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SULFONYL CHLORIDE ACTIVATION OF HYDROXYLIC MATERIALS

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SUMMARY

We have discovered that chromophoric sulfonyl chlorides and affinity ligand-containing sulfonyl chlorides prepared based upon fluorinated carbon skeletons are excellent activating agents for agarose. The primary basis for development of such reagents has been pentafluorobenzenesulfonyl chloride and tresyl chloride, both of which are excellent activating agents.

Activation using the sulfonyl chlorides whose synthesis is described here has yielded matrices that are very reactive to nucleophilic displacement by amines and thiols and, thus, should be excellent agents for the immobilization of affinity ligands, enzymes, cells, etc. The resulting material is based on covalent coupling and, thus, is more stable than affinity ligands or enzymes immobilized by other methods.

INTRODUCTION

The discovery by Axen et al. [1] of activation of agarose by cyanogen bromide provided the breakthrough necessary for the development of much of what we now call solid-phase biochemistry, as it permitted the immobilization of enzymes, antibodies, other proteins, affinity ligand, etc., to agarose. Unfortunately, cyanogen bromide activation creates a material which, although satisfactory for the initial development of immobilized enzymes and affinity chromatography, has in recent years proved to be less desirable. Two particular qualities of cyanogen bromide-activated coupled materials are at the basis of this observation. First, affinity matrices based on cyanogen-activated supports tend to yield immobilized ligands that slowly leak from the agarose or other hydroxylic material to which they are attached [2]. This is because the isourea linkage between the agarose and the affinity ligand or immobilized enzyme is subject to attack by hydroxyl groups and/or other nucleophilic functions. The second disadvantage of cyanogen bromide activation and coupling is that the resulting material contains an extra ionic function, namely, again, the isourea group [3]. This is not terribly important in the case of most enzyme immobili-

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zations but is a problem in affinity chromatography as it confers anion-exchange properties to the material, in addition to any affinity chromatographic properties that might exist. Such a combination of ion exchange and affinity can be either fortunate or unfortunate, depending upon charge and types of impurities, etc., to be removed by the chromatographic process.

Numerous chemistries have been investigated to obviate the problems with cyanogen bromide. Among the many activation methods that have been attempted, one of the most recent and most successful has been that of activating agarose using sulfonyl chlorides [4—11]. The sulfonyl chloride reacts with the hydroxylic matrix to produce a sulfonyl ester. Sulfonyl esters of primary hydroxyl functions are easily displaced by nucleophilic groups such as nucleophilic ligands and lysyl and cysteinyl residues of proteins. The resulting ligands are coupled via a secondary amine or by a thioether linkage, both of which are very stable and do not introduce new ionic charges. This activation method was first utilized by Gribnau [4] using tosyl chloride and mesyl chloride but, unfortunately, was performed under conditions that led to the reaction of both primary and secondary hydroxyl groups of the agarose. The secondary hydroxyl sulfonyl esters are not easily displaced by nucleophiles. Gribnau [4] found that, while proteins were easily coupled to the matrix, one could not remove all of the sulfonyl ester groups. He thus concluded that the method was not a method of choice and continued his development along other lines. Fortunately, Nilsson and Mosbach [5, 6] independently activated agarose with tosyl chloride and tresyl chloride under relatively mild conditions for a short period of time. This yielded predominantly primary hydroxyl activation, and the result was an excellent matrix for coupling protein and affinity ligands. The fluorine groups of tresyl chloride (trifluoroethanesulfonyl chloride) are highly electron-withdrawing and thus confer excellent "leaving-group" properties to the resulting sulfonyl ester. For this reason, tresyl chloride has been thought to be a reagent of choice in recent times for the activation of agarose, and tresyl chloride-activated agarose has, therefore, been commercialized [7].

This laboratory has been engaged in the investigation of two derivatives of the tresyl and tosyl activation procedures, namely (1) the activation of hydroxylic materials with colored sulfonyl chlorides [8—10], whereby one could, with ease, follow the activation and coupling procedures visually or spectrophotometrically, and (2) the coupling of sulfonyl chlorides containing affinity ligand substituents, which would yield "affinity-directed immobilization", where the immobilized enzyme would be coupled through relatively few sites near the active site. This should allow the formation of a more highly active immobilized enzyme preparation with a higher degree of homogeneity and, therefore, a superior material for both research and synthetic purposes.

