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Synthesis and protein-binding properties of spacer-free thioalkyl agaroses

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ABSTRACT

Thioalkyl agaroses were synthesized by the tresyl chloride method. Activation of beaded agarose (Sepharose 4B) with tresyl chloride is a non linear function of the tresyl chloride concentration in the activation mixture. The coupling of alkanethiols to tresyl chloride-activated Sepharose was successful in an alkaline medium containing 0.64 *M* sodium hydroxide. For the synthesis of thioalkyl agaroses (alkyl-S-Sepharose) with different surface concentrations of immobilized residues the activation time and not the tresyl chloride concentration should primarily be varied. At high tresyl chloride concentrations and long activation times the agarose matrix is destroyed. It was found that optimum concentrations of tresyl chloride lie between 0.05 and 0.15 *M* if activation times of 60 min are not exceeded. Alkyl-S-Sepharoses differ significantly in their protein adsorption properties from alkyl-N-Sepharoses prepared by the cyanogen bromide or carbonyl diimidazole (CDI) methods. Linkage of the alkyl residue via a thioether bridge enhances the adsorption of proteins by at least an order of magnitude. The results indicate a much stronger influence of the base (sulphur atom) of an immobilized alkyl residue in comparison with its tip (methyl group) than has hitherto been realized. The higher affinity of binding may be due to an interaction of the π-electrons of the sulphur atom with π-electrons in an aromatic amino acid of the protein in addition to the expected hydrophobic interaction between alkyl residue and protein.

INTRODUCTION

Alkyl agarose derivatives have been employed for the chromatographic separation of macromolecules (for reviews, see refs. 1 and 2) and for quantitative protein adsorption studies (for a review, see ref. 3). For the analysis of protein adsorption the interpretation of results is greatly facilitated if the residues are directly coupled to the inert hydrophilic agarose matrix as the interference of spacers with protein binding can never be fully ruled out. As cross-linked agarose gels pose a similar problem as spacer-containing gels only non-cross-linked agarose (Sepharose 4B) was employed.

As an alternative to the cyanogen bromide method of activation of agaroses (which may also introduce some charges [4,5]), the *p*-toluenesulphonyl chloride (tosyl chloride) method of activation [6,7] was introduced. Owing to the relatively low reactivity of tosyl chloride Mosbach and Nilsson [8] later introduced 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride) as a more potent activator of agarose for

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subsequent coupling reactions. Tresylates of agarose (tresyl agarose) can react with primary amino groups or with thiol groups [8]. In the latter instance uncharged agarose derivatives are obtained. Except for preliminary reports [9–11], no method for the preparation of thioalkyl agaroses (alkyl-S-Sepharose) by the tresyl chloride method has been reported, probably owing to the difficulty of bringing alkanethiols into optimum solution for coupling to tresyl agarose. Since our initial report [9], thioether-bonded alkyl derivatives of agarose have been reported by Porath's group [12]. In their work thioalkyl derivatives (C_6–C_{14}) were coupled to Sepharose 6B by the bis-epoxide method via a spacer backbone consisting of ten carbon and three oxygen atoms.

In this paper we report the coupling of homologous thioalkyl residues (C_2–C_4) without a spacer directly to the agarose matrix leading to alkyl-S-Sepharoses which can be employed for protein adsorption experiments and hydrophobic chromatography. These alkyl-S agaroses have profoundly different protein-binding properties than the corresponding alkyl-N-agaroses produced by either the cyanogen bromide [13] or the carbonyl diimidazole [14] methods.

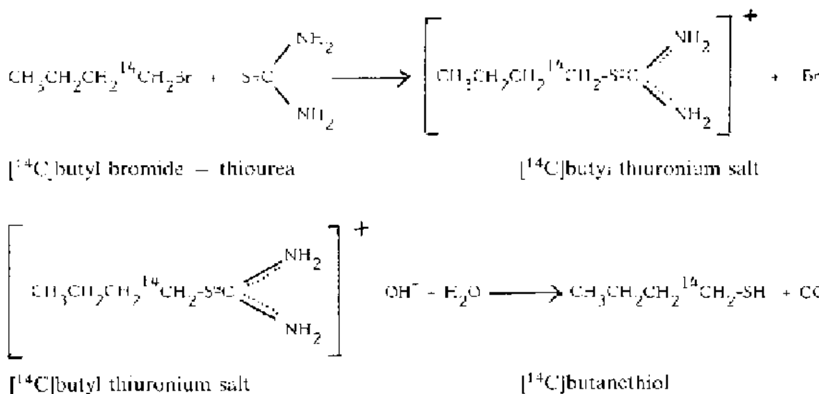
EXPERIMENTAL

Preparation of calmodulin

Bovine testis calmodulin was isolated according to ref. 15 in conjunction with affinity chromatography [16]. The biological activity of purified calmodulin was tested with phosphorylase kinase [17] in the AutoAnalyzer test [18]. The calmodulin concentration necessary or half-maximum activation of phosphorylase kinase was 30–50 n*M*.

Preparation of alkyl-S-Sepharose

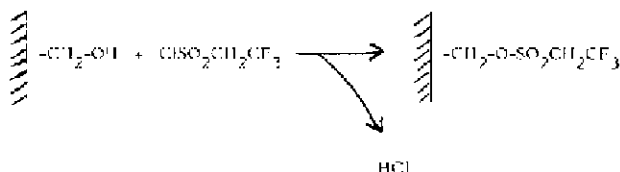
Synthesis of n-[1-^{14}C]butanethiol. The method is based on the synthesis of thiols via S-alkyl thiuronium salts from thiourea [19].



