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REVIEW

The Use of Cyanogen Bromide and Other Novel Cyanylating Agents for the Activation of Polysaccharide Resins

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Index Entries: Cyanogen bromide, in the activation of polysaccharide resins; cyanylating agents, in the activation of polysaccharide resins; activation of polysaccharide resins by cyanogen bromide; polysaccharide resins, activation by cyanylating agents.

1. INTRODUCTION

In 1964 the successful synthesis of a true ester of cyanic acid was reported (*1*), employing an alcohol and cyanogen bromide (CNBr).† This reaction was applied in 1967 to the "activation" of polysaccharides by treatment of the resin with CNBr in basic medium (*2,3*). Such "activated" resins could then be used for the covalent linkage of amino-containing ligands to the resin. Contrary to all previously known methods of immobilization (*4*), coupling of ligand to CNBr-activated polysaccharides proceeded smoothly in aqueous medium and at near-physiological pH. This method was therefore eminently suitable for the immobilization of fragile, biologically active molecules, such as enzymes, antibodies, peptide hormones, and so on. Clearly, the possibility of immobilizing such

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†Abreviations: CNBr, cyanogen bromide; CDAP, 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate; CTEA, N-cyanotriethylammonium tetrafluoroborate; DMF, dimethylformamide; pNPC, p-nitrophenylcyanate; TEA, triethylamine.

TABLE 1
Survey of Cyan-Based Activation Procedures for Polysaccharides

Category	Literature references
Conventional procedures	Axen (*2,9*) Cuatrecasas (*5,13*) March (*15*) Nishikawa (*12*) Porath (*3,10*) Stage (*11*) Wilchek (*14*)
Cyano-transfer procedures	Kohn (*16*)
Novel cyanylating agents	Kagedal (*18*) Kohn (*17,33*)

molecules in a facile fashion, without appreciable loss of their biological activity, constituted an important breakthrough. Therefore it is not surprising that with the introduction of affinity chromatography (*5*), hydrophobic chromatography (*6*), and the rapid expansion of solid-state enzymology, activation of polysaccharides by CNBr became one of the most widely used techniques in modern biochemistry (*7,8*).

Consequently a large variety of different activation procedures have emerged. As shown in Table 1, these procedures can be classified into three major categories. *Conventional procedures* employ CNBr as activating agent and a strong inorganic base such as NaOH or Na_2CO_3 as reaction catalyst. In *cyano-transfer procedures,* CNBr is employed in conjunction with a tertiary amine such as triethylamine (TEA) that serves as a reaction catalyst. The third category comprises procedures in which various *cyanylating agents* are used instead of CNBr. These procedures yield resins that are identical to (or at least similar to) the commonly employed CNBr-activated resins, thereby avoiding the use of the hazardous CNBr itself.

It is the aim of this article to provide a detailed account of the presently available techniques for the cyan-based activation of polysaccharide resins.

2. CONVENTIONAL PROCEDURES

2.1. Theoretical Considerations

As defined here, *conventional activation procedures* are characterized by the use of CNBr as activating agent in a strongly basic reaction medium. All CNBr activation procedures published before 1982 belong into this category. These procedures share many common features and can all

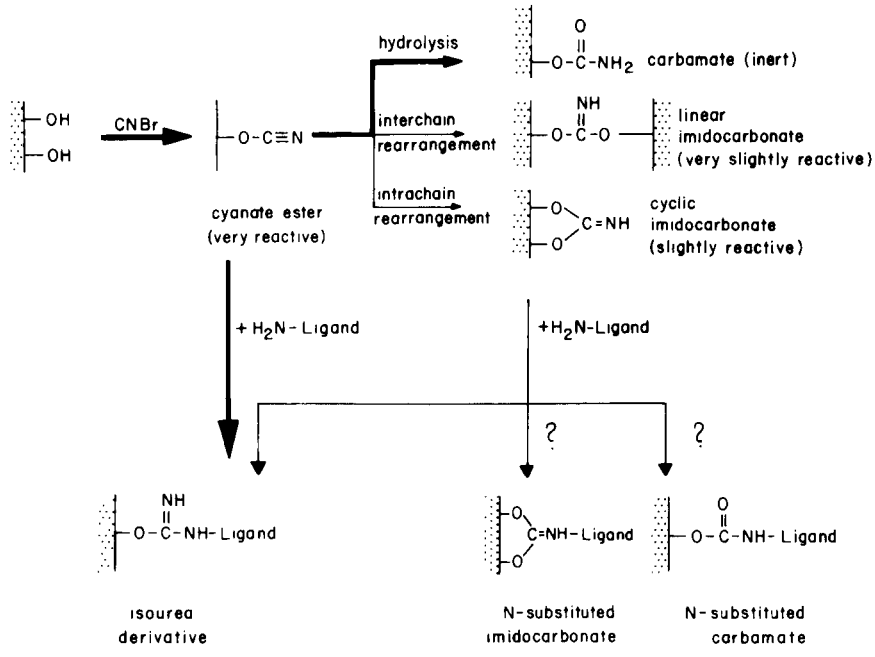

Fig. 1. Mechanism for the activation of agarose-based resins by conventional procedures (*19*).

be regarded as modification of the original CNBr activation technique that was developed in 1967 by Axen et al. (*2*).

The strongly basic reaction medium employed in all conventional activation procedures causes the rapid hydrolysis of CNBr to the inert cyanate ion (OCN^-).‡ In this way over 90% of the initially present amount of CNBr is deactivated without ever reacting with the resin. The reaction of the remaining CNBr results in the formation of cyanate esters on the resin (Fig. 1). These cyanate esters are also susceptible to hydrolysis by base, and most cyanate esters are inevitably hydrolyzed to less active imidocarbonates or totally inert carbamates in the course of the activation reaction (Figs. 1 and 2). Hence for all conventional procedures the *overall activation yield* (moles of ligand coupled /moles of CNBr employed for activation) is only about 0.5–2%.

