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p-Nitrophenylcyanate—An Efficient, Convenient, and Nonhazardous Substitute for Cyanogen Bromide as an Activating Agent for Sepharose

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Abstract

The reaction of aromatic cyanates with agarose-based resins was investigated. Phenylcyanate yielded aliphatic–aromatic imidocarbonates as the major product, whereas *p*-nitrophenylcyanate acted as a cyanylating agent, yielding mainly cyanate esters on the resin. Such cyanate esters were recently also shown to be the active group on cyanogen bromide-activated Sepharose; hence, the stable and nonvolatile *p*-nitrophenylcyanate was found to be a very convenient substitute for the highly hazardous cyanogen bromide. Activations with *p*-nitrophenylcyanate could be done safely outside a hood. Employing triethylamine instead of the commonly used inorganic bases, an optimized activation procedure was developed that is about 10 times more efficient than conventional cyanogen bromide activation. Since both cyanogen bromide and *p*-nitrophenylcyanate-activated resins contain cyanate esters as active groups, the coupling of ligands proceeded in an identical fashion in both cases.

Index Entries: *p*-Nitrophenylcyanate, as activating agent for agarose; *p*-nitrophenylcyanate, mechanism of reaction with agarose; phenylcyanate, mechanism of reaction with agarose; agarose, activation of; Sepharose, activation by cyanogen bromide, phenylcyanate, and *p*-nitrophenylcyanate; activation, of agarose by cyanogen bromide and aromatic cyanates; cyanogen bromide, *p*-nitrophenylcyanate as a substitute activating agent for.

Introduction

For the preparation of affinity chromatography columns or the immobilization of biologically active proteins, activation of polysaccharide resins by cyanogen bro-

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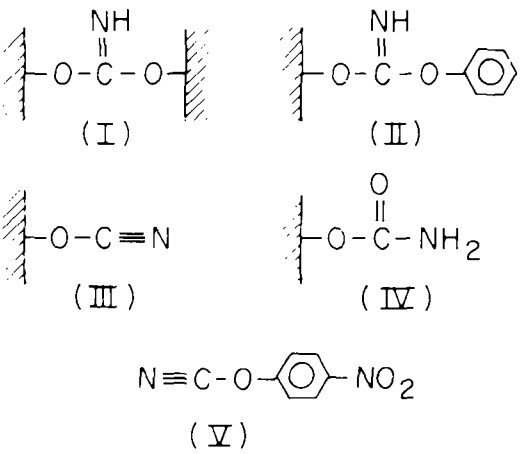

Fig. 1. Molecular structures of aliphatic imidocarbonates (I), aliphatic–aromatic imidocarbonates (II), cyanate esters (III), carbamates (IV), and the activating agent pNPC (V). The vertical lines indicate the resin backbone.

mide (CNBr)‡ has been widely employed (*1*). Compared with several alternative activation procedures, the use of CNBr is convenient, except that it is a highly hazardous reagent that cannot be handled without special precautions.

The possibility of using aromatic cyanates (Ar-OCN) as alternative activation agents has already been investigated (*2*). Although in these studies several aromatic cyanates were screened, none was found to activate polysaccharide resins to a higher extent than CNBr itself. Based on the presence of phenyl derivatives on phenylcyanate-activated Sephadex G-25 (the only case investigated in some detail), coupling of ligand was proposed to proceed via aliphatic–aromatic imidocarbonates (Fig. 1, II). However, studies on the chemistry of aromatic cyanates (*3*, *4*) indicate that these compounds may act as true cyanylating agents. Hence, the reaction of an aromatic cyanate with agarose could conceivably result in the formation of *cyanate esters* (Fig. 1, III) on the resin, analogous to the reaction of CNBr with agarose (*5*, *6*). Consequently certain aromatic cyanates could possibly be excellent substitutes for CNBr.

These considerations prompted us to investigate the interaction of aromatic cyanates with agarose, employing phenylcyanate and *p*-nitrophenylcyanate (pNPC) as model compounds. The primary objectives of our studies were to establish the mechanism by which aromatic cyanates react with agarose, and to examine the possibility of using a stable and nonhazardous aromatic cyanate as a substitute for CNBr.

Materials and Methods

Reagents were of analytical grade, except CNBr, which was obtained in technical grade from Schuchardt & Co., W. Germany. p-*Nitrophenylcyanate* was obtained

‡Abbreviations used are: CNBr, cyanogen bromide; DMF, dimethylformamide; pNPC, *p*-nitrophenylcyanate; TEA, triethylamine.

from Makor Chemicals Ltd, 91064 Jerusalem, POB 6570, Israel and was used without further purification. *Phenylcyanate* was synthesized as described by Grigat and Pütter (*7*).