In a 100-ml spherical flask, 15.5 g (0.2 mol) of thiourea and 10.2 ml of 95% ethanol were mixed with 25.5 g (0.18 mol) of butyl bromide to which *ca.* 2.5 mCi of *n*-[1-^{14}C]butyl bromide were added and boiled for 6 h in a reflux column. After

cooling, the butyl thiuronium salt crystallized. The liquid was removed by suction and the thiuronium salt was hydrolysed to butanethiol by the addition of 56 ml of 5 *M* sodium hydroxide solution under nitrogen and heating at 100°C for 2 h under reflux. After cooling, the solution was neutralized with 2 *M* hydrochloric acid. The thiol phase was isolated, dried with magnesium sulphate and fractionated on a Vigreau column. Finally 6.3 ml (5.48 g) of pure butanethiol was obtained, corresponding to a total yield of *ca.* 33%. The boiling point of the end product was 96–98°C.

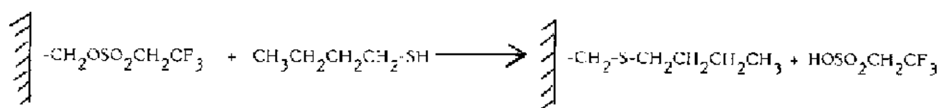
Activation of agarose matrix with tresyl chloride. The method of activation of agarose (Sepharose 4B; Pharmacia, Uppsala, Sweden) with tresyl chloride is essentially derived from ref. 8 and can be described by the following reaction:



Accordingly, 40 g "wet weight" [4] of Sepharose 4B (Sepharose from which the exterior water had been removed by suction [4]) were washed at very low vacuum on a Büchner funnel with 20 volumes each of water, water–dioxane (3:1), water–dioxane (1:3) and finally water-free (dry) dioxane, which was then removed by suction (suction of air through the gel was avoided), yielding a cake of water-free Sepharose. To 10 g of water-free Sepharose, 0.5 vol. of dioxane and pyridine (2 ml/ml tresyl chloride) were added. Tresyl chloride in an amount of 10–40 μl/g water-free gel was added in the first minute in 3 vol. of dioxane with stirring. The gel was activated for 120 min with the temperature being held constant at 20°C (water-bath). Activation was stopped by washing the gel on a Büchner funnel with 20 volumes each of water-free (dry) dioxane, water–dioxane (1:3), water–dioxane (3:1) and finally water. The activation product, tresyl-Sepharose, can be stored in water at 4°C for several days without loss of reactivity [8].

For the measurement of time curves, 40 g "wet weight" of Sepharose 4B were activated by 0.052, 0.104, 0.156 and 0.208 *M* tresyl chloride respectively. At the indicated times samples of 1/4 to 1/8 volume of the incubation mixture with the activated gel were taken from the mixture, washed (see above) and incubated for 1 h with the alkaline alkanethiol solution as described below.

Coupling of alkanethiol (C_2–C_4) to tresyl-Sepharose. First experiments showed that tresyl-Sepharose could not be derivatized with butanethiol according to the general procedure described by Mosbach and Nilsson [8]. However, as will be shown (see Results), this problem was overcome by using another medium. The following reaction scheme describes the coupling reaction:



In a typical preparation, 10 g (wet weight) of tresyl-Sepharose were added to 18 ml of a solution containing 0.43 *M* alkanethiol in 1 *M* sodium hydroxide solution in a closed flask at room temperature. The final concentrations in the mixture after adding the Sepharose were 0.28 *M* alkanethiol and 0.64 *M* sodium hydroxide. The preparations can be scaled up by a factor of 25 without substantial changes in the coupling yield.

For the synthesis of a homologous series, *e.g.*, of three different thioalkyl agaroses of identical degree of substitution, a 3-fold amount (wet weight) of Sepharose 4B was activated with tresyl chloride as described above. After activation and washing (see above), three equal portions of the gel were added at room temperature to the corresponding alkaline alkanethiol solution, one of which was butanethiol solution containing the tracer *n*-[1-^{14}C]butanethiol. As the degree of substitution with butanethiol corresponded quantitatively to the degree of activation with tresyl chloride (see Results), an identical degree of substitution of the tresyl-Sepharose in the non-tracer-labelled alkanethiol solutions could be concluded from the *n*-[1-^{14}C]-butanethiol experiment.

After coupling, the gel was washed on a Büchner funnel with *ca.* 20 volumes each of water, acetone–water (1:3, v/v), acetone–water (3:1, v/v) and finally pure acetone until the thiol smell had disappeared. This acetone wash is essential for the removal of disulphides (as will be shown). By a subsequent reversal of the just described washing procedure, the gel was transferred back into an aqueous medium.

As has been discussed [7,20], the S–C bond is very resistant to acidic and alkaline conditions so that stable gel derivatives are obtained. In our experience, butyl-S-Sepharose gels can be stored in neutral aqueous solutions for up to 7 years without a significant loss of immobilized residues.

Micrographs of washed Sepharose 4B and Sepharose 4B derivatives (suspended in water containing bovine serum albumin, 1 mg/ml) were made with a Leitz microscope and a magnification of 140-fold [we thank Dr. F. Mehnert (University of Bochum) for taking the micrographs of Sepharose].

Synthesis of alkyl-N-Sepharoses

Uncharged alkyl-N-Sepharose was prepared according to the carbonyl diimidazole (CDI) method of Bethell *et al.* [14]. [^{14}C]Butylamine and [^{14}C]ethylamine (New England Nuclear) were employed as tracers, yielding identical substitution results with the CDI method (see also refs. 4 and 21 for results with the cyanogen bromide method).

Solutions and reagents

Dioxane was distilled and subsequently stored in the presence of sodium wire. Highly pure commercial dioxane stored in the presence of sodium wire gave similar results. Water was first deionized and then distilled before use. All other reagents were of analytical-reagent grade. Tresyl chloride was obtained from Fluka.

Analysis of tresyl- and butanethiol-Sepharose

Sulphur determination. Tresyl- and butyl-Sepharose were dried under vacuum at 40°C for *ca.* 2 days (see below). About 20 mg of the dried gel were transferred to a heat stable vial and solid sodium was melted and dripped into the gel. The vial was heated to

a red glow and then transferred into 5 ml of water, where it burst. The aqueous solution of sodium salts was filtered and employed for sulphur determination by iodimetric titration according to Kimball *et al.* [22]. The analyses were done in triplicate.

