*Journal of Chromatography*, 219 (1981) 361–372
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,195

# INVESTIGATION OF THE ACTIVATION OF VARIOUS INSOLUBLE POLYSACCHARIDES WITH 1,1′-CARBONYLDIIMIDAZOLE AND OF THE PROPERTIES OF THE ACTIVATED MATRICES

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(Received July 13th, 1981)

## SUMMARY

This report further characterises a new procedure for the preparation of affinity chromatographic supports, namely the activation of hydroxylic solid-phase supports with 1,1′-carbonyldiimidazole (CDI). Matrices with a controlled degree of substitution can be synthesised by the use of CDI, and if required, a high level of activation can readily be achieved *e.g.* up to 5 mmol/dry gram with cross-linked agarose. The CDI-activated agarose was found to have a half-life of greater than fourteen weeks when stored in dioxane. The conditions for coupling simple amines of differing $pK_a$ values to these active matrices were evaluated and the coupling yields analysed. Based on these results, conditions suitable for the coupling of proteins were established. The linkage of the amino group of the ligand to the support (an N-alkylcarbamate) was shown to possess good stability over a wide pH range. This stability was much greater than that of the isourea linkage obtained with the cyanogen bromide activation method. It is expected that this new activation procedure should prove to be particularly useful for a variety of affinity chromatographic experiments, including those which cannot tolerate the hydrolytic release of small amounts of the insolubilised ligand.

The CDI method has been extended to other polysaccharide matrices *e.g.* cellulose and dextran derivatives, and to glycophase-coated glass beads. The activated glass bead derivative provides a suitable support for attachment of ligands for high-performance affinity chromatography.

## INTRODUCTION

Recently we introduced a new procedure for the preparation of activated ma-

0021-9673/81/0000–0000/$02.50 

trices suitable for the attachment of affinity chromatography ligands or leashes[1]. This procedure involved the reaction of cross-linked agarose with 1,1′-carbonyldiimidazole (CDI) to form an N-alkylcarbamate (Scheme A, Fig. 1), which could then be smoothly coupled with N-nucleophiles . A number of other carbonylating reagents, such as 1,1′-carbonyl-1,2,4-trizaole, were also shown to allow the preparation of activated matrices[3], although CDI remains the reagent of choice, particularly in terms of convenience and activation yields. An important advantage of this new activation method compared with the standard cyanogen bromide method[4,5] is the absence of charged groups in the pH range normally used in affinity chromatography. Other advantages are the ease of handling of the reagent, the ability to achieve a range of substitutions under a variety of readily controlled activation conditions, and the stability of the activated product.