Because of the low efficiency of the conventional procedures, large amounts of CNBr have to be employed, constituting a severe health hazard. Conventional procedures have two additional disadvantages: the activated resin contains both cyanate esters and imidocarbonates in varying proportions (*19,20*). The simultaneous presence of two different active moieties is highly undesirable from a theoretical point of view, since coupling to such resins is essentially ill-defined. The presence of carbamates on activated resins represents another complication, since carbamates have been identified as possible sources of interference dur-

‡Not to be confused with the highly poisonous cyanide ion (CN^-).

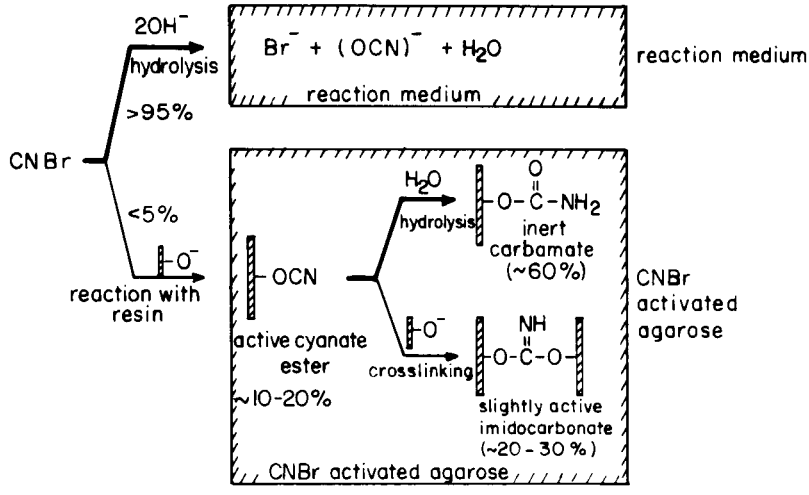

Fig. 2. Side reactions during CNBr-activation by conventional activation procedures.

ing affinity chromatography (*21*). In addition, carbamates block potentially reactive sites on the resin, and thereby reduce the amount of ligand that can be coupled to such CNBr-activated resins.

Ideally, the active moiety should be the only functional group introduced into the resin during activation. However, when agarose is activated by one of the conventional procedures, highly contaminated resins are obtained. As shown in Table 2, the active cyanate esters constitute only about 10–15% of all nitrogen derivatives that are found on activated agarose.

In spite of these disadvantages conventional activation procedures and in particular the procedures of Axen et al.(*2*) and March et al.(*15*) are widely used. These procedures are well established and have proven themselves in numerous applications (*7*). When performed in an accurate fashion, reproducible resin characteristics are obtained.

TABLE 2
Average Composition of Sepharose 4B Activated by a Conventional Activation Procedure[a]

	Amount of activation product	
Activation product	μmol/g dry resin	% of total nitrogen content
Imidocarbonates	400	20
Cyanate esters	250	12
Carbamates	1400	68

[a]Activation procedure: March et al. (*15*).

2.2. Practical Considerations

Workers in the field have repeatedly stressed that the activation procedure should be tailored to suit the intended application. "Trial and error" and personal preferences have often dictated the choice of a specific procedure. With the more precise understanding of the mechanism of activation that has recently emerged, a more rational approach to the problem of optimization of activation should be possible.

The conventional activation procedures can be subdivided into two classes, based on the method of maintaining the high pH of the reaction medium: The pH can be kept constant either by the continuous addition of base (*titration technique*) or by the use of a strongly buffered reaction medium (*buffer technique*). In general, procedures employing the buffer technique are characterized by short reaction times (1–3 min) compared with procedures employing the titration technique (5–12 min).The buffer technique is reliable and easy to perform, whereas the titration technique requires the availability of an automatic titrator or considerable experimental skill in order to perform the titration manually in a satisfactory way. It seems that the titration technique yields resins with a slightly higher coupling capacity. Good results are obtained by adding CNBr as solid material, and several such procedures have been published (*13,14,22*). Since aqueous solutions of CNBr cannot be stored at all, and since concentrated solutions of CNBr in organic solvents can under certain circumstances decompose explosively, adding solid CNBr could be the method of choice in laboratories where activations are performed only very rarely.

2.2.1. Illustrative Procedure for the Use of the Titration Technique (12)

— Wash the resin (e.g., Sepharose 4B) with distilled water on a coarse, sintered glass funnel. Obtain a packed cake of resin by applying mild vacuum suction.
— Transfer the resin into the reaction vessel and add enough distilled water to obtain a thick but stirrable slurry (at this stage the volume of the reaction mixture should be approximately 1.2 times the settled bed volume).
— Cool this slurry to 10°C.
— For 10 g of suction-dried Sepharose 4B, dissolve 1 g of CNBr in 2 mL of *N*-methylpyrrolidone, DMF, or acetonitrile. Add the solution of CNBr with vigorous stirring to the reaction mixture (some CNBr will precipitate).
— Adjust the pH to 11.0 by adding a 2–4*N* aqueous solution of NaOH. Keep the pH constant by the controlled addition of more NaOH solution. Stir vigorously to permit rapid mixing and to facilitate the resolubilization of CNBr.
— After 5–15 min, depending on the exact reaction temperature, base consumption will noticably slow down, indicating

that the resin has obtained its highest level of activation. (The reaction should be interrupted at this stage rather than waiting for complete cessation of base consumption.)
— Transfer the activated resin rapidly to a coarse, sintered glass funnel and wash the resin with ice-cold, distilled water until all CNBr has been removed (approximately 500 mL).
— Use the activated resin immediately for coupling of ligand.