Radioactive tracer analysis. The degree of substitution was monitored by adding *n*-[1-^{14}C]butanethiol to the coupling mixture in an amount of 0.05–0.25 μCi/ml [4]. The amount coupled was determined after acid hydrolysis [4] of the *n*-[1-^{14}C]butanethiol-labelled agarose (colourless hydrolysate) followed by liquid scintillation counting [4]. The analyses were done in triplicate.

Determination of gel parameters (packed gel, dry gel, surface area)

The volume of packed gel was determined as previously described [4], giving the degree of substitution in μmol/ml packed gel [4]. The dry weight of the agarose was either determined as described previously [23] or after drying in vacuum for *ca.* 2 days at 40°C (see above). The degree of substitution can then also be expressed in μmol/g dry weight, correcting for errors due to shrinking of the gel during activation or coupling [24]. All analyses were done in triplicate. In general, 1 ml of packed Sepharose leads to *ca.* 30 mg (29–32 mg) of agarose mass after drying [23]. With substituted gels the weight of the dry agarose was normalized, *i.e.*, corrected to net dry weight (= total weight of dried alkyl gel minus the weight of incorporated thioalkyl residues) [24]. Finally, the degree of substitution can either be expressed as a molecular substitution ratio in mol alkyl residue/mol anhydrodisaccharide [24] based on a molecular weight of 306 for an anhydrodisaccharide unit, or as a surface concentration in nmol/m^2 [25,26]. The values for the surface concentration in nmol/m^2 are based on a concentration of anhydrodisaccharide units of *ca.* 100 μmol per 30 mg of dry agarose (see also ref. 23) and a mean surface area of *ca.* 1.3 nm^2 per anhydrodisaccharide unit (with the assumption that this corresponds to the water-accessible surface area) in the final quaternary structure of agarose [27]. From these values a specific surface area of the agarose strands of 2610 m^2/g dry agarose can be calculated. Accordingly, for alkyl-substituted agarose 1 μmol/ml packed gel corresponds to 12.8 nmol/m^2 for non-shrunken agarose [26].

Protein was determined according to Lowry *et al.* [28] on an AutoAnalyzer.

Analytical hydrophobic affinity chromatography of calmodulin [16]

The chromatographic analysis of alkyl agaroses with calmodulin was performed on columns (12 × 0.9 cm I.D.) containing 2 ml of packed gel at room temperature. The gel was washed and equilibrated with buffer A (20 m*M* Tris–HCl, 1 m*M* $CaCl_2$, pH 7.0). A sample of 1 mg of purified calmodulin was applied in a volume of 1 ml (buffer A). Unless stated otherwise, fractions of 1.5 ml were collected. The column was then washed with 9 ml of buffer A and subsequently with 9 ml of buffer B (= buffer A + 0.3 *M* NaCl). Adsorbed calmodulin was eluted with buffer C (20 m*M* Tris–HCl, 0.3 *M* NaCl, 10 m*M* EGTA, pH 7.0). Flow was achieved by gravity. Only fresh, unregenerated gel was used.

RESULTS

Synthesis of butyl-S-Sepharoses

Coupling mixture. Table 1 demonstrates that even at high tresyl chloride

TABLE I

DEPENDENCE OF THE COUPLING OF [^{14}C]BUTANETHIOL TO TRESYL-SEPHAROSE 4B ON THE COUPLING MEDIUM

Sepharose was activated with tresyl chloride (0.208 *M*) for 24 h at room temperature as described and then coupled as follows. (a) In the first coupling experiment, 10 g (wet weight) of tresyl-Sepharose were washed with water–dioxane–acetone (1:2:6, v/v/v) and then added to 14.4 ml of solution A (4 ml of [^{14}C]butanethiol plus 10.4 ml of acetone). To the washed gel in solution A were added 3.6 ml of solution B (1.2 ml of 0.5 *M* Na_2CO_3 plus 2.4 ml of dioxane) and this mixture was incubated for 15 h at room temperature. (b) In the second experiment, 10 g (wet weight) of tresyl-Sepharose were added to 18 ml of solution C (1 *M* NaOH, 0.43 *M* [^{14}C]butanethiol) and incubated for 15 h at room temperature. In both experiments after 15 h the coupled gel was washed with acetone and analysed for incorporation of [^{14}C]butanethiol as described

Coupling medium	[^{14}C]Butanethiol (mol/l)	Degree of substitution (μmol butyl residues/ml packed gel)
Carbonate–acetone–dioxane	1.3	2
0.64 *M* NaOH	0.28	56

concentrations (0.208 *M*) in the incubation mixture, only very little (2 μmol/ml packed gel) butanethiol can be coupled to tresyl-Sepharose in a carbonate–acetone–dioxane medium, a solution which is capable of solubilizing high concentrations (1.3 *M*) of butanethiol. In contrast, solubilizing butanethiol for coupling in pure 0.64 *M* sodium hydroxide (= alkaline alkanethiol) leads to a nearly 30-fold increase in the degree of substitution (Table I).

Washing procedure. The "coupling values" obtained can, however, still be misleading as they depend strongly on the washing procedure, as is shown in Table II. Failure to wash with an organic solvent (e.g., acetone) leads to highly erroneous degrees of substitution. This is most easily demonstrated by incubating non-activated,

TABLE II

INFLUENCE OF THE WASHING PROCEDURE ON THE RETENTION OF [^{14}C]BUTANETHIOL ON NON-ACTIVATED SEPHAROSE 4B AND ON THE DEGREE OF SUBSTITUTION OF TRESYL-SEPHAROSE WITH [^{14}C]BUTANETHIOL

5 g (wet weight) of non-activated Sepharose 4B was added to 1.8 vol. of 1 *M* NaOH–0.43 *M* [^{14}C]butanethiol ($\pm$0.107 *M* H_2O_2) and incubated at room temperature for 7 h. Tresyl-Sepharose (activated with 0.156 *M* tresyl chloride for 1 h) was coupled by the same method (but without H_2O_2) for 20 h at room temperature. After coupling the gels were washed either with 20 vol. of 1 *M* NaOH or 20 vol. of water, water–acetone (3:1), water–acetone (1:3) and acetone (followed by a wash in the reverse order to return to the aqueous medium).

