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# SYNTHESIS OF NEW HYDROPHOBIC ADSORBENTS BASED ON HOMOLOGOUS SERIES OF UNCHARGED ALKYL SULPHIDE AGAROSE DERIVATIVES

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## SUMMARY

A homologous series of uncharged thioalkyl derivatives of agarose were prepared by a simplified synthetic route and their adsorption behaviour towards human serum proteins was evaluated and compared with that of a commercially available alkyl ether derivative of agarose. The influence of the spacer arm length on the adsorption efficiency was also investigated. The degree of substitution of the derivatives can be estimated conveniently by sulphur analysis.

The four different types of thiolkyl derivatives ($C_6$, $C_8$, $C_{12}$ and $C_{14}$) investigated here behave in all respects like hydrophobic adsorbents. The coupling yield obtained is high (75% or more) and is better than that obtained by alternative synthetic routes reported so far. The adsorption capacity towards serum proteins of the various derivatives increases with increasing alkyl chain length and degree of substitution. Desorption is achieved by a progressive decrease in the polarity of the eluent and the recovery of the applied material is in the range 80–90%. The role played by the thioether as a possible modulator of the observed hydrophobic adsorption is discussed. For the group separation of serum proteins the optimum adsorbent, as regards capacity combined with ease of elution of adsorbed material, should be substituted with chains of six or eight carbon atoms and have a ligand concentration in the range 80–120 $\mu$mole $g^{-1}$ dry gel.

## INTRODUCTION

The historical aspects of the development of various hydrophobic adsorbents for the separation of biological macromolecules were extensively documented by Hjertén[1] in 1973. The factors governing hydrophobic adsorption and subsequent desorption were also discussed. The adsorbents produced at that time were of a mixed hydrophobic–ionic type[2–5] prepared by activating the matrix with cyanogen bromide[6] and coupling various alkyl- or arylamines or amino acids. The inherent disadvantages of such adsorbents for hydrophobic interaction chromatography have been discussed by Porath *et al.*[7], Hjertén[1] and Wilchek and Miron[8]. The positive charge on such adsorbents[9] was later neutralized by acetylation[8,10], resulting in essentially charge-free adsorbents.

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Porath *et al.*[7] and Hjertén *et al.*[11] introduced for the first time a series of well defined, charge-free hydrophobic adsorbents. Their adsorption characteristics were such that they fulfilled the basic requirements for hydrophobic adsorbents enumerated by Hjertén[1], namely, adsorption of proteins was favoured at high salt concentrations ("salting-out" effect[7,12]), at low pH and at elevated temperatures. Desorption was achieved by a reversal of the above conditions or by decreasing the polarity of the eluent[4].

It should be emphasized, however, that the synthesis of almost all of the adsorbents mentioned above is either tedious or complicated. In some instances, the hydrophobic ligand must be pre-synthesized and the coupling reaction performed in an essentially water-free medium[11]. The coupling yield is at best 50%. In other instances, a large excess of the hydrophobic ligand was used during coupling[3,13], with the result that the degree of substitution could not be manipulated at will. In addition, a further step is required if one needs to abolish the positive charges by acetylation[8].

In this paper, the synthesis of homologous series of charge-free alkyl sulphide derivatives for use in hydrophobic interaction chromatography is reported. The synthetic route adopted gives a high coupling yield (75% or higher). The degree of substitution can thus be varied as required to produce adsorbents with varying ligand densities at a constant alkyl chain length. The process can also be scaled up as required without affecting the degree of substitution. The reactants are readily available from commercial sources and the adsorbents are relatively cheap to produce, with the obvious advantages that they can be synthesized easily at other research laboratories or be produced commercially to make them available to a wider scientific community*

## EXPERIMENTAL

### *Chemicals*

Sepharose 6B, octyl-Sepharose CL-4B, gradient polyacrylamide gels (PAA 4/30) for electrophoresis and reference protein kit were bought from Pharmacia (Uppsala, Sweden). Bis-epoxide (1,4-butanediol diglycidyl ether) and 1-dodecanethiol were obtained from Polysciences (Warrington, PA, U.S.A.), 1-tetradecanethiol from ICN Pharmaceuticals (Plainview, NY, U.S.A.); 1-hexanethiol, 1-octanethiol and sodium borohydride from Fluka (Buchs, Switzerland) and epichlorohydrin from Merck Schuchardt (Hohenbrunn bei München, F.R.G.). Normal human serum was obtained from the blood bank at the University Hospital (Uppsala, Sweden). All other chemicals were of analytical-reagent or reagent grade and were used as purchased.

### *Synthesis of Sepharose-S-$C_n$** adsorbents*

The activation of Sepharose with bis-epoxide was performed essentially as described by Sundberg and Porath[14] with minor modifications. Thus, 1 g of thoroughly

* Patent pending.