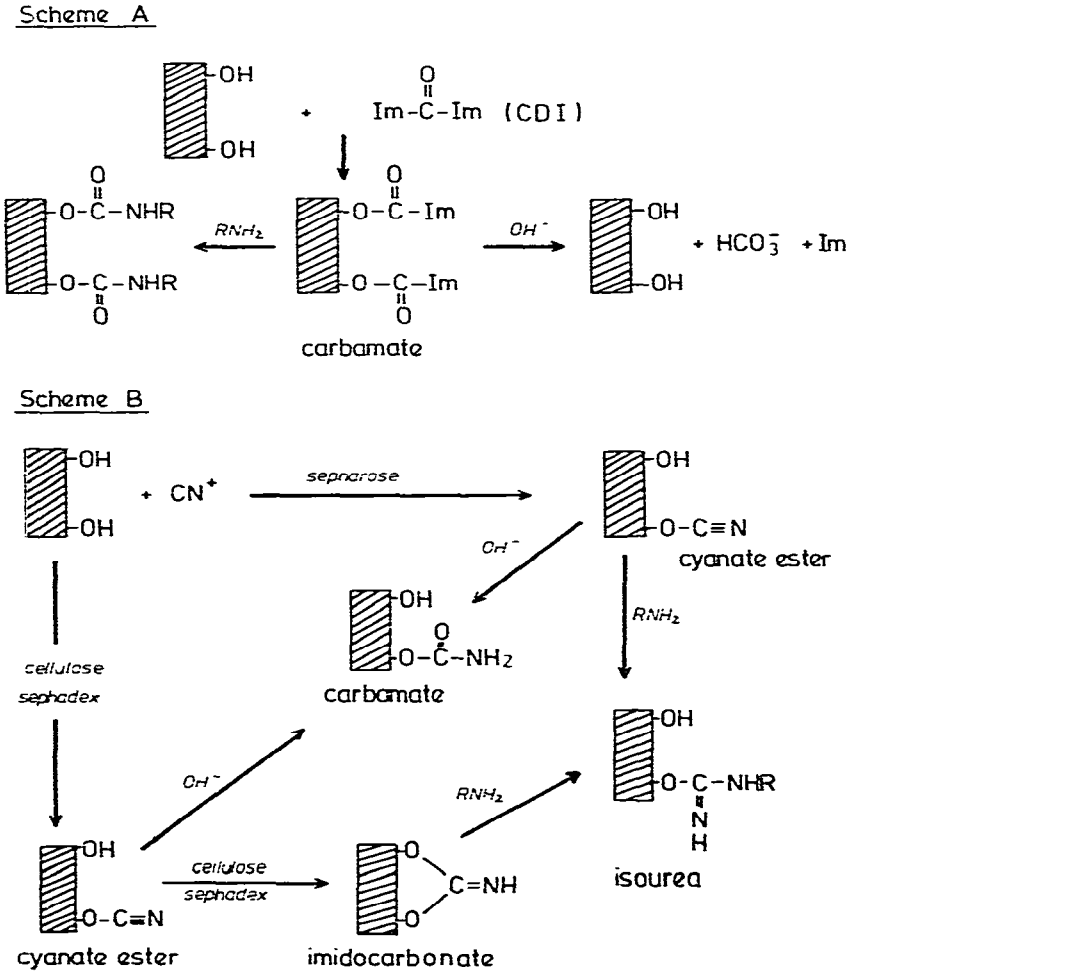


Fig. 1. The chemical reactions involved in the carbonylation of a polysaccharide (Scheme A) and the activation of a polysaccharide with CNBr (Scheme B).

In this report the CDI approach has been extended to other insoluble polysaccharide matrices, such as cellulose derivatives and glass beads. Additional data on the derivatisation of cross-linked agarose are discussed. Experiments demonstrating the stability of both the activated and coupled products are also described.

## EXPERIMENTAL

### *Reagents*

CDI, Reactigel™, (6X, 20–25 μmoles active groups/ml gel) and carbohydrate-coated glass beads (glycophase-G™/CPG40) were purchased from Pierce (Rockford, IL, U.S.A.); cross-linked allyl dextran (Sephacryl S-200, superfine), cross-linked agarose (Sepharose CL-6B), and cross-linked dextran (Sephadex G-25) from Pharmacia (Uppsala, Sweden); regenerated cellulose (75–150 μm) from Viscose Group Ltd. (Great Britain); cellulose powder (Whatman cellulose CC-31) from Whatman Ltd. (Great Britain); agarose–polyacrylamide copolymer (Ultrogel AcA-44) from Industrie Biologique Francaise (France); and cellulose beads from the Institute of Macromolecular Chemistry (Czechoslovakia). Trypsin (type III) and soybean trypsin inhibitor (type IIs) were purchased from Sigma (St. Louis, MO, U.S.A.). Acetone was analytical-reagent grade and all other chemicals were of reagent grade. Radioactivity was measured on a Packard Model 2002 Tricarb scintillation counter.

### *Methods*

The procedures used for solvent exchange of the matrices from water, a typical activation procedure, a typical coupling procedure for simple amines and analysis of all carbonylated matrices except glass beads are described in the companion publication[3].

### *Analysis of carbonylated carbohydrate-coated glass beads*

Since the active beads are not stable to the previous analytical method, the beads were hydrolysed at pH 3 for 4 h. The pH was maintained by automatic addition of acid during this period. The suspension was then titrated between the limits of pH 9 and 4 to obtain the amount of imidazole present. This figure was used to calculate the number of active groups present. The method does not allow for the presence of cyclic carbonates; however, previous studies have indicated that negligible amounts of cyclic carbonate are formed under these conditions[1].

