed through the blank column and analyzed on a cation-exchanger; and (b) passed through the column prepared in the presence of hemoglobin and analyzed on the ion-exchanger. The chromatogram traces indicate that the T6, C5/T20, C3 column adsorbed hemoglobin. Accordingly, entrapment of the T6, C5 granules into small, rigid T20, C5 gel grains did not affect the selective recognition of hemoglobin. Owing to the rigidity of these grains the column had the advantage to permit high flow rates.

sulfate and TEMED solutions were increased 2.5-fold. About 40 min following polymerization the gel was disintegrated by forcing it once through a 100-mesh net and then packed into a Pasteur pipette. The column was washed with 6 % SDS in 10 % acetic acid and equilibrated with 0.02 M sodium phosphate, pH 7.0. The sample consisted of 20 µL of a solution of hemoglobin (10 mg $mL^{-1}$), cytochrome C (2 mg $mL^{-1}$) and lysozyme (2 mg $mL^{-1}$) in 0.001 M sodium phosphate, pH 7.0. The flow resistance of this bed was considerably lower than that obtained with the original T6 C5 gel. The selectivity test (Figure 6), performed as described for the experiment presented in Figure 4, demonstrates that it is possible to combine high specificity with high flow rate on beds with small gel particles which have the advantage to permit shorter run times and less zone broadening.

## Results and Discussion

In a recently published article we described a method for the preparation of gels with the property to permit selective recognition of hemoglobin, cytochrome C and transferrin [1]. From the chromatograms in Figures 1, 2 and 4 one can conclude that the same method can be employed to adsorb specifically ribonuclease, growth

hormone and myoglobin from horse. No doubt, similar beds can be designed for other proteins. However, it should be stressed that we do not yet have a method which without modifications can be used for any protein. Variations in pH, ionic strength and gel concentration can have a strong influence on the adsorptive properties of the gel. Several experiments show that the nature of the monomers also affect the affinity, although in this study we have solely used acrylamide and N,N'-methylenebisacrylamide. The degree of crosslinking is another important parameter, which we have kept at a value much lower than that used in entrapment of proteins and conventional molecular imprinting [2–12].

It is also interesting to note that one can synthesize a gel which can recognize several proteins (Figure 3). Such a gel can be of interest for rapid "negative purification" of a certain protein by suspending the gel particles in the protein sample and decanting.

Although this is the second paper in a series of articles on gels with selective recognition, many experiments remain to be done before several obvious questions can be answered. Only some of the problems will be addressed in the discussion below.

***Do some protein molecules become attached covalently to the gel matrix?*** This might be so because "hemoglobin columns" are somewhat colored even after extensive washing with different buffers and a 10 % solution of acetic acid containing 10 % SDS. Since monomer radicals form in the polymerization process one cannot exclude the possibility that these react with hemoglobin molecules which thus may become linked to the gel network. To investigate whether such a reaction occurs a "hemoglobin column" was prepared as described in Ref. [1] with the exception that many amino acids were present at high concentrations along with hemoglobin during the polymerization. The free radicals should react also with the free amino acids if they react with the hemoglobin molecules and one should expect a weaker color of this "hemoglobin column" which, however, was not the case. The experiment seems to indicate that the proteins are not linked covalently to the gel matrix.

***What conclusions can be drawn from the finding that (bio)affinity methods not always give the selectivity theoretically expected?*** Selective interactions between different constituents in the living cell form the basis for its function. This biorecognition has been utilized also in non-biological systems, for instance, in methods utilizing biosensors and in bio-affinity chromatography, including molecular imprinting [4–12]. These artificial systems have been employed with success in many investigations in biochemistry and related disciplines. However, the specificity is not always as high as expected, the main reason being that the ligands (for instance, enzyme substrates, antibodies) and the functional monomers used for imprinting (for instance methacrylic acid) are charged or/and non-polar and, therefore, create beds which to a greater or lesser extent also behave as conventional media for ion-exchange or/and hydrophobic-interaction chromatography, i.e., the selectivity is lost because *many strong* bonds is often not compatible with high specificity (see below). The situation is particularly anxious when electrostatic and hydrophobic interactions occur at the same, time, since an increase in the ionic strength of the eluent decreases the former type of interaction but increase the latter [15]. In such cases it may be impossible to find a buffer concentration such that the adsorbed sample can be released [16]. There are, accordingly, reasons to investigate whether methods can be designed in which ligands and functional monomers are omitted, i.e., methods based solely on interactions with the (chromatographic) matrix, which may be equivalent to the "nonspecific interactions" which in conventional, chromatographic experiments are obnoxious. The experiments described herein should be considered against this background.

