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COUPLING OF EFFECTOR MOLECULES TO SOLID SUPPORTS

Development of an Alternative to the Cyanogen Bromide Activation of Polysaccharides

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In view of the limited stability of the isourea bond, formed in ligand coupling to CNBr-activated polysaccharides, an alternative to this current activation method has been developed. 2,4,6-Trifluoro-5-chloropyrimidine (FCP), known as a reactive group in reactive dyes, was used to activate Sepharose. Under appropriate conditions a thermally stable product with unimpaired beaded structure was obtained, which was reactive toward amines and mercaptans. Coupling with hexamethylenediamine, aniline, and ethanethiol, respectively, yielded an incorporation of 0.2–2.7, 0.9–1.7, and 1.1 mmol ligand/g dry agarose.

The stability of immobilized ligands based on FCP-Sepharose between pH 4 and 8 was about 200 times higher as compared to products originating from CNBr-Sepharose; ligand leakage was only 0.5×10^{-3}%/h. The possibility of obtaining a high degree of substitution is a further advantage of the FCP activation. In addition, the FCP-activated Sepharose can be stored in the wet state at 4°C without substantial decrease in coupling capacity.

The FCP analogs 2,4,5,6-tetrachloro- and 2,4,5,6-tetrafluoropyrimidine, and other polymers (cellulose, Sephadex, aminomethylpolystyrene) appeared to be applicable also.

INTRODUCTION

Although the principle of affinity chromatography (1) is quite simple it has become clear that purifications by this method require more than routine chemical immobilization of a ligand, adsorption of the complementary binding component, and elution by some change in the composition of the eluent. Difficulties can arise from limited stability of the ligand–matrix bond and from possible, nonspecific interactions caused either by the ligand itself or by the connector or spacer. Moreover, conservation of the beaded matrix structure may not be compatible with the conditions which are necessary for the chemical derivatization. For reviews, see Jakoby and Wilchek (2), Lowe and Dean (3), Porath and Kristiansen (4), and Gribnau (5).

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Cuatrecasas and Parikh (6) demonstrated the instability of alanine-agarose, prepared by coupling the amino acid to the CNBr-activated matrix. The half-life of the ligand released in 0.05 M sodium bicarbonate, pH 8.0, at room temperature was about 40 days. Albumin-substituted agarose showed a substantially higher stability, presumably because of multivalent linkage of the protein to the matrix. Since then an increasing number of publications have appeared concerning ligand leakage of agarose or porous glass-supported steroids (7–9), catecholamines (10), alkylamines (11), enzymes (12), and hormones (13,14).

Tesser et al. (15,16) investigated the chemistry of the detachment process in the case of ligands coupled to polyacrylamide or to CNBr-activated Sepharose. The release of ligands from a polyacrylamide support was ascribed to hydrolysis of the amide linkage, facilitated by anchimeric assistance of neighboring carboxyl and carboxamide groups. Model studies with ε-aminocaproic acid *p*-nitroanilide coupled to CNBr-activated Sepharose revealed that the ligand itself, a guanidine derivative, or a urea derivative was released depending on the pH and the composition of the eluent. Enhanced stability of the ligand–matrix conjugate may be achieved by coupling ligand molecules polyvalently to the CNBr-activated support. This enhancement can be quantified by mathematical treatment of the leakage phenomenon (17,18). However, coupling procedures resulting in linkages with an inherently higher stability than the isourea bonds are to be preferred.

A number of problems have to be considered in the development of novel activation procedures for agarose. The potential application of chemical reactions is limited by the fact that agarose is present as an aqueous suspension of swollen beads which have a limited thermal and mechanical stability. In addition, the differing chemical reactivities of homogeneous versus heterogeneous systems will be relevant.

Inspection of the structure of agarose (Fig. 1) reveals the possibility of forming a cyclic acetal derivative by reacting the hydroxyl functions at C_4 and C_6 of the galactopyranosyl units with an aldehyde. An amino function, with possibilities for further coupling reactions, might be introduced by performing the acetalization with a nitro-substituted aldehyde, followed by reduction, or with an *N*-protected amino acetal, followed by deprotection. Attempts to synthesize such cyclic acetal derivatives were, however, unsuccessful.

Amino functions can be introduced directly into a polysaccharide chain by displacement of previously introduced *p*-toluenesulfonyl or methanesulfonyl groups with ammonia or a diamine (19). For agarose, however, the low incorporation of amino functions, the drastic reaction conditions required, the high residual tosyl and mesyl content, and the damage caused to the

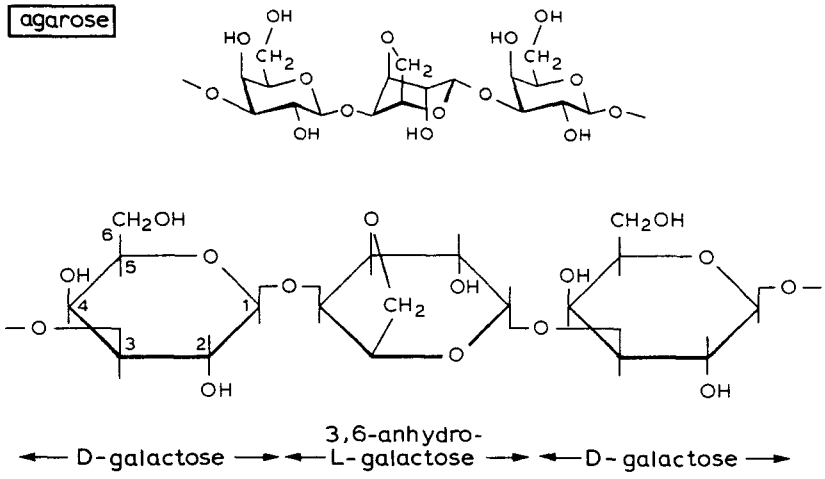


FIG. 1. Structure of agarose.

matrix structure during the isolation of the sulfonate derivatives necessitated the investigation of other alternatives.

In the textile industry elaborate chemical investigations have been performed in order to develop suitable methods for the covalent coupling of dyes to fibers (especially cellulose), with concomitant preservation of fiber strength. Special attention has been paid to two types of halogen-substituted heterocycles, namely 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride, Fig. 2a) and halogenated diazines (e.g., tetrachloropyrimidine).

Cyanuric Chloride

The high reactivity of cyanuric chloride arises from the presence of the three nitrogen atoms in the heterocyclic ring; they cause electron deficiency at the carbon atoms, as appears from the charge density distributions (Fig. 2b) for various nitrogen heterocycles (20). The chlorine atoms further increase the relatively positive charge on the carbon atoms. The combination of both effects facilitates a nucleophilic attack in which chlorine is replaced. The reactivity of the resulting dichlorotriazinyl residue depends on the substituent introduced. The rate constants for successive substitutions of two chlorine atoms by aniline at 25°C are 14 and 0.11 liters $\cdot$ mol^{-1} $\cdot$ min^{-1}, respectively (21). Such a difference enables a stepwise derivatization. Cellulose activated with cyanuric chloride still contains chlorine, capable of further reactions with suitable groupings (22). On the other hand, the trifunctional molecule can be substituted, e.g., with a water-soluble dye, and

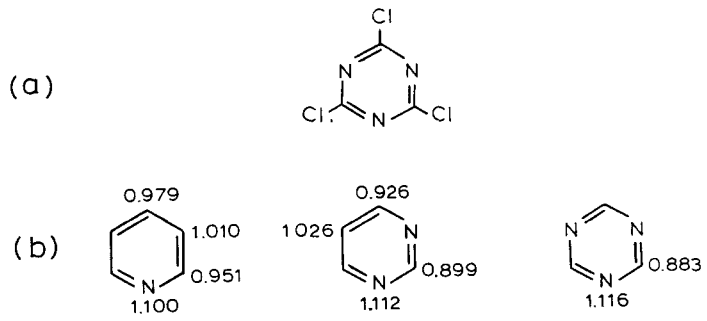


FIG. 2. (a) Cyanuric chloride (2,4,6-trichloro-*s*-triazine); (b) charge density distribution in nitrogen heterocycles. After Peacock (20).

the resulting reactive dye can be coupled to cellulose under alkaline conditions (23).

The preferential coupling of the reactive dye in aqueous alkali to cellulose instead of rapid hydrolysis can be ascribed to the higher nucleophilicity of the cellulose anion in comparison with the hydroxyl ion (24,25), and to the higher dissociation constant of cellulose (pK 13.7) compared to water (pK 15.7), which results in a higher concentration of cellulose anions than of hydroxyl ions. In addition, the concentration of the dye in the cellulose phase can be as much as 500 times higher than in solution, depending on its adsorptive affinity (24). The order C_6–OH > C_2–OH > C_3–OH has been found for the point of attachment (26,27).