** Sepharose-S-$C_n$ (abbreviated henceforth to Seph-S-$C_n$) denotes Sepharose 6B on to which is coupled an alkyl mercaptan via a thioether linkage. The corresponding adsorbents prepared according to Hjertén *et al.*[11] will be designated Seph-O-$C_n$, and those described by Er-el *et al.*[3] will be called Seph-N-$C_n$, regardless of the nature or length of the spacer arm.

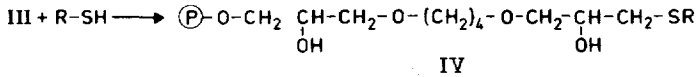
washed and suction-dried Sepharose 6B was mised with 1 ml of 0.6 *M* NaOH (containing 2 mg of sodium borohydride) and 0.5 ml of 1,4-butanediol diglycidyl ether in a round-bottomed flask and the slurry was gently stirred overnight (15 h) at room temperature. The slurry was washed thoroughly with deionized water (at least 50 ml) and transferred back into a clean round-bottomed flask. To this was added 0.8 ml of 1 *M* NaOH, 10 mg of sodium borohydride and 0.8 ml of 95% ethanol (containing 2–100 μmole of the alkyl mercaptan to be coupled). The solution of the alkyl mercaptan in ethanol was always prepared immediately before use. The slurry was stirred gently for 20 h at room temperature. The coupled product was washed on a Büchner funnel with deionized water followed by 95% ethanol (about 3 ml of each). This washing cycle was repeated five times or until the smell of mercaptan from the washed gel had virtually disappeared. This procedure can be scaled up as required without affecting the degree of substitution or the coupling yield.

The following reaction scheme summarizes the steps employed for the synthesis:

(1) Activation of the polymer matrix, Ⓟ-OH[14]:

I II III

(2) Coupling of the alkyl mercaptan (RSH) to the activated Sepharose (III) proceeds according to the reaction



IV

Note that the final product IV has a long hydrophilic spacer arm containing ten carbon atoms.

An alternative synthetic route has also been adopted using epichlorohydrin for activating the gel instead of the bis-epoxide.

The gel was activated with epichlorohydrin according to the procedure of Porath and Olin[15] and was reacted with 1-octanethiol (10 μmole/g suction-dried wet gel) as described above. The coupled product has a short spacer arm composed of three carbon atoms and its ligand concentration is about 120 μmole $g^{-1}$ dry gel.

*Determination of the dry weight of the gel, the degree of substitution and the coupling yield*

The dry weight of the gel was determined, in triplicate, as follows. A sample of coupled gel was washed exhaustively with distilled water on a Büchner funnel under suction and the gel suspension in water was deaerated under vacuum (water pump) and packed into a glass column (20 × 1 cm I.D.) to give a bed volume of 10 ml. The packing of a column was routinely performed at a fixed flow-rate of 25 ml $cm^{-2}$ $h^{-1}$ to ensure reproducibility of the bed volume. The contents of the column were quantitatively transferred into a Büchner funnel and, after suction drying, transferred to a desiccator. The gel was then dried under vacuum (water pump) over $CaCl_2$ for 18 h and again over fresh $CaCl_2$ for 5 h. The drying was continued over $Mg(ClO_4)_2$ at a vacuum of 2–7 mmHg for 5 days. This drying procedure was suffi-

cient to achieve a constant weight. The weight of the dry gels was determined to establish the relationship between the volume of a packed gel sample and its dry weight.

The degree of substitution of a gel sample was calculated from elementary sulphur analysis performed according to Gustafsson[16] and expressed as %S per dry weight of gel. This was recalculated on a molar basis to give the degree of substitution as the number of moles of ligand per millilitre of packed gel. The coupling yield was also calculated and is expressed as the ratio between the amount of sulphur (ligand) coupled to the gel and the amount of alkyl mercaptan added to the coupling reaction mixture.

*Chromatography*

Routinely, a 20 × 1 cm I.D. glass column was packed with the appropriate gel to obtain a bed volume of 8 ml. The packing was performed at a flow-rate of 25 ml $cm^{-2}$ $h^{-1}$ to ensure as reproducible bed volume. The column was then equilibrated with the initial eluent buffer [0.1 *M* Tris–HCl, containing 0.5 *M* $K_2SO_4$ (pH 7.6) which will be referred to as buffer I]. The sample of human serum was exhaustively dialysed against buffer I prior to application. Routinely, a 1-ml (total $A_{280}$ = 60) sample of the dialysed and clarified human serum was applied to the column by means of a peristaltic pump maintained at a flow-rate of 16.6 ml $cm^{-1}$ $h^{-1}$. The experiments were performed at room temperature and fractions of 3.3 ml were collected. The effluent was monitored continuously with a recording photometer (Uvicord; LKB, Bromma, Sweden) and fractions were read manually with a Pye Unicam SP 8–100 spectrophotometer.

Elution of adsorbed material was performed using the following desorption buffers in sequence: buffer II, 0.1 *M* Tris–HCl (pH 7.6); buffer III, 40% (v/v) ethylene glycol in buffer II; buffer IV, 30% (v/v) isopropanol in buffer II. The fractions corresponding to each peak were pooled and the total absorbance at 280 nm was measured. Electrophoretic analysis of the pooled and concentrated fractions was performed in slabs of a 4–30% polyacrylamide gradient gel according to the manufacturer's manual (Pharmacia).