Comments: The reported coupling capacity (as tested for ϵ-aminocaproic acid) is 20 μmol/g drained Sepharose 4B. This is one of the highest values for the coupling capacity reported for any heretofore published conventional activation procedure.

2.2.2. Illustrative Procedure for the Use of the Buffer Technique (15)

— Wash the resin with distilled water on a coarse, sintered glass funnel and obtain a packed cake of resin by applying mild vacuum suction.
— Mix 10 g of suction dried resin with 10 mL of water and 20 mL of a 2*M* sodium carbonate (or potassium carbonate) solution.
— Cool this mixture to 0°C by means of an ice-bath.
— Prepare an approximately 10*M* solution of CNBr in acetonitrile, DMF, or *N*-methylpyrrolidone by dissolving 1 g of CNBr in 1 mL of organic solvent. (Acetonitrile, DMF, and *N*-methylpyrrolidone give identical results.)
— With vigorous stirring, add 1 mL of CNBr solution all at once to the reaction mixture.
— After exactly 2 min, transfer the reaction mixture onto a coarse, sintered glass funnel and wash the resin until all CNBr has been removed.
— Use the activated resin immediately for coupling.

Comments: The procedure is easy to perform and fairly reproducible. However, the resin obtained is not highly active. The reported coupling capacity for alanine is only 10 μmol/g drained Sepharose 4B. The concentrated stock solutions of CNBr in organic solvent have a tendency to explode, especially upon aging or when contaminated. The CNBr solutions should be stored at −20°C for not more than a few weeks. Solutions that acquire a yellow tinge should be immediately destroyed.

2.2.3. The Use of Solid CNBr

In both procedures described above, the appropriate amount of CNBr may be added as a solid. In this case the reaction times are longer because of the slow solubilization of CNBr. Since the rate of dissolution depends on the crystal size, the exact reaction times have to be deter-

mined by trial and error. It is helpful to add 10–20% (v/v) of organic solvent, such as acetonitrile, DMF, or *N*-methylpyrrolidone, to the reaction mixture. This increases the solubility of CNBr. Likewise, it is advantageous to wash the activated resin first with a 10–20% (v/v) mixture of organic solvent in water in order to efficiently remove traces of undissolved CNBr. The use of solid CNBr instead of CNBr stock solutions reportedly yields resins with a slightly improved coupling capacity (*23*).

Caution: CNBr is highly toxic. When using solid CNBr, place a balance into a well-ventilated hood and weigh out the required amount of CNBr in a closed weighing vessel.

3. THE CYANO-TRANSFER TECHNIQUE

3.1. Theoretical Considerations

It is well known that aliphatic hydroxyl groups are not nucleophilic enough to react directly with CNBr. Therefore, no reaction occurs when CNBr is added to a suspension of polysaccharide resin in distilled water. In all conventional activation procedures, the addition of strong base to the reaction medium serves to enhance the nucleophilicity of the resin, by transforming some of the resin's hydroxyl groups into the more nucleophilic alkoxide ions. Since the pK_a of polysaccharide hydroxyl groups is about 12, the reaction medium has to be at a pH of 11 or higher in order to ionize a substantial fraction of the resin's hydroxyl groups. However, as outlined in Section 2.1, in such a strongly basic reaction medium several base-catalyzed side reactions reduce the efficacy of the activation reaction to less than 2%.

An alternative approach involves the enhancement of the *electrophilicity of CNBr* by means of a suitable "cyano-transfer" agent. This approach eliminates the need for the presence of strong base in the reaction medium and therefore avoids the base-dependent side reactions mentioned above. Cyano-transfer procedures are based on the formation of a highly reactive, salt-like complex between CNBr and a tertiary amine [von Braun reaction (*29*)]. Although most tertiary amines will form active complexes with CNBr, the cheap and easily available triethylamine (TEA) seems to be most suitable. The corresponding cyano-transfer complex, *N*-cyanotriethylammonium bromide (Fig. 3), is formed *in situ* by adding TEA to a suspension containing both CNBr and the resin. Contrary to CNBr itself, the cyano-transfer complex is able to cyanylate the resin's hydroxyl groups (Fig. 4).

If the cyano-transfer activation reaction is performed in a mixture of acetone and water (6:4) at −15°C, the overall activation yield approaches 20%. This represents approximately a 15-fold increase compared to most conventional procedures. Consequently, only about 50 mg of CNBr are

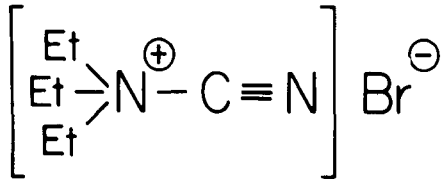

Fig. 3. *N*-cyanotriethylammonium bromide.