The hydrolytic stability of the dye–fiber bond in the case of mono- and dichlorotriazinyl dyes has been shown to be maximum between pH 6 and 7. It decreases by about tenfold per pH unit (28). The ease of hydrolysis depends strongly on the type of substituents on the triazine nucleus (24,26,29).

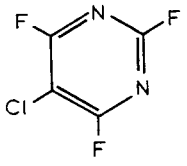
Halogenated Pyrimidines

The primary substitution in 2,4,5,6-tetrachloropyrimidine by nucleophiles proceeds at C_4 (30). Trichloropyrimidinyl dyes can be coupled to cellulose by attack of the cellulose anions at C_2 and C_6. The chlorine atom at C_5 modifies the reactivity of the other positions, but does not undergo displacement.

The reactivity of this type of dyes is lower than that of corresponding dyes based on the triazine nucleus [see Fig. 2b and Hildebrand (26)]. The hydrolytic stability of the dye–fiber bond is accordingly higher. It appeared to be approximately 100 times higher for a trichloropyrimidinyl derivative

than for a corresponding dichlorotriazinyl derivative coupled to cellulose (28).

A combination of the reactivity of the dichlorotriazinyl dyes and the final dye–cellulose bond stability of the trichloropyrimidinyl dyes was achieved by the application of 2,4,6-trifluoro-5-chloropyrimidine (FCP) as the reactive group (31,32). Fluorine is far more rapidly displaced in nucleophilic aromatic substitution reactions than other halogens, because of its extreme electronegativity. Difluorochloropyrimidinyl dyes are about 170 times more reactive than the corresponding trichloropyrimidinyl dyes (26), whereas both compounds yield essentially the same endproduct after reaction with alkali cellulose.



2,4,6,-Trifluoro-5-chloropyrimidine (FCP)

Reactivity of Halogenated Diazines and Triazines toward Frequently Occurring Functional Groups in Ligands

The reactivity of difluorochloropyrimidinyl dyes toward the side chain functional groups of cysteine, histidine, lysine, and the α-amino groups of *N*-terminal amino acids has been thoroughly investigated (26,33,34). Very detailed data of the reactivity of chlorotriazinyl dyes with respect to the functional groups mentioned previously have been published (35,36).

Calculations based on experiments with appropriate model compounds yielded the following order of decreasing reactivity: cysteine thiol, *N*-terminal α-amino, histidine imidazolyl, lysine-ε-amino, serine hydroxyl, tyrosine hydroxyl, arginine guanidino, and threonine hydroxyl group. The indolyl group of tryptophan appeared to react only at very high pH values, and the cysteine disulfide and methionine thioether groups showed no reactivity. The relative reactivity is particularly dependent on the pH of the reaction mixture.

Application of FCP, FCP Analogs, and Reactive Dyes Based on FCP, to Ligand Immobilization

The application of FCP to ligand immobilization merited a thorough investigation (5,37). Two activation procedures were developed: (I) Coupling of a reactive azo dye, based on FCP, to agarose, followed by reduction

and diazotation. This reactive support could be used for the immobilization of tyrosine- or histidine-containing proteins, or for the coupling with low-molecular-weight ligands, containing an activated aromatic ring. (II) Application of the FCP group as such. Two general procedures are possible for the coupling reactions. Introduction of a reactive group into the matrix ("activation"), followed by coupling with the ligand, or activation of the ligand, followed by coupling to the matrix. An eventual spacer can be similarly introduced by previous coupling to the matrix or by coupling the presynthesized spacer ligand derivative.

Preactivation of the matrix offers a more universal procedure, avoiding the synthesis of a reactive ligand derivative for each specific case. It has the simultaneous advantages and disadvantages of solid phase syntheses. The excess of reactive groups should be blocked with noninterfering low-molecular-weight compounds. Coupling of the preactivated ligand to the support gives a more uniform and chemically well-defined affinity matrix. The former method and several intermediate ones are currently in use. Investigations on the preactivation of matrices were emphasized.

The reactivity of the FCP-agarose toward amines and mercaptans was examined, and the ratio between hydrolysis and substitution during the coupling with amines and ω-aminocarboxylic acids was determined by elemental analysis and potentiometric titration.

The stability of the FCP-agarose bond was determined in two ways: (a) incubation of agarose coupled with a reactive dye based on FCP at room temperature in buffers of different pH, followed by spectrophotometric investigation of the supernatants; (b) incubation of hexamethylenediamine-substituted FCP-agarose at room temperature in buffers of different pH, followed by thin-layer chromatographic screening of the concentrated supernatants.

Finally, the FCP activation was applied to other types of matrices, and FCP analogs (2,4,5,6-tetrachloropyrimidine and 2,4,5,6-tetrafluoropyrimidine; TCP and TFP, respectively) were also investigated.

EXPERIMENTAL SECTION

Sephadex and Sepharose were obtained from Pharmacia; the latter matrix was checked by microscopic investigation before use. Polyacrylamide and cellulose were from Bio-Rad and Whatman, respectively.

Samples of the ion exchange resins NKZ-2 and NKZ-4 (cross-linked aminomethylpolystyrene) and of reactive dyes, tetrafluoropyrimidine (TFP), tetrachloropyrimidine (TCP), and 2,4,6-trifluoro-5-chloropyrimidine (FCP), were a gift from Bayer AG (Leverkusen, West Germany).

The last-named compound is an almost colorless liquid (density 1.675) with a boiling point of 116°C/760 mm, and a melting point of about 2°C. It is flammable (flash point between 21° and 55°C), and experimental use in advised to be performed in a well-ventilated hood.

Most of the other reagents and solvents used were of analytical grade quality; xylene (mixture of the three isomers, and free of thiophene) and dioxan were of a practical grade.

Ninhydrin Test for Primary Amino Functions in Polymers

A small sample of the dry polymer and a crystal of ninhydrin were suspended in a little water, and heated. The presence of primary amino groups was demonstrated by a blue coloration (the dye was adsorbed by the polymer).

Lassaigne's Test for Sulfur

A small sample of the dry polymer was fused with a small piece of metallic sodium in an ignition tube, and maintained at red heat for 1 min. The hot tube was plunged directly into 1–2 ml of distilled water in a test tube, and the total content was boiled for 1 min and filtered. The presence of sulfide in the filtrate was demonstrated by the addition of a few drops of a solution of sodium nitroprusside (pink to purple coloration), or by addition of a lead(II) acetate solution after previous acidification with acetic acid (black precipitate).

Detection of Compounds in TLC

Detection was performed by quenching of fluorescence after exposure of the plates (Merck, F_{254}) at the excitation wavelength (254 nm), by coloration with ninhydrin, or according to Reindel and Hoppe (38).

Standard Dry Sepharose

The following method was applied to prepare samples of Sepharose 4B with a standardized water content: the wet suspension was transferred into a sintered glass funnel (Schott & Gen, Mainz; 3D–3), and then allowed to "drip dry"; a small tube (length 40 cm, internal diameter 1 mm) was put at the end of the funnel to accelerate the filtration. Amounts of this Sepharose, corresponding to an arbitrary but constant mark on the filter (corresponding to 22.5 ml), were taken as starting material. The weight of these samples varied generally between 23 and 25 g; the agarose content was approximately 4%.

Coupling of Reactive Dyes to Agarose

Sepharose 4B was washed free from sodium azide, and 25 g of the standard dry material was suspended in a solution of 0.1 g of dye in 11 ml of water. The suspension was stirred for 10 min (not with a magnetic stirrer bar!) and 15 ml of a NaCl solution (50–100 g/liter) was added. The pH was increased subsequently to 10.5–11.0 by addition of 1 M NaOH. The reaction mixture was kept at this constant pH with a pH stat and stirred for 24 h at room temperature.

The product was filtered off, washed thoroughly with water, and packed into a column; the latter was eluted slowly with water (about 250 ml). The colored Sepharose was stored suspended in a 0.05% aqueous sodium azide solution at 4°C.

The degree of substitution was determined colorimetrically (Metrohm Spektralkolorimeter E-1009). A sample of the wet product was lyophilized and dried *in vacuo* over concentrated sulfuric acid. An aliquot was dissolved in warm 0.1 M HCl and measured together with standard solutions of the dye, also in 0.1 M HCl. For Levafix Brilliant Red E-4BA ($\lambda = 512$ nm) a degree of substitution of 12.4% was found. This value should be corrected, however, for the amount of additives which are included in the commercial dye.

Coupling of Reactive Dyes to Cross-Linked Agarose

Cross-linking was performed according to the method of Porath et al. (39). The procedure for dye coupling was as just described except that the reaction was allowed to proceed for 1 h at 60°C. A degree of substitution of 11.3% was found for Levafix Brilliant Red E–4BA, again uncorrected.