This report concerns our investigation into a series of fluorinated chromophoric sulfonyl chlorides. This is based upon our discovery that pentafluorobenzenesulfonyl chloride [10] was an excellent activating material, with leaving-group properties very similar to tresyl chloride but at considerably lower cost and with less volatility, both of which are difficulties associated with laboratory activation of agarose with tresyl chloride. Therefore, we have synthesized a series of activating reagents designed as derivatives of either

pentafluorobenzenesulfonyl chloride or tresyl chloride. Our initial results suggest that these are excellent activating agents.

EXPERIMENTAL

Materials

Pentafluorobenzenesulfonyl chloride and 1,2,2,2-tetrafluoroethylsulfonyl fluoride were purchased from SCM Specialty Chemicals (Gainesville, FL, U.S.A.). 2,3,5,6-Tetrafluoroaniline and trifluoromethanesulfonic acid anhydride were purchased from Aldrich (Milwaukee, WI, U.S.A.), fuming sulfuric acid (27—33%) was purchased from Baker (Phillipsburg, NJ, U.S.A.), and Sepharose (agarose) CL-4B was purchased from Sigma (St. Louis, MO, U.S.A.). Triethylamine and pyridine were dried and kept over potassium hydroxide, acetone was dried over magnesium sulfate and Drierite, and all the solutions were filtered before use. All other chemicals were of analytical grade and used as supplied.

Synthesis of tetrafluorosulfanilic acid

Tetrafluorosulfonic acid [12] forms the basis for a series of pentafluorosulfuric acid-like chromophoric and affinity-directed sulfonyl chloride-activating agents. To synthesize tetrafluorosulfanilic acid, fuming sulfuric acid (30 ml) was added to 2,3,5,6-tetrafluoroaniline (15 g) which contained a few pieces of crystalline iodine. This was kept at 130°C for 1 h. The mixture was poured into 1500 ml of ice water and neutralized with barium carbonate. The barium sulfate was filtered off using charcoal-coated filter paper (Schleicher and Schuell, analytical paper No. 508), and the filtrate was treated with ion-exchange resin in acid form (Dowex 50W-X8), after which the water was evaporated on a rotary vacuum evaporator. The water was evaporated at less than 60°C on a rotavap, initially using a water aspirator; a vacuum pump was used to evaporate the last trace of water from the residue, such that the temperature was never allowed to go above 60°C. The product was purified by washing with a solution of acetone—chloroform (3:1, v/v) (50 ml). The resultant white solid was dried under vacuum in a vacuum desiccator. The tetrafluorosulfanilic acid was stored desiccated in the freezer (−20°C). Recrystallization was not necessary nor advisable.

The yield of tetrafluorosulfanilic acid after purification was 73%. The white compound decomposed at 277°C. It is important to evaporate the last traces of water using a vacuum pump, as long exposure to ± 90—100°C causes desulfonation of the tetrafluorosulfanilic acid. When this takes place, white fine crystals collect in the condenser of the rotary evaporator.

Synthesis of tetrafluorodabsyl chloride (4-dimethylaminobenzene-2′,3′,5′,6′-tetrafluorobenzene-4′-sulfonyl chloride)

Dabsyl chloride has been used as a good chromophoric (red) activating agent [9]. Its tetrafluoro derivative is synthesized as follows. Tetrafluorosulfanilic acid (10 g) and sodium nitrite (3.5 g) were dissolved in 30 ml of water and cooled on ice. This solution then was added to a solution of 150 ml of 50% hydrochloric acid, and the mixture was stirred on ice for 15 min. After this, ca.

1 g of urea was added until no more nitrogen bubbles were evolved. The mixture was kept on ice for 15 min. Then N,N-dimethylaniline (25 g) in 125 ml of glacial acetic acid was slowly added dropwise, while stirring the mixture on ice. After addition of the dimethylaniline, the mixture was stirred for 1 h on ice, after which 25% (v/v) potassium hydroxide was added slowly to neutralize the solution to pH 7, as checked by pH paper. The mixture was kept at room temperature for 1 h, and the brown-red precipitate that formed was filtered off and dried under vacuum.