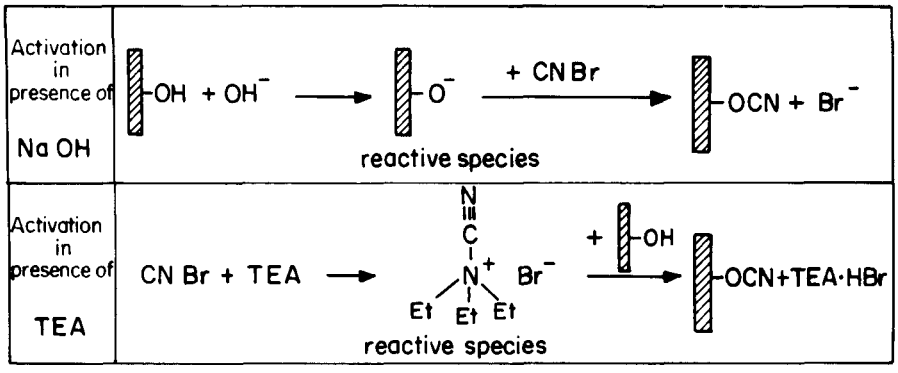

Fig. 4. Mechanism of activation in presence of NaOH compared to that in the presence of TEA (*16*).

required for the activation of 10 g suction-dried Sepharose 4B. This represents a significant reduction of the health hazard associated with CNBr activation. These conditions gave the highest activation yield (cyanate esters). The highly volatile nature of acetone makes it rather unpleasant to use as a solvent. For convenience and daily use it is possible to employ less volatile solvents such as DMF or acetonitrile, and carry out the reaction at 0°C even though these modifications slightly reduce the activation yield.

Unfortunately, it is not presently clear whether noncrosslinked agarose resins (such as Sepharose 4B) can withstand the recommended activation conditions (60% acetone, −15°C) without suffering some irreversible, conformational changes. It was observed on several occasions that cyano-transfer activated Sepharose 4B was "sticky" and had a tendency to form lumpy aggregates during coupling.

It is well known that the imidocarbonates, introduced into the resin during conventional activation, partly crosslink the resin and thereby enhance its chemical and physical stability. The lack of imidocarbonate formation during cyano-transfer activation could actually be disadvantageous for certain applications. Hence, it seems preferable to employ crosslinked agarose resins, such as CL-Sepharose 4B, when a cyano-transfer activation is performed. Good results were obtained when alkaline phosphatase was coupled to cyano-transfer-activated CL-Sepharose 4B (Dr. R. Feldberg, personal communication), while for bilirubin oxi-

dase, initial experiments suggest better results with conventionally activated resins (C. Sung, personal communication).

The convenience of the cyano-transfer technique suggests that it will be worthwhile to examine its application to the immobilization of yet more enzymes. Based on initial experimental experience the cyano-transfer activation technique seems to be the method of choice for the preparation of highly activated resin derivatives, particularly for the coupling of low-molecular weight ligands such as spacer arms, single amino acids, or small peptides. As shown in Table 3, the maximal coupling capacity of cyano-transfer activated Sepharose 4B is 75 μmol/g drained resin, as compared to a maximal coupling capacity of only 20 μmol/g drained resin obtained by conventional activation.

3.2. Practical Considerations

Conventional activation by CNBr has the advantage that a purely aqueous reaction medium can be employed, making it possible to activate noncrosslinked agarose resins without damage to the resin. On the other hand, several alternative activation techniques have been developed that require an absolutely anhydrous reaction medium and can therefore only be used for the activation of crosslinked agarose resins [e.g., carbonyldiimidazole activation (*24,25*), *p*-nitrophenylchloroformate activation (*26*), or sulfonyl chloride activation (*27*)]. The cyano-transfer technique seems to represent an intermediate situation: it employs as reaction medium 60% acetone in water, making it possible to activate

TABLE 3
Comparison of Cyano-Transfer Activation and Conventional Activation

	Cyano-transfer activation[a]	Conventional CNBr activation[b]
pH of reaction medium	pH 8	pH 11
Overall activation yield	15–20%	1–2%
Amount of CNBr employed for 10 g Sepharose 4B	50–100 mg	1–2 g
Resin composition:		
Cyanate ester	99%	15%
Imidocarbonate	0	25%
Carbamate	1%	60%
Maximal coupling capacity	>75 μmol	20 μmol
CNBr stock solutions	1*M*	10*M*
Washings	Free of CNBr	Contain CNBr
Health hazard	Reduced	Very significant

[a]The procedure described in Section 3.2.1. was used (*16*).
[b]The procedure described in Section 2.2.1. was used (*12*).

noncrosslinked agarose resins, although the possibility of a slight degree of damage to the noncrosslinked resin can presently not be excluded.

The inclusion of acetone in the cyano-transfer reaction medium serves a dual purpose. First of all, the presence of an organic solvent prevents the freezing of the reaction mixture when the reaction temperature is lowered to its optimum value at −15°C. Secondly, some organic solvent is required to stabilize the highly water-sensitive cyano-transfer complex. In a purely aqueous medium, *N*-cyanotriethylammonium bromide is hydrolyzed too rapidly to be an effective activating agent. Attempts to use the cyano-transfer technique in a purely aqueous medium resulted in irreproducible and generally low activation yields. The presence of at least 50% (v/v) of organic solvent (acetone, DMF, and so on) in the reaction medium is therefore absolutely necessary.

On the other hand, the reaction temperature is not as critical a parameter. Optimal results clearly require a reaction temperature below −10°C. Only at this temperature are activated resins obtained, which are virtually free of imidocarbonates and carbamates. However, resins activated at 0°C will be only slightly less active, and even at room temperature activated resins are obtained that are greatly superior over conventionally activated resins (*16*).