Reduction and Diazotation of Azo-Dye-Linked Matrices

One gram of standard dry, colored, Sepharose 4B (Levafix Brilliant Red E-4BA) was suspended in 20 ml of water, and small amounts of solid sodium dithionite were added until the color had changed from dark red to a light yellow-brown. The product was filtered off and washed with water. Diazotation was performed by suspending the reduced agarose derivative in 3 ml of 0.1 M HCl at 0°C, followed by dropwise addition of 1 M sodium nitrite solution; the presence of nitrite in the reaction mixture was checked with KI starch paper. The suspension was stirred for 30 min, and then the treated agarose was filtered off and washed with ice-cold water. The presence of reactive diazonium functions was demonstrated by reaction with β-naphthol in alkaline solution, resulting in an immediate dark red coloration of the agarose.

FCP Activation of Agarose

Reactions were performed in a 100-ml three-necked round-bottomed flask with a cooling mantle (Fig. 3). The interior of the flask was previously silanated: the flask was rinsed after thorough cleaning with a 5% (wt/vol) solution of Dow–Corning 200 Fluid 350 CS in chloroform; after evaporation of the chloroform it was heated for 30 min at 300°C. The agarose was suspended in the reaction medium by means of a "Vibro-Mixer."

Method A. Approximately 25 g of standard dry Sepharose 4B was suspended in 50 ml of 3 M NaOH and rotated slowly for 15 min at room temperature. The alkali Sepharose was filtered off on the standard filter and sucked dry by vacuum to the original mark. This material was suspended in 40 ml of a mixture of xylene–dioxan (1 : 1; wt/wt) and the suspension was agitated with the Vibro-Mixer at maximum frequency, and cooled to 0°C.

A solution of 4–5 g (2.5–3.0 ml) of FCP in 4 ml of the same xylene–dioxan mixture was added dropwise at such a rate that the temperature did not rise above 0.5°C; this procedure required about 1 h. The reaction was quenched by addition of concentrated acetic acid (4.2 ml). The activated agarose was filtered off on a sintered glass funnel and washed with xylene–dioxan (200 ml), dioxan (200 ml), and finally water (1 liter). The wet "cake" was stored at 4°C.

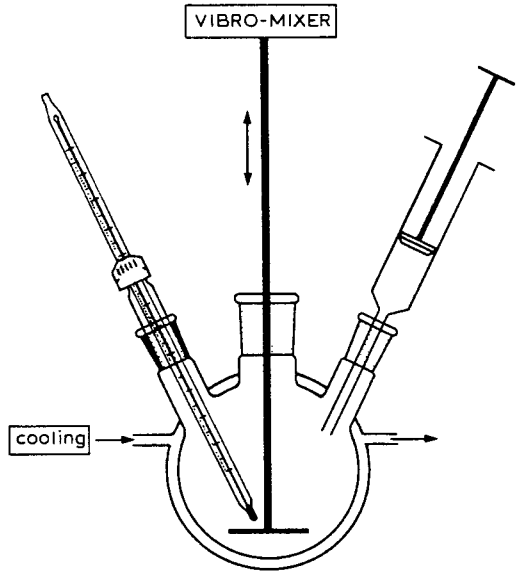


FIG. 3. Experimental setup for the FCP activation of polysaccharides.

The FCP-agarose did not dissolve in water at higher temperatures (> 40°C), in contrast with native Sepharose 4B. An aliquot was washed with acetone, sucked dry by vacuum, and dried *in vacuo* over concentrated sulfuric acid. The N, F, and Cl contents were determined by elemental analysis. Some representative examples are given in Table 1.

Method B. Alkali Sepharose 4B, prepared as in method A, was packed into a plastic syringe (20 ml, Brunswick), the needle of which was replaced by a Teflon tube (length 7 cm, internal diameter 3 mm), cf. Fig. 3.

The Sepharose was added to a solution of FCP (4–5 g) in 40 ml of xylene–dioxan (1 : 1; wt/wt), which was cooled previously to 0°C, at such a rate that the temperature did not rise above 4°C; this required about 25 min. The reaction was allowed to proceed for another 15 min at 0°C, and concentrated acetic acid (4.2 ml) was added at the end of this period. The FCP-agarose was isolated as described in method A. Representative examples are given in Table 2.

Method C. Approximately 25 g of standard dry Sepharose 4B was washed with 250 ml of a solution of NaOH or Na_2CO_3 (cf. Table 3), and sucked dry by vacuum to the original mark on the standard filter. The alkali-Sepharose was packed into a plastic syringe, as described in method B. The total amount of alkali Sepharose was injected in one portion into a solution of FCP in 40 ml of xylene–dioxan (1 : 1; wt/wt), which had been cooled previously to −10°C. The temperature rose to +9°C, but then dropped quite rapidly; the reaction was allowed to proceed for 15 min at +3°C. A slight excess of acetic acid (≥ standard volume times alkali concentration) was added after that period. The isolation of the activated agarose was performed as described in method A. Representative results are given in Table 3.

TABLE 1

Batch	%N	%F	%Cl
a	4.6	2.9	5.9
b	4.7	2.8	5.7
c	4.6	2.9	6.0

TABLE 2

Batch	%N	%F	%Cl
d	5.8	5.3	6.5
e	5.7	5.4	6.6
f	5.7	5.05	6.4

TABLE 3

Batch	Alkali	FCP (g)	%N	%F	%Cl
g	3.00 M NaOH	1.07	0.8	0.6	1.0
h	0.27 M NaOH	1.07	1.7	1.3	1.9
i	0.10 M NaOH	1.00	0.9	0.9	1.15
j	0.27 M Na_2CO_3 (pH 11.4)	1.03	0.6	0.5	0.55
k	0.27 M Na_2CO_3 (pH 11.4)	1.09	0.55	0.4	0.6
l	0.27 M Na_2CO_3 (pH 11.4)	1.13	0.5	0.4	0.55
m	0.27 M Na_2CO_3 (pH 11.4)	0.95	0.5	0.4	0.5
n[a]	0.27 M $Na_2CO_3/NaHCO_3$ (pH 10.0)	1.01	—	—	—
o[a]	0.27 M $NaHCO_3$ (pH 8.4)	1.08	—	—	—
p	3.00 M NaOH	4.87	4.1	3.2	4.6
q	1.32 M NaOH	5.06	4.2	4.0	4.7
r	1.32 M NaOH	4.86	4.1	3.5	4.4

[a] Samples of these batches dissolved in hot water; HMDA-treated products reacted negatively to ninhydrin. Therefore, elemental analysis was omitted.

Coupling of FCP-Agarose with Hexamethylenediamine

FCP-activated Sepharose 4B, sucked dry by vacuum, was suspended in an aqueous solution of hexamethylenediamine (HMDA), and the suspension was rotated slowly for 15–20 h at room temperature. The product was filtered off, washed thoroughly with double-distilled water, 0.05 M HCl (two times the gel volume), double-distilled water, 0.1 M NaOH (five times the gel volume) and again with double-distilled water until the eluate was neutral. A qualitative check on the presence of primary amino functions was performed with the ninhydrin reagent. An aliquot of the HMDA-Sepharose was washed with methanol, sucked dry, and dried finally *in vacuo*. The N, F, and Cl contents were determined by elemental analysis. Representative results are given in Table 4.

Coupling of FCP-Agarose with Aniline

Sepharose 4B (23.04 g, standard dry) was activated according to method A. Half of the activated product was suspended in a solution of aniline (1.74 g) in 25 ml of 0.07 M phosphate buffer (pH 7.0) and 10 ml of peroxide-free dioxan (I). The suspension was rotated for 26 h at room

TABLE 4

	FCP-Sepharose (g)	HMDA	Before HMDA %N	%F	%Cl	After HMDA %N	%F	%Cl
I	17.25	4 g/50 ml	4.4	—	—	6.95	—	—
II	14.80 (d)	2 g/50 ml	5.8	5.3	6.5	9.4	0.9	5.7
III	8.85 (a)	2 g/25 ml	4.6	2.9	5.9	7.7	0.3	5.3
IV	5.00 (h)	1.2 g/10 ml[a]	1.7	1.3	1.9	3.4	0.2	1.75
V	2.70 (i)	0.4 g/10 ml	0.9	0.9	1.15	2.0	<0.1	1.2
VI	5.00 (l)	0.4 g/10 ml	0.5	0.4	0.55	0.9	<0.1	0.45

[a] Adjusted to pH 10.

temperature. The product was filtered off, washed with water–dioxan (2.5 : 1; vol/vol) and water. An aliquot was washed with acetone, sucked dry, and dried *in vacuo*; N, F, and Cl contents were determined by elemental analysis. Half of the aniline-Sepharose was suspended in a solution of HMDA (2 g) in water (25 ml); the mixture was rotated for 40 h at room temperature. Isolation of the product was performed as described for HMDA-agarose.

