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# APPLICATION OF NEUTRAL HYDROPHOBIC GELS IN HYDROPHOBIC CHROMATOGRAPHY[1]

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## INTRODUCTION

Many processes in biological systems occur on surfaces, e.g., recognition of foreign cells, binding of acceptor molecules on surface receptors, deposition of proteins and blood components on the walls of blood vessels and on artificial implants. As has been reported (1–3), most materials in contact with blood very quickly adsorb a protein film and then at a lower rate the elementary constituents (4). The composition of the adsorbed protein film is dependent on the structure and chemistry of the adsorbing surface. The deposition of a protein layer and activation of serum proteins during or after the adsorption process has been found to be responsible for the start of the clotting cascade and the generation of thrombi (see, e.g., ref. 5).

The investigations described here are aimed at identifying the chemical structural elements that yield minimum protein adsorption for a maximum number of blood proteins. To ascertain the adsorptive forces of different groups, Sepharose was substituted with different substituents (Table 1). Sepharose gels were substituted with epoxides to avoid the introduction of charged or polar groups, which would have been the case in the substitution with cyanogen bromide (6).

## MATERIALS AND METHODS

The following instruments were used: for UV spectroscopy a Varian Techtron 635, for NMR spectra a Varian T60, for mass spectra a MAT 311, and for infrared spectra a Perkin-Elmer 221. The ball-type distillation was performed on a Custilator (Chemophor, Zürich). Chromatography was performed at 35°C in Multichrom columns (bore 0.5 cm, length 25 cm,

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TABLE 1. **Substituted Gels**[a]

| Gel | R | Gel | R |
|---|---|---|---|
| 2a | $CH_3$ | k[1] | $(CH_2)_2-O-H$ |
| b | $n-C_4H_9$ | l[1] | $(CH_2)_2-O-(CH_2)_2-O-H$ |
| c | $i-C_5H_{11}$ | m | $C_6H_{12}-O-H$ |
| d | $C_6H_{13}$ | n | $C_6H_{12}-O-CH_3$ |
| e | $C_7H_{15}$ | o | $C_6H_{12}-CN$ |
| f | $C_8H_{17}$ | p[1] | $CH_2-C(=O)-OH$ |
| g[b] | $C_{10}H_{21}$ | q | $(CH_2)_3CN$ |
| h | $CH_2-C_6H_5$ | r | $[(CH_2)_3-\overset{\oplus}{N}(CH_3)_3]CH_3SO_4\ominus$ |
| i | $CH_2-C_3F_7$ | s | $(CH_2)_3COOC_2H_5$ |
| j | $(CH_2)_3-Si(CH_3)_2-O-Si(CH_3)_2-CH_3$ | | |

[a]Structure:

$$\text{Seph.}-O-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-O-R.$$

Substitution degree approx. 200 $\mu$mol $g^{-1}$ gel.

[b]Most proteins were adsorbed so strongly that they could not be recovered from the gel, not even with very chaotropic solvents.

Serva Technik, Heidelberg) using silicone tubes and Teflon fittings with a Gilson Minipuls II pump. Elution volumes were measured using the UVICORD II absorptiometer (LKB Producter, Bromma, Sweden) at 284 nm.

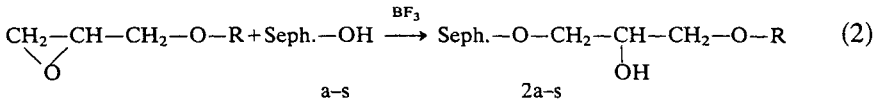
The buffer (pH 7.3) consisted of 500 $cm^3$ 0.01 mol $dm^{-3}$ $KH_2PO_4$, 370 $cm^3$ 0.01 mol $dm^{-3}$ NaOH, 5.59 g NaCl, 0.175 g $NaN_3$, $\rho_L$ = 1.073 g/$cm^3$. The void volumes $v_0$ were determined using Dextran Blue 2000 (Deutsche Pharmacia, Frankfurt). The internal volume $v_i$ was calculated using the following equation (7):

$$v_i = \frac{W_r \cdot \rho_G}{\rho_L(W_r/\rho_L + 1)} \cdot (v_t - v_0) \tag{1}$$

where $W_r$ is the solvent regain, $\rho_L$ is the density of solvent (1.073 g/$cm^3$), $\rho_G$ is the density of gel, and $v_t$ is the total volume of the packed column.