3.2.1. Illustrative Procedure for the Use of the Cyano-Transfer Activation Technique (16)

— Dissolve 10 g of CNBr in 100 mL acetone. Likewise, dissolve 15.2 g of *analytical grade* TEA in 100 mL of acetone.
— Place the resin in a coarse, sintered glass funnel and wash it first with water, then with acetone:water (3:7), and finally with acetone:water (6:4).
— Drain the washed resin *partly* by applying mild suction. (Caution: Do not obtain a packed cake of resin. Because of the volatility of acetone, the resin tends to dry up rapidly. This would cause irreversible damage to beaded agarose resins).
— Transfer 10 g of drained resin into a suitable reaction vessel and add 10 mL of acetone:water (6:4). A dense, but easily stirrable slurry should be obtained.
— Cool the reaction mixture to −15°C. (A suitable cooling mixture can be prepared by mixing 500 g of ice cubes with 100 g of sodium chloride).
— Use Fig. 5 to determine the volume of CNBr solution, required for activation.
— Add the required volume of CNBr solution. While stirring vigorously, add an *identical volume* of the TEA solution dropwise over a period of 1–2 min.
— Transfer the resin rapidly onto a coarse, sintered glass funnel and wash extensively with cold water. During the washing process, avoid complete draining of the resin, since cyano-

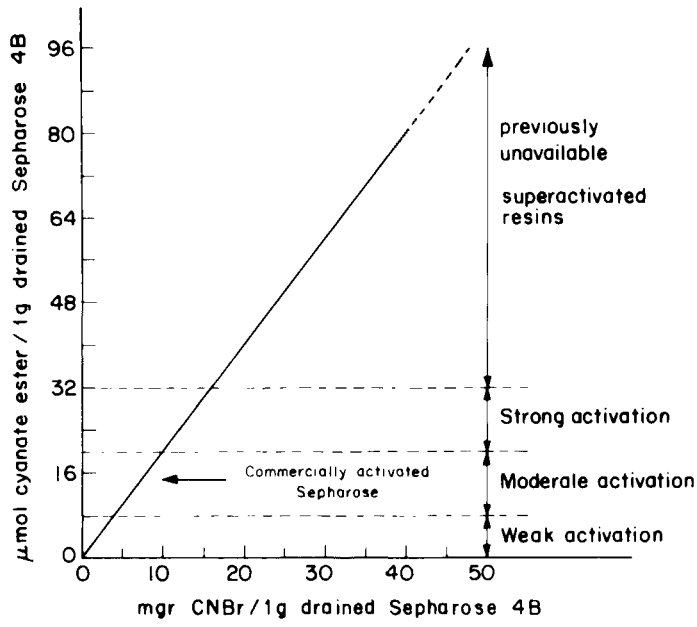

Fig. 5. Degree of activation as function of the amount of CNBr employed for the activation of 1 g drained Sepharose 4B by the cyano-transfer procedure (*16*).

transfer activated resins have a tendency to form lumps. (Caution: since acetone absorbs at 280 nm, incomplete removal of all traces of acetone will interfere with the spectrophotometric determination of protein in the coupling medium).
— Use the washed resin immediately for coupling (see Section 5.1 for storage of activated agarose resins).

Comments: Very highly activated resins require special care during activation and washing in order to prevent the formation of lumps. It is advantageous to transfer the resin after activation first into a large volume of ice cold water (500 mL for 10 g resin). This diluted suspension can then be passed through a sintered glass funnel with less danger of formation of lumpy aggregates.

Although acetone was used as solvent in the original cyano-transfer procedure, the volatile nature of acetone, and in particular its strong absorption at 280 nm, make acetone a difficult solvent to work with.

These problems can be avoided by employing DMF instead of acetone throughout the entire activation procedure. Other organic solvents such as *N*-methylpyrrolidone may be used as well. However, acetonitrile and dioxane are unsuitable, since 6: mixtures of these solvents with water will freeze at −10°C.

4. NOVEL CYANYLATING AGENTS

4.1. *N*-Cyanotriethylammonium Tetrafluoroborate (CTEA)

The first reaction step in cyano-transfer activations between CNBr and TEA results in the formation of *N*-cyanotriethylammomium bromide (Fig. 4). Unfortunately, this compound is unstable, decaying at temperatures above −10°C (*28*). In accordance with the mechanism of the von Braun reaction (*29*), the nucleophilic attack of the Br^- ion can be identified as the main reason for the inherent instability of *N*-cyanotriethylammonium bromide. Therefore, it is not surprising that stable compounds were obtained, when the Br^- ion was replaced by nonnucleophilic anions, such as tetrafluoroborate (BF_4^-), or perchlorate (ClO_4^-)(*28,30*). From a practical point of view the use of tetrafluoroborate as counterion is advantageous, since perchlorate-containing compounds have a tendency to be explosive. *N*-cyanotriethylammonium tetrafluoroborate (CTEA) is a white, crystalline solid. Under dry nitrogen, it can be stored at room temperature for several months; however, when left uncovered, it will rapidly absorb moisture and decay within a few hours.

Since CTEA is nonvolatile and not severely toxic, no special precautions are necessary when handling this compound. Activations with CTEA can therefore be performed safely without a hood. Kinetic studies revealed that CTEA is equivalent to an equimolar mixture of CNBr and TEA (*31*). This fact confirms the proposed mechanism of cyano-transfer activation. In addition, it explains the striking similarity between the CTEA-activation procedure and the cyano-transfer activation procedure described in Section 3.2.1. Detailed analysis of CTEA-activated resins showed that these resins are identical in all aspects to cyano-transfer-activated resins.

4.1.1. Illustrative Procedure for the Use of CTEA as Activating Agent (17)

— Prepare a 0.2*M* aqueous solution of TEA.
— Determine the amounts of CTEA and TEA required by use of Table 4.
— Weigh out the required amount of CTEA in a closed weighing vessel, avoiding exposure of the compound to moisture.
— Place the resin in a coarse, sintered glass funnel and wash first with water, then acetone:water (3:7), and finally with acetone:water (6:4).
— Drain the resin shortly by mild suction. (Caution: Do not drain the resin to dryness, since this will damage the structure of agarose resins.)
— Transfer 10 g of drained resin into a 50 mL glass beaker and add 10 mL of acetone:water (6:4). A dense, but easily stirrable slurry should be obtained.