The same procedure was repeated with another batch of FCP-Sepharose; the composition of the reaction mixtures was 0.18 g of aniline, 2 ml of peroxide-free dioxan, and 10 ml of 0.2 M borate buffer pH 8.0 (II) and 9.0 (III), respectively. Coupling with aniline was performed for 86 h, and with HMDA for 21 h.

All of the aniline-Sepharose derivatives were positive to the ninhydrin test, after treatment with HMDA. Analyses are given in Table 5.

Coupling of FCP-Agarose with Imidazole

FCP-Sepharose 4B (sucked dry by vacuum; 5 g) was washed with 50 ml of 0.2 M borate buffer, pH 9.0, suspended in 5 ml of a solution of imidazole

TABLE 5

	FCP-Sepharose %N	%F	%Cl	Aniline-Sepharose %N	%F	%Cl	After HMDA treatment %N
I (a)	4.6	2.9	5.9	5.7	1.2	5.55	6.9
II (b)	4.7	2.8	5.7	6.0	—	—	7.1
III (b)	4.7	2.8	5.7	5.4	—	—	7.5

(0.5 M) in the same buffer, and the suspension rotated for 17 h at room temperature. The product was filtered off, washed with water, 0.1 M HCl, water, 0.1 M NaOH, and double-distilled water; an aliquot was washed with methanol, sucked dry, and dried *in vacuo*. N and F contents were determined by elemental analysis.

Analyses:	FCP-Sepharose	%N	5.7	%F	5.05
	(batch f; Table 2)	%Cl	6.4		
	Imidazole-Sepharose	%N	10.75	%F	1.28

Coupling of FCP-Agarose with Ethanethiol

FCP-Sepharose 4B (sucked dry by vacuum: 2 g) was suspended in a mixture of ethanethiol (1 ml), 0.2 M borate buffer, pH 9.2 (5 ml), and ethanol (7 ml). The suspension was rotated for 21 h at room temperature. The product was filtered off, washed thoroughly with ethanol–water (1 : 1; vol/vol), ethanol, and acetone. The material gave a positive Lassaigne test for sulfur.

Analyses:	FCP-Sepharose	%N	3.8	%F	2.55
		%Cl	4.8	%S	<0.2
	EtSH-Sepharose			%S	2.8

Stability of the Agarose Derivatives

(I) Samples (2 g) of standard dry Sepharose 4B and epichlorohydrin cross-linked Sepharose 4B, coupled with Levafix Brilliant Red E-4BA were incubated for 66 h at room temperature in 3-ml volumes of buffers (0.1 M citric acid/0.2 M Na_2HPO_4, according to McIlvaine; pH 2.50, 4.12, 6.20, 8.25) and aqueous ammonia (1 M; pH 11.8). The samples were washed previously with 10 ml of the respective buffers.

The absorption of the clear supernatants was determined on a Metrohm Spektralkolorimeter E-1009, at 512 nm, against appropriate blanks.

(II) Samples (0.1 g) of standard dry HMDA-Sepharose (batch I, Table 4) and blank Sepharose 4B were incubated for 46 h at room temperature in 0.5-ml volumes of the following solutions: NaOAc–HOAc (0.005 M; pH 5.00 and 6.15), triethylamine–HOAc (0.08 M; pH 8.50), triethylamine (0.08 M; pH 11.80), ammonia (1 M; pH 11.71), sodium azide (0.05%; pH 6.00). The samples were washed previously with 2 ml of the respective solutions.

Samples (0.1 ml) of the clear supernatants were dried *in vacuo*, the residues were dissolved in 0.01 ml of methanol, and the total volumes were spotted on a precoated silica gel plate (Merck, F_{254}). The plate was

developed in 1-butanol–acetic acid–water (4:1:1), and the components were detected by means of ultraviolet irradiation (245 nm), ninhydrin, and chlorine–*o*-tolidine.

FCP-Agarose-HMDA; Substitution versus Hydrolysis

Sepharose 4B was activated according to method C (batch q, Table 3).

Coupling with HMDA. FCP-Sepharose (sucked dry by vacuum; 13 g) was suspended in a solution of HMDA (3.75 g; sevenfold molar excess) in water (50 ml), and under a nitrogen atmosphere. The pH of this solution was adjusted previously with concentrated HCl to pH 10.00, and kept at this value by means of a pH-stat apparatus.

Hydrolysis. FCP-Sepharose (sucked dry by vacuum; 13 g) was suspended in 50 ml of a carbonate–bicarbonate buffer (0.28 M; pH 10.00).

Both suspensions were stirred at room temperature, and samples (5 ml) were taken at $t = 0$, 0.25, 1, 4 and 21 h. The samples were filtrated and the agarose was washed with water (100 ml) and acetone (50 ml), sucked dry, and dried *in vacuo*. The N and F contents were determined by elemental analysis (Table 6).

Characterization of Agarose Derivatives by Potentiometric Titration

Sepharose 4B was activated according to method C (batch p, Table 3). Samples of the standard dry material (7 g; "S-FCP") were suspended in 20 ml of buffered solutions (0.2 M carbonate–bicarbonate, pH 10.00), and rotated for 17 h at room temperature:

"S-Aca": 2.14 g of ε-aminocaproic acid/20 ml (tenfold molar excess). The product was filtered off and washed with water (500 ml), 1 M HCl (25 ml), and double-distilled water (250 ml).

"S-HMDA": 1.91 g of hexamethylenediamine/20 ml (tenfold molar excess). The product was filtered off, and washed with water (500 ml), 1 M HCl (20 ml), water (200 ml), 1 M NaOH (25 ml), and double-distilled water (250 ml).

TABLE 6

t (h)	%N[a]	%N[b]	%F[a]	%F[b]
0	4.2	4.2	4.0	4.0
0.25	6.1	4.2	1.7	3.3
1	6.6	4.3	1.4	3.1
4	6.8	4.4	1.0	2.75
21	7.2	4.2	0.6	2.5

[a] Coupling with HMDA.
[b] Hydrolysis.

TABLE 7

Sample (standard) dry; 3 g)	%N[a]	%F[a]	%Cl[a]	Matrix content of standard dry gel (%)	mEq OH^- (pH 3.0 → 11.5)
S	—	—	—	3.36	0.085
S-FCP	4.1	3.2	4.6	3.93	—
S-Aca	4.5	1.3	4.1	4.44	0.285
S-HMDA	6.3	0.8	4.3	3.93	0.230
S-OH	4.2	2.3	4.5	3.66	0.184

[a] Of the dry gel material.

"S-OH": 20 ml of buffer only. The product was filtered off and washed with water (500 ml), 1 M HCl (20 ml), and double-distilled water (250 ml).

Samples (1 g) of standard dry S-Aca, S-HMDA, S-OH, S-FCP, and blank Sepharose ("S") were washed with 25 ml of acetone, sucked dry, and dried *in vacuo* (*note*: a smaller standard filter was used; Schott & Gen Mainz, 15aD-3, with 20 cm of tube with an internal diameter of 1 mm at the end). The weight of the dry materials and the N, F, and Cl contents were then determined (Table 7).

Samples (3 g) of S, S-FCP, S-Aca, S-HMDA, and S-OH were prepared using the same standard filter, suspended in 100 ml of 1 M KCl, and adjusted to pH 1.8 with concentrated HCl. Potentiometric titration was performed with 0.0998 M NaOH.

Methylsulfonylethyloxycarbonyl HMDA-Agarose

Standard dry S-HMDA (1 g) from the preceding experiment was washed with 20 ml of DMF–water (1 : 1; vol/vol) and suspended in 2.5 ml of the same mixture. The pH was adjusted to 9.8 with triethylamine. Methylsulfonylethyl succinimidocarbonate (Msc-ONSu, 97 mg), dissolved in 0.5 ml of DMF, was then added to the agarose derivative. The pH was maintained at 8.5–9.5 for 5 min and then lowered to 6.8 by the addition of 0.1 M acetic acid.. The product was filtered off, washed with DMF–water (100 ml), water (100 ml), and acetone (50 ml), sucked dry and then dried *in vacuo*.

Analysis: %N 5.81 %S 1.98

TCP Activation of Agarose; Coupling with HMDA, EtSH

Sepharose 4B (25 g, standard dry) was activated with TCP according to method B; 4.90 g of TCP was used. The alkali Sepharose was added at such a rate that the temperature of the reaction mixture did not rise above 30°C;

about 20 min were required for the addition. The reaction was allowed to proceed for a further 15 min at room temperature, whereupon concentrated acetic acid (4.2 ml) was added. The activated agarose was isolated as described for FCP-Sepharose. The TCP-Sepharose did not dissolve in hot water.