The resulting potassium salt of tetrafluorodabsic acid (12 g) was dried by adding a few drops of thionyl chloride and then ground with phosphorous pentachloride (12 g) containing 3 ml of phosphorous oxychloride at room temperature for 20 min. The mixture was poured into 1200 ml of crushed ice, filtered, and washed with cold water. The dark-red product was dried under vacuum. The tetrafluorodabsyl chloride was stored desiccated in a freezer (−20°C).

Tetrafluorodabsyl chloride: $C_{15}H_{10}N_3O_2SClF_4$, MW 395, deep-red crystals, decomposes at 310°C.

Synthesis of tetrafluorodipsyl chloride (N-2,4-dinitrophenyl-4-amino-2',3',5',6'-tetrafluorobenzenesulfonyl chloride)

Using micro glassware, tetrafluorosulfanilic acid (0.6 g), synthesized as described above, in 7 ml of dioxane was added to 60% sodium hydride (mineral in oil) (0.2 g). The mixture was boiled, with stirring, for 140 min, and after cooling, 2,4-dinitrofluorobenzene (0.44 g) was added dropwise with stirring to this sand-like, light-brown mixture. The resulting orange mixture was refluxed for 3.5 h, and the resulting brown solid was filtered off and dried under vacuum. The reaction of tetrafluorosulfanilic acid and sodium hydride seemed to be complete after 50 min of refluxing, and the reaction of this mixture and the Sanger reagent (2,4-dinitrofluorobenzene) was complete after 2.5 h of reflux. The sodium salt (0.55 g) was washed with 4 *M* hydrochloric acid, dried with thionyl chloride, and ground for 5 min with a mixture of phosphorous pentachloride (1.75 g) and 1.45 ml phosphorous oxychloride. This mixture was poured into ice water and filtered, and the yellow-brown tetrafluorodipsyl chloride was dried under vacuum in a vacuum desiccator. The tetrafluorodipsyl chloride was stored desiccated in a freezer.

Tetrafluorodipsyl chloride: $C_{12}H_9N_2O_2SCl$, MW 429, yellow-brown, decomposes at 130°C.

Synthesis of α-(2,4-dinitrobenzene)tetrafluoroethanesulfonyl fluoride

A series of reagents was also developed, based on the fluoroalkylsulfonic acids, as, for example, tresyl chloride. The synthesis of α-(2,4-dinitrobenzene)-tetrafluoroethanesulfonyl fluoride is an example of such a reagent. To triethylamine (2 g), which was stirred on ice, was slowly added 3.7 g of α-hydrotetrafluoroethanesulfonyl fluoride [13—15]. After 10 min of stirring, 2,4-dinitrofluorobenzene (3.8 g) was added in drops, while maintaining the stirred mixture on ice. After a further 50 min of stirring on ice, the reaction mixture was allowed to warm to room temperature and kept there with continuous stirring for another 300 min. The dark-red syrupy mixture (10 ml) that re-

sulted was dissolved in 10 ml of benzene and washed with 10 ml of aqueous hydrochloric acid solution (HCl—water, 1:3, v/v). The red oily organic layer was washed four times with 10 ml of water (with ca. 30 mg of sodium chloride added to improve the separation), dried over magnesium sulfate, and filtered. The benzene was evaporated using ethanol and a stream of dry nitrogen blown over the mixture. To the resulting dark syrup was added, on ice, ethanol and water in a proportion so that yellow-brown crystals were formed. The crystals of α-(2,4-dinitrobenzene)tetrafluoroethanesulfonyl fluoride were filtered and dried under vacuum. The α-(2,4-dinitrobenzene)tetrafluoroethanesulfonyl fluoride was stored desiccated in a freezer.

α-(2,4-Dinitrobenzene)tetrafluoroethanesulfonyl fluoride: CF_3CFHSO_2F, MW 350.1, yellow-brown; m.p. 41—42°C.