## RESULTS AND DISCUSSION

### *Comparison of the activation of cross-linked agarose with the CDI and CNBr methods*

Table I shows the efficiency of carbonylation of cross-linked agarose with CDI and compares this procedure with the CNBr method[4,5]. In the CDI method the activated matrix contains imidazolyl carbamates which couple smoothly with N-nucleophiles to yield N-alkylcarbamates (Scheme A, Fig. 1). The CNBr method produces a cyanate ester on reaction with agarose[6], and probably an imidocarbonate with Sephadex or cellulose[7]. Both "active species" couple with N-nucleophiles to give affinity chromatography ligands attached via N-substituted isoureas (Scheme B, Fig. 1). However these groups can, prior to coupling, undergo hydrolysis to unreactive

TABLE I

COMPARISON OF ACTIVATION YIELDS FOR THE CDI AND CNBr METHOD ON CROSS-LINKED AGAROSE

| *Reagent* | *Solvent* | *Amount (mmol)* | *Yield of active groups* | |
|---|---|---|---|---|
| | | | *(mmol/3 g)* | *Percent* |
| CDI* | Acetone | 0.93 | 0.45 | 49 |
| CDI* | Dioxane | 0.93 | 0.4 | 43 |
| CDI* | Dioxane | 2.7 | 1.73 | 65 |
| CNBr** | Water | 6 | 0.1 | 2 |
| CNBr** | Water | 24 | 0.11 | 0.46 |

* The activation procedure was carried out as described in the *Methods* section on 3 g of moist cake.
** This was carried out by the method of March *et al.*[5], on 3 g of moist cake, with 1 *M* $Na_2CO_3$ as the buffer.

carbamates (see Scheme B, Fig. 1). The cyanate esters present were estimated by Kjeldahl nitrogen analysis before and after hydrolysis with 1 *M* HCl (see Scheme B, Fig. 1). The ratio of inactive to active groups was shown to be *ca.* 2.5 to 1. The level of active groups obtained with CNBr activation, as shown in Table I, has been corrected for the presence of unreactive carbamates. The concentration of active groups obtained with CDI activation was determined by titration analysis of the carbonate and imidazole liberated by basic hydrolysis (see Scheme A, Fig. 1)[3]. The comparison shown in Table I clearly establishes that even small amounts of CDI can be used to produce much higher levels of activation than with CNBr.

We found that under the reaction conditions described by March *et al.*[5] for the CNBr method, the activation yield can be improved only 10% even with a four-fold excess of CNBr (Table I). By contrast, the following study showed that the CDI method can be used to prepare highly substituted matrices by a proportional increase in the amount of reagent. Cross-linked agarose was activated in dioxane with varying amounts of CDI and the yields obtained are shown in Fig. 2. The maximum activ-

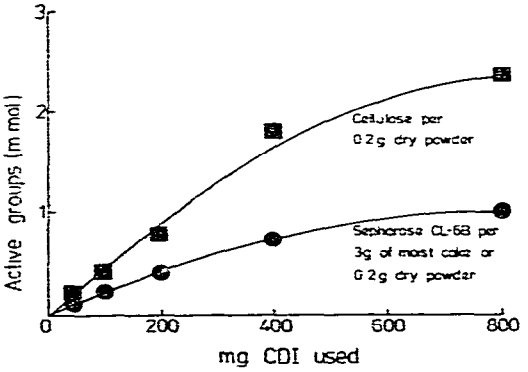


Fig. 2. The yield of formation of active matrix from the reaction of cross-linked agarose or cellulose with varying amounts of CDI. The dry weight of 3 g of moist cake of Sepharose CL-6B is *ca.* 0.2 g. The cellulose derivative used was HP-Regenerated Cellulose.

ation potential is *ca.* 5 mmol per dry gram of matrix (or 1 mmol per 3 g of moist cake). This is about ten times the value obtained for CNBr activation. Such a highly activated matrix is not always desirable in the case of cross-linked agarose where shrinkages can occur, and the original gel volume is not regained even after an aqueous coupling has taken place. For most affinity chromatography work only the lowest point on Fig. 2 (100 μmol per 3 g of moist cake) is relevant as greater activation levels can lead to multi-attachment of peptide and protein ligands, with consequent reduction in biological activities. However, with the attachment of a simple ligand for use as a leash or spacer, a high substitution may be useful.