Analysis: %N 2.2 %Cl 5.7

TCP-Sepharose (sucked dry by vacuum; 1.3 g) was suspended in a 0.35 M aqueous HMDA solution (10 ml), and the suspension was rotated for 69 h at room temperature. The product was isolated as described for HMDA–FCP-Sepharose. The material reacted positively to ninhydrin.

Analysis: %N 3.3 %Cl 4.7

TCP-Sepharose (sucked dry by vacuum; 1.3 g) was washed with tetrahydrofuran (THF, 10 ml) and suspended in a mixture of triethylamine (0.5 ml), ethanethiol (0.5 ml), and THF (3 ml). The suspension was rotated for 69 h at room temperature. The product was filtered off, washed with ethanol, packed in a small column and eluted with ethanol, and finally dried. The Lassaigne test for sulfur was positive.

Analysis: %Cl 4.8 %S 1.0 (blank %S <0.2)

TFP Activation of Agarose; Coupling with HMDA, EtSH

Sepharose 4B (25 g, standard dry) was activated with TFP (5.13 g) according to method B. The alkali Sepharose was added at such a rate that the temperature of the reaction mixture did not rise above 0°C, which required approximately 25 min. The reaction was allowed to proceed a further 10 min at −2°C, whereupon concentrated acetic acid (4.2 ml) was added. The product was isolated as described for FCP-Sepharose. The TFP-Sepharose did not dissolve in hot water.

Analysis: %N 7.3 %F 13.2

TFP-Sepharose (sucked dry by vacuum; 2 g) was suspended in a 0.7 M aqueous HMDA solution (5 ml), and the suspension was rotated for 20 h at room temperature. The product was isolated as described for HMDA–FCP-Sepharose, and reacted positively to the ninhydrin reagent.

Analysis: %F 11.65 %F 6.2

TFP-Sepharose (sucked dry by vacuum; 2 g) was coupled with EtSH as described for TCP-Sepharose. The Lassaigne test for sulfur was positive.

Analysis: %F 8.7 %S 6.5 (blank %S <0.2)

FCP Activation of Sephadex; Coupling with HMDA, EtSH

Sephadex G–50 was swollen in water for 24 h, and activated according to method A. The Sephadex beads showed a somewhat fractured surface after the activation. The wet product was washed with acetone, dried *in vacuo*, and stored, cool and dry.

Analysis: %N 0.4 %F 0.2 %Cl 0.5

FCP-Sephadex (standard dry; 3 g) was suspended in a 0.7 M aqueous HMDA solution (5 ml), and then rotated for 16 h at room temperature. The product was isolated as described for HMDA–FCP-Sepharose, and gave a positive ninhydrin test.

Analysis: %N 0.7 %F <0.1 %Cl 0.6

FCP-Sephadex (standard dry; 3 g) was suspended in a mixture of 0.2 M borate buffer (pH 9.2; 5 ml), ethanol (7 ml), and EtSH (1 ml), and the suspension was rotated for 17 h at room temperature. The product was isolated as described for FCP-agarose, and gave a faintly positive Lassaigne test.

Analysis: %S 0.3 (blank Sephadex: %S <0.2)

FCP Activation of Cellulose; Coupling with HMDA, EtSH

Cellulose (Whatman CF-11; 4 g) was suspended in 3 M NaOH (50 ml) and the suspension was rotated for 18 h at room temperature. The alkali-cellulose was filtered off, and added gradually to a solution of 7.91 g of FCP in 40 ml of xylene–dioxan, at such a rate that the temperature did not rise above 5°C, which required about 10 min. The reaction was allowed to proceed for a further 15 min; 5 ml of concentrated acetic acid was then added and the product was isolated as described for FCP-Sepharose. A part of it was washed with acetone, dried *in vacuo*, and stored, cool and dry.

Analysis: %N 2.4 %F 2.4 %Cl 2.4

FCP-cellulose (sucked dry by vacuum; 1 g) was suspended in a 0.35 M HMDA solution (5 ml), and the suspension was rotated for 22 h at room temperature. The product was isolated as described for HMDA–FCP-Sepharose, and gave a positive ninhydrin test.

Analysis: %N 4.0 %F 0.4 %Cl 2.5

FCP-cellulose (sucked dry by vacuum; 0.5 g) was washed with tetrahydrofuran, suspended in a mixture of tetrahydrofuran (1 ml), EtSH (0.5 ml), and triethylamine (0.1 ml), and the suspension was rotated for 21 h

at room temperature. The product was isolated as described for TCP-agarose, and reacted positively to the Lassaigne test.

Analysis: %S 2.35 %F 0.9 (blank cellulose: %S <0.2)

FCP Activation of Cross-Linked Aminomethylpolystyrene; Coupling with EtSH

The ion exchange resin NKZ-2 was dried for 2 days at 40°C and for 2 days *in vacuo*; 10 g (20–30 mEq NH_2) was suspended in a solution of FCP (10 g) in 50 ml of toluene at 0–5°C. Triethylamine (5.6 ml) was then added gradually, followed by concentrated acetic acid (3 ml) after 1 h. The product was filtered off, washed with toluene (250 ml), diethyl ether (250 ml), sucked dry, and then dried *in vacuo*.

A sample (2 g) was suspended in a mixture of tetrahydrofuran (3 ml), EtSH (1.5 ml), and triethylamine (1.6 ml), and the suspension was rotated for 28 h at room temperature. The product was filtered off, washed with ethanol (250 ml), dried and ground in a mortar, washed again with ethanol, and dried. Lassaigne's test for sulfur was positive.

Analysis: %S 0.4 (blank NKZ-2: %S <0.2)

DISCUSSION

Coupling with Reactive Azo Dyes

Reactive dyes can be coupled to agarose under relatively mild conditions and in aqueous medium. Addition of sodium chloride to the reaction mixture is necessary to decrease the negative surface potential of the polysaccharide, thereby reducing the repulsion of the dye anions. This results in a higher adsorption of the dye, thus facilitating the final covalent coupling (40). Under these conditions the degree of substitution obtained, determined colorimetrically and based on dry gel weight, varied between 11 and 12% (uncorrected for additives present in the commercial dye).

The ease of reducing the colored support with sodium dithionite was dependent on the type of dye used. Reduction by addition of solid sodium dithionite to an aqueous suspension of the dyed agarose occurred considerably faster than reduction with the same reagent under alkaline conditions.

The diazotized aminoaryl-Sepharose appeared to be a very stable compound; diazonium groups were detectable even after 60 h at room temperature. The product couples with phenols under alkaline conditions (sodium carbonate), with amines at slightly acidic or neutral pH.

Performance of ligand coupling by the successive reactions: coupling of agarose with dye–reduction–diazotation–coupling with ligand, permits a qualitative, visible control of the reaction sequence owing to the characteristic color changes occurring during the different steps.

FCP Activation of Agarose

The choice of the solvent system in which the reaction between FCP and agarose has to be performed is rather critical owing to the hydrophobicity of FCP and the hydrophilicity of the agarose gels. Several systems were investigated and the best results were obtained with a 1 : 1 (wt/wt) mixture of xylene and dioxan (cf. 22, 42).

The order in which the reactants are added may also influence the composition of the final product. Three procedures were therefore investigated. In method A, Sepharose 4B was treated with 3 M NaOH, and then suspended in the solvent system just described; the FCP was added gradually to the thoroughly mixed suspension.

The degree of substitution of the resulting activated agarose can be calculated from the formula $100/(306+148.5n) = \%N/28n = \%Cl/35.5n = \%F/38n$, where n represents the number of difluorochloropyrimidinyl residues (DFP) per anhydro-(galactopyranosyl-anhydrogalactopyranose) unit ("ag", mol. wt. 306). Partial hydrolysis and cross-linking, occurring to an unknown extent, were not taken into account. Values based on the N and Cl content of the matrices (n_N and n_{Cl}, respectively) are summarized in Table 8 (cf. Table 1).

These values average out at the empirical formula $(ag)_3(DFP)_2$ (mol. wt. 1215) with respect to %N and %Cl values. The fluorine content was considerably less (54%) than that calculated, however, resulting in a N/F ratio of about 2 instead of 1. This must be explained by partial cross-linking and hydrolysis during the activation reaction. Two tentative, limiting structures for the activated polymer may be represented as follows: MFP(OH)-ag-ag-ag-MFP(OH) (mol. wt. 1211) and ag-MFP-ag-MFP-ag (mol. wt.

TABLE 8

Batch	n_N	n_{Cl}	Mol. wt.	%F		N/F
				Exp.	Calc.	
a	0.66	0.68	403	2.9	6.19	2.15
b	0.69	0.65	408	2.8	6.41	2.26
c	0.66	0.69	404	2.9	6.22	2.11

TABLE 9

Batch	n_N	n_{Cl}	Mol. wt.	%F		N/F
				Exp.	Calc.	
d	0.92	0.77	443	5.3	7.91	1.50
e	0.90	0.78	439	5.4	7.76	1.45
f	0.89	0.76	438	5.05	7.72	1.53

1175), with MFP(OH) and MFP representing monofluoromonohydroxychloropyrimidinyl and monofluorochloropyrimidinyl residues, respectively. The calculated nitrogen contents for these two structures are 4.62 and 4.77, respectively, both of which are in agreement with the experimental data.