Gel	Degree of substitution (μmol butyl residues/ml packed gel)	
	Wash with 1 *M* NaOH or KOH	Wash with acetone
Sepharose 4B (non-activated):		
Incubation with [^{14}C]butanethiol + NaOH	10	0.17
Incubation with [^{14}C]butanethiol + NaOH + H_2O_2	64	0.64
Tresyl-Sepharose: coupling with [^{14}C]butanethiol + NaOH	37	22.2

control Sepharose 4B in alkaline [^{14}C]butanethiol followed by a wash with 1 *M* sodium hydroxide or potassium hydroxide (Table II). Apparently 10 μmol butyl residues/ml packed gel are coupled. In a second experiment, non-activated Sepharose was incubated in alkaline [^{14}C]butanethiol together with hydrogen peroxide as oxidant of the thiol. The apparent degree of substitution of the gel with butanethiol increases to over 60 μmol/ml packed gel. The non-covalent nature of thiol retention on the gel is easily demonstrated by washing it with acetone, which reduces the amount retained (probably insoluble disulphide) by a factor of 100. Acetone-stable radioactivity was therefore taken as evidence for covalent coupling of the thiol to the tresyl-activated Sepharose. In the present case with tresyl-Sepharose (Table II) a true coupling yield of *ca.* 22 μmol butyl residues/ml packed gel is obtained.

Time dependence of coupling. Fig. 1 shows the time dependence of the coupling of *n*-[1-^{14}C]butanethiol to tresyl-Sepharose (activated with 0.208 *M* tresyl chloride) for 15 h. Maximum coupling is achieved after 1 h. As the amount of butanethiol is in *ca.* 10-fold excess over the amount of immobilized tresyl leaving groups on the activated gel, a quantitative substitution of these groups and not a limitation of available butanethiol seems likely to be the reason for termination of the reaction. As a consequence of these results, all further coupling mixtures (Tables II–V, Figs. 2–5) were incubated for 60 min at room temperature only.

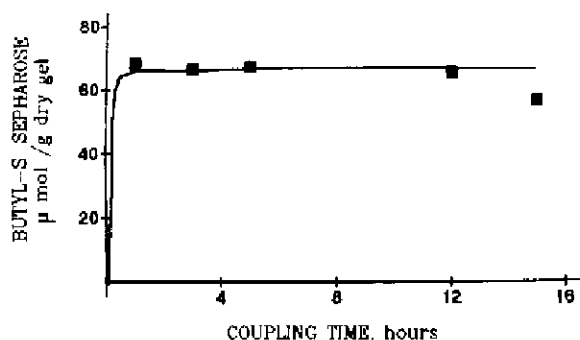


Fig. 1. Time dependence of the coupling of [^{14}C]butanethiol to tresyl-Sepharose at room temperature. 40 g (wet weight) of tresyl-Sepharose (activated by 0.208 *M* tresyl chloride) was added to 72 ml of 1 *M* NaOH–0.43 *M* [^{14}C]butanethiol. At the indicated times *ca.* one fifth of the coupling mixture was taken as a sample and the reaction was stopped by washing with acetone on a Büchner funnel as described. The degree of substitution was determined by measuring the ^{14}C incorporation (0.028 μCi/ml) as described.

Dependence of activation on tresyl chloride concentration. Table III shows the non-linear dependence of the degree of substitution with tresyl and butyl residues on the initial concentration of tresyl chloride in the activation mixture. The immobilized tresyl chloride and butanethiol were determined in parallel by a determination of sulphur in the activated gel before and after coupling (Table III, series I) and by radioactivity measurements of the coupled gel (Table III, series I and II). The amount of tresyl chloride covalently incorporated into the gel from the incubation mixture was between 3 and 8%. Therefore, the initial concentrations of tresyl chloride only change insignificantly as a result of the activation procedure itself. Table III demonstrates that

TABLE III

DEPENDENCE OF THE DEGREE OF SUBSTITUTION OF SEPHAROSE 4B ON THE TRESYL CHLORIDE CONCENTRATION IN THE INCUBATION MIXTURE

Sepharose 4B was activated with tresyl chloride at the given concentration for 60 min. The degree of substitution was measured by determination of elemental sulphur in tresyl-Sepharose and butyl-S-Sepharose (series I) and by tracer analysis of butyl-S-Sepharose with [^{14}C]butanethiol (series I and II). In series I activation was performed in dioxane distilled and stored over sodium wire; in series II it was performed in commercial dioxane stored over sodium wire.

Tresyl chloride (mol/l)	Sulphur determination (μmol/ml packed gel)		[^{14}C]tracer analysis (μmol/ml packed gel)
	Tresyl-Sepharose	Butyl-Sepharose	
Series I:			
0.052	—	·	2.6
0.104	9.6	9.1	12.6
0.156	21.8	26.4	22.5
0.208	59.0	63.2	54.7
Series II:			
0.040			3.5
0.060			3.0
0.080			1.9
0.090			19.3
0.100			32.0
0.150			37.0

the sulphur and the butyl residue determinations yield almost identical results within the experimental error. From this experiment, coupling yields of *ca.* 100% can be calculated.

Progress curves of tresylation. As the coupling yield is *ca.* 100%, tresylation can be measured via ^{14}C incorporation of alkanethiol into tresyl-Sepharose. The time dependence of tresylation as measured in this manner is shown in Fig. 2. At low tresyl chloride concentrations (Fig. 2A) there is a primary incorporation phase which reaches a plateau region after 40–120 min. However, as the concentration of tresyl chloride is increased (Fig. 2B), a secondary incorporation phase can be seen to occur from the previous plateau phase after *ca.* 80 min, leading to a 2–3-fold additional increase in immobilized butanethiol residues over the plateau region. Finally, at very high tresyl chloride concentrations (0.208 *M*) a true plateau phase is absent and the degree of substitution increases continuously from the onset (Fig. 2B). Tresyl chloride concentrations above *ca.* 0.21 *M* lead to a destruction of the agarose beads (not shown).