*Regeneration*

The following procedure was found adequate for regenerating the adsorbents after each cycle of adsorption and desorption. The regeneration can be performed either in the column or, when handling large amounts of adsorbent, on a Büchner funnel. The adsorbent is washed with 1 *M* NaOH (three times the total bed volume) and the column is allowed to stand overnight. The washing with distilled water is continued until the effluent is almost neutral, followed by equilibration with the starting buffer to be used during the ensuing adsorption experiment. Alternatively, the adsorbent is washed with 95% ethanol (2–3 column volumes) followed by distilled water (at least 5 column volumes) and equilibrated with starting buffer. Very tightly bound substances (*e.g.*, certain pigments and lipids found in some extracts from biological tissues) can be removed by using the above washing procedures in sequence. In extreme instances, the use of a small pre-column is advisable for trapping solutes that tend to be bound irreversibly. For chromatography of serum proteins, washing with NaOH or ethanol was found to be sufficient to regenerate the adsor-

bent, allowing it to be used for several cycles of adsorption and desorption without any detectable change in its qualitative or quantitative adsorption properties.

## RESULTS AND DISCUSSION

### *Degree of substitution and coupling yield*

Table I shows the results of the sulphur analyses of the various alkyl agaroses. As the amount of sulphur found is equivalent to the amount of alkyl group coupled to the gel, the ligand density in each series can readily be calculated. This procedure is simpler and more convenient than the alternative ones used by other workers[10,11,13]. For the octyl derivative, which we investigated in greater detail, the relationship between the degree of substitution and the octyl mercaptan added during the coupling step is illustrated in Fig. 1. A linear relationship is observed for the ligand concentrations up to about 350 $\mu$mole S $g^{-1}$ dry gel. This might suggest, as postulated by Jennissen[17], that the ligands are distributed uniformly on the gel derivatives for degrees of substitution up to 350 $\mu$mole $g^{-1}$ dry gel. Such a uniform distribution might be a direct consequence of the high solubility of the alkyl mercaptans in alkaline solution. When the alkyl mercaptan is present in large excess, a plateau is approached which apparently corresponds to the number of available oxirane groups on the gel.

The results further show that the coupling yield in the linear portion of the curve is in the range 85–95%, which is much higher than that for the corresponding

TABLE I

RELATIONSHIP BETWEEN COUPLING YIELD AND THE AMOUNT OF ALKYL MERCAPTAN ADDED TO THE ACTIVATED GELS

The activation procedure results in gels with an oxirane content of about 550 $\mu$mole $g^{-1}$ dry gel as calculated from the data of Sundberg and Porath[14]. For purposes of comparison, the concentration of alkyl mercaptan in the coupling mixture is expressed on a dry weight basis, although in practice the concentration was calculated per weight of the suction-dried wet gel. The conversion to dry weight is achieved on the basis that 1 g of coupled and suction-dried wet gel corresponds to 85 mg of dry gel.* The sulphur content of the blank gel (amounting to 20 $\mu$mole $g^{-1}$ dry gel for the batch we employed) has been accounted for in each of the values reported here. The concentration of ligand or alkyl mercaptan is excpressed in $\mu$mole S $g^{-1}$ dry gel.

| *Concentration of alkyl mercaptan* | *Concentration of coupled ligand* | | | | *Coupling yield (%)* |
|---|---|---|---|---|---|
| | *Hexyl* | *Octyl* | *Dodecyl* | *Tetradecyl* | |
| 2000 | 445 | 440 | 430 | 430 | ** |
| 400 | 340 | 330 | 330 | 310 | 78–85 |
| 200 | 170 | 170 | 180 | 150 | 75–90 |
| 120 | ns*** | 100 | ns | ns | 83 |
| 40 | 37 | 36 | 32 | 35 | 80–93 |
| 20 | 16 | 16 | 17 | 17 | 80–85 |

* The results of the analyses showed that: (a) 1 g of suction-dried wet gel corresponds to 1.27 ml of packed gel and (b) 1 ml of packed gel corresponds to 67 mg of dry gel.