In addition, the CNBr method requires both the reactions and washing of the active matrix to be carried out quickly. Activation time is not critical with the CDI method and the washing of the activated matrix is carried out under conditions whereby negligible hydrolysis occurs.

In all cases, the coupled products derived from the carbonylated agaroses and 6-aminohexanoic acid were devoid of charged groups at alkaline pH values (as determined by titration analysis). By contrast the corresponding product from the CNBr-activated matrix exhibited on titration analysis a second charged group ($pK_a$ 9.5), which was attributed to the N-substituted isourea linkage arising from the coupling of the ligand to the activated matrix. The chemical complexity of the CNBr reaction has been also documented by other groups[8–10], and leads to a certain amount of non-specific binding of contaminants to the matrix during an affinity purification.

*Stability of CDI-activated cross-linked agarose to storage and to aqueous coupling conditions*

The activated cross-linked agarose can be readily stored for extended periods (*e.g.* 6 months) in the refrigerator at 4°C under dry acetone. Dioxane is unsuitable for this purpose because of its high freezing point. The stability of the activated matrix was also investigated by storing a sample under fresh dioxane at room temperature in the dark. Samples were withdrawn at different time intervals and coupled with 6-aminohexanoic acid. Despite the fact that the matrix was stored at 20°C rather than 4°C, the product still had some 60% of its initial activity after fourteen weeks of storage. It is therefore a practical proposition to activate a large amount of reswollen agarose and store the product until required. This is an advantage which the cyanogen bromide method does not afford.

*Comparison of the coupling efficiency of the CDI-activated matrix with that of the CNBr-activated matrix*

Fig. 3 shows that at the same excess of ligand (in the case glycine), the CDI-activated matrix is at least as reactive as the CNBr-activated matrix. The coupling of 6-aminohexanoic acid is a good model for attachment of proteins to the CDI-activated matrix, since the $pK_a$ value of the ε-amino group of the amino acid lysine is similar to that of 6-aminohexanoic acid. Many proteins are sensitive to extremes of pH. This lability precludes couplings being carried out at pH 9–10, which would give the most efficient coupling conditions for CDI-activated gels[3]. However, coupling can be carried out in the range pH 7.5–9. Under these conditions a high coupling yield can still be obtained provided an activated matrix of moderately high substitution is used[2]. This approach is particularly valuable for the coupling of proteins which are

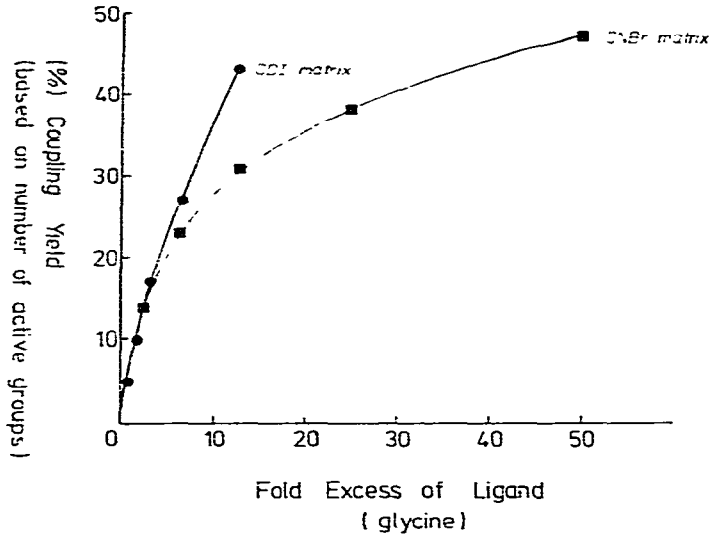


Fig. 3. The coupling yield of different amounts of glycine with either the CNBr- or CDI-activated matrix (0.28 mmol of active groups in each case). In each study the activated matrix (moist cake) was treated in 5.3 ml of water with 1 *M* N,N,N′,N′-tetramethylethylenediamine at pH 9. The calculated excess of glycine was added as a solid to the reaction immediately after the addition of the aqueous buffer to the activated matrix. The amount of coupled ligand was determined by titration analysis.