TABLE 4
Amounts of CTEA and TEA Employed for the Activation of 10 g Drained Sepharose 4B

Degree of activation	Approximate coupling capacity, μmol ligand/g resin	CTEA, mg	TEA solution,[a] mL
Weak	5	60	0.6
Moderate	15	180	1.8
Strong	30	360	3.6

[a]0.2*M* aqueous solution

— Cool the reaction mixture to 0°C or lower (*see* Section 3.2).
— While stirring vigorously, add the required amount of CTEA. Immediately thereafter start adding the corresponding amount of TEA solution dropwise over a period of 1 min (for 0°C reaction temperature) or 3 min (for −15°C reaction temperature).
— Transfer the entire reaction mixture rapidly into 200 mL ice-cold water, filter this diluted suspension through a coarse, sintered glass funnel, followed by several additional washings with ice-cold water.
— Use the activated resin immediately for coupling (see Section 5.1 for storage of activated resins).

Comments: CTEA is commercially available from Sigma, Chemical Company, PO Box 14508, St. Louis, MO 63178, USA. It can also be prepared in a fairly laborious two-step reaction according to the published procedure (*30*).

4.2. 1-Cyano-4-(Dimethylamino)-Pyridinium Tetrafluoroborate (CDAP)

CDAP is an aromatic *N*-cyano complex with chemical properties similar to CTEA. However, CDAP (Fig. 6) is far more stable than CTEA. This enhanced stability is caused by the resonative dislocation of the positive charge over the entire aromatic ring.

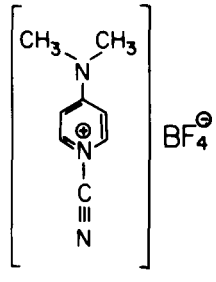

Fig. 6. Chemical structure of CDAP. Because of resonance, the positive charge is delocalized over the entire aromatic ring.

CDAP is a very convenient reagent. It is a nonvolatile solid that can be stored at room temperature for extended periods. Since CDAP is only very slightly hygroscopic, it can be weighed in an open vessel. Stock solutions of CDAP in dry acetonitrile (or similar organic solvents) can be prepared and remain stable for several weeks when stored at −20°C. In aqueous solution the stability of CDAP is strongly pH-dependent. At pH 1 (0.1*N* HCl) complete hydrolysis requires several weeks, at pH 7 (0.1*M* phosphate) complete hydrolysis requires several hours, and at pH 14 (0.1*N* NaOH) CDAP is hydrolyzed instantaneously (*31*).

CDAP has a strong UV absorption at 301 nm (ϵ in 0.1*N* HCl = 28,600 L $mol^{-1}cm^{-1}$). This characteristic peak makes it possible to monitor the concentration of CDAP in a very convenient fashion, making CDAP an ideal cyanylating reagent for kinetic studies (Fig. 7).

CDAP is a more selective cyanylating agent than CTEA or CNBr. CDAP does not react with methionine, a fact that has been exploited for the selective cyanylation of cysteine residues in intact proteins (*32*). The

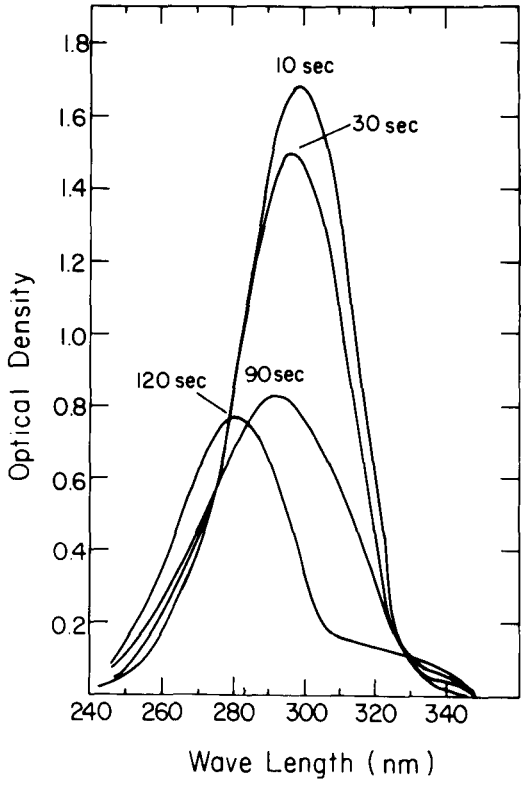

Fig. 7. Spectral changes during activation of Sepharose 4B by CDAP. At the indicated times aliquots of the reaction medium were withdrawn, diluted 10-fold with 0.1*N* HCl and analyzed on a Cary spectrophotometer. The disappearance of the characteristic peak at 301 nm (CDAP) with the concomitant formation of a peak at 280 nm (4-dimethylaminopyridinium hydrochloride) indicate the progress of the activation reaction.

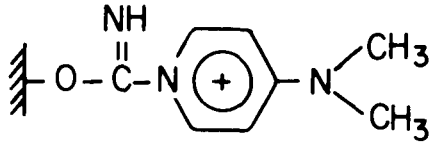

Fig. 8. The pyridinium-isourea structure proposed for the pyridine derivatives incorporated into Sepharose 4B during activation by CDAP (*31*).

stability and selectivity of CDAP give rise to extraordinarily high activation yields. Depending on the exact procedure used, activation yields between 20 and 80% have been obtained. This represents a nearly 100-fold increase over the yields obtainable with conventional CNBr activation procedures. This feature makes CDAP the most effective activating agent known so far.