** The amount coupled corresponds apparently to the available bis-epoxide groups attached to the gel.

*** ns = Not synthesized.

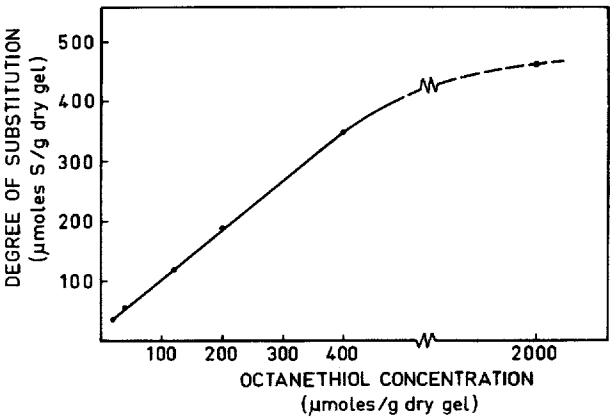



Fig. 1. Relationship between the octanethiol concentration used during coupling and the degree of substitution obtained for the Seph-S-$C_8$ derivative. The degree of substitution is calculated from the sulphur content of each gel derivative without correcting for the contributions from the blank gel (amounting to 20 μmole S $g^{-1}$ dry gel). Extrapolation of the linear portion of the curve to zero octanethiol concentration gives a measure of the sulphur content of the blank gel and corresponds very well to the value found in an independent sulphur analysis of the blank gel. Note the linear correspondence up to a ligand concentration of about 350 μmole $g^{-1}$ dry gel.

Seph-O-$C_n$[11] or Seph-N-$C_n$[3,13] derivatives. The coupling reaction is thus almost quantitative, indicating the higher reactivity of the mercaptans compared with the corresponding amines or glycidyl ethers. For all practical purposes, therefore, the degree of substitution can be calculated conveniently from the amount of mercaptan used during the coupling step, thus rendering an independent analysis of the ligand density almost redundant. This simplifies the synthesis of a series of carefully graded hydrophobic adsorbents (*i.e.*, adsorbents with different degrees of substitution at a constant alkyl chain length or *vice versa*) in order to achieve optimal conditions for the group separation of proteins in a given sample or the isolation of a particular protein.

In the standard procedure adopted here, 50% ethanol is used as the solvent for the alkyl mercaptans. This has been optimized so that the longer chain mercaptans can be solubilized. When smaller water-soluble molecules are to be coupled, the ethanol can be omitted without any adverse effect on the degree of substitution or on the coupling yield.

A large excess of 1,4-butanediol diglycidyl ether has been used throughout to ensure that coupling is limited only by the amount of added mercaptan. The excess of unreacted oxirane groups need not be blocked, as more than 90% are hydrolysed during the coupling procedure which extends over a period of 16–24 h and high pH. This is illustrated by the following series of control experiments: three different gel samples were treated as indicated and their relative oxirane contents were determined by manual titration with thiosulphate according to Sundberg and Porath[14]. The activation and coupling procedures were performed as described above. The gels were as follows: gel A, freshly activated Sepharose 6B; gel B, tetradecyl-Sepharose 6B with the lowest degree of substitution (*ca.* 1.7 μmole $g^{-1}$ suction-dried gel); gel C, the same as gel B, but after deactivation in a solution of 0.2 *M* $Na_2CO_3$ containing

1% $NaBH_4$ for 24 h at 45°C. The following results were obtained for the oxirane content: gel A, 27; gel B, 4; and gel C, 3 $\mu$mole $g^{-1}$ suction-dried wet gel.

The results show that the difference in oxirane content between the deactivated gel C and gel B is only 1 $\mu$mole $g^{-1}$. More than 96% of the unreacted oxirane groups are thus hydrolysed during the coupling procedure. The persistence of oxirane groups, even after the extensive and deliberate deactivation step, indicates that they are inaccessible or "inert" and are therefore not expected to interfere with the chromatographic experiments. This conclusion is consistent with the results of Sundberg and Porath[14].

*Adsorption capacity vs. degree of substitution*

The relationship between the adsorption capacity for serum proteins and the degree of substitution is shown in Fig. 2. The amount of adsorbed materials is expressed as the difference between the total $A_{280}$ of the serum applied to each column and the total $A_{280}$ of the fraction eluted unadsorbed by the equilibrating buffer. In every instance, the amount of serum applied was much lower than the total available capacity of the column.

The results show that the capacity of the gel derivatives for proteins is a sigmoidal function of the alkyl-residue density and that, for a given degree of substitution, the adsorption capacity increases in the order hexyl < octyl < dodecyl < tetradecyl. Gels with higher degrees of substitution (above 350 $\mu$mole $g^{-1}$ dry gel) tend to float in aqueous buffers and are therefore difficult to pack. This is particularly so with the octyl and higher derivatives. Their chromatographic performance is also diminished, probably owing to channelling and wall effects. Consequently, the adsorption capacity of such gel derivatives could not be estimated accurately. Moreover, it was observed that proteins adsorbed to highly substituted gels are more difficult to elute completely than they are from gels with lower degrees of substitution.