available only in small amounts. Table II illustrates the excellent linkage yields which can be achieved for a variety of proteins, when the coupling reaction is carried out at pH 8.5 with a matrix at a moderate level of activation. At activation levels much higher than 0.5 mmol per gram of moist cake, the possibility of multipoint attachment of a protein becomes more significant. The use of highly activated matrices could lead in some cases to a lower biological activity of the immobilised protein. However, with more robust proteins, particularly protein antigens, multipoint attachment may prove advantageous as weaker affinity interactions can result in a more facile desorption of retained components. The immobilised proteins listed in Table II demonstrate that a variety of other materials can be bound to cross-linked agarose with good retention of biological activity[2].

The CDI method is clearly very attractive for the coupling of leashes. This can be achieved most readily and efficiently with aqueous buffers at pH 11 for bis-amines of the form $NH_2(CH_2)_nNH_2$ (refs. 1 and 2). Alternatively dioxane can be used as a solvent for 1,6-diaminohexane and other diamines soluble in this solvent. For alkali-sensitive matrices such as glass beads, glycylglycine, which can be coupled at pH 8, has proved an excellent leash. The protein can be attached to the matrix under mildly acidic conditions via a diimide-mediated coupling to a preformed leash with either a free amino or carboxyl group. This procedure has been used in the preparation of high-capacity trypsin affinity columns[1]. In this earlier communication, we described the immobilisation of trypsin inhibitor via CDI-activated cross-linked agarose at pH 9, and the subsequent use of the generated affinity support for the purification of bovine trypsin.

TABLE II

SOME EXAMPLES OF THE COUPLING YIELDS OF LIGANDS TO A CDI-ACTIVATED MATRIX USING MILD COUPLING CONDITIONS

Sepharose CL-6B (3 g of moist cake) was activated with 1 mmol of CDI. The ligands were added to the activated matrix which was buffered with 0.1 *M* borate buffer, pH 8.5. The coupling yield was estimated by decrease in optical density (at $\lambda_{max}$ of the sample) of the washings relative to the coupling reaction. The protein concentration on the matrix was determined by amino acid analysis of a 1 ml sample which had been hydrolysed with 6 *N* HCl, 110°C, 24 h.

| *Ligand* | *Coupling yield (%)* | *Protein concentration on the matrix (μmol/ml)* |
|---|---|---|
| Sulphanilic acid azo-bovalbumin | 90 | 1.0 |
| Bovine thyroglobulin | 94 | 0.4 |
| Bovine thyroid stimulating hormone | 88 | 2.3 |
| Porcine insulin | 100 | 2.9 |
| Human immunoglobulin | 87 | 1.0 |
| 3,3',5'-Thyronine | 78 | 33 |

*Stability of the coupled matrix*

It is well known that ligands coupled to agarose through amino groups by the CNBr method are slowly released into solution, particularly at alkaline pH values[11]. It has been demonstrated that this release occurs mainly as a result of hydrolysis at

TABLE III

RELEASE OF [$^{14}$C]GLYCINE FROM COUPLED CROSS-LINKED AGAROSE, VIA CDI METHOD

Cross-linked agarose (6 g of moist cake) was activated in dioxane with CDI (0.9 g) in the usual way. It was coupled to glycine (0.04 g) containing [$^{14}$C(U)]glycine (28.2 mCi/mmol) at pH 9 in the usual way at 4°C. The coupled agarose was washed, divided into six equal portions, which were suspended in 10 ml of the following 0.1 *M* buffer solutions: 1, potassium hydrogen phthalate, pH 3.5; 2, potassium phosphate, pH 6; 3, potassium phosphate, pH 7; 4, TRIS, pH 8; 5, potassium carbonate, pH 10; 6, sodium phosphate, pH 11.5. The samples were stored at 4°C and aliquots of each (0.5 ml) were withdrawn after 1, 3, 7, 14 and 42 days.