***Classification of chromatographic techniques in terms of the number of bonds between the solute and the stationary phase and the strength of these bonds.*** Proteins and other macromolecules with many charged or/and non-polar groups bind strongly to a bed which has a high density of ligands of the same types of groups by virtue of electrostatic and hydrophobic interactions, respectively. However, the adsorption is not selective because such a bed can, via different combinations of the ligands, strongly bind *many* proteins *of varying* structure. This alternative *(several, strong* bonds) is used in conventional gradient elution of macromolecules (proteins) in ion-exchange and hydrophobic-interaction chromatography. For isocratic elution of proteins these separation methods require *few, strong* bonds [17, 18). As to low-molecular weight compounds, the strength of the bonds can (or perhaps, must) be *high,* since the number of bonds is necessarily *small.* An example of this alternative for small molecules is the molecular imprinting method which has been employed with great success for the separation of enantiomers and other low-molecular weight compounds, often using strong electrostatic bonds created by functional monomers [4–12]. In ideal molecular imprinting *all* functional monomer molecules in the bed have positions close to the complimentary groups in the solute molecules. However, a gel in which only some fraction of the functional monomer molecules fulfills this requirement also exhibits (a certain degree of) selectivity because the binding caused by the rest of these monomer molecules will be weaker, more or less resembling that occurring in conventional ion exchangers (when charged functional monomers are used) or beds for hydrophobic-interaction chromatography (when nonpolar functional monomers are used). The difficulty to synthesize a gel for ideal molecular imprinting (or the latter semi-ideal approach) increases with the number of binding sites (for isochemical substances often equivalent to an increase in mo-

lecular weight). Therefore, it is not surprising that no molecular-imprinting experiments with high selectivity for proteins have been reported. These considerations indicate that monomers which give rise to very strong bonds should be replaced by those interacting more *weakly*. To attain a strong over-all binding the number of bonds must, accordingly, be *large*. Examples of weak interactions are those originating from hydrogen bonds, charge transfer, faint induced dipoles and slightly non-polar groups. Acrylamide and N,N'-methylenebisacrylamide represent appropriate monomers. Gels synthesized from these monomers are widely used in many standard methods for electrophoresis and chromatography. All accumulated experience shows that these gels are very inert to biopolymers, including proteins. A very close contact between a protein molecule and polymer chains in the gel is, accordingly, a prerequisite for the generation of bonds. This proximity is created when the monomers polymerize around the protein molecule (and may increase the strength of each bond).

If the discussed hypothesis for selective recognition is correct one should expect a protein of a relatively small size (and therefore fewer adsorption sites) to interact only weakly with gels of a composition similar to that employed in this investigation. Therefore, we studied the behavior of insulin (molecular weight: 5 700) on a T6, C5 gel prepared in the presence of insulin. The protein was much less adsorbed than were larger proteins, such as growth hormone and transferrin, but the interaction was nevertheless specific because the elution volume was 40 % larger than that obtained on a blank gel synthesized in the absence of insulin (not shown herein).

***Some comments on the mechanism of the selective recognition.*** In the sixties it was shown that proteins could be immobilized in a gel for bio-affinity chromatography if they were included in the monomer solution [2, 3]. It was postulated that the immobilization consisted of occlusion of the protein molecules in the polymeric network. In view of the properties of the gels described herein it is likely that at least part of the immobilization originated from selective adsorption and not by entrapment caused by occlusion.

Visually one can see that it is very difficult to remove the last traces of adsorbed hemoglobin molecules, as mentioned above. In fact, beds with a gel composition such that all hemoglobin could be removed by washing (as judged by the naked eye) did not exhibit selective recognition of this protein. Can this finding be explained by specific protein-protein interactions? One cannot at present exclude completely this possible adsorption mechanism, since proteins can be precipitated in the presence of polymers, such as polyacrylamide, dextran, polyethyleneglycol, etc., i.e., the proteins have a tendency to aggregate in contact with polymers [19–21] and also when they are cross-linked to form a gel [22–23]. It should be emphasized that continuous addition of a polymer (or a saturated ammonium sulfate solution) to a mixture of proteins does not give rise to a precipitate where all the different proteins have the same concentrations as in the original mixture. Instead, a stepwise precipitation occurs: each protein precipitates at a certain polymer (ammonium sulfate) concentration, characteristic of each particular protein [19–21]. The underlying selectivity mechanism could perhaps operate also in the proposed, alternative protein-protein interaction. Therefore, it is not self evident that the above hypothesis based on modified imprinting (without functional monomers) is correct. For this reason we prefer not to give the method presented herein any particular name.

