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AFFINITY ADSORBENT PREPARATION

CHEMICAL FEATURES OF AGAROSE DERIVATIZATION WITH TRICHLORO-*s*-TRIAZINE

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SUMMARY

Dichloro-*s*-triazinyl-agarose and a model analogue, 2-methoxy-4,6-dichloro-*s*-triazine, were prepared and found to hydrolyze readily. Key hydrolysis products were titrated potentiometrically and found to be acidic. Formation of such ionic groups was avoided by performing nucleophilic displacement reactions in non-aqueous media in the presence of an efficient base. The choice of base was critical since most of those typically used in acyl-halide type reactions formed insoluble adducts with trichloro-*s*-triazine in organic solvents. Remaining triazinyl chlorides of the activated support could be displaced rapidly and quantitatively in stepwise fashion by nucleophiles in organic solution.

INTRODUCTION

The typical bioaffinity adsorbent is prepared by coupling an affinity ligand to an insoluble hydrophilic support. Although the inventory of useful ligands increases rapidly, certain drawbacks of the support or coupler can be serious. A noteworthy example is cyanogen bromide-activated agarose[1], in which the N-substituted isourea bonds formed with alkylamines are only moderately stable[2] and cationic at neutral pH[3]. The former condition can allow ligands to "bleed" from the support[4,5], while the latter can cause non-specific adsorption[6,7].

Reagents of a type that can provide strong covalent bonds have been employed in the dye industry since the introduction of reactive dyes[8,9]. The most versatile and widely employed of these reagents is the symmetrical, conjugated heterocycle, TsT**. This reagent and substituted forms react like acyl-chlorides and have been used for coupling enzymes and affinity ligands to supports like cellulose and agarose[10–12]. The

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** Abbreviations: TsT = 2,4,6-trichloro-*s*-triazine; DAE = 1,2-diaminoethane; MDsT = 2-methoxy-4,6-dichloro-*s*-triazine; DsT-agarose = dichloro-*s*-triazinyl-agarose; MsT-agarose = monochloro-*s*-triazinyl-agarose (in this case one site on the ring is linked to aniline).

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procedures usually employed are similar to those involving cyanogen bromide, including support activation in alkaline aqueous media. Under these circumstances, however, the coupling reagent must react competitively with water[13] and support alkoxyl groups, making it difficult to predict and control the degree of support activation.

As shown in this report, hydrolysis is not confined to TsT but includes triazinyl chlorides of the activated support. Comparisons are made with model compounds, including titrimetric analysis of hydrolysis products. Previously outlined reactions[14] which avoid hydrolysis are detailed in this report, including controlled support activation and step-wise displacement of triazinyl chlorides. Results are discussed in terms of the relevant chemistry of triazine to the preparation of affinity and ion-exchange type adsorbents.

EXPERIMENTAL

Materials

Sepharose CL-4B and CL-6B were purchased from Pharmacia (Uppsala, Sweden). DAE, 1,3-diaminopropane, 1,4-diaminobutane, 1,6-diaminohexane, N,N-diisopropylethylamine and TsT (cyanuric chloride, 99%) were obtained from Aldrich (Milwaukee, WI, U.S.A.). The TsT was recrystallized two times from chloroform before use. MDsT was prepared as described by Dudley *et al.*[15]. Aniline (99 mole%) and 1,4-dioxane (99 mole%) were obtained from Fisher. Ninhydrin and hydrindantin were purchased from Pierce (Rockford, IL, U.S.A.). All other chemicals and reagents were of the highest grade commercially available.

Support activation

The agarose as an aqueous gel was washed on a sintered glass funnel exchanging dioxane for water as described by Hjertén *et al.*[16]. The agarose–dioxane gel was allowed to stand overnight in glass-stoppered graduated cylinder to permit accurate determination of the bed volume. With a measure of dioxane commensurate with bed volume, the support was rinsed into a round bottom flask equipped with a glass-sealed stirrer and water condenser. The flask was immersed in a thermostatted oil bath, and the contents were stirred at 100 rpm. N,N-Diisopropylethylamine in dioxane was added and the temperature allowed to equilibrate for at least 30 min. The reaction was initiated by the addition of TsT dissolved in dioxane. The tertiary amine concentration was twice that of TsT, and the final volume was twice the bed volume. Total volumes were used to calculate reagent concentrations.