| *Reaction time (days)* | *Loss of [$^{14}$C]glycine (% of total)*,*** | | | | | |
|---|---|---|---|---|---|---|
| | *pH 3.5* | *pH 6* | *pH 7* | *pH 8* | *pH 10* | *pH 11* |
| 1 | 0.3 | 0.6 | 0.5 | 0.2 | 0.4 | 0.8 |
| 3 | 0.3 | 0.4 | 0.3 | 0.3 | 0.6 | 1.0 |
| 7 | 0.4 | 0.3 | 0.3 | 0.7 | 0.8 | 1.4 |
| 14 | 0.3 | 0.2 | 0.2 | 0.3 | 0.9 | 4.0 |
| 42 | 0.4 | 0.3 | 0.3 | 1.1 | 2.0 | 6.7 |

* The amount of glycine remaining attached to the matrix was determined by digesting the matrix with 10 *M* HCl at 60°C. After cooling, a 0.5-ml aliquot was neutralised and the cpm of $^{14}$C was measured. This value was used to calculate the total $^{14}$C cpm in the sample.

** Expressed as a percentage of total counts per gram of moist cake of coupled agarose.

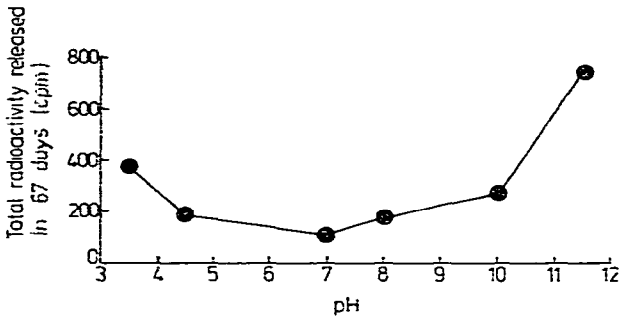


Fig. 4. The relative stability of agarose-OCONHCH$_2$COOH when left for up to 42 days at 4°C at different pH values. The experimental procedure was described in the footnotes to Table III and the values shown in this graph are the total of all values obtained at a given pH.

the isourea groupings[11] particularly in the presence of buffers which contain amines[7]. It was therefore of interest to compare the CNBr and CDI activation methods in terms of their abilities to produce products resistant to ligand leakage. The stability of the CDI-activated matrix was determined by measuring the release of [$^{14}$C(U)]glycine from the coupled matrix at various pH values over a period of six weeks. The results, expressed as percentages of the total [$^{14}$C(U)]glycine present, are shown in Table III. The linkage shows good stability at the pH values normally used in affinity chromatography. Fig. 4 shows the relative pH stability for the glycine-matrix, which again illustrates that the carbamate linkage is stable except at extremes in pH.

Tesser *et al.*[11] have studied the solvolytic detachment of [$^{14}$C(U)]alanine and cAMP from CNBr-activated matrices. Fig. 5 compares the data from their study with

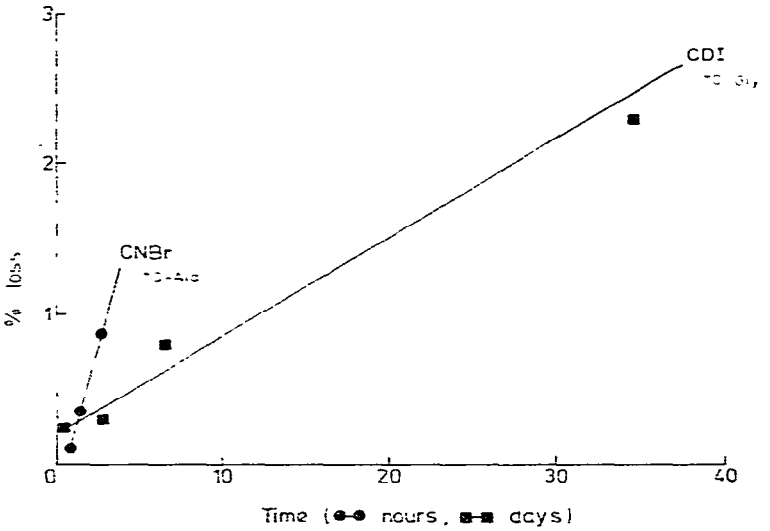


Fig. 5. A comparison of the rates of solvolytic detachment at pH 8 of [$^{14}$C(U)]-glycine which was insolubilised by the CDI method and [$^{14}$C(U)]-alanine which was insolubilised by the CNBr method. The CNBr data were derived from Fig. 2 in a study by Tesser *et al.*[11].