Sepharose 4B was purchased from Deutsche Pharmacia, Frankfurt; all other chemicals, which were analytical grade, were supplied by Aldrich, Merck, Fluka, and Boehringer. The following proteins were used for chromatography: human albumin, prothrombin (concentrate), fibrinogen (bovine) (all Behring-Werke, Marburg), IgG, $\gamma$-globulin (Fr. II) (Miles Laboratories Ltd., Slough, England), thrombin (Merck).

Sepharose 4B was substituted by reaction with epoxides catalyzed by boron trifluoride according to the procedure described in ref. (8) (for R, see Table 1):



(2)

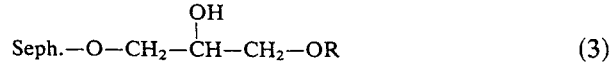
The synthesis and analytical characterization of the epoxides as well as the determination of the degree of substitution of the gels and the chromatographic characterization is described in detail in (9). Only gels with a comparable degree of substitution (approx. 200 $\mu$mol/g) were used for the investigation described here.

The elution volumes $v_e$ and $v_0$ (for Dextran Blue) were measured in drops. During the running time of each chromatography (about 2 weeks), the gels were compacted, and the void volume $v_0$, as well as in some cases the internal volumes $v_i$, decreased. To calculate $K_i$, the corresponding $v_i$ had to be used. This could be done by plotting the $v_i$ as a function of column height, as described earlier (9). All elution volumes were measured at least twice, in most cases more often. The results are given in Table 2 and are plotted in Fig. 1 (average values).

On the most columns thrombin showed two peaks, which were very broad and difficult to measure. Thus, the results for thrombin are not as accurate as for the other proteins. All data concerning this protein are for the peak with the lower $v_e$.

## RESULTS

The gels listed in Table 1 were synthesized. For comparison of the adsorptive forces of serum proteins on different gels, the latter had to have the same degree of substitution (approx. 200 $\mu$mol/g gel). All gels were represented by the general formula



(3)

The substituents R are listed in Table 2. The exponent 1 means that protected substituents on gels [as described in (9), Table 1] were hydrolyzed to the hydroxyl compound, and chromatography was performed on the hydroxyl-containing substituted gels.

Substitution degrees higher than approx. 200 $\mu$mol/g were not possible on gels 2a, 2j, and 2n, because they had lost their pore structures and had a noncomparable and extremely small $v_i$ and very low liquid flow. In this case, the hydrophobic interaction of the substituents forced the gel to contract and

TABLE 2. **Chromatographic Results**[a]

| Substituent | Protein | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Albumin | Globulin | Fibrinogen | IgG | Prothrom. | Thrombin | TAI | μmol/g |
| $K_{2a}$ | 0.670 | 0.632 | 0.380 | 0.603 | 0.693 | 0.790 | — | |
| Sepharose 4B | 0.041 | 0.031 | 0.061 | 0.070 | −0.031 | −0.041 | +0.131 | |
| 2b | 0.102 | 0.037 | 0.089 | 0.168 | 0.116 | 0.248 | 0.760 | |
| 2c | 0.065 | 0.007 | 0.103 | 0.024 | 0.029 | 0.01 | 0.238 | |
| 2d | 0.054 | 0.137 | 0.242 | 0.019 | 0.034 | 0.205 | 0.692 | |
| 2e | 0.278 | 0.215 | 0.555 | −0.04 | 0.075 | 0.395 | 1.478 | |
| 2f(1)[b] | 0.328 | 0.062 | 0.349 | 0.089 | 0.054 | 0.152 | 1.034 | 100 |
| 2f(2) | >1.00 | 0.125 | 0.373 | 0.131 | 0.015 | 0.028 | >1.672 | |
| 2g[c] | | | | | | | | |
| 2h | 0.003 | 0.022 | 0.135 | 0.020 | 0.024 | −0.058 | 0.146 | |
| 2i | 0.136 | −0.053 | 0.379 | 0.111 | 0.023 | 0.075 | 0.671 | |
| 2j(1) | −0.008 | −0.117 | −0.023 | −0.126 | −0.170 | −0.121 | −0.565 | 100 |
| 2j(2) | 0.023 | 0.012 | 0.003 | 0.049 | 0.042 | −0.041 | 0.088 | |
| $2k^1(1)$ | −0.162 | −0.217 | 0.116 | −0.115 | −0.181 | −0.291 | −0.850 | |
| $2k^2(2)$ | 0.000 | −0.044 | 0.116 | −0.017 | −0.153 | −0.002 | −0.100 | 360 |
| 2l | −0.0015 | −0.004 | 0.146 | −0.036 | 0.002 | 0.053 | 0.159 | |
| 2m(1) | 0.008 | −0.013 | 0.236 | −0.037 | −0.175 | −0.241 | −0.222 | 105 |
| 2m(2) | 0.053 | 0.059 | 0.132 | 0.055 | 0.085 | −0.022 | 0.362 | |
| 2n | 0.072 | 0.095 | 0.119 | 0.125 | 0.070 | −0.035 | 0.446 | |
| 2o | −0.038 | 0.034 | 0.276 | 0.056 | −0.036 | −0.191 | 0.101 | |
| $2p^1$ | −0.033 | −0.062 | 0.311 | −0.007 | (0.006) | −0.068 | ~0.147 | |
| 2q | −0.052 | 0.039 | −0.009 | −0.091 | −0.010 | −0.114 | −0.237 | |
| 2r | 0.250 | 0.120 | 0.481 | 0.126 | 0.198 | 0.239 | 1.414 | |
| 2s | 0.106 | 0.133 | 0.343 | 0.114 | 0.115 | 0.093 | 0.904 | |