Fig. 3 illustrates that the secondary incorporation phase of tresylation and coupling is not due simply to a volume decrease (shrinkage) of the substituted agarose. A recalculation of the original data for the degree of substitution (Fig. 3A) on the basis of the determined dry weight (Table IV) of the agarose leads to lower values but does not abolish the secondary incorporation phase (Fig. 3B).

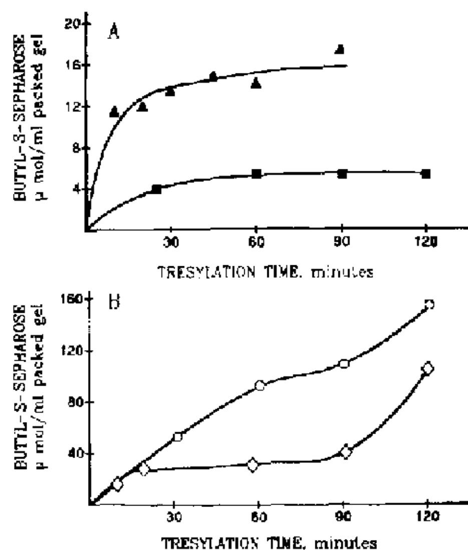


Fig. 2. Progress curves of the tresylation of Sepharose 4B at different concentrations of tresyl chloride in the activation mixture. At each concentration of tresyl chloride (see below) 40 g (wet weight) of Sepharose 4B was activated. At the indicated times *ca.* 1/4–1/8 vol. of the incubation mixture was taken as a sample, washed with dioxane and transferred to aqueous medium as described. The obtained gel (*ca.* 5–10 g, wet weight) was then added to 1.8 vol. of 1 *M* NaOH–0.43 *M* [^{14}C]butanethiol and incubated for 1 h at room temperature. The degree of substitution was determined by measuring the incorporation of [^{14}C]butanethiol. (A) ■ = 0.052 *M*; ▲ = 0.104 *M*. (B) ◇ = 0.156 *M*; ○ = 0.208 *M*.

TABLE IV

SYNTHESIS OF ALKYL-S-SEPHAROSES OF DIFFERENT DEGREES OF SUBSTITUTION AND DIFFERENT ALKYL CHAIN LENGTH IN PREPARATIVE AMOUNTS

For the butyl-S-Sepharose series, *ca.* 210 g (wet weight) of Sepharose 4B were incubated with the given concentration of tresyl chloride for the specified time and then coupled to [^{14}C]butanethiol for 1 h. For the homologous series (C_2–C_4), *ca.* 50 g (wet weight) of Sepharose 4B were activated and coupled as described. For definition of µmol/ml packed gel, µmol/g dry gel, mol/mol anhydrodisaccharide and nmol/m², see Experimental.

Gel	Tresyl chloride (mol/l)	Activation time (min)	Corrected dry weight (mg/ml packed gel)	Degree of substitution			
				µmol butanethiol/ml packed gel	µmol/g dry gel	mol butanethiol/mol anhydrodisaccharide	nmol/m²
Sepharose 4B			32.0				
Butyl-S-Sepharose	0.052	15	33.6	1.7	50.6	0.015	19.4
	0.156	1	30.1	5.2	172.7	0.053	66.2
	0.156	2	32.0	8.9	278.1	0.085	106.6
	0.156	3.5	37.1	12.0	323.4	0.099	123.9
	0.156	5	33.6	20.7	616.1	0.189	236.1
	0.156	20	38.4	29.0	755.2	0.219	289.3
	0.156	60	36.5	28.4	778.1	0.238	298.1
	0.156	90	37.6	40.7	1082.4	0.331	414.7
	0.156	120	62.4	107.0	1714.7	0.524	657.0
Ethyl-S-Sepharose	0.156	20	34.1	25.0	733.1	0.224	282.0
Propyl-S-Sepharose	0.156	20	36.6	25.0	683.1	0.209	262.7
Butyl-S-Sepharose	0.156	20	36.5	25.0	684.9	0.210	263.4

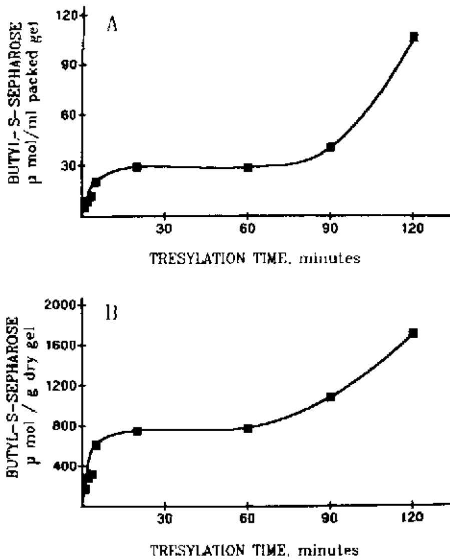


Fig. 3. Biphasic activation of Sepharose 4B by the tresyl chloride method. The degree of substitution with tresyl residues was determined by the quantitative substitution of tresyl leaving groups with [^{14}C]butanethiol as described. The concentration of tresyl chloride in the incubation mixture was 0.156 *M*. The curves are composed of a primary incorporation phase, a plateau phase and a secondary incorporation phase. The data demonstrate that the secondary incorporation phase is not a result of the dimensions employed for expressing the data and thus is not a result of gel shrinkage. (A) Degree of substitution expressed in μmol/ml packed gel. (B) Degree of substitution expressed in μmol/g dry gel.

Microscopic analysis. Fig. 4 demonstrates that macroscopically tresyl- and butyl-S-Sepharose do not change their morphology significantly in comparison with the unsubstituted gel. At high degrees of substitution polygonal beads occur.