Activation of agarose

The following procedure is applicable for all agarose activations (except trifluoromethane sulfonyl) and can be scaled up or down if needed. Wet Sepharose CL-4B (bead size 40—190 μm) was washed successively by suction filtration twice with 10 gel vols. each of demineralized water, 3:1, 1:1, 1:3 (v/v) water—acetone, and finally three times with 5 gel vols. of anhydrous acetone. The purpose of the solvent exchange described above was to remove all the water that might hydrolyze the sulfonyl chloride.

To the acetone-treated agarose beads was added an amount of the desired sulfonyl chloride, and this mixture was suspended in 0.5 gel vol. of dry acetone, which contained different amounts of dry pyridine. For example, activation was performed with dansyl chloride and dansyl fluoride concentrations of 3.0 mmol/g wet beads. Activation with dansyl chloride was performed in 0.02% pyridine in dry acetone (v/v), while activation with dansyl fluoride was performed in 0.2, 20 and 50% pyridine in dry acetone (v/v).

The activation by fluorescent sulfonyl chloride was followed using a handheld long-wavelength UV light (λ_{ex} 366 nm), viewing it in dry acetone. For quantitative fluorometric determination, a Varian SF 330 spectrophotofluorometer was used. Instrument settings employed for dansyl derivatives were λ_{ex} 325 nm, selector × 1/10, read mode 0.25, time constant 0.25, λ_{em} 485 nm.

The filtered beads, with interstitial fluid removed, were suspended in a glycerol—water mixture (87:13, v/v). Beads, which underwent the same activation procedures but without the fluorescent sulfonyl chloride/fluoride, served as a control.

Activation of agarose with pentafluorobenzenesulfonyl chloride

Wet agarose (25 g) was exchanged into dry acetone as described above. The acetone-treated agarose was then transferred into a 100-ml round-bottom flask containing 3 ml of dry acetone and 2 ml of dry pyridine. The mixture was vigorously stirred while 2.0 g of pentafluorobezenesulfonyl chloride were added dropwise over ca. 1.5 min. The reaction was continued for an additional 15 min in a shaker bath at 24°C. The beads, washed as in the tresyl chloride procedure, were stored at 4°C in 1 m*M* hydrochloric acid.

Activation of agarose with trifluoromethanesulfonic acid anhydride

Wet agarose (2 g) was exchanged into dry acetone. The acetone-treated

agarose was then added to a 100-ml round-bottom flask in an ice bath and allowed to cool for ca. 2 min. The flask contained 6 ml of dry acetone. Under nitrogen conditions, 1.5 ml of trifluoromethanesulfonic acid anhydride were added very slowly, under vigorous magnetic stirring, with a syringe. To neutralize the trifluoromethanesulfonic acid liberated during the reaction, ca. 100 mg of anhydrous potassium carbonate were added before the addition of anhydride was started. The reaction was continued for another 2 h in a shaker bath at 4°C. After the activation, the beads were washed and stored in dry acetone at 4°C.

Assay of activated agarose with p-aminothiophenol

Wet agarose (5 g) was added to 10 ml of a 0.5 *M* *p*-aminothiophenol solution in buffer—ethanol (1:1; pH 9). The buffer contained 0.2 *M* sodium bicarbonate and 0.5 *M* sodium chloride.

During the coupling process, samples of beads (0.3 g) were taken at different times and sequentially washed with 2 × 30 gel vols. of coupling buffer and 1.0 *M* sodium carbonate. To remove any untreated aminothiophenol, the beads were further washed extensively, first with distilled water, then with 30 gel vols. each of 30:70, 60:40 and 80:20 ethanol—distilled water, and finally 2 × 30 gel vols. of ethanol. Subsequently, to remove the ethanol, the beads were washed with 30 gel vols. each of 80:20, 60:40 and 30:70 ethanol—distilled water, 30 gel vols. of distilled water, and, finally, 30 gel vols. of 1 m*M* hydrochloric acid.