our data on the corresponding CDI-activated matrix. This comparison shows that the urethane linkage (CDI method) is *ca.* 25 times more stable than the isourea linkage (CNBr method). This figure is probably a minimum as the slight amount of radioactivity present in the supernatant of glycine matrix prepared by the CDI method and stored at pH 8 did not increase significantly with time of incubation. The low level of radioactivity in the supernatant (up to 1 % of total) is probably due to the presence of fines in the sample rather than glycine released by hydrolysis of the carbamate linkage. The greatly improved stability of attachment of a ligand to the matrix activated by CDI should prove to be invaluable in experiments which are adversely affected by the presence of free ligands, *e.g.* the localisation of hormone receptors on a cell surface. No ligand leakage was observed when sulphanilic acid azo-bovalbumin linked to Sepharose by the CDI method (see Table II) was stored at pH 7 at 4°C for 2 months. Tesser *et al.*[11] have already commented on the unsuitability of the CNBr-activated matrix with such ligands. Similar leakage has been noted for deoxycortico-

TABLE IV

ACTIVATION YIELDS OF POLYSACCHARIDE MATRICES WITH CDI

| *Dry matrix presoaked in DMF** | | *Matrix presoaked in water and solvent-exchanged*** | | | |
|---|---|---|---|---|---|
| *Matrix* | *Yield*** (0.93 mmol of CDI)* | *Solvent* | *Yield**** | | |
| | | | *0.47 mmol CDI* | *0.93 mmol CDI* | *1.86 mmol CDI* |
| Cellulose (Whatman cellulose powder CC-31) | 0.1 | A | 0.08 | 0.1 | 0.14 |
| | | B | 0.06 | 0.08 | 0.14 |
| CL-Dextran (Sephadex G-25) | 0.4 | A | 0 | 0 | 0 |
| | | B | 0.16 | 0.38 | 0.56 |
| Regenerated powder cellulose | 0 | A | 0.12 | 0.20 | 0.28 |
| | | B | 0.08 | 0.12 | 0.34 |
| HP-Regenerated cellulose (Regenerated cellulose after hydroxypropylation) | 0.44 | A | 0.20 | 0.32 | 0.56 |
| | | B | 0.16 | 0.24 | 0.50 |
| Glass beads (carbohydrate-coated glass beads, Glycophase-G-CPG40) | 0.06 | A | 0.06 | 0.07 | – |
| *Matrix as aqueous slurry*[§] | | | | | |
| CL-Agarose (cross-linked Sepharose 6B) | | A | 0.16 | 0.30 | 0.48 |
| Agarose–polyacrylamide (Ultrogel AcA-44) | | A | 0.12 | 0.18 | 0.24 |
| CL-Allyl dextran (Sephacryl S-200) | | A | 0.10 | 0.22 | 0.38 |
| Cellulose beads (Cellulose regenerated in bead form) | | A | 0.16 | 0.26 | 0.38 |

* In all cases 0.2 g of the dry matrix was presoaked in DMF for 16 h and then treated with CDI for 0.5 h.

** The dry matrix (0.2 g) was preswollen in water for 16 h and then solvent-exchanged into either dioxane (A) or DMF (B).

*** Yield of active groups determined by titration analysis and expressed as mmol/0.2 g dry wt. of matrix.

[§] The wet equivalent of 0.2 g dry weight was used for those matrices supplied as aqueous slurries.

steroids[12], estradiol[13], catecholamines[14], and ε-DNP lysine[15] from agarose conjugates prepared via the CNBr method.

*Activation of other matrices by CDI*

Although agarose in bead form is one of the most commonly used matrices for affinity chromatography, it was necessary to use cross-linked agarose beads for this activation procedure with CDI because of the requirement of an organic non-protic solvent for the activation step. Agarose beads without cross-linking lost their mechanical stability and flow-rates deteriorated after activation with CDI in dioxane. However, there are many other hydroxyl-containg matrices available which are amenable to organic solvents. All of those examined and reported in Table II were found to be reactive under optimal conditions. In all cases the active groups on the matrices were shown to be imidazolyl carbamates as found for cross-linked agarose.