[a] Relative adsorptivities $\Delta K_i = K_i - K_{2a}$.
[b] Numbers in brackets indicate different experiments with gels of differing substitution degree; see Table 3.
[c] The proteins were adsorbed irreversibly and could not be eluted.

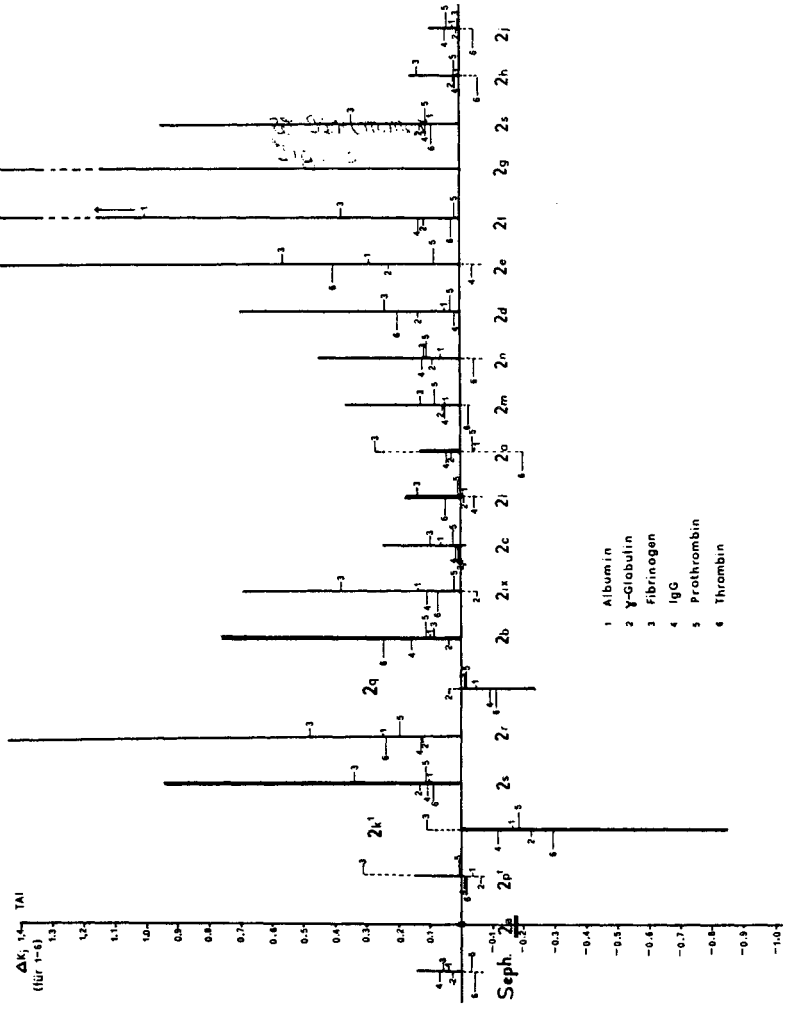



FIG. 1. Adsorptivities of substituted gels (approx. 200 $\mu$mol/g) expressed in $K_i$ and TAI values). The numbers 1–6 indicate the $K_i$ values for proteins 1–6 on gels 2a–2s. For gel 2g, see footnote into Table 2.

finally collapse. The term $v_i$ is a measure of the unchanged pore structure of the gels, because destroying the pore structure would result in smaller internal volumes and consequently in a change in the exclusion limits of the gels compared to Sepharose 4B. This would result in gel chromatographic effects, i.e., in a dependence of the elution volume $v_e$ of the proteins on the molecular weights. We did not find such dependence. As we showed in (9), the internal volumes of the compared gels remained unchanged by the substitution reaction. The results of the chromatography are given in Table 2.