Coupling reactions

The activated support, DsT-agarose, was washed with 10 bed volumes of dioxane on a sintered glass funnel. It was transferred to a reaction flask with one bed volume of dioxane and, to start the coupling reaction, with one additional bed volume of reactant (alkyl or arylamine) dissolved in dioxane. Following reaction with arylamine, the support was washed with 5 bed volumes of dioxane and transferred to a reaction flask with one bed volume of dioxane. One additional bed volume of alkylamine dissolved in dioxane was added to start the coupling reaction.

Analyses

Alkanediamines coupled to DsT- or MsT-agarose were measured with ninhydrin reagent[17] as described previously[14]. Equivalence between ninhydrin values for coupled amines and a standard of 6-aminohexanoic acid was established by potentiometric formol titrations[18]. Following coupling reactions, gels were washed on a sintered glass suction funnel sequentially with excess dioxane, acetic acid (1 *M*), NaCl (1 *M*), water and acetone. Washed samples were dried *in vacuo* over silica gel.

Potentiometric and pH-stat titrations

An automatic titrator (Radiometer TTT2/SBR3) was used to examine the hydrolysis of chloro-*s*-triazines and the hydrogen ion equilibria of hydrolytic products. The titrations were carried out by adding standard 0.1 *N* HCl (potentiometric titrations) or 0.1 *N* KOH (pH-stat) from an automatic syringe (Radiometer ABU 12) to 5–10 ml of solution containing 0.1 *M* KCl and other solutes as required. The pH was measured with combined glass/calomel electrodes (Radiometer GK 2322C), standardized before each determination with commercial buffer solutions having pH values of 4.0, 7.0 and 10.0. The thermostatted titration vessel was provided with a motor driven glass stirrer and a flow of nitrogen to prevent carbon dioxide from entering.

For pH-stat experiments, TsT or MDsT (0.5–1.0 *M*) in dioxane was added to temperature- and pH-equilibrated 0.1 *M* KCl solution to initiate hydrolysis. The final dioxane concentration of reaction mixtures was 2% or less. At various stages of hydrolysis, certain samples were titrated potentiometrically with standard acid. Appropriate blank tirations and volume corrections were made. The titration rate ranged from 0.05 to 0.2 pH units per min, depending upon the rate of sample self-acidification due to hydrolysis. The titration error due to self-acidification was 1% or less at or below pH 8.0 when one chloride of MDsT had been hydrolyzed.

For experiments involving both chloride ion and hydrogen ion determination, MDsT was hydrolyzed with pH-stat control in 0.1 *M* KNO_3. At various stages of hydrolysis, samples were titrated with a manual microburette containing standard 0.1 *M* $AgNO_3$. End points were measured with a chloride ion selective electrode (Beckman 39652), standardized with KCl solution.

DsT-Agarose gels in dioxane were washed on a sintered glass funnel exchanging water for dioxane[16]. The gels were washed with 0.1 *M* KCl (5 bed volumes), drained to form a wet cake, and 6 g added to titration vessels. After addition of 4 ml of 0.1 *M* KCl, hydrolysis was conducted under pH-stat control. At various stages of hydrolysis, gels were washed with 5 bed volumes of 0.1 *M* KCl, and 5 g of the wet cake was added to 5 ml of 0.1 *M* KCl in a titration vessel. Gels were titrated potentiometrically with standard acid at 0.05 pH units per minute. Blank titrations consisted of nonderivatized gels treated in the same way.