The order in which FCP and alkali agarose were mixed was inverted in method B, to reduce the extent to which reactive fluorine was lost during the activation procedure according to method A. The results are summarized in Table 9 (cf. Table 2).

There is an increase in the degree of substitution and a decrease in N/F ratio, compared with the results of method A, although the same total amounts of FCP, Sepharose, and alkali had been used. This is the result of a more favorable competition between coupling with agarose and mere hydrolysis.

Warren et al. (22) found that optimal activation of cellulose with cyanuric chloride was obtained after pretreatment of the cellulose with 13–14% (~3 M) NaOH. This is in the range of alkali concentration in which the swelling of the cellulose has reached a maximum and the internal structure of the cellulose has been considerably changed. This corresponds roughly to the inflection point in Vieweg's curve, representing the adsorption of NaOH by cellulose from aqueous solutions, as a function of the NaOH concentration (41). Smith and Lenhoff (42) applied the same conditions to the activation of Sepharose with cyanuric chloride.

Agarose in the wet state is already a highly swollen polymer, however; 94–98% of the bead weight consists of water. Therefore pretreatments with lower amounts of alkali were investigated. Furthermore, a shorter reaction time was used and the total amount of alkali-agarose was added in one portion to the FCP-xylene–dioxan solution, to obtain a uniform substitution of the matrix (method C). The results are summarized in Table 10 (cf. 3).

The influence of the change in reaction conditions is apparent from the experiment in which again 3 M NaOH and approximately 5 g of FCP were used. The degree of substitution is lower than that found in methods A and

TABLE 10

Batch	Alkali	FCP (g)	n_N	n_{Cl}	Mol. wt.	%F Exp.	%F Calc.	N/F[a]
g	3.00 M NaOH	1.07	0.10	0.09	320	0.6	1.14	1.82
h	0.27 M NaOH	1.07	0.20	0.18	335	1.3	2.27	1.75
i	0.10 M NaOH	1.00	0.10	0.10	321	0.9	1.22	1.31
j	0.27 M Na_2CO_3 (pH 11.4)	1.03	0.06	0.05	315	0.5	0.76	1.60
k	0.27 M Na_2CO_3 (pH 11.4)	1.09	0.06	0.05	315	0.4	0.75	1.70
l	0.27 M Na_2CO_3 (pH 11.4)	1.13	0.05	0.05	314	0.4	0.62	1.44
m	0.27 M Na_2CO_3 (pH 11.4)	0.95	0.06	0.05	314	0.4	0.68	1.54
p	3.00 M NaOH	4.87	0.57	0.50	390	3.2	5.55	1.74
q	1.32 M NaOH	5.06	0.59	0.51	394	4.0	5.73	1.44
r	1.32 M NaOH	4.86	0.57	0.46	391	3.5	5.58	1.60

[a] Molar ratio.

B, owing to the shorter reaction time. The value of the N/F ratio is lower than in method A because of the reversed addition of the reagents, but higher than in method B because of the direct presence of the total amount of alkali already at the beginning of the reaction.

Lowering the concentration of NaOH to 1.32 M, resulting in equimolar amounts of FCP (5 g = 29.7 mmol) and alkali (standard dry volume of the Sepharose multiplied by 1.32 = 29.7), did not change the degree of substitution, but lowered the N/F ratio slightly. The difference between 3 and 1.32 M NaOH was clearly a surplus, which was consumed only by hydrolysis of the excess of FCP.

An approximately sixfold decrease in the degree of substitution was obtained by application of the original concentration of alkali (3 M) but with a reduced amount of FCP (1 g); the N/F ratio increased slightly, due to a larger excess of alkali. Reducing the concentration of NaOH from 3 to 0.27 M, with a constant amount of FCP (1 g), again resulted in equimolar amounts of FCP and NaOH. The degree of substitution was doubled with a simultaneous slight decrease of the N/F ratio, both effects being due to a more favorable competition between coupling and hydrolysis.

With even lower concentrations of alkali, a lower degree of substitution and a lower value for the N/F ratio were obtained. A further lowering of the pH gave an even lower degree of substitution but with an increase in the N/F ratio. Finally, at pH values equal to or lower than 10 no detectable

substitution occurred; the number of ionized agarose-hydroxyl functions had become minimal, resulting merely in hydrolysis of the FCP.

Matrices of a wide range of degree of activation can be synthesized by controlled variation of the amounts of alkali and FCP, yielding reproducible results. Approximately one difluorochloropyrimidinyl residue was introduced per agarose unit, at the maximum degree of substitution, obtained in method B by application of 29.7 mmol of FCP per 3.3 mmol of agarose units (ninefold excess). The achievement of a higher degree of substitution is obviously impeded by steric factors and the incompatibility of the hydrophilic polymer with the hydrophobic reagent. The C_6 position of the D-galactopyranosyl moieties (cf. Fig. 1) are, mainly for steric reasons, the presumable points of attachment.

Reactive matrices with an unimpaired beaded structure are obtained by all three methods. The activated agarose does not dissolve in water at higher temperatures ($\geq 40°C$), in contrast to the native material, permitting further derivatization under more drastic conditions. A change from water to organic solvents does not affect the matrix structure. The FCP-treated agarose is reactive toward amines and mercaptans, and the coupling capacity does not diminish upon prolonged storage at 4°C in the wet state.

Coupling of FCP Agarose with Amines and Mercaptans

Reaction of FCP-agarose with hexamethylenediamine (HMDA) can lead to monosubstitution, cross-linking and hydrolysis. In all experiments an excess of HMDA was used to diminish the possibility of cross-linking. The degree of substitution, assuming that there will be no cross-linking with HMDA, can be calculated from $100/(306+148.5n+96p) = \%N/28(n+p)$, with n representing the original number of DFP groups and p the number of HMDA groups, per agarose unit. The maximum value of p will be $2n$ divided by the original N/F ratio; the correction, introduced by dividing with this ratio, is necessary in view of the previous hydrolysis and cross-linking during the FCP activation. The results, summarized in Table 11 (cf. Table 4), indicate that the maximum value of p is achieved in all batches except batch II; in this case a relatively lower excess of HMDA was used. Apparently both fluorine atoms of the DFP residues have been substituted.

An indication of the extent to which hydrolysis and cross-linking occur during substitution with HMDA can be obtained by comparing the increase in nitrogen content (ΔN) with the decrease in fluorine content (ΔF), both expressed in atom percent. ΔN should be equal to $|2\ \Delta F|$ if only monovalent coupling occurs. The best agreement was found (Table 12) in batch IV, where HMDA coupling was performed at pH 10.

TABLE 11

Batch	n_N	p	p_{max}	Mol. wt.	%Cl	
					Exp.	Calc.
I	0.63	0.48	—	446	—	5.02
II	0.92	0.84	1.23	523	5.7	6.24
III	0.66	0.62	0.61	463	5.3	5.06
IV	0.20	0.24	0.23	359	1.75	1.98
V	0.10	0.13	0.15	334	1.2	1.06
VI	0.05	0.06	0.07	319	0.45	0.56

TABLE 12

Batch	ΔN (at%)	|2 ΔF| (at%)
II	0.26	0.46
III	0.23	0.27
IV	0.12	0.11
V	0.08	0.10
VI	0.03	0.05

Substitution versus hydrolysis as a function of time for the coupling of HMDA to FCP-agarose was investigated by incubation of FCP-agarose at pH 10 with and without HMDA; samples were taken at different points of time, and the N and F contents were determined (Fig. 4). The N content was not affected by hydrolytic detachment of fluorine, since F and OH have nearly equal equivalent weights (curve N_b). The F content was diminished by about one-half during the first 15 min of the reaction with HMDA (curve F_a). This corresponds with monosubstitution; the second fluorine of each DFP residue will have a diminished reactivity due to the deactivating influence of the alkylamino group.

The equation $N_a - N_b = 2(F_b - F_a)$ should be valid, assuming no additional cross-linking by HMDA. The two respective curves show fairly good agreement, indicating that cross-linking had occurred only to a limited extent.

The reaction of aniline with FCP-agarose will not be complicated by additional cross-linking. The degree of substitution can be calculated from $100/(306+148.5n+73p) = \%N/(28n+14p)$, with p representing the number of aniline molecules per agarose unit. The results are summarized in Table 13 (cf. Table 5).