The washed beads (0.3 g), coupled to *p*-aminothiophenol, were added to a vial and diazotized with 0.87 ml of 1.2 *M* hydrochloric acid and 27 ml of 1% sodium nitrite for half an hour. After this, the beads were washed three times with 10 vols. each of distilled water and incubated in 1 ml of 1% 2-naphthol in 1% sodium hydroxide on ice for 1 h.

The wet, red-colored beads (100 mg) were suspended in 2.9 ml of a glycerol—water solution (87:13, w/v), and the absorbance was measured at the desired wavelength (usually 520 nm) against a blank containing untreated beads.

RESULTS AND DISCUSSION

The fluorinated sulfonyl chloride compounds, whose synthesis is described in the previous section, have proved to be excellent activating agents for agarose and similar hydroxylic matrices. They have activation properties similar to tresyl chloride in that approx. 80% of the chromophoric sulfonyl chloride can be displaced by a nucleophilic ligand or group on an enzyme. It remains to be seen if conditions can be developed whereby the chromophoric activating residue can be 100% displaced. The problem in displacement appears to be two-fold, namely (1) the leaving-group nature of the sulfonyl chloride and (2) the directing of the sulfonyl chloride reaction to primary and not secondary hydroxyl groups on the matrix. The first problem, namely, leaving-group nature, appears to have been chiefly solved via the use of fluorinated sulfonyl chlorides based upon pentafluorobenzenesulfonyl chloride or tresyl chloride. The second problem, that of directing the reaction to primary hydroxyl groups,

is being approached in two ways. First, one can employ matrices such as Tris-acryl, which have predominantly primary hydroxyl groups. Conversely, one can employ conditions such that the reaction of the sulfonyl chloride or other sulfonyl derivative will occur with high selectivity toward primary, rather than secondary, hydroxyl groups. To date, we have found that performing the reactions in the presence of small amounts of pyridine at very low temperatures and for a limited period of time permits the maximum direction of the reaction toward primary hydroxyl. An attempt was made, as detailed in the experimental

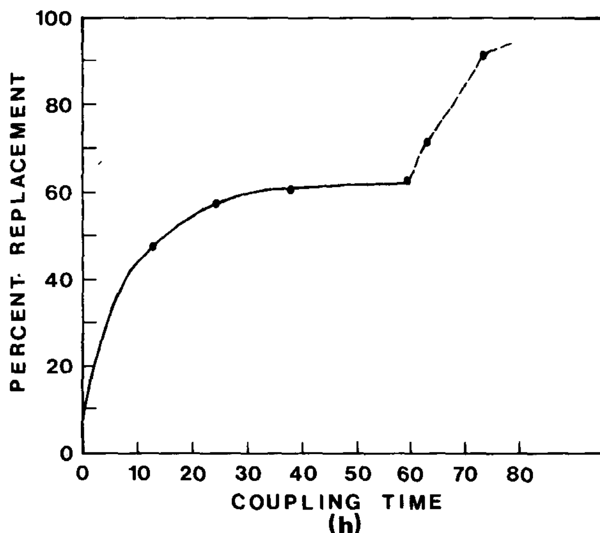

Fig. 1. Replacement of tetrafluorodabsyl chloride by ethanolamine at pH 9.0 and room temperature; the dotted line is the result of an increase in the coupling temperature to 55° C. Activation was performed at −10° C for 15 min, in 2% pyridine and 1.25 mol sulfonyl chloride per g dry beads; activation yield 32 μmol/g of dry agarose.

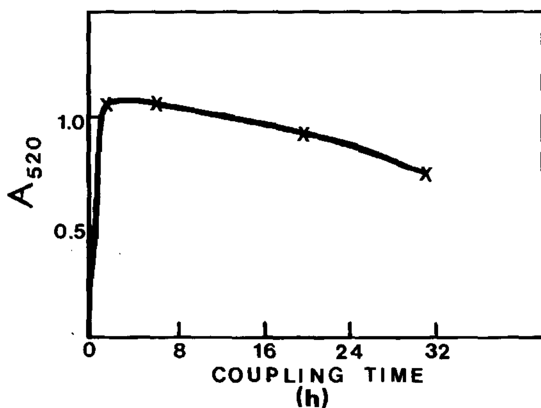

Fig. 2. Coupling of *p*-aminothiophenol to agarose Cl-4B beads, activated with pentafluorobenzenesulfonyl chloride at 24° C, for various times. Coupling was performed at 30° C using 0.5 *M* *p*-aminothiophenol in buffer (pH 9)—ethanol (1:1). The absorbance was determined after diazotizing the coupled beads and reacting with 2-naphthol, as described in Experimental.