During the activation by CDAP, pyridine derivatives are incorporated into the resin (*17*), for which the *pyridinium-isourea* structure shown in Fig. 8 has been proposed (*31*). Most of the resin-bound pyridine derivatives can be removed from the resin by hydrolysis in dilute HCl. However only during the coupling step the last traces of the pyridinium-isourea derivatives are decomposed, resulting in the release of 4-dimethylaminopyridine into the coupling medium. Fortunately, 4-dimethylaminopyridine is a rather inert compound that will not react with proteins. Hence its release into the coupling medium is not expected to have any adverse effect on the ligand. However, since 4-dimethylaminopyridine absorbs very strongly at 280 nm, its presence in the coupling medium will interfere with the spectrophotometric determination of free ligand.

4.2.1. Illustrative Procedure for the Use of CDAP as Activating Agent (17)

— Prepare a 0.2*M* aqueous solution of TEA and a stock solution of CDAP in dry, analytical grade acetonitrile (0.1 g CDAP/mL).
— Determine the amounts of CDAP and TEA required by use of Table 5.

TABLE 5
Amounts of CDAP and TEA Employed for the Activation of 10 g Drained Sepharose 4B

Degree of activation	Approximate coupling capacity, μmol ligand/g resin	CDAP, solution[a] mL	TEA solution,[b] mL
Weak	5	0.25	0.2
Moderate	15	0.75	0.6
Strong	30	1.50	1.2

[a]0.1 g CDAP/mL in dry acetonitrile.
[b]0.2*M* aqueous solution of TEA.

— Wash and drain the resin as described for CTEA activation (Section 4.1.1.).
— Transfer 10 g of drained resin into a 50 mL glass beaker and add 10 mL of acetone:water (6:4). A dense, but easily stirrable slurry should be obtained.
— Cool the reaction mixture to 0°C.
— While stirring vigorously, add the required volume of CDAP stock solution, wait 30 s, then add the corresponding volume of TEA solution dropwise over a period of 1–2 min.
— Transfer the entire reaction mixture rapidly into 200 mL ice-cold 0.05*N* HCl and allow the resin to settle for 15 min. Then filter the suspension through a coarse, sintered glass funnel, followed by extensive washings of the activated resin with ice-cold water.
— Use the resin immediately for coupling (see Section 5.1 for storage of activated resins).

Comments: CDAP is commercially available from Sigma, Chemical Company, POB 14508, St. Louis, MO 63178, USA. In addition, it can be prepared in a fairly facile two step reaction (*32*). Because of the higher stability of CDAP, the activation reaction proceeds only sluggishly when the temperature is lowered below 0°C. This temperature seems optimal, although the activation can also be performed at room temperature. By exposing the activated resin to dilute acid, a large fraction of the pyridinium-isourea derivatives are hydrolyzed and thus removed from the resin. Since cyanate esters are stable toward dilute mineral acid, the coupling capacity of the resin is not reduced by the recommended acid treatment step.

4.3. p-Nitrophenylcyanate (pNPC)

pNPC is a commercially available, nonhygroscopic, nonhazardous substance that can be stored at 0°C for several months. Its use as activating agent for agarose resins was first investigated by Kagedal and Akerström (*18*), who simply replaced CNBr by pNPC without significantly changing the activation procedure. Under these conditions neither pNPC nor any of the other organic cyanates screened resulted in improved resin activation when compared to conventional CNBr activation. In addition, Kagedal and Akerström erroneously proposed the formation of aliphatic-aromatic imidocarbonates (Fig. 9-II) as active moiety on pNPC activated resins.

Recent studies showed that pNPC acts as *cyanylating agent* when reacted with agarose. Hence, cyanate esters are the major functional groups formed on the resin, while aliphatic-aromatic imidocarbonates constitute only a minor side product. Because of the relative insolubility

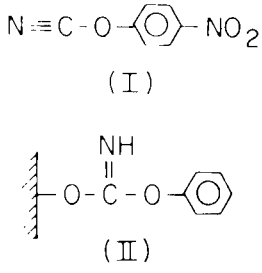

Fig. 9. Structure of the activation agent pNPC (I) and structure of the aliphatic-aromatic imidocarbonates (II) that were erroneously proposed as the main product on pNPC-activated agarose.

of pNPC in water, the use of a partly organic reaction medium is advantageous. Since pNPC is rapidly hydrolyzed by strong, inorganic bases, the activation reaction is best catalyzed by a weak, organic base such as TEA. In order to minimize the possible interference by *p*-nitrophenol derivatives during the coupling step, the activated resin can be exposed briefly to ice-cold, diluted hydrochloric acid. In this way most of the resin-bound aliphatic-aromatic imidocarbonates are removed prior to coupling. These considerations were incorporated into the pNPC activation procedure described below.

4.3.1. Illustrative Procedure for the Use of pNPC as Activating Agent (33)

— Prepare a 1.4*M* solution of pNPC by dissolving 2.3 g pNPC in 10 mL dry acetonitrile.
— Wash and drain the resin as described for CTEA-activation (Section 4.1.1).
— Transfer 10 g of drained resin into a 50 mL glass beaker and add 10 mL acetone:water (6:4). A dense but easily stirrable slurry should be obtained.
— Cool the reaction mixture to 0°C.
— Use Table 6 to determine the amounts of pNPC and TEA required.