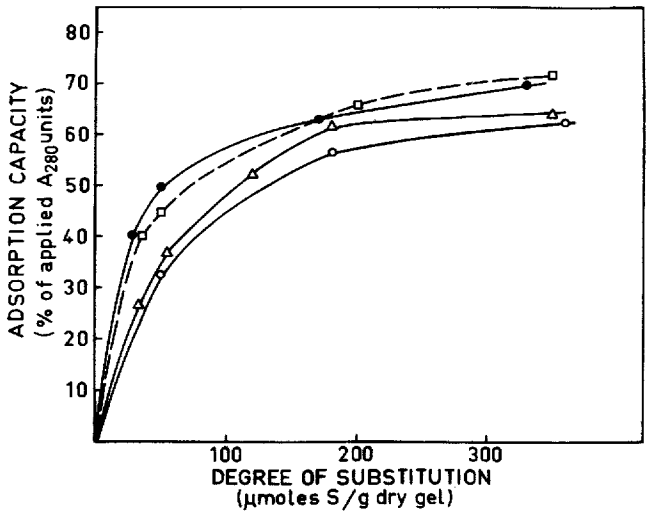



Fig. 2. Adsorption capacity for serum proteins of the various Seph-S-$C_n$ derivatives as a function of the concentration of fixed ligand. For experimental details, see text. ○, Hexyl; △, octyl; □, dodecyl; ●, tetradecyl derivative.

The family of curves shown in Fig. 2 seem to represent the behaviour of other hydrophobic adsorbents reported so far[10,13,17,18]. Such a relationship has been attributed to multi-point attachment of the proteins to the adsorption sites on the gels[17] and to the apparent temperature dependence of the minimum number of binding sites on the macromolecules[19]. However, the relationship obtained here is fairly linear up to a ligand concentration of about 80 $\mu$mole $g^{-1}$ dry gel. This would suggest that, as the concentration of fixed ligands is increased, the already adsorbed proteins overshadow the adsorption sites in their vicinity, thereby sterically hindering their accessibility to other adsorbable proteins. It further suggests that an adsorbent with a ligand density of about 80 $\mu$mole $g^{-1}$ dry gel has a fairly high capacity combined with an increased ease of desorption, with the obvious advantage that the recovery is high and the risk of denaturation of sensitive proteins is minimized.

The results shown in Fig. 3 give further support to the conclusions reached above and also show that the strength of the adsorption increases with increasing *n*-alkyl chain length. This is based on the finding that desorption of bound proteins

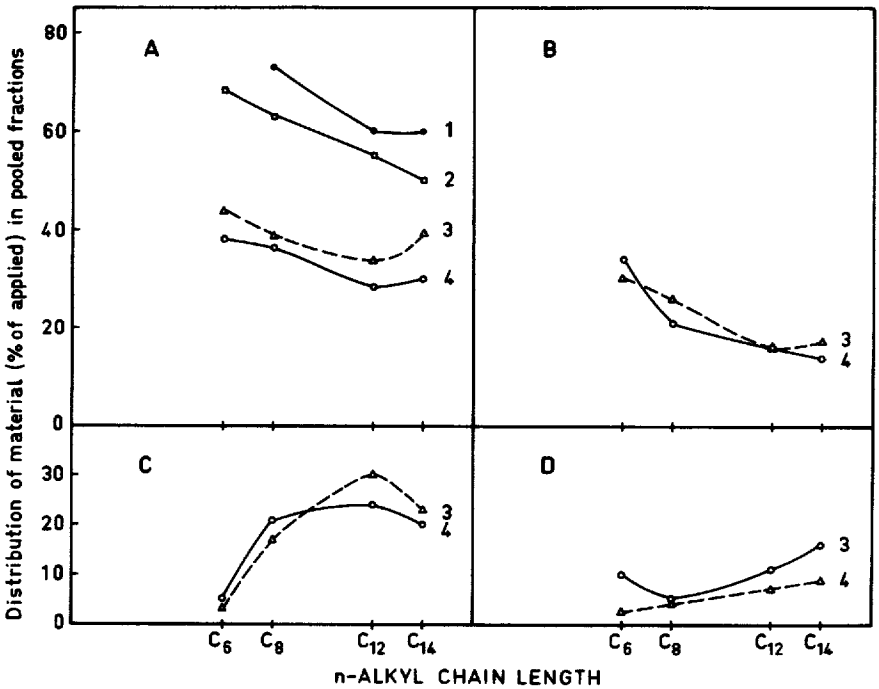



Fig. 3. Effect of the *n*-alkyl chain length (at various ligand concentrations) on the amount of serum proteins adsorbed and on the relative amounts of bound proteins that are successively displaced by eluents of decreasing polarity. For experimental details, see text. In each series of experiments, the percentage distribution of the unadsorbed material (A) and the fractions that were eluted by desorption buffers II (B), III (C) and IV (D) was calculated relative to the total amount of serum ($A_{280}$ units) applied in each instance. The family of curves was obtained for ligand concentrations of 19.6 (curve 1), 39 (curve 2), 196 (curve 3) and 392 (curve 4) $\mu$mole $g^{-1}$ dry gel. The total recovery of applied material was in the range 80–90%. Note: (1) with increasing alkyl chain length, the amount of unabsorbed material progressively decreases (*cf.*, Fig. 2); (2) the behaviour of the $C_{14}$ adsorbent is anomalous; and (3) the strength of the binding (adsorption) increases as the *n*-alkyl chain length increases (see curves B–D).