RESULTS

Hydrolysis of chloro-s-triazines

Hydrolysis was studied by pH-stat titration of liberated HCl. As a model for activated supports such as DsT-agarose, MDsT was prepared. The course of MDsT hydrolysis at pH 9.0, 25°C is shown in Fig. 1. The rapid production of two proton equivalents per MDsT does not represent hydrolysis of both triazinyl chlorides. The

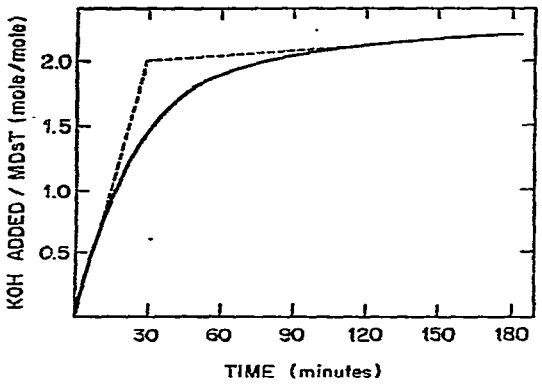
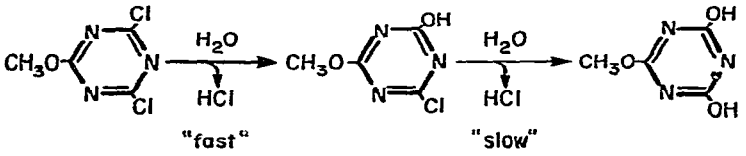


Fig. 1. Hydrolysis of MDsT at pH 9.0, 25°C. A 100-μl volume of 0.5 *M* MDsT in dioxane was added to 5.0 ml of 0.1 *M* KCl and the pH maintained by recording pH-stat titration with 0.1 *N* KOH.

asymptotes illustrate biphasic hydrolysis: rapid reaction at the first acyl-chloride followed by slow reaction at the second (Scheme 1). Measurements of both proton and chloride ion production (Table I) confirm that hydrolysis of the first acyl-chloride generates an acidic group (*i.e.*, one proton equivalent of HCl and one of ionization).



Scheme 1.

Potentiometric titrations at various stages of MDsT hydrolysis show the appearance of a weak acid (pK_a 3.99 $\pm$ 0.09, five determinations) during the first phase reaction. This group is transformed into one less acidic (pK_a 6.43 $\pm$ 0.07, five determinations) as hydrolysis of the second acyl-chloride progresses. Typical blank and sample curves for hydrolyzed MDsT are presented in Fig. 2 (insert) for comparison with those of hydrolyzed DsT-agarose.

Titrations of hydrolyzed DsT-agarose indicate that the respective triazinyl

TABLE I

CHLORIDE ION AND HYDROGEN ION DETERMINATIONS FOR MDsT HYDROLYSIS

Hydrolysis of MDsT at pH 8.0, 25°C. A 0.1-ml volume of 1.0 *M* MDsT in dioxane was added to 5.0 ml of 0.1 *M* KNO_3 and the pH maintained by recording pH-stat titration with 0.1 *M* KOH. At various intervals, samples were titrated for chloride ion with 0.1 *M* $AgNO_3$.

Time (min)	$\frac{(H^+)}{(MDsT)}$	$\frac{(Cl^-)}{(MDsT)}$	$\frac{(H^+)}{(Cl^-)}$
30	0.321	0.160	2.01
50	0.485	0.242	2.00
70	0.608	0.311	1.95
120	0.957	0.488	1.96
210	1.544	0.770	2.01