Those matrices supplied as aqueous slurries were shown to activate readily by following the same procedure used for cross-linked agarose, *i.e.* solvent exchange into dioxane (see lower half of Table IV). Matrices supplied in the dry form were most conveniently activated by presoaking them directly in dimethylformamide (DMF) for 16 h, followed by activation with CDI for 30 min. The one exception to this was the regenerated cellulose which had been produced by the viscose process, dried and ground to a powder. For activation of this matrix it was necessary to presoak it first in water and then solvent exchange into dioxane or DMF. In all other cases there was no advantage in preswelling the matrix in water first. Presoaking in DMF directly was sufficient to swell these matrices for activation with CDI (see Table IV). However, all matrices failed to react with CDI if the dry forms were put directly into dioxane.

Dioxane and DMF were equally effective for solvent exchanging the matrices from their aqueous slurries. The one exception was Sephadex G-25, which did not

TABLE V

COUPLING YIELDS OF VARIOUS CDI-ACTIVATED MATRICES WITH 6-AMINOHEXANOIC ACID AT pH 10

All matrices (0.2 g dry wt.) were solvent exchanged from water and treated with 0.93 mmol of CDI for 0.5 h. The activated matrix was isolated and treated with 5.4 mmol of 6-aminohexanoic acid at pH 10 for 5 h. No additional buffer was used.

| *Matrix** | *Solvent** | *Active groups*** (mmol/0.2 g)* | *Coupled groups*** (mmol/0.2 g)* | *Yield (%)**** |
|---|---|---|---|---|
| Powdered Cellulose | A | 0.14 | 0.042 | 30 |
| Regenerated Cellulose | A | 0.28 | 0.084 | 30 |
| Regenerated Cellulose | B | 0.34 | 0.114 | 34 |
| HP-Reg. Ccell. | B | 0.50 | 0.222 | 44 |
| Cellulose Beads | A | 0.38 | 0.096 | 25 |
| Cross-linked Agarose | A | 0.48 | 0.168 | 35 |
| Agarose-polyacrylamide copolymer | A | 0.24 | 0.090 | 38 |
| Cross-linked Dextran | B | 0.56 | 0.150 | 27 |
| Cross-linked Allyl-dextran | A | 0.38 | 0.20 | 52 |

* As in Table IV.

** Determined from duplicate activation and titration analysis.

*** Percentage of active groups coupled.

activate in dioxane. In this case there was a pronounced shrinkage of the cross-linked dextran beads upon removal of the water with dioxane. This did not happen with DMF.

The carbohydrate-coated glass beads were similarly activated with CDI and this gives a new route to supports for high-performance affinity chromatography[16].

Regenerated cellulose powder which had been previously treated with propylene oxide to introduce hydroxypropyl groups[17] could be activated to very high levels when treated with large amounts of CDI. This is a result of the well-known lipophilic effects of the hydroxypropyl groups which enable it to swell effectively in organic solvents. Greater than 10 mmol of active groups per gram of dry matrix were introduced in this way (see Fig. 1). As in the case of cross-linked agarose, noticeable shrinkage of the matrix occurred at such high degrees of substitution, but these readily obtainable high levels of activation demonstrate the utility of the CDI method. Specialised supports for hydrophobic chromatography and ion exchange chromatography have been produced by this method.

Table V shows that the same range of matrices, after activation with CDI, coupled readily to 6-aminohexanoic acid at pH 10. The coupling efficiencies were determined by titration of the carboxylic end groups introduced and the yields were generally in the range of 30–50 %. The carbohydrate-coated glass beads were coupled to glycylglycine at pH 8 and gave similar yields of carboxylic end-groups.

As a result of these studies it has been shown that CDI is a reagent of general utility for activating hydroxylic supports for affinity chromatography. The active group was always the imidazolyl carbamate, which reacts readily with nucleophilic amines providing stable, unchanged N-alkylcarbamate linkages to ligands and spacer molecules for affinity chromatography.

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