from gels with increasing *n*-alkyl chain length requires eluents of progressively decreasing polarity. Thus, for the $C_6$ derivative, most (55%) of the adsorbed proteins were eluted by desorption buffer II (*i.e.*, simply by omitting the added salt from the equilibrating buffer), whereas for the $C_{12}$ and $C_{14}$ derivatives most (45 and 35%, respectively) of the adsorbed proteins were eluted by buffer containing 40% of ethylene glycol. The adsorption/desorption characteristics of the $C_8$ derivative lie between those of the $C_6$ and $C_{12}/C_{14}$ derivatives, as would be expected. The $C_8$ derivative thus appears to combine a sufficiently high adsorption capacity with a relatively easy desorption of bound proteins. Considering the relative ease with which these derivatives can be synthesized, one can conveniently "tailor-make" hydrophobic adsorbents to suit the requirements of a particular group separation problem. The results presented here provide a good basis for such a choice.

*Comparison of the adsorption behaviour towards serum proteins of Seph-S-$C_n$ derivatives and Seph-O-$C_8$*

For purposes of comparison, the commercially available octyl-Sepharose CL-4B (Seph-O-$C_8$) was used as the reference "pure" hydrophobic adsorbent. According to the manufacturer, the ligand density is 40 $\mu$mole $ml^{-1}$ packed gel, which corresponds to about 600 $\mu$mole $g^{-1}$ dry gel. For the Seph-S-$C_n$ derivatives, the concentration of coupled ligand is 8 $\mu$mole $ml^{-1}$ packed gel, which corresponds to 121 $\mu$mole $g^{-1}$ dry gel. The ligand concentration of the commercial product is thus about five times higher than that of the Seph-S-$C_n$ derivatives used here.

The electrophoretic patterns of the fractions obtained after chromatography of human serum on these gels are shown in Fig. 4. The results show that the patterns of the proteins that are not adsorbed by the Seph-S-$C_n$ series of gels are remarkably similar. Some differences are apparent in lanes B-3 and 4, where some low-molecular-weight proteins at the bottom of the plate seem to be bound more strongly by the $C_{12}$ and $C_{14}$ columns. Characteristic of all the adsorbents in this series is that albumin, the major component and one of the "hydrophobic marker proteins" in serum, is not detected in the unadsorbed fractions, whereas with the Seph.O-$C_8$ column a significant amount of albumin is detectable in the unadsorbed fraction (see lane A-1). This finding is unexpected, considering the much higher ligand density in octyl-Sepharose CL-4B and in view of the fact that the amount of serum applied to each of the columns is much lower than the total available capacity. This would suggest that the Seph-S-$C_n$ series of adsorbents are comparatively more selective in terms of the types of proteins that they bind, a selectivity which might be attributed to some modulating influence of the thioether bond or the relatively long spacer arm situated between the gel surface and the attached alkyl groups. An alternative plausible explanation might be that the low degree of substitution in the Seph-S-$C_n$ series imparts a higher degree of selectivity simply due to possible competition between the adsorbable solutes for the available adsorption sites whereby the more hydrophobic ones are bound preferentially at the expense of the weakly hydrophobic solutes in the mixture.

Differences in the adsorption behaviour within the Seph-S-$C_n$ series becomes more apparent and can be deduced indirectly from the patterns obtained for the fractions desorbed by decreasing the salt concentration (plate C) or the polarity (plates D and E) of the eluent buffer. Thus, most of the proteins that are adsorbed

to the Seph-S-$C_6$ derivative are apparently displaced by elution with desorption buffers II and III, whereas with the Seph-S-$C_8$ derivative, for example, several proteins still remain adsorbed to the column and are apparently displaced by further elution with desorption buffer IV (see plate E-2). These findings are consistent with, and give further support to, the results shown in Fig. 2.

*The role of the spacer arm*

Two batches of Seph-S-$C_8$ adsorbents that were identical in all respects except the length of their respective spacer arms were synthesized to investigate the possible influence of the spacer arm on the adsorption characteristics of such gel derivatives. In each instance, the ligand concentration was about 120 $\mu$mole $g^{-1}$ dry gel. As in all previous experiments, human serum was used as the sample.

The results obtained are shown in Fig. 5, and indicate that there is at least a qualitative difference between the patterns of the proteins eluted in the corresponding fractions from the two adsorbents. The most distinct difference is the presence of a significant amount of albumin among the unadsorbed proteins from the column with the shorter spacer arm. This would suggest that the capacity of this adsorbent for