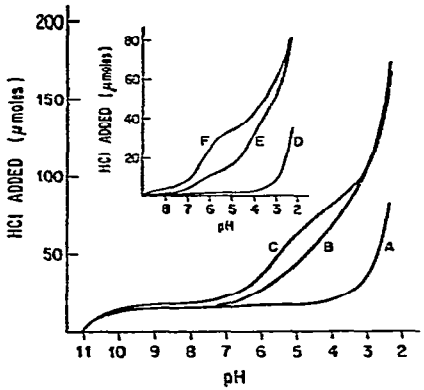


Fig. 2. Potentiometric titration curves of hydrolyzed DsT-agarose (Sepharose CL-6B). Curve A is the control titration of non-derivatized agarose. Curve B is the titration of DsT-agarose immediately after transfer of the gel from dioxane to aqueous phase. Curve C is that of DsT-agarose after 1 h hydrolysis at pH 11.9, 25°C. Titrations with 0.1 *N* HCl at 25°C were conducted at 0.05 pH unit per min (curves A and C) and 0.2 pH unit per min (curve B). Insert: Potentiometric titration curves of hydrolyzed MDsT. Curve D is the blank titration (5 ml of 0.1 *M* KCl). Curve F is that of MDsT (80 μmoles) after 8 h hydrolysis at pH 11.0, 25°C. Titrations with 0.1 *N* HCl at 25°C were conducted at 0.05 pH units per min (curves D and F) and 0.1 pH unit per min (curve E).

hydroxyls are slightly more acidic than those of the model compounds. Curve C (Fig. 2), typically found after extensive DsT-agarose hydrolysis, shows a group with an approximate pK_a of 5.5. Curve B is typically found after simple exchange of water for dioxane in the activated support. The titration (curve B) was initiated at pH 8.0 because sample self-acidification (hydrolysis) was too rapid at higher pH; for illustration purposes the curve has been displaced to the blank value at pH 8.0. The broader titration curves observed for hydrolyzed DsT-agarose might reflect the inductive effects of different carbohydrate alkoxyl substituents, but this interpretation is complicated by possible steric effects and associations with neighboring hydroxyl groups of the support. Analogous studies with MsT-agarose failed to reveal any dissociable group in the pH range studied (pH 2.5 to 11.0), a result of significant practical import.

The comparative hydrolysis rates for TsT and MDsT were studied in order to evaluate the deactivating effect of the alkoxyl substituent (Table II). The accelerating effect of pH was expected from other studies of TsT hydrolysis[13], but the more pronounced effect upon MDsT was not. Although aqueous phase activation procedures with TsT are generally performed at pH greater than 10, at pH 10 the rate constants

TABLE II

PSEUDO FIRST ORDER CONSTANTS (min^{-1}) FOR THE HYDROLYSIS OF TsT AND MDsT

pH-stat titrations were conducted in 0.1 *M* KCl at 15°C with KOH titrant. The triazine reactants were added as dioxane solutions, the volume of which was always less than 1% of the aqueous phase.

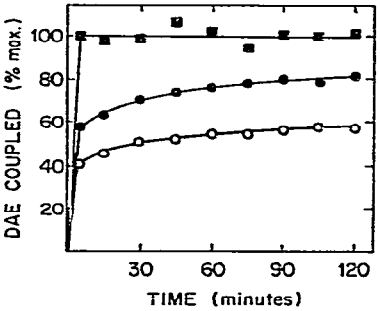
pH	*TsT*	*MDsT*
7.0	0.034	0.0005
8.0	0.044	0.0008
9.0	0.104	0.0036
10.0	0.25	0.018

differ only by a factor of 9.3 when statistical corrections are made for identical chlorides (*i.e.*, 3 for TsT and 2 for MDsT). It is not clear, therefore, how activation of supports in alkaline aqueous media can proceed without concomitant hydrolysis of the activated support structures.

Organic phase reactions

To avoid hydrolysis of TsT and DsT-agarose during support activation, conditions for organic phase reactions were investigated. Efficient neutralization of the HCl produced during the reaction between TsT and polyol support was considered essential in order to prevent alkyl halide formation[19]. However, many of the organic soluble bases commonly used in acyl-halide type reactions were observed to form insoluble adducts with TsT in organic solvents. These bases included pyridine, 4-dimethylaminopyridine, 2,6-lutidine, N-ethylmorpholine and triethylamine. The adducts were not the result of base impurities, since an equivalent of TsT was precipitated by an equivalent of base. Bases which did not form insoluble adducts were N,N-dimethylaniline, N-phenylmorpholine and N,N-diisopropylethylamine. The strongest of these bases, N,N-diisopropylethylamine, was selected for use in support activation reactions.