TABLE 6
Amounts of pNPC and TEA Employed for the Activation of 10 g Drained Sepharose 4B

Degree of activation	Approximate coupling capacity, μmol ligand/g resin	pNPC solution,[a] mL	TEA,[b] mL
Weak	5	0.20	0.20
Moderate	15	0.70	0.70
Strong	30	2.00	2.00

[a]1.4*M* solution of pNPC in dry acetonitrile. The amount of activating agent is not linearly related to the coupling capacity of the resin.
[b]Analytical grade TEA.

— Add the required amount of pNPC solution to the reaction mixture. While stirring vigorously, add an equal volume of analytical grade TEA all at once.
— Continue stirring for 10 min. Then transfer the entire reaction mixture into 300 mL of ice-cold acid treatment medium (acetone:0.5*N* HCl = 1:1).
— Keep the activated resin in the acid treatment medium at 0°C for 20 min. Then transfer the resin onto a coarse, sintered glass funnel and wash extensively with ice-cold water.
— Use the activated resin immediately for coupling (*see* Section 5.1 for storage of activated agarose).

Comments: pNPC is commercially available from Sigma, Chemical Company, POB 14508, St. Louis, MO 63178, USA.
Stock solutions of pNPC in dry acetonitrile can be stored at −20°C for several weeks. The presence of pNPC can very easily be detected by the pyridine-dimethylbarbituric acid reagent described in Section 5.2. This reagent can also be used for the quantitative determination of pNPC (*31*).
Even after extensive treatment with acid, traces of *p*-nitrophenol derivatives remain on the resin. These derivatives decompose during coupling, releasing small quantities (<1 μmol/g resin) of the intensely yellow *p*-nitrophenolate ion into the coupling medium. Therefore, the coupling medium acquires a slight yellow tinge. After coupling, the resin is perfectly white and free of further phenol derivatives.

4.4. Comparison of CTEA, CDAP, and pNPC as Activating Agents for Agarose

CTEA, CDAP, and pNPC are excellent substitutes for the hazardous and inconvenient CNBr. All these reagents make it possible to obtain highly activated resins by simple and convenient procedures. The properties of CTEA, CDAP, and pNPC are summarized in Table 7.

5. AUXILLARY PROCEDURES FOR WORKING WITH CYAN-ACTIVATED RESINS

5.1. Short-Term Storage of Activated Resins

Cyanate esters are the active group responsible for coupling of ligand on all cyan-activated agarose resins. Since cyanate esters are fairly stable in dilute acid, activated agarose resins can be stored for several hours in 0.05–0.1*N* HCl at 0°C, without diminuation of the coupling capacity.

Procedure: Immediately upon completion of the activation reaction, transfer the slurry of activated resin into ice-cold 0.05*N* HCl

TABLE 7
Comparison of the Properties of CTEA, CDAP, and pNPC as Activating Agents for Agarose

	p-Nitrophenyl cyanate	*N*-cyanotriethyl ammonium	1-Cyano-4-dimethylamino p.
Activation yield	10%	10–20%	60–80%
Reaction temperature	0°C	−15 → 0°C	0 → 20°C
Resin impurities	Carbamates, phenol	Carbamates	Carbamates, pyridine derivatives
Stability	Stable for months, nonhygroscopic	Stable for months, hygroscopic	Stable for years, nonhygroscopic
UV absorption	—	—	strong at λ = 301
Assessment	Cheapest	Ideal substitute for CNBr, purest resins	Ideal for kinetic studies, highest yield

(20 mL of 0.05*N* HCl for each gram of activated resin). Filter this suspension through a coarse, sintered glass funnel, wash the resin several times with ice-cold 0.05*N* HCl, then resuspend the washed, activated resin in 0.05*N* HCl (5 mL/g of resin). Keep the temperature at 0°C.

For coupling, transfer the resin into a coarse, sintered glass funnel and wash the resin briefly with ice-cold water followed by ice-cold *coupling buffer*.

5.2. Qualitative Test for Active Groups

Since all cyan-activated resins contain at least some cyanate esters, testing for cyanate esters can be used as a qualitative test for the resin's state of activation. The same test procedure can also be used for the qualitative detection of the activating agents CNBr, CTEA, CDAP, or pNPC.

Preparation of the pyridine-dimethylbarbituric acid reagent: Dissolve 0.75 g *N,N'*-dimethylbarbituric acid (available from Chemical Dynamics Corporation, PO Box 395, 3001 Hadley Road, South Plainfield, New Jersey 07080, USA) in 45 mL analytical grade pyridine and make up the volume to 50 mL with distilled water. (The reagent turns dark brown at room temperature. It should be stored at −20°C.)

Test procedure: Add 1–2 mL of reagent to about 100 mg drained resin (or to a drop of sample solution containing one of the activating agents) and observe the formation of an intense, purple color.

5.3. Determination of the Coupling Capacity

On cyano-transfer activated resins, and on resins activated by CTEA, CDAP, or pNPC cyanate esters are exclusively responsible for

coupling of ligand. For these resins, the quantitative determination of cyanate esters can be used to accurately predict the coupling capacity of the activated resin.

Test procedure: Add 3 mL of pyridine–dimethylbarbituric acid reagent to approximately 100 mg drained resin. Shake or stir vigorously in a stoppered test tube for 15 min, then dilute the intense purple color to any convenient volume with distilled water. Allow the resin to settle and measure the absorption of the purple solution at 588 nm. Use a molar absorption coefficient of 137,000 L $mol^{-1}cm^{-1}$ to calculate the amount of cyanate esters according to Beer-Lambert's Law. (For moderately active resins dilution to a final volume of 500 mL is usually necessary in order to reduce the absorption to below 1.5).

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