Storage of pNPC

Stored air-tight and dry at −18°C, pNPC is stable. Slow trimerization was observed at 25°C in the course of several months. Solutions of pNPC in acetone could be stored for several weeks at −18°C. At room temperature, such solutions deteriorated within a few days.

Analysis of Activated Resins

Cyanate esters, imidocarbonates, carbamates, total nitrogen content, and the coupling capacity of activated resins were determined as described previously (*5*).

For the determination of phenol derivatives on activated resins, weighed samples of resin were hydrolyzed in 0.1*N* NaOH at 40°C for 30 min. All phenol derivatives on the resin are thereby quantitatively liberated as phenolate ion (a_{max} = 287 nm, ϵ = 2600 L mol^{-1} cm^{-1}) or *p*-nitrophenolate ion (a_{max} = 400 nm, ϵ = 18000 L mol^{-1} cm^{-1}), both of which were determined spectrophotometrically in the hydrolysis medium.

Quantification of Sepharose Resins

All interstitial water was removed from samples of resin by suction. The weight of such drained resin preparations was recorded as "wet weight." After drying *in vacuo* over P_2O_5, 1 g "wet" Sepharose 4B was consistently found to contain 50 ± 5 mg of agarose.

Activation in Presence of Strong, Inorganic Base

Solutions of activating agent were prepared by dissolving either CNBr (1 g), or pNPC (0.5 g), or phenylcyanate (0.5 mL) in 10 mL of dimethylformamide (DMF). Wet Sepharose 4B (10 g), resuspended in 1*M* K_2CO_3 (15 mL), was cooled to 0°C. To this suspension the desired, cooled solution of activating agent (10 mL) was added. After 3 min of vigorous stirring, the resin was extensively washed with cold DMF: H_2O (1:1), followed by cold water. Aliquots of resin were immediately weighed and analyzed.

Optimized Prodcedure for Activation by pNPC in the Presence of TEA

Wet Sepharose 4B (10 g), washed successively with acetone : H_2O (20% v/v), acetone : H_2O (50% v/v), and finally acetone : H_2O (70% v/v), was resuspended in 10 mL acetone : H_2O (70% v/v) and cooled to 0°C. To this solution the desired volume of pNPC reagent (2.3 g pNPC dissolved in 10 mL acetone to give 1400 μmol pNPC/mL) was added with vigorous stirring, followed by an *equal* volume of neat TEA (in this way a fivefold molar excess of base over activating agent was obtained). After 10 min the entire reaction mixture was poured into 200 mL of ice-cold acid-treatment medium (acetone : 0.5*M* HCl, 1 : 1), and allowed to stand for

20 min at 0°C. Thereafter the resin was washed with ice-cold acetone : H_2O (1 : 1), followed by ice-cold H_2O, and used for coupling as described for CNBr-activated resins (*1*).

Storage of Activated Resins

Activated resins could be stored in ice-cold acid-treatment medium (acetone : 0.5*M* HCl, 1 : 1) for up to 1 h without significant reduction of the coupling capacity. For prolonged storage, activated resins were extensively washed with storage medium (acetone : dioxane : H_2O, 60 : 35 : 5). When resuspended in storage medium and kept at −18°C, the coupling capacity decreased at an approximate rate of less than 10% per month. The reswelling capacity of Sepharose 4B resins remained unchanged.

Results

The reaction of aromatic cyanates with agarose was investigated under conditions identical to those used for CNBr activation (*8*). Several activations were performed, employing either phenylcyanate, pNPC, or CNBr as activating agent. The composition of the resulting activated resins was determined. The data recorded in Table 1 revealed that the interaction of phenylcyanate with agarose resulted predominantly in the formation of aliphatic–aromatic imidocarbonates (Fig. 1, II). In contrast, when pNPC was used as the activating agent, cyanate esters (Fig. 1, III) were formed. Only traces of aliphatic–aromatic imidocarbonates were present on pNPC activated resin. Thus the composition of pNPC activated resins was found to correspond closely to the composition of CNBr-activated resins.

Since both the activating agents and the active cyanate esters formed on the resin are unstable in strong base, the presently employed activation conditions resulted in very low overall activation yields (for definitions, see Table 1). Consequently we attempted to improve the efficacy of the activation reaction by substituting the weaker base TEA for the commonly used strong inorganic bases, such as NaOH or K_2CO_3. An additional modification was the inclusion of acetone in the reaction medium in order to prevent precipitation of the water-insoluble pNPC.

With these modified reaction conditions, a marked improvement in the efficacy of the activation process was obtained, as indicated by the formation of highly activated resins at comparably low quantities of pNPC. Systematic optimization of the reaction conditions resulted in the procedure described in Materials and Methods.