Formation of DsT-agarose was followed conveniently by coupling alkane diamines at remaining triazinyl chlorides and measuring the pendent amine by ninhydrin. These coupling reactions proceeded readily in organic solvent at room temperature, and substitution for both chlorides of the activated support occurred with excess diamine (Fig. 3). At lower diamine concentration the biphasic nature of these successive substitutions reflects the well known deactivation effect of the first substitution[20]. Nucleophilic substitution also appears to be progressively more



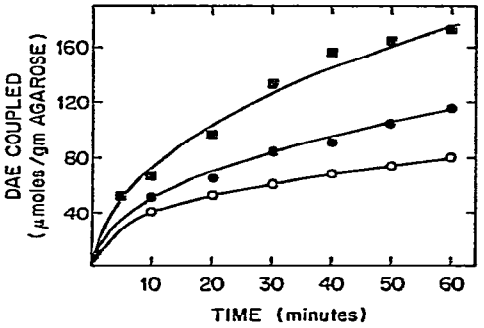
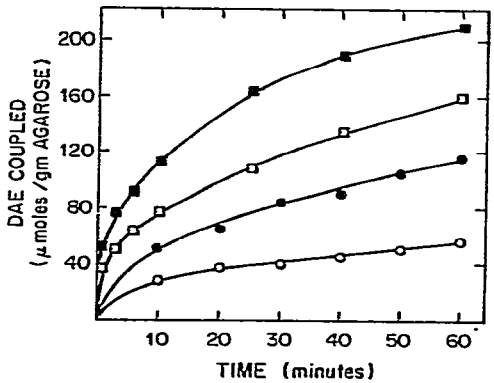


Fig. 3. Effect of DAE concentration on coupling with DsT-agarose. Reactions were performed at room temperature in 15 ml of dioxane containing 5 ml of DsT-agarose (Sepharose CL-6B) and DAE. DAE concentrations were: ○, 0.025 *M*; ●, 0.10 *M*; ■, 0.50 *M*. Each curve represents a different sample of freshly prepared DsT-agarose. Values for maximum coupling were those obtained after 2 h reaction with 2 *M* DAE in dioxane. The data, the averaged triplicates of ninhydrin assay with washed and dried samples, are given as percentages of maximum coupling.

Fig. 4. Effect of Temperature on the course of agarose activation. Sepharose CL-6B (100 ml in dioxane, bed volume) was reached with TsT (0.1 *M*) in dioxane at 30°C (○), 50°C (●), and 70°C (■). Aliquots representing 1% of the reaction mixture were removed at various stages of the reaction, quickly washed with excess dioxane, and reacted with DAE (1 *M*) in dioxane for 1 h at room temperature. The data are the averaged triplicates of ninhydrin assay with washed and dried gels.

sensitive to the developing HCl, since in these reactions the diamine also must act as the general base.

This analytical procedure was utilized to examine the effects of temperature and TsT concentration on the course of activation. The effects of temperature are shown in Fig. 4, where the amount of triazine bound per gram of dry support is simply half the value of coupled diamine. These reactions were limited for convenience to one hour but can be prolonged to achieve higher degrees of support modification. The effects of the other reaction parameter, TsT concentration, are demonstrated in Fig. 5. Both variables can be altered to achieve the desired degree of modification. A series of modified supports can be prepared by removing portions of the suspension during the course of an activation. This strategy would be useful in quantitative binding studies where variations in ligand density are important. For batch preparations, however, it is expedient to fix the reaction time and temperature while varying the TsT concentration (Fig. 6).



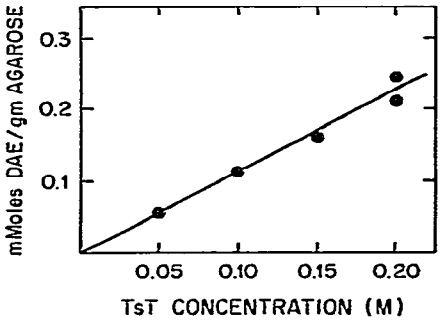


Fig. 5. Effect of TsT concentration on the course of agarose activation. Sepharose CL-6B (100 ml in dioxane, bed volume) was reacted at 50°C in dioxane with various concentrations of TsT: ○, 0.05 *M*; ●, 0.10 *M*; □, 0.15 *M*; and ■, 0.20 *M*. Aliquots representing 1% of the reaction mixture were removed at various intervals, washed with excess dioxane, and reacted with DAE (1 *M*) in dioxane for 1 h at room temperature. The data are the averaged triplicates of ninhydrin assay with washed and dried gels.