## PRESENTATION OF EXPERIMENTAL DATA

Methyl Sepharose 2a (R=$CH_3$; see Table 2), being the gel with the smallest organic substituent, was chosen as reference gel. Gel 2a also proved to be less adsorbing than the more hydrophilic Sepharose 4B. Table 2 shows the results of the chromatography. For comparison of the adsorptive properties of the gels, $K_i$ ($i$ = running index) is compared to the standard $K_{2a}$ by a relative adsorptivity scale, defined by the equation

$$\Delta K_i = K_i - K_{2a}$$

[$K_i > 0$ therefore means that the adsorption strength of the considered protein on gel $i$ is stronger, and $K_i < 0$, that it is smaller than on gel 2a. The first line in Table 2 gives the $K_i$ values of Methyl sepharose 2a, all others are $\Delta K_i$ values.

As a measure for the total adsorptivity, we defined a *total adsorptive index* (TAI), which is the total of the adsorptivities of all proteins:

$$\text{TAI} = \sum aj \cdot \Delta K_i$$

where $aj$ is a "physiologic index" describing the importance of the protein $j$ for the generation of a thrombus by contact of an implant material in the bloodstream with the functional groups at the surface as chosen in substituent $i$. The $aj$ index is definitely different for each protein, because some adsorbed proteins (e.g., albumin) seem to prevent formation of a clot on a surface, while others play a role in the activation of the clotting cascade. Glycoproteins like fibrinogen are of importance for the adhesion platelets.

Table 3 shows the influence of the degree of substitution. The ΔTAI values describe the difference of the TAIs between two gels carrying the same substituent in different concentrations (see below). It was shown (11) that the capacity of the columns was sufficient, the internal volumes were unchanged by the substitution reaction, and the distribution constants $K_i$ did not correlate with the molecule mass of the proteins, i.e., gel chromatographic effects were excluded.

TABLE 3. **Influence of Degree of Substitution**

| Protein | Silicon 100 ($K_i$, $\mu$mol $g^{-1}$) I | Silicon 200 ($K_i$, $\mu$mol $g^{-1}$) II | ΔTAI 2j | Glycol 200 ($K_i$, $\mu$mol $g^{-1}$) I | Glycol 360 ($K_i$, $\mu$mol $g^{-1}$) II | Glycol ΔTAI $2k^1$ | Octyl 100 ($K_i$, $\mu$mol $g^{-1}$) I | Octyl 180 ($K_i$, $\mu$mol $g^{-1}$) II | Octyl ΔTAI 2g | Hexyl—OH 105 ($K_i$, $\mu$mol $g^{-1}$) I | Hexyl—OH 230 ($K_i$, $\mu$mol $g^{-1}$) II | Hexyl—OH ΔTAI 2m |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Albumin | −0.008 | 0.023 | | −0.162 | 0 | | | 1.0[a] | | 0.008 | 0.093 | |
| γ-Globulin | −0.117 | 0.012 | | −0.217 | −0.044 | | 0.034 | 0.125 | | −0.013 | 0.059 | |
| Fibrinogen | −0.023 | 0.003 | | +0.116 | +0.116 | | 0.349 | 0.373 | | 0.236 | 0.132 | |
| IgG | −0.126 | 0.049 | | −0.115 | −0.017 | | 0.089 | 0.131 | | −0.037 | 0.055 | |
| Prothrombin | −0.170 | 0.042 | | −0.181 | −0.153 | | 0.054 | 0.015 | | −0.157 | 0.085 | |
| Thrombin | −0.121 | −0.041 | | −0.291 | −0.002 | | 0.152 | 0.028 | | −0.241 | −0.022 | |
| TAI | −0.565 | +0.088 | 0.653 | −0.850 | −0.100 | 0.750 | +1.672 | 1.672 | 0.638 | −0.222 | 0.362 | 0.584 |

[a] Could not be determined exactly because albumin was not eluted from gel 2t (II).