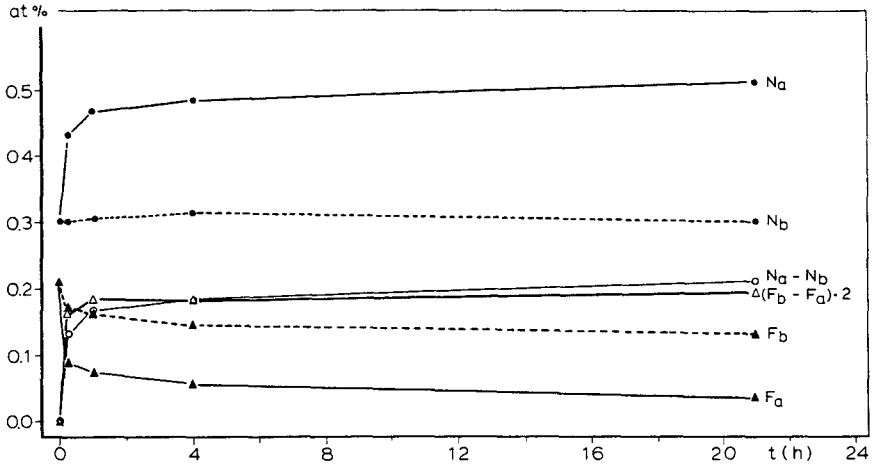


FIG. 4. The coupling of hexamethylenediamine to FCP-activated agarose; substitution versus hydrolysis. N_a, increase of nitrogen content due to substitution with HMDA; N_b, nitrogen content during hydrolysis; F_a, decrease of fluorine content due to substitution with HMDA and hydrolysis; F_b, decrease of fluorine content due to hydrolysis.

The value of p did not reach its maximum, as in the coupling reactions with HMDA, which must be ascribed to the substantial lower nucleophilicity of the arylamino group. Total displacement of fluorine was not achieved, even after 40 h of coupling; aftertreatment with HMDA yielded ninhydrin-positive products. The increase in nitrogen content (0.08 at% for I) is somewhat smaller than the corresponding decrease in fluorine content (0.09 at% for I), which is presumably due to hydrolysis (cf. Table 5).

Coupling of FCP-agarose with imidazole was performed at pH 9; the degree of substitution, calculated from $100/(306+148.5n+48p) = \%N/28(n+p)$, was 0.97 ($p_{max}$ 1.17). The increase in nitrogen content (0.36 at%) agreed with the corresponding loss of fluorine (2×0.20 at%), indicating that only minor hydrolysis had occurred during the coupling reaction.

TABLE 13

Batch	n_N	p	p_{max}	Mol. wt.	%Cl	
					Exp.	Calc.
I	0.66	0.47	0.61	438	5.55	5.34
II	0.69	0.53	0.61	447	—	5.48
III	0.69	0.28	0.61	428	—	5.71

The reactivity of FCP-agarose toward mercaptans was exemplified by the reaction with ethanethiol. The degree of substitution, calculated from $100/(306+148.5n+42p)=\%S/32p$, was 0.34 ($p_{max}$ 0.51).

These results demonstrate the applicability of FCP-activated Sepharose to the immobilization of aliphatic and aromatic amines and of compounds containing a thiol group or an imidazole moiety. The beaded matrix structure is not impaired by the conditions of the coupling reactions, as observed microscopically. The FCP-Sepharose affords, therefore, a versatile material for ligand immobilization.

Stability of the Agarose Derivatives

Incubation of the Levafix Brilliant Red E-4BA derivatives of native and cross-linked Sepharose 4B in buffers of different pH, followed by spectrophotometric measurement of the clear supernatants, yielded the leakage curves represented in Fig. 5. Leakage was absent or negligible in the range of pH 4–8. Prior cross-linking with epichlorohydrin resulted in higher acid stability of the final agarose derivative.

Chromatography of the concentrated acidic incubation media (pH 2.50) on Sephadex G–10 demonstrated the presence of colored, high-molecular-weight components in addition to the free dye. The matrix itself apparently had started hydrolyzing, the stability of the link between agarose and the "ligand" no longer being the limiting factor.

The amounts of colored agarose which were incubated corresponded to 8 mg of commercial dye (degree of substitution 12%, matrix content of the gel 3.4%). The amount of dye detached at pH 4.12 during 66 h from colored

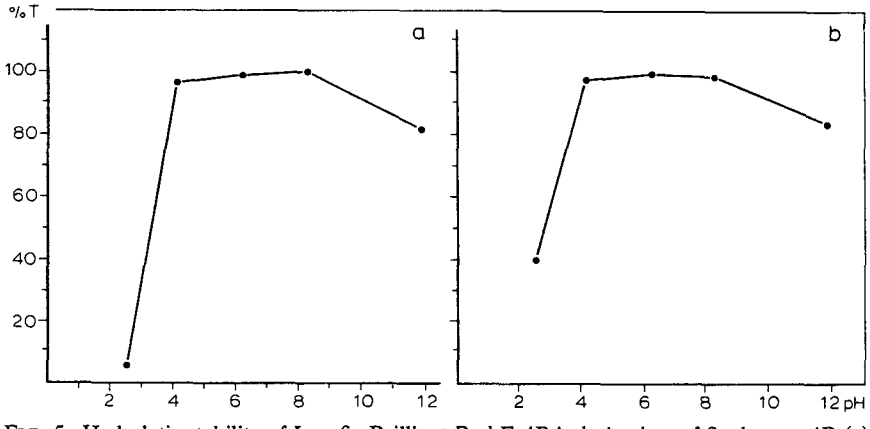


FIG. 5. Hydrolytic stability of Levafix Brilliant Red E-4BA derivatives of Sepharose 4B (a) and epichlorohydrin cross-linked Sepharose 4B (b). For explanation see text.

native Sepharose corresponded to 0.003 mg of commercial dye, resulting in a leakage of 0.04%/66 h.

Treatment of blank and HMDA-substituted agarose, as described in the leakage-stability test, yielded the same results for blank and substituted matrices, indicating that no detectable detachment of HMDA or derivatives had occurred during the period of incubation. The amounts of HMDA-Sepharose incubated corresponded to 0.42 mg of HMDA; the detection limit of HMDA with the chlorine–*o*–tolidine reagent was determined to be 20 ng. This means that the leakage of the agarose derivative in the described experiment was less than 0.02%/46 h, or on the average 15.7 pmol/h/0.1 g of standard dry gel.

Both experiments demonstrate that highly stable immobilized "ligands" are obtained by coupling these molecules to FCP-activated agarose. The mean leakage was about 0.5×10^{-3}%/h, which compares favorably with leakage from products based on CNBr-activated agarose: 0.17%/2 h at pH 8 in Tris–HCl at room temperature (15, 16); 15%/3 months at pH 8 in sodium bicarbonate at room temperature (43).

Characterization of Agarose Derivatives by Potentiometric Titration

The titration profiles of ε-aminocaproic acid and hexamethylenediamine-substituted FCP-activated Sepharose 4B (S-Aca, S-HMDA), and of the activated matrix treated with coupling buffer without amino components (S–OH), are presented in Fig. 6. The titrations were performed in 1 M KCl (cf. 44) in the pH range 1.8–12, using unsubstituted Sepharose as a blank. The results are given in a differential form, which has the advantage that the distribution of titratable groups over the whole pH range is readily seen (cf. 45). Owing to an increasing error at the extremes of the pH range, only data between pH 3 and 11.6 are shown.

The S-OH matrix clearly has acidic properties, which is not unexpected in view of the pK_a values of compounds with a comparable structure, e.g., cyanuric acid (7.2) or barbituric acid (4.0). The presence of amino and carboxyl groups in S-HMDA and S-Aca, respectively, already apparent from Fig. 6, is more clearly illustrated in Fig. 7; a correction for the presence of the acidic hydroxychloropyrimidinyl residues has been applied by subtracting the differential curve of S-OH. The occurrence of negative values in the curve of S-HMDA indicates that hydrolysis during coupling with HMDA occurs to a smaller extent than during treatment with coupling buffer only. The pK_a value of S-Aca is 5–6 (ε-aminocaproic acid 4.43), and of S-HMDA 10–11 (hexamethylenediamine 10.76).