Fig. 6. The effect of TsT concentration on agarose activation at fixed reaction time and temperature. Sepharose CL-6B (100 ml in dioxane, bed volume) was reacted at 50°C for 1 h with various concentrations of TsT in dioxane. Coupling of DAE (1 *M*) in dioxane was performed for 1 h at room temperature. The values given for 0.05, 0.10 and 0.15 *M* TsT were obtained with the same commercial batch of agarose; values of 0.20 *M* TsT were obtained with two separate batches.

Data for 1 h of reaction (Fig. 6) showed that the proportion of added TsT consumed in the reaction was a small constant (3.8%). The unknown components reflected in Figs. 4 and 5 include steric effects, the number of "available" support hydroxyls and their inherent differences in reactivity toward TsT. It is possible that some of the reagent commercially used to cross-link the support also provides reactive hydroxyls, since different commercial batches of cross-linked support (Sepharose CL-6B) gave slightly different degrees of activation under identical reaction conditions (see Fig. 6, legend). It should be noted, however, that only cross-linked agarose was

TABLE III

COUPLING OF DAE TO DsT-AGAROSE WITH AND WITHOUT PRIOR REACTION WITH ANILINE

Coupling reactions with aniline (1 *M*) and DAE (2 *M*) were performed in dioxane for 1 h at room temperature. Experiments Nos. 1 through 5 were performed with Sepharose CL-6B and experiments No. 6 and 7 with Sepharose CL-4B. Experimental numbers represent separate batches of activated support.

Exp. No.	*Aniline*	*μmoles amine/g agarose*	*%*
1-A	(−)	213	100
1-B	(+)	113	53
2-A	(−)	199	100
2-B	(+)	112	56
3-A	(−)	279	100
3-B	(+)	148	53
4-A	(−)	231	100
4-B	(+)	119	52
5-A	(−)	235	100
5-B	(+)	115	49
6-A	(−)	235	100
6-B	(+)	128	54
7-A	(−)	267	100
7-B	(+)	137	51

studied, since the non-cross-linked form dissociated in organic solvents, especially at elevated temperature.

Stepwise reactions at individual chlorides of DsT-agarose with different alkylamines may be feasible in organic solvents. While the data (Fig. 3) suggested that this may be achieved at low alkylamine concentration, they also indicate that the first alkylamine substituent strongly deactivated the remaining triazinyl chloride. It seemed more practical to limit the reaction to a single triazinyl chloride with a weaker, less deactivating nucleophile. We have found aniline to be a model nucleophile in this respect. At room temperature it reacted with the DsT-agarose to leave a single triazinyl chloride for subsequent coupling reaction. Evidence for this is presented in Table III for DsT-agarose reacted with DAE with and without prior reaction with aniline.

TABLE IV

COUPLING OF ALKANE DIAMINES TO MsT-AGAROSE

Sepharose CL-6B was activated with 0.1 *M* TsT for 1 h at 50°C in dioxane. Coupling reaction with aniline (1 *M*) was performed in dioxane for 1 h at room temperature. The MsT-agarose was reacted in triplicate with each of the diamines (1 *M*) in dioxane for 1 h at room temperature.