Most of our experiments aimed at designing columns with selective recognition have been performed on polyacrylamide gels, although other gels can also be employed. It appeared that relatively small variations in the structure of the monomers affected more or less the degree of selectivity. A few experiments with gels of 1–6 % (w/v) agarose were not successful. Maybe these gels with their large pores are more suitable for very large proteins or particles, such as viruses. It is known that crosslinking of agarose gels with divinyl sulfone causes some nonspecific interaction with proteins [24]. Since our working hypothesis on the selective recognition is partly based on non-specific interactions, an agarose gel was prepared in the presence of hemoglobin and at the same time crosslinked with divinyl sulfone [24]. This gel showed specific adsorption of hemoglobin, although the loading capacity was low. It should be stressed that crosslinking of agarose only slightly affects the pore size of the gel [25].

In free electrophoresis the net charge of the analyte determines its migration rate. The observation that the order of elution of serum proteins from an anion-exchanger is reversed compared to that of their migration rates in electrophoresis [26] indicates that the net charge is the parameter that determines the degree of interaction with the column bed. In other words, the orientation of the charged groups of a protein should be of no importance, nor even the number of positive and negative groups, but only the difference in number between them. Some support for this view is the fact that anionic (cationic) proteins cannot be adsorbed onto a cation (anion) exchanger, although both kinds of proteins have positive, as well as negative groups. If the hypothesis that the position of the charged amino acid residues in a protein is less important upon interactions than the net charge, then the generally accepted mechanism of molecular imprinting can be questioned. Or should the electrostatic interactions in ion exchange and molecular imprinting be treated differently because of the (non-proven) much closer contact between the protein molecules and the polymer chains in the latter method? Or is the shape of the complimentary cavity formed during the polymerization more important than the bonds?

***The specificity.*** A comparison between the chromatograms in Figure 4a and Figure 4b (obtained from analyses with a cation exchanger of the eluate from the

"blank column" and the "horse myoglobin" column, respectively) shows that only horse myoglobin was adsorbed onto the latter column but none of the other proteins, not even whale myoglobin, notwithstanding that the amino acid sequence of the two myoglobins differs in only twenty of the 153 amino acid positions and in such a way that the 3-dimensional structure is only slightly affected (Figure 5). The specificity of the bed was, accordingly, high.

During a test period of one month a "hemoglobin column" was used in fourteen experiments without loss in specificity.

***Potential applications.*** A comparison of the three chromatograms in Figure 2 shows that the human growth hormone was adsorbed only on the gel prepared in the presence of the hormone. The gel grains thus behave as beads to which antibodies have been attached and can, therefore, probably be used in RIA (radioimmunoassay) or ELISA (enzyme-linked immunosorbent assay). If so, animal experiments aimed at the generation of antibodies can be avoided not only in these assays but also in other experiments, including receptor studies. The high specificity of the gels described herein makes it tempting to use them also in the area of biosensors, where the binding can be studied by rapid response from transducers based on different principles. It is relatively easy to investigate whether the gels, in practice, can be utilized in such analytical systems. More time-consuming experiments are required if the gels are to be designed to function as artificial enzymes. Those interested in these and related areas should read an excellent article by Mosbach and Ramström [6].

***Forthcoming studies.*** The mechanism of the selective recognition is but one of many questions which importunately insist upon an answer. Others are: how to increase the protein capacity, to improve the reproducibility (we cannot always repeat a successful experiment); to elute the protein adsorbed specifically in a small volume and in a gentle way; to modify the nature and the composition of the gel so that all types of particles (for instance viruses) and molecules, including all proteins, can be adsorbed specifically (we have not yet found general conditions which can be used for any protein); does a high specificity require a high rigidity of the solute; if so, should the solute molecules be cross-linked (reversibly). Analogous electrophoretic methods should also be developed.

## Conclusions

The experiments described in this paper strengthen the hypothesis that it is possible to design gels which resemble chemically and biologically antibodies in the sense that they recognize selectively and bind strongly a particular protein. A prerequisite seems to be that the number of bonds between the gel and the protein is large and that each bond is relatively weak, although other binding mechanisms cannot be excluded. All experiments have been performed with proteins, but there are no reasons to believe that the application range of the method is limited to this class of substances. The ease and simplicity with which the gels can be prepared render them potentially attractive for many different techniques based on selective recognition.

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