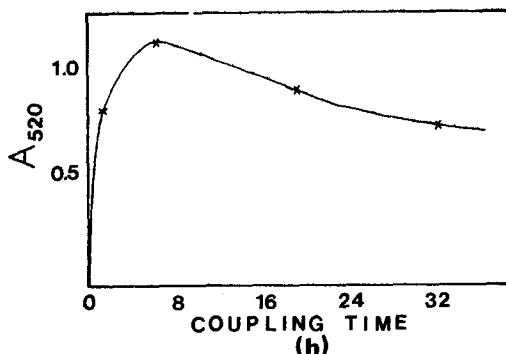

Fig. 3. Coupling of *p*-aminothiophenol to agarose CL-4B beads, activated with tresyl chloride at 30° C, for various times. Coupling was performed at 30° C using 0.5 *M* *p*-aminothiophenol in buffer (pH 9)—ethanol (1:1). The absorbance was determined after diazotizing the coupled agarose and then reacting it with 2-naphthol, which produced a red chromophore.

section of this paper, to direct the reaction by using sulfonyl fluorides instead of sulfonyl chlorides. The rather unreactive sulfonyl fluorides were unable to activate hydroxyl-containing matrices except under the most rigorous and undesirable conditions. It remains to be seen as to whether other active sulfonyl compounds, for example, sulfonyl azides, sulfonyl imidazoles, etc., will prove to have the desired selectivity. Such experiments are currently in progress.

As can be seen from Fig. 1, the reaction of ethanolamine leads to the displacement of tetrafluorodabsic acid from the agarose ester under mild conditions. This is comparable to tresyl chloride or pentafluorobenzenesulfonyl choride-activated matrices, as depicted in Figs. 2 and 3.

The use of perfluorinated ethanesulfones may allow the synthesis of bifunctional sulfonyl fluorides. Subsequently, these can be converted to sulfonyl chlorides and used to prepare affinity-directed immobilization materials. The principle of such a material is shown in Fig. 4. The basic idea is that a sulfonyl chloride containing a covalently bound ligand is reacted with agarose. The ligand recognizes the enzyme to be immobilized and binds it tightly, but non-covalently, in such a position that the active site is blocked from reacting with the activated matrix. While the enzyme is in this particular position, nucleophilic residues (particularly lysine residues) near the active site of the enzyme will react with the sulfonyl ester, displacing the sulfonyl ester ligand molecule and immobilizing the enzyme in a fairly homogeneous fashion and with high reactivity. To date, only a few examples of such immobilization have been attempted. For example, the dye residues that we are currently employing have an affinity for bovine serum albumin fatty acid binding sites. We have immobilized serum albumin using such affinity-directed immobilization matrices, although it remains to be proved that the immobilization is, indeed, "affinity-directed".

In conclusion, fluorinated chromophore sulfonyl chlorides based upon pentafluorobenzenesulfonyl chloride or tresyl chloride have proved to be excellent activating agents for agarose. The only deleterious effect of their application is that a percentage of the activating sulfonyl chloride reacts with secondary hydroxyls and cannot be displaced. When the reagent employed is