Diamine	*μmoles amine/g agarose*
1,2-Diaminoethane	59.7 ± 0.5
1,3-Diaminopropane	59.3 ± 4.3
1,4-Diaminobutane	61.7 ± 0.2
1,6-Diaminohexane	60.9 ± 0.4

For many affinity adsorbent preparations diaminoalkanes are particularly useful as "extension arms"[21]. The data in Table IV demonstrate that different chain-length diamines reacted readily and quantitatively in organic phase. Clearly, there is opportunity to choose from a wide variety of substituted alkylamines to produce other useful derivatives, but each will be subject to appropriate solvent conditions. Dioxane was used for the activation and coupling reactions partly for convenience. It dissolves TsT to high concentrations, and its miscibility with water facilitates solvent exchange. Although other solvents can be employed and may be required by particular alkyl or arylamines, care must be taken to ensure that the solvent is inert toward acyl-halides[22,23].

DISCUSSION

Hydrolysis

TsT contains equivalent acyl-like chlorides. Substitution deactivates the remaining triazinyl chlorides, and experience dictates that less deactivating nucleophiles should be reacted first[15]. In general, nitrogen substitution produces greater deactivation than does oxygen, and reaction control with the latter requires greater precision[15]. This is complicated further by the aqueous media in which most reactions are conducted, particularly pH-dependent competitive hydrolysis[13]. Hydrolysis will be significant, for example, during alcoholysis, given the usual similarity of alcohol and water proton dissociations. As a consequence, alcoholysis of TsT and chloro-*s*-triazines is performed solvolytically wherever possible[15]. This is obviously impossible with insoluble polyols such as agarose and cellulose, and the problem is proportionately greater. Few quantitative data are available to this point, but, for example, reaction of a dichlorotriazinyl dye with cellulose anion and hydroxide showed nearly equivalent bimolecular reaction constants in the pH range 9.46 to 12.73 (ref. 24).

Whether the reagent is CNBr or TsT, competing hydrolysis in aqueous phase will make it difficult to control and predict the extent of support activation, and this will be exacerbated by concomitant hydrolysis of activated structures. The well-known weak deactivating effects of alkoxyl substituents made the latter a likely event for DsT-agarose. This expectation was supported by the pseudo-first-order rate constants for MDsT hydrolysis, especially at alkaline pH (Table II). The observations (Fig. 2) that hydrolysis of DsT-agarose and the model compound, MDsT, produce acidic hydroxyl substituents also is consistent with weak alkoxyl inductive effects and dissociation constants for other triazinyl hydroxyls[13]. Formation of such ionic groups must be avoided to prevent non-specific adsorption phenomena during affinity chromatography[6,7].

Organic phase reactions

To avoid hydrolytic reactions, conditions were established for support activation in organic phase. Since the nucleophile is that of an insoluble polymer, the acylation reaction occurs in a two-phase, heterogeneous system requiring a soluble base for efficient HCl neutralization. Study of organic bases commonly employed in acyl-halide type reactions revealed unusual precipitation phenomena with TsT. For one of these, triethylamine, Schroeder[25] observed similar phenomena and suggested that quarternary ammonium salts were formed. We have avoided this by using the sterically hindered base, N,N-diisopropylethylamine, as proton sponge during support activation. However, few quantitative data are available concerning the mechanisms of TsT acylations, expecially those occurring in non-aqueous media. The problem is

greater in the present circumstance, because agarose does not constitute a well-defined nucleophile in terms of the accessibility and reactivity of its hydroxyl groups. Nonetheless, support activation occurs smoothly (Figs. 4 and 5) and predictably (Fig. 6) in organic media.

The availability of two triazinyl chlorides in the activated support structure presents challenging possibilities for the design of affinity as well as ion exchange type adsorbents. One or both positions may be used to incorporate determinants for adsorption and/or for additional synthetic steps. Although the DsT support was reacted in non-aqueous media to avoid the hydrolytic production of ionic groups, the MsT support can be reacted in either organic solvents or, as previously shown[14], in aqueous media. However, reactions in aqueous media require additional study, particularly the circumstances under which quantitative reactions can be performed with various types of nucleophiles. The use of aniline to convert DsT to MsT support is not obligatory. It represents both a convenience and a model reaction for which other nucleophiles may be substituted. However, care must be taken to insure that alternative nucleophilic substitutions sufficiently deactivate the triazine ring so that any subsequent hydrolysis of the remaining chloride does not introduce an ionic hydroxyl.

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