Controlled variation of the degree of substitution. The foregoing data show that the synthesis of butyl-S-Sepharoses with different degrees of substitution is complex and depends non-linearly (Table II) on the tresyl chloride concentration in the activation mixture. Therefore, a series of gels with different degrees of substitution (without disturbance by the secondary incorporation phase) are not easily obtained if the tresyl chloride concentration is varied. These problems can be circumvented by varying the activation time at a constant tresyl chloride concentration of, *e.g.*, 0.156 *M* (Table III) to obtain any degree of substitution above 5 μmol/ml packed gel. If one adheres to the primary incorporation phase only (*i.e.*, times below the second increment 1–60 min, Fig. 2B), homogeneity of the gels appears to be obtainable.

In order to avoid activation times shorter than 1 min, gels below 5 μmol/ml packed gel were synthesized at a lower tresyl chloride concentration, *e.g.*, 0.052 *M*. Table III also demonstrates that between 1 and 20 min of activation the mean dry weight of the agaroses (corrected for the weight of the immobilized alkyl residues) increases by 4–6% compared with the control, indicating a negligible gel shrinkage (50–755 μmol/g dry agarose). A maximum gel shrinkage to *ca.* 50% of the initial agarose volume is indicated by a doubling of the dry weight/ml packed gel for the gel

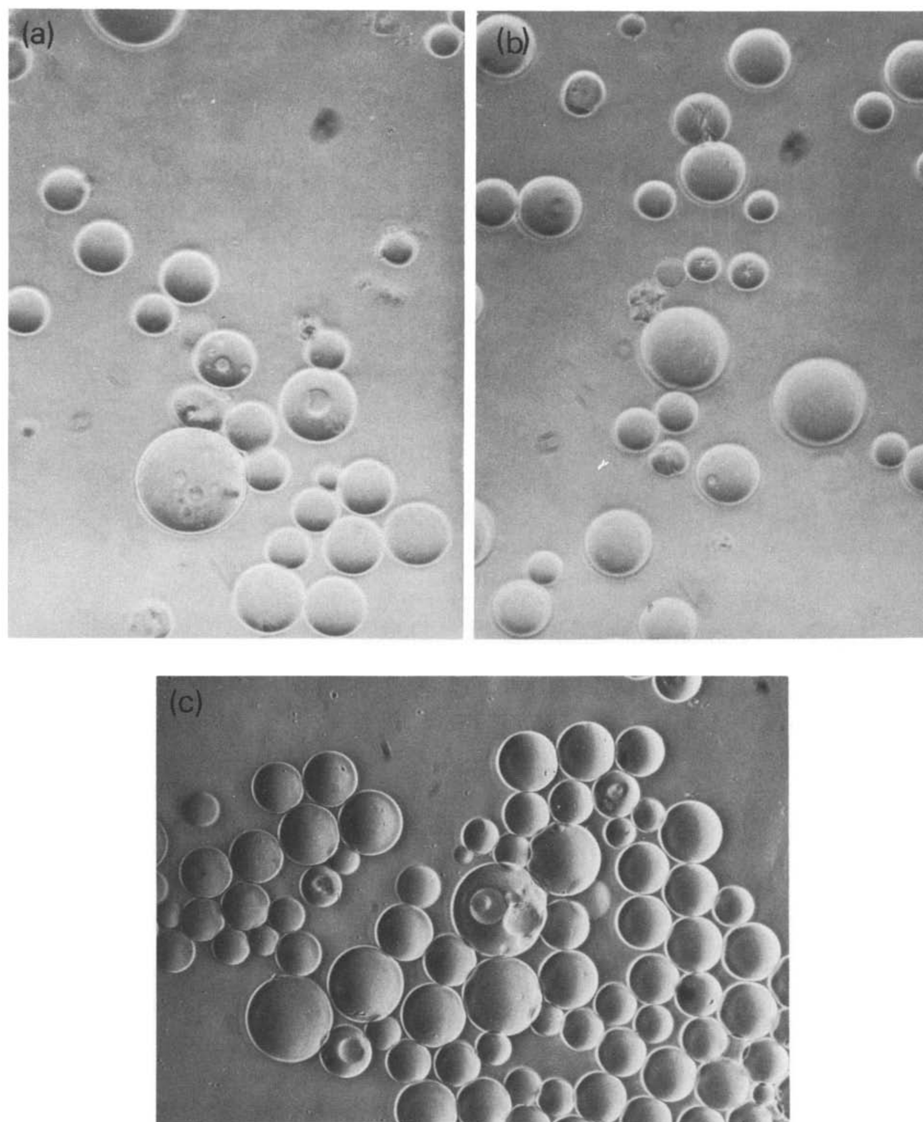


Fig. 4. Micrographs of tresyl- and butyl-S-Sepharose 4B. Magnification *ca.* 140-fold. The diameter of the spheres is between 40 and 140 μm (Pharmacia). About 100 μl of packed gel of the respective Sepharose were suspended in 1 ml of 1% bovine serum albumin to eliminate aggregation of the spheres. The gel suspension was degassed under vacuum for *ca.* 5 min and then spread on a microscope plate for analysis. Photographs were taken with a Leica camera mounted on a Leitz microscope (Agfa-Ortho 25 film). The degrees of substitution were determined by (A) elemental sulphur determination and (B) [^{14}C]butanethiol incorporation. (A) Tresyl-Sepharose 4B (22 μmol/ml packed gel). (B) butyl-S-Sepharose 4B (23 μmol/ml packed gel); (C) control Sepharose 4B.

activated for 120 min (1715 μmol/g dry agarose). Therefore, except for very high degrees of substitution (over 1000 μmol/g dry weight), data can be expressed either on the basis of μmol/ml packed gel or μmol/g dry gel without introducing large errors. A maximum of 0.5 mol alkyl residue/anhydrodisaccharide was introduced into the agarose, indicating that only about half of the available primary OH groups are derivatized on the most highly substituted gel. In Table IV the data are also expressed as surface concentrations (range 19–650 nmol/m^2), making a comparison with other synthetic surfaces possible.