## DISCUSSION

### *General Considerations*

The relative adsorption energies $E_i$ of the gels toward a protein can be expressed as the sum of three partial energies [$\Delta E_i$ is the difference between the adsorption energy toward gel $i$ ($E_i$) and the adsorption energy toward Sepharose 4B ($E$)]:

1. Adsorption energy of the functional end group ($\Delta E_s$) of the substituent
2. Adsorption energy of the alkyl chain ($\Delta E_c$) of the substituent
3. Adsorption energy of the matrix (Sepharose 4B, $\Delta E_M$)

Agarose gels cause very little denaturation or adsorption of sensitive biochemical substances because of their hydrophilic nature and the nearly complete absence of charged groups.

Since the matrix was Sepharose 4B in all cases and the reaction conditions for the substitution of Sepharose were the same for all gels, $\Delta E_M$ is only dependent on the substituent and the protein. A change of exclusion limits was shown to be unlikely (9,11), and the microenvironment may be assumed to be very much alike in all gels, since the method of preparation and the degree of substitution were the same for all gels.

$\Delta E_S$ and $\Delta E_C$ are dependent on the protein. The total relative adsorption can be expressed as follows:

$$\Delta E_i = \Delta E_C + \Delta E_S + \Delta E_M \tag{4}$$

$\Delta E_C$ as well as $\Delta E_S$ are of the greatest interest, especially for the design of protein nonadsorbing polymers, or at least their surfaces. For the measurement of the influence ($\Delta E_S$) of the functional group on the adsorption of proteins, $\Delta E_C$ = constant would be advantageous. An alkyl chain has to be chosen that is just long enough to bring the functional end groups into optimum contact with the protein. By using larger alkyl chains, the influence of the functional group on the adsorption of proteins can be superceded by the influence of the alkyl chain.

$\Delta E_C$ is not linearly dependent on the chain length; it is a function of the chemical environment in the bound state. If $\Delta E_C$ were linearly dependent on the chain length, the binding forces would increase linearly with the chain length. The results showed, however, that the increase of the binding force (represented by the distribution coefficient $K_i$) was neither linear nor unlimited. A "critical chain length" with a maximum adsorptivity was observed. Similar effects have been observed by others. Davey, Sulkowski, and Carter (15), for example, found that both human as well as rabbit interferons are bound irreversibly to a $C_8$-substituent, but that rabbit

interferon does not bind to $C_4$ and is irreversibly bound by $C_{10}$. By contrast, human interferon binds considerably to $C_4$, but reversibly to $C_{10}$. Longer alkyl chains showed a decrease in binding affinity (see Table 2). This can readily be explained by the observation that alkyl chains tend to fold (12) from a certain chain length onward due to intermolecular hydrophobic binding.

$\Delta E_S$ and $\Delta E_M$ probably are complicated functions, too. The adsorption energy is a function of the contact area of the protein with the adsorbent. Very short substituents can hardly interact with the protein, so that the adsorption energy is almost completely represented by the terminus $E_M$. Adsorption on the matrix surface itself may result in protein deformation.

The contact area and thus the partial energy $\Delta E_C$ increases with increasing chain length. During the binding to the substituent, the protein may deform to obtain maximum hydrophobic contact with the alkyl chain. Longer and more hydrophobic alkyl chains may even cause a denaturation of the proteins, due to the "detergentlike" action of the hydrophobic ligand (10,12). This effect may explain the irreversibility of the binding of albumin on gels containing $C_8$ and longer alkyl chains (Table 2, gel 2g).

*Influence of Hydrophobic Substituents*

The replacement of the H atoms in the Sepharose-OH groups by a hydrophobic and covalently bound $CH_3$ group will drastically change the state of order of the water, which will lead to a different adsorption capacity. In fact, we found that the TAI and the adsorptivity (expressed by $K_i$), respectively, were lower for gel 2a than for Sepharose itself. The replacement of a $CH_3$ group by another longer alkyl group will probably have a less dramatic impact on the state of order of the bound water molecules in the pores than the replacement of relatively mobile H atoms by the $CH_3$ group.