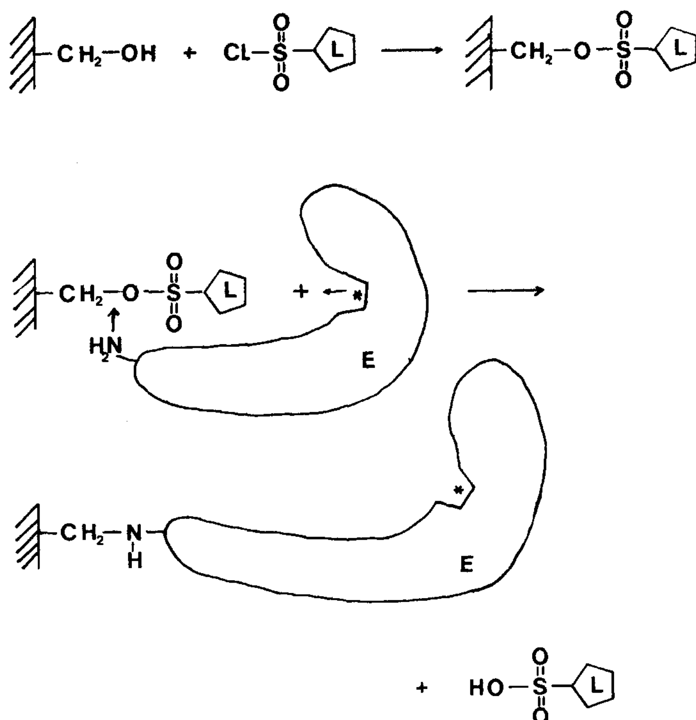

Fig. 4. The principle of affinity-directed enzyme immobilization. An immobilized ligand binds the enzyme non-covalently near the sulfonyl ester. Subsequently, a nucleophile adjacent to the active site, but not in it, displaces the ester, yielding the immobilized enzyme. * = Active site; E = enzyme; L = ligand.

colorless, as is tresyl or tosyl chloride, the presence of such unreacted residue goes unnoticed and does not disturb the investigator. The presence of the colored residues, on the other hand, confers some degree of permanent colorization to the matrix, even after the enzyme has been immobilized. This problem will be obviated when the activation is done briefly and with an appropriate reagent directing the activation to the primary hydroxyl groups. Enzymes and ligands immobilized by this method, however, possess certain specific advantages over those immobilized by classical cyanogen bromide coupling; for example, the bond between the ligand or enzyme is a secondary amine and is thus much more stable than the isourea bond created by the cyanogen bromide coupling method.

In addition, an ancillary property of sulfonyl chloride activation by the methods described here is the creation of affinity-directed immobilization of enzymes where a sulfonyl chloride, preferably based on tresyl chloride or pentafluorobenzenesulfonyl chloride, is coupled to a ligand and this ligand-containing sulfonyl chloride reacted with agarose. The resulting material has the potential property of being able to immobilize enzymes via residues near the active site of the enzyme.

REFERENCES

1 R. Axen, J. Porath and S. Ernback, Nature, 214 (1967) 1302.
2 M. Wilchek, T. Oka and Y. Topper, Proc. Natl. Acad. Sci., U.S.A., 71 (1974) 1630.
3 J. Kohn and M. Wilchek, in W.H. Scouten (Editor), Solid Phase Biochemistry, Wiley, New York, 1983, p. 599.
4 T.C.J. Gribnau, Ph.D. Thesis, University of Nijmegen, Nijmegen, 1977.
5 K. Nilsson and K. Mosbach, Biochem. Biophys. Res. Commun., 102 (1981) 449.
6 K. Nilsson and K. Mosbach, Acta Chem. Scand., 35 (1981) 19.
7 K. Mosbach, Pharmacia, patent pending.
8 W.H. Scouten and W. van den Tweel, in I. Chaiken, M. Wilchek and I. Parikh (Editors), Affinity Chromatography and Biological Recognition, Academic Press, New York, 1983, p. 299.
9 W.H. Scouten and W. van den Tweel, Ann. N.Y. Acad. Sci., 434 (1985) 249.
10 W.H. Scouten, M. Dekker and H. Kranenberg, in K. Mosbach (Editor), Methods Enzymol., in press.
11 W.H. Scouten, patent application pending.
12 P. Sartori and G. Bauer, J. Fluorine Chem., 12 (1978) 203.
13 I.L. Knunyants and G.A. Sokol'skii, Angew. Chem. Int. Ed. Engl., 11 (1972) 7.
14 D.C. England, M.A. Dietrich and R.V. Lindsey, Jr., J. Am. Chem. Soc., 82 (1960) 6181.
15 D.C. England and H. Oak, U.S. Pat., 2,852,554 (1958).