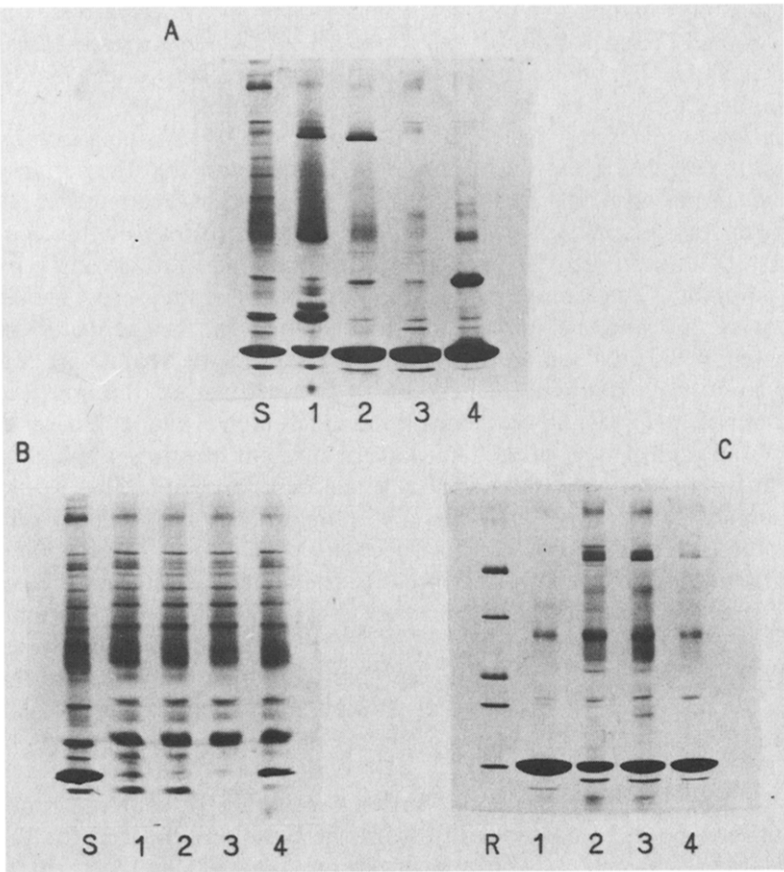



Fig. 4.

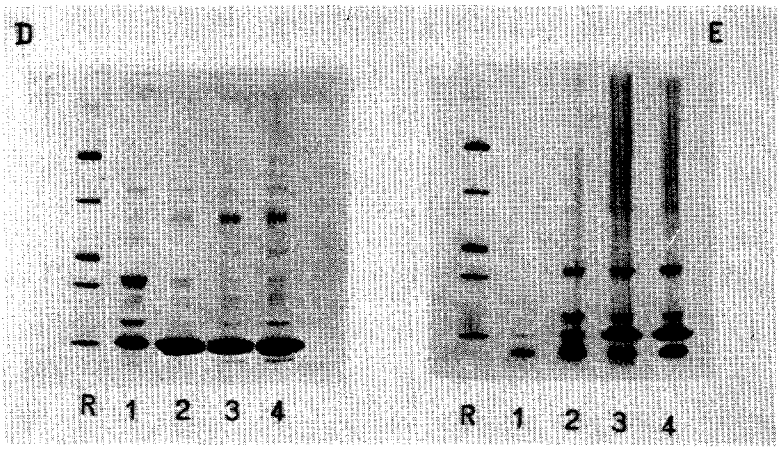



Fig. 4. Polyacrylamide gel (PAA 4/30) electrophoretic patterns of fractions obtained after chromatography of human serum on Seph-O-$C_8$ (plate A) and Seph-S-$C_n$ (plates B–E). In each instance, 8–10 $\mu$l of the concentrated fractions ($A_{280} \approx 10$) were applied and electrophoresis was performed for 16 h at 150 V according to the manufacturer's recommendations. Plate A: the patterns of the fractions obtained from octyl-Sepharose CL-4B (Seph-O-$C_8$). S = applied serum sample; 1 = unadsorbed fraction; 2,3 and 4 = fractions eluted by desorption buffers II, III and IV, respectively. Plates B–E: patterns of the fractions obtained from hexyl (1), octyl (2), dodecyl (3) and tetradecyl (4) derivatives of agarose (Seph-S-$C_n$ series). Plate B shows the patterns of the unsorbed fractions, plate C the fractions eluted by desorption buffer II, plate D the fractions eluted by desorption buffer III and plate E the fractions eluted by desorption buffer IV. R = reference proteins representing (from top to bottom) thyroglobulin ($M_r$ = 669,000), ferritin ($M_r$ = 450,000), catalase ($M_r$ = 232,000), lactate dehydrogenase ($M_r$ = 140,000) and albumin ($M_r$ = 67,000). Note: (a) the quantitative and qualitative diffrences in the patterns of the proteins unadsorbed by Seph-O-$C_8$ (lane A-1) on the one hand and those unadsorbed by the Seph-S-$C_n$ series on the other. Despite the fact that Seph-O-$C_8$ apparently adsorbs more proteins, a significant amount of albumin is found among the unadsorbed fractions. This is interesting considering that none of the columns was overloaded and also that the comparatively high ligand density in the Seph-O-$C_8$ (about five times higher) should have been sufficient to adsorb all of the albumin in the applied sample. (b) Gels of the Seph-S-$C_n$ series efficiently adsorb the albumin regardless of the alkyl chain length, (c) The adsorption of proteins to the Seph-S-$C_n$ series increases gradually with increase in the alkyl chain length (*cf.*, plates C–E).