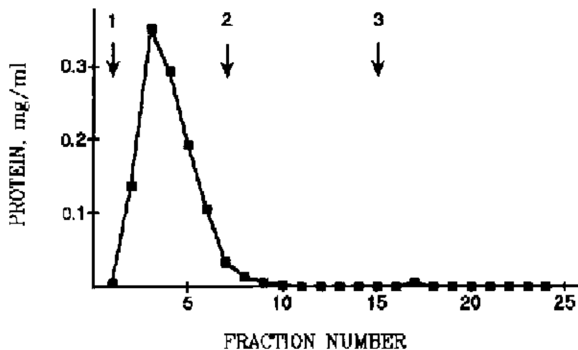


Fig. 5. Adsorption of calmodulin on butyl-N-Sepharose as measured by hydrophobic affinity chromatography. Calmodulin (1 mg) was applied to 2 ml of packed gel of uncharged butyl-N-Sepharose (33 μmol/ml packed gel) on a column (12 cm × 0.9 cm I.D.) in a sample volume of 1 ml in buffer A (with 1 mM $CaCl_2$). Arrow 1, wash with buffer A; arrow 2, wash with buffer B (with 300 mM NaCl); arrow 3, elution with buffer C (with 10 mM EGTA). The fraction volume was 1.5 ml. Flow through the column was driven by gravity.

Protein binding properties of alkyl-S-Sepharoses

Influence of alkyl chain length [29]. Alkyl-S-Sepharoses are very efficient adsorbents of proteins, as can be exemplified by experiments with the Ca^{2+}-binding protein calmodulin. In a control experiment (Fig. 5), calmodulin is not adsorbed on a butyl-N-Sepharose containing 33 μmol/ml packed gel. In the homologous series (C_2–C_4) of alkyl-S-Sepharose in Fig. 6 (constant degree of substitution: 25 μmol/ml packed gel; Table IV), it can be seen that the adsorption of calmodulin is dependent on the chain length of the immobilized thioalkyl residue. Butyl-S-Sepharose (Fig. 6C) is a very strong adsorbent of calmodulin and even propyl-S-Sepharose (Fig. 6B) adsorbs over 50% of the applied calmodulin (42% "reversibly"). Only ethyl-S-agarose adsorbs poorly at the defined surface concentration and thus has similar properties to the butyl-N-Sepharose control.

Influence of degree of substitution [4,26]. Calmodulin binding also depends strongly on the degree of substitution of Sepharose with thiobutyl residues. Below *ca.* 1 μmol/ml packed gel very little binding occurs (not shown). At 1.7 μmol/ml packed gel significant binding can be measured (Table V). The total amount adsorbed (= difference between applied and recovered amount in eluates) increases *ca.* 5-fold as the degree of substitution increases *ca.* 3-fold from 1.7 to 5.7 μmol/ml packed gel

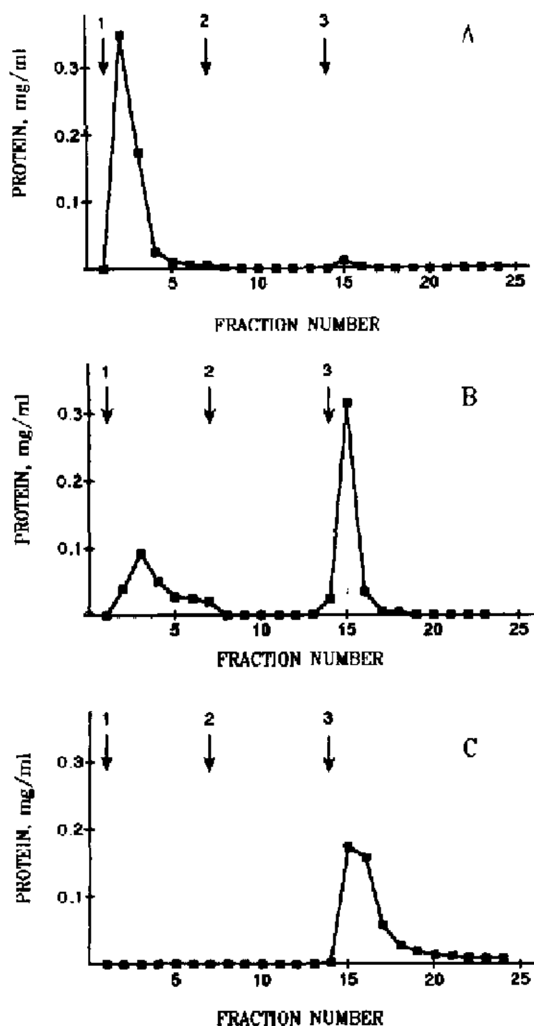


Fig. 6. Adsorption of calmodulin to a homologous series of alkyl-S-Sepharose as measured by hydrophobic affinity chromatography. Calmodulin (1 mg) was applied to 2 ml of packed gel of uncharged butyl-S-Sepharose on a column (12 cm × 0.9 cm I.D.) in a sample volume of 1 ml in buffer A (with 1 m*M* $CaCl_2$). Arrow 1, wash with buffer A; arrow 2, wash with buffer B (with 300 m*M* NaCl); arrow 3, elution with buffer C (with 10 m*M* EGTA). The fraction volume was 1.0 ml. (A) Ethyl-S-Sepharose (25 μmol/ml packed gel); (B) propyl-S-Sepharose (25 μmol/ml packed gel); (C) butyl-S-Sepharose (25 μmol/ml packed gel).

(Table IV). At very high degrees of substitution *ca.* 40% of the adsorbed protein cannot be eluted with EGTA (EGTA-irreversible binding). This fraction of calmodulin can, however, be eluted with detergents (*e.g.*, sodium dodecyl sulphate) (not shown).

TABLE V

QUANTITATIVE HYDROPHOBIC AFFINITY CHROMATOGRAPHY OF CALMODULIN ON ALKYL AGAROSES

The data were derived from column experiments as described. Calmodulin was applied in buffer A ($+Ca^{2+}$), the column was then washed with buffer B (+300 m*M* NaCl) and finally eluted with buffer C (+EGTA). The excluded amount of calmodulin corresponds to the amount washed from the column in buffers A and B. The eluted amount corresponds to the amount eluted with buffer C. The total yield corresponds to the amount recovered in buffers A–C as a percentage of the amount applied. The difference between amount applied and amount recovered is the non-EGTA-elutable fraction ("irreversibly" bound protein), which can, however, be eluted by detergents.