The degree of substitution (DS) of S-Aca and S-HMDA can be calculated from the elemental analysis data, using the equations 100/(306+

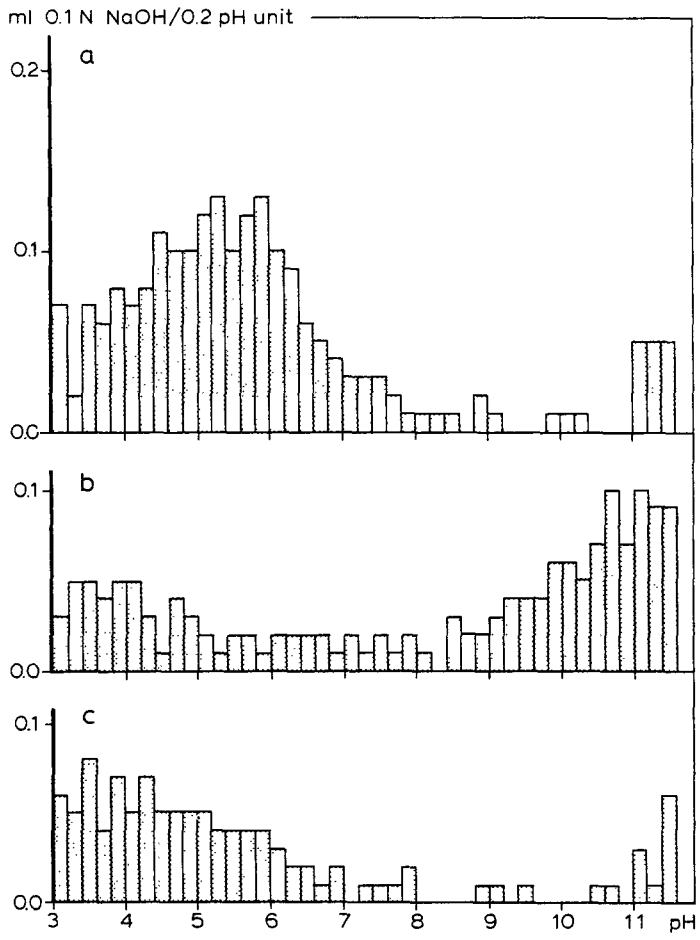


FIG. 6. Differential titration of samples (3 g, standard dry) of (a) S-Aca, (b) S-HMDA, and (c) S-OH in the range pH 3.0–11.6. The curves have been corrected for the titration of blank Sepharose in the same pH range.

$148.5n+111p)=\%N/(28n+14p)$ and $100/(306+148.5n+96p)=\%N/28(n+p)$, respectively, with n representing the original number of DFP groups, and p the number of ε-Aca or HMDA groups per agarose unit (Table 14).

Values of the degree of substitution can also be calculated from the titrimetric data of S-HMDA and S-Aca (cf. Table 7). A correction must be applied for the presence of the acidic hydroxychloropyrimidinyl residues, which are introduced during the original FCP activation and the subsequent

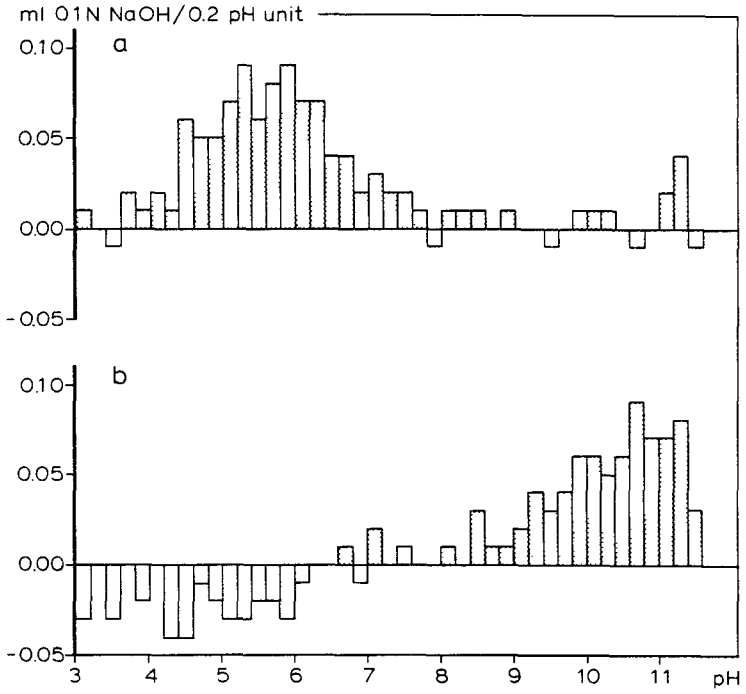


FIG. 7. Differential titration of samples (3 g, standard dry) of (a) S-Aca and (b) S-HMDA in the range pH 3.0–11.6. The curves have been corrected for the titration of S-OH in the same pH range.

coupling. The application of unsubstituted agarose as a blank will give values of DS that are too high; using S-OH as a blank will yield values of DS that are too low, owing to a presumably lower extent of hydrolysis during the coupling than during the incubation with buffer only. The application of the original FCP-agarose as a blank neglects the hydrolysis, which has occurred during the coupling; furthermore, titration of this material is impeded by its reactivity. Therefore, only the two limits, which are determined by unsubstituted Sepharose and S-OH, are given (Table 14). The DS of

TABLE 14

	DS(mEq/g dry gel) (elemental analysis)	DS(mEq/g dry gel) (titration)
S-HMDA	0.92	0.4–1.2
S-Aca	0.44	0.7–1.5

S-HMDA calculated from the elemental analysis agrees with the range determined by the titrimetric data. The DS of S-Aca, calculated from the elemental analysis, is even lower than the lower limit calculated from the titration. This may be ascribed to a catalytic influence of the carboxyl moiety of ε-Aca on the hydrolysis. A similar phenomenon has been found during the hydrolysis of cyanuric chloride (46). S-OH will therefore present a blank value which is too low for S-Aca.

Moreover, the DS of S-HMDA can be calculated directly from the number of groups dissociating between pH 8.0 and 11.6, yielding a value of 0.8 mEq/g dry gel. A similar calculation of the DS of S-Aca from the number of groups dissociating between pH 3.0 and 7.0 again must include, however, a correction for the presence of acidic hydroxychloropyrimidinyl residues, dissociating in the same range (a value of 0.6 mEq/g dry gel is obtained by using the curve of S-OH between pH 3.0 and 7.0 for this correction).

Additional characterization of S-HMDA was achieved by reacting the polymer with methylsulfonylethyl succinimidocarbonate (Msc-ONSu) (47), followed by determination of the sulfur content. For x equiv NH_2/g dry S-HMDA and p equiv S/g dry S-HMDA-Msc: $x = p/(1-150p)$. This results in a value of 0.68 mEq NH_2/g dry S-HMDA, which is lower than calculated from the titration. This must be ascribed to partial inaccessibility of amino groups for Msc-ONSu.

Titration and Msc derivatization both give values for the effective number of primary amino groups, in the case of S-HMDA; the calculation based on the nitrogen content, determined by elemental analysis, presents a theoretical upper limit. The lack of a suitable blank impedes the titrimetric determination of the carboxyl content of S-Aca; a parallel experiment with sodium acetate may offer a solution. Derivatization by carbodiimide coupling with a suitably labeled compound (e.g., cystamine), followed by elemental analysis, may afford another alternative.

Application of FCP Analogs, and FCP Activation of Other Polymers

Activation of agarose was also performed with TCP and TFP, being less and more reactive, respectively, than FCP. The experimental conditions were adjusted accordingly. The number of trichloro- and trifluoropyrimidinyl residues per agarose unit was 0.28 and 1.22, respectively, based on the nitrogen content of the products and assuming monosubstitution only. The calculated halogen contents were higher than the experimental values, indicating partial hydrolysis and cross-linking. The activated matrices had an unimpaired beaded structure, were thermostable, and were reactive toward amines and mercaptans.

Preliminary experiments indicated that other polysaccharides (cellulose, Sephadex) and aminomethylpolystyrene could also be activated with FCP. Cellulose was comparable with agarose with respect to reactivity; the other polymers were substituted to a smaller extent.

Application of the Described Methods to Solid-Phase Biochemistry

FCP-activated matrices (polysaccharides, aminomethylpolystyrene) have been applied to the immobilization of several enzymes (trypsin, glucose oxidase, penicillinase). The relative activities of the resulting products were highly dependent on the type of enzyme used, and varied between 8 and 98% (5,37). The reaction sequence: azo dye coupling–reduction–diazotation–enzyme coupling, was also applied successfully.

Insulin was coupled to FCP-activated Sepharose 4B, yielding preparations with antilipolytic activity without disturbing leakage effects (5). In affinity chromatography, ligand immobilization onto FCP-Sepharose was applied to the preparation of a biospecific adsorbent for trypsin. *p*-Aminobenzamidine was used as ligand in combination with a hydrophilic glycylglycine spacer (5).

Full experimental details concerning these investigations will be given in subsequent papers. Recently, Hofmann et al. (48) described the application of the FCP activation procedure to the synthesis of high capacity avidin-Sepharose, to be used for the immobilization of biotin-labeled hormones.

Note: During the preparation of this communication an interesting paper by Lang and co-workers appeared, which describes the use of agarose-triazine derivatives in affinity chromatography: LANG, T., SUCKLING, C. J., and WOOD, H. C. S. (1977) J. Chem. Soc. Perkin I: 2189.

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