Replacement of the $CH_3$-group of gel 2a by a longer alkyl group generally increased the relative adsorptivity (Table 2). The "critical chain length," where the proteins showed maximum adsorption on the gels, was different for each protein: fibrinogen, thrombin, and $\gamma$-globulin showed the strongest adsorption forces, with a chain length of seven C atoms, whereas the maximum adsorption forces for IgG and albumin occurred with a longer chain (see Table 2). The maximum adsorbing chains for these two proteins were not determined, but the critical chain length, at least for albumin, was somewhat higher than $C_8$. Albumin was bound so strongly to a decyl-substituted gel that it could not be desorbed from the gel, not even with denaturing buffer systems such as "blood buffer"/glycol, 1:1. Similar results were found by Hofstee (13,14). These results for the single proteins are also expected to be found in the total adsorptivities for all proteins (TAI)

(see Fig. 1; TAI for 2a < 2b < 2d < 2e < 2f < 2g). They also show the effect of the branching of the alkyl chain (compare, e.g., 2b v 2c, 2b v 2d, and 2e, 2j v 2e), which makes the substituent more bulky and less able to enter very small hydrophobic crevices in the protein surface. Branching effects have also been observed by others (13,15).

*Influence of Hydrophilic and Polar Substituents*

The influence of the hydrophilic or polar functional end group of the alkyl chains can be observed directly without considering the influence of the alkyl chains by comparing the hexyl-alkyl-substituted gels 2d (substituent $C_6H_{12}$—H), 2e ($C_6H_{12}$—$CH_3$), and 2f ($C_6H_{12}$—$C_2H_5$) with gels where the alkyl group of a hexyl moiety is replaced by a functional group, i.e., 2m ($C_6H_{12}$—OH), 2n ($C_6H_{12}$—$OCH_3$), or 2o ($C_6H_{12}$—CN). The gels 2m, 2n, and 2o are much less adsorptive than the hydrophobic hexyl-alkyl-substituted gels 2d, 2e, and 2f, as expressed in TAI values (Fig. 1). Increasing the polarity and hydrophilicity in the chain also reduces the adsorptivity compared to an alkyl chain of identical length (2s v 2e).

The influence of the functional group on gels with a shorter alkyl chain is not so easy to interpret because the influence of the matrix surface on the adsorption ($\Delta E_M$) may be of the same magnitude, and the former might be screened. In many cases, however, at least the trend of their impact on the adsorptivity could be seen. Thus, a positive surface charge (produced by a quaternary ammonium group) in 2r produced a strong adsorptivity toward all proteins, whereas a gel with negative charge ($2p^1$) (the carboxyl groups are partly ionized at the pH of the buffer) showed relatively low affinity for the proteins.

Very interesting is the low adsorptivity of CN groups containing substituents (see Fig. 1, 2q, 2o). Especially in the case of hexyl-substituted gels (2d v 2o), the adsorptive forces reducing influence of the CN group can easily be observed.

*Influence of Degree of Substitution*

The influence of the degree of substitution is demonstrated in Table 3. Here the $(TAI)_i$ for two different degrees of substitution for the same substituent are compared by subtracting $(TAI)_{low}$ of the lower substituted gel from the $(TAI)_{high}$ of the higher substituted gel:

$$\Delta(TAI) = (TAI)_{high} - (TAI)_{low}$$

that is, $\Delta(TAI) > 0$ means higher adsorption on the higher substituted gel. For the proteins investigated here, the higher substituted gels generally

showed a higher total adsorptivity. It has been demonstrated by Hofstee (13), however, that the hydrophobicity of the substituent, and not the degree of substitution, is the limiting factor (16).

## SUMMARY

The adsorption of different serum proteins was chromatographically measured under standardized conditions. The results show that Sepharose 4B adsorbs serum proteins more strongly than a methyl-substituted sepharose gel (gel 2a). Gels with substituents with chain lengths from $C_1$ to $C_5$ show only relatively low adsorptivity. A protein dependent critical chain length of the substituents with stronger binding forces toward these proteins could be identified. A branching of the substituents and very voluminous groups reduce hydrophobic binding forces. Among the functional end groups investigated in this work, hydrophilic moieties, carboxylate, and cyano groups proved to be low serum protein adsorbing functional chemical groups.

## ACKNOWLEDGMENT

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## NOMENCLATURE

| | |
|---|---|
| $v_i$ | internal volume of gels |
| $v_0$ | void volume |
| $v_t$ | total volume of packed column |
| $v_e$ | elution volume for the particular protein |
| $K_i$ | distribution coefficient for protein between gel ($i$) and mobile phase (buffer); $K_i = (v_e - v_0)/v_i$ |

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