albumin is considerably less than that of its counterpart with the longer spacer arm. Herein lies probably the most obvious difference between these two "essentially identical" adsorbents. The long spacer arm apparently enhances the hydrophobicity of the *n*-alkyl ligand covalently attached to it. By itself, however, the long spacer arm seems to be "inert" and, for all practical purposes, does not adsorb proteins. This has been shown by coupling mercaptoethanol to Sepharose 6B that had been activated with bis-epoxide according to the standard procedure adopted here. On chromatography of serum on such a derivative, a maximum of about 1% of the applied material was weakly adsorbed. This finding is contrary to the proposal of Shaltiel and Er-el[20] that long hydrocarbon extensions themselves contribute to binding of proteins via hydrophobic interactions. A possible explanation of the discrepancies mentioned here might be that the long spacer arm which Shaltiel and Er-el refer to might have more hydrophobic character than the one we used in this work.

The long spacer arm also seems to impart a significant degree of selectivity to the adsorbent. The one with a short spacer arm seems to be less selective than its

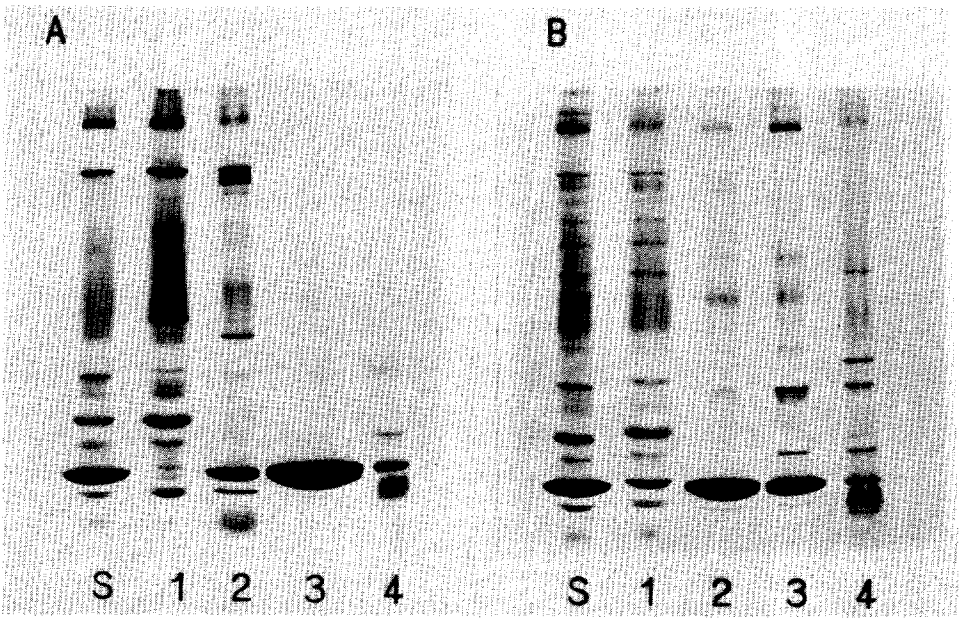



Fig. 5. Electropherogram of fractions obtained after chromatography of serum on Seph-S-$C_8$ derivatives with a long spacer arm (plate A) and a short spacer arm (plate B). The short spacer arm is derived from epichlorohydrin activation and the long one from 1,4-butanediol diglycidyl ether activation of the gel. S = whole serum; 1 = unadsorbed fraction; 2, 3 and 4 = fractions eluted by desorption buffers II, III and IV, respectively. Note: (1) the presence of a significant amount of albumin in the unadsorbed fraction (lane B-1) from the column with short spacer arm. As in the previous instances, none of the columns was overloaded with sample. (2) Most of the albumin is eluted in fraction 3 (lane A-3) when the spacer arm is long. However, most of it elutes in fraction 2 (lane B-2) when the spacer arm is short. (3) There is a qualitative difference between the patterns of proteins adsorbed on these "essentially identical" adsorbents. (4) The pattern obtained on plate B is reminiscent of that obtained with octyl-Sepharose CL-4B (see Fig. 4A).

long spacer counterpart, as evidenced by distinctive differences in the patterns of the proteins that are eluted by 40% ethylene glycol and 30% isopropanol (Fig. 5, lanes 3 and 4). This might be due to a possible relaxation of steric restrictions that arise from the matrix backbone and a concomitant increase in the flexibility of the ligand as it protrudes further out into the solvent. Its advantages in this respect are analogous to those observed in affinity chromatography in general[21–23].

*Stability*

The alkyl sulphide ligand is stable within the pH stability range (pH 3–14) of the parent gel matrix to which it is coupled. It is also stable towards chaotropic salts such as KSCN and denaturing agents such as urea and guanidine hydrochloride. Further, the concentration of ligand was essentially unchanged after desulphating[24] a series of gel derivatives with varying degrees of substitution. Treatment of the cross-linked blank gel in the same manner reduced its sulphur content from 29 to 20 $\mu$mole $g^{-1}$ dry gel, which is due to the hydrolysis of the sulphate ester groups that are present in the parent gel.

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