Type of gel	Degree of substitution (μmol/ml packed gel)	Calmodulin			Total yield (%)
		mg applied	mg excluded (unbound)	mg eluted (boud)	
Control:					
Butyl-N-Sepharose	28	1	0.94	0	94
Alkyl-S-Sepharose:					
Homologous series:					
Ethyl-S-	25	1	0.85	0.01	86
Propyl-S-	25	1	0.34	0.42	76
Butyl-S-	25	1	0	0.66	66
Degree of substitution series:					
Butyl-S-	1.7	1	0.79	0.15	94
Butyl-S-	5.2	1	0.19	0.70	89
Butyl-S-	40.7	1	0	0.61	61

DISCUSSION

By working in a strongly alkaline medium, alkanethiols can easily be coupled to tresyl-Sepharose. The method is complicated by the tresylation procedure itself. which is a non-linear function of the tresyl chloride concentration (Table III). This may be due to inactivation of tresyl chloride at low concentrations in the incubation mixture owing to impurities (water?) in the dioxane medium, although this is improbable considering the distillation process employed for the purification of this solvent. The behaviour therefore remains unclear.

In comparison with other activation procedures, the tresylation of agarose as performed here warrants other special considerations. A primary incorporation phase followed by a plateau region is observed (Figs. 2 and 3). As the amount of butanethiol is *ca.* 10-fold in excess of the number of tresyl residues on the activated gel, a limitation of butanethiol cannot be the reason for the plateau region. From this plateau it may be concluded that a single reaction type is involved. At high tresyl chloride concentrations and long activation times a second increment of substitution occurs (secondary incorporation phase). This might be an indication of a second reaction type or possibly of an unfolding of the stacked and double helical agarose structure [27] exposing new reactive hydroxyl groups (possibly also secondary hydroxyl groups) for activation

after a critical exposure time to tresyl chloride. These changes need not be seen on the micrographs of Sepharose 4B, which appears intact after activation and coupling (Fig. 4).

Owing to these abnormal properties of tresyl chloride, the synthesis of structurally homogeneous alkyl-S-Sepharoses with different degrees of substitution poses a special problem. Therefore, a constant, low to intermediate tresyl chloride concentration should be chosen and the time varied to obtain different degrees of tresylation. One should work in the primary incorporation phase and plateau region well below the secondary incorporation phase.

Butyl-S-Sepharose is very efficient in the adsorption of proteins. As measured in batch experiments, the binding capacity of butyl-S-Sepharose for phosphorylase *b* is over an order of magnitude higher than that of butyl-N-Sepharose (synthesized by the cyanogen bromide method) [9–11] at low degrees of substitution (1–5 μmol/ml packed gel). At high degrees of substitution similar binding capacities for both gel types are obtained (*ca.* 20 mg/ml packed gel at an apparent equilibrium concentration of free enzyme of 0.5 mg/ml) [11].

With calmodulin, C_8-agarose derivatives (octyl- and aminooctyl-) have been reported as strong adsorbents [30,31]. As shown here (Table V, Fig. 6), even a C_3 derivative, propyl-S-Sepharose, is capable of adsorbing calmodulin in a Ca^{2+}-dependent manner. The results also show that the length of the immobilized alkyl residue on the Sepharose is not as decisive as expected, as the shorter propyl-S- derivative is a much better adsorbent than the longer butyl-N- derivative (Table V). This strongly indicates a special function of the sulphur base of the immobilized alkyl residue as compared with its tip (methyl group) and it also makes it very improbable that the dependence of calmodulin adsorption on the surface concentration of immobilized residues is due to changes in their availability (*i.e.*, further extension from the gel of a certain population of residues).

Because of these different protein-binding properties, alkyl-S-Sepharoses should prove useful in hydrophobic chromatography and possibly also in the preparation of selective adsorbent surfaces for biomaterials.

Why do the alkyl-S-Sepharoses bind calmodulin and phosphorylase *b* [9–11] more tightly than the alkyl-N-Sepharoses? Theoretically, the sulphur in the thioalkyl residue could complex to metals, especially in metal-binding proteins. In fact, a number of sulphur–metal complexes in proteins, *e.g.*, Fe^{2+}, Cu^{+} and Zn^{2+}, have been described (for a review, see ref. 32). Comparable complexes between Ca^{2+} and sulphur have to our knowledge not been described for proteins. Experimentally the adsorption of the Ca^{2+}-binding protein fibrinogen to alkyl-S-Sepharose cannot be influenced by Ca^{2+} or EGTA [33]. It is therefore very improbable that complexation between metal and sulphur play an important role in the binding of proteins, specifically Ca^{2+}-binding proteins, to alkyl-S-agaroses.

However, two other possible explanations for the enhanced protein binding to alkyl-S-agaroses exist. In the first it could be reasoned that the sulphur atom displays properties similar to a carbon atom, as sulphur is also non-polar [34]. As sulphur increases the free energy of the system as it is transferred, *e.g.*, from the interior of a protein to water [34] it may have similar water-structuring properties to carbon. Based on the free energies of transfer, neutral oxygen and nitrogen (N/O) belong to a different (polar) class of atoms than the sulphur atom [34], which might explain the different properties of butyl-S- and butyl-N-agaroses.

On the other hand, a major difference between the sulphur atom, a carbon atom and the N/O class atoms lies in the π-electrons of the sulphur atom. A plausible conclusion is therefore that there might be an aromatic amino acid near the hydrophobic pocket or area of calmodulin binding to the base of the thioalkyl residue (this may also hold for other proteins tested in our system, phosphorylase *b* and fibrinogen). Owing to the large differences in the adsorption properties of the two gel types, we feel that the latter interpretation warrants careful consideration. Similar conclusions have been reached by Porath [35] for other sulphur-bearing agarose derivatives.

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