The kinetics of the activation of Sepharose 4B by pNPC in the presence of TEA is shown in Fig. 2. A close correspondence between the disappearance of pNPC from the medium and the concomitant formation of cyanate esters on the resin was observed. Cyanate ester incorporation reached a maximal level about 7 min after initiation of the reaction and remained constant for up to 15 min. This feature obviates the need for exact timing of the activation reaction.

The composition of resins activated by pNPC in the presence of TEA is recorded in Table 1. The most striking feature of the TEA-mediated activation by pNPC is

Table 1
Comparison of Activated Sepharose 4B Obtained by Different Activation Procedures

	Resin characteristics after activation in presence of K_2CO_3 employing			Resin characteristics after activation[a] in the presence of TEA employing pNPC	
	Phenyl cyanate	CNBr	pNPC	Prior to acid treatment	After acid treatment
Overall activation yield[b]	4%	1.5%	5%	17%	15%
Resin purity factor[c]	—	0.11	0.21	0.49	0.52
Coupling capacity[d]	17	15	15	50	45
Aliphatic imidocarbonates[e]	Traces	25	10	6	<1
Aliphatic-aromatic imidocarbonates[e]	22	0	2	7	<1
Cyanate esters[e]	Traces	12	15	53	50

[a]The optimized procedure described in Materials and Methods was followed, employing 300 μmol pNPC/g wet resin.
[b]Defined as μmol ligand coupled/μmol activating agent used.
[c]Defined as cyanate esters on resin/total nitrogen content of resin.
[d]Defined as μmol ligand coupled/g wet resin.
[e]Data recorded in μmol/g wet resin.

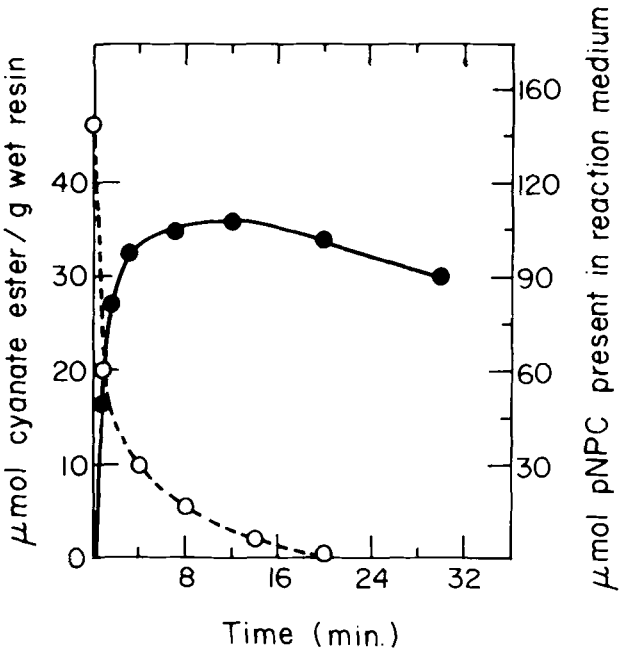

Fig. 2. Formation of cyanate esters on Sepharose 4B (—●—●—) and disappearance of pNPC from the reaction medium (–○– –○– –○–) as function of time. Wet Sepharose 4B (10 g) was activated with pNPC (1400 μmol) as described in Materials and Methods.

the over threefold increase in the "overall activation yield" compared to pNPC activation in the presence of strong, inorganic bases. When compared to conventional CNBr activation, TEA-mediated activation by pNPC is over 10 times more efficient. In order to assess the composition of activated resins in a quantitative fashion, the molar ratio of "cyanate esters on resin" to "total nitrogen content of resin" was defined as "resin purity factor." Conventional CNBr activation yields resins that are highly contaminated by inert carbamates (*6*). On such resins the active cyanate esters constitute only a small fraction of the total amount of nitrogen derivatives, and correspondingly CNBr-activated resins have a low resin purity factor of about 0.1. TEA-mediated activation by pNPC resulted in a fivefold increase of the resin purity factor, thereby diminishing possible interference by inactive nitrogen derivatives, which "contaminate" the activated resin (*9*).

Analysis of pNPC-activated resins revealed the presence of a small, but significant, amount of aliphatic–aromatic imidocarbonates on the resin (Table 1). Activated resins practically free of these phenol derivatives can readily be obtained by taking advantage of the extreme liability of imidocarbonates toward acid. By exposure of pNPC-activated resins to cold, diluted HCl, nearly all phenol-derivatives are removed from the resin, whereas the acid-stable cyanate esters remain unaffected (Table 1). In such acid-treated resins cyanate esters and carbamates constitute over 99% of the total nitrogen content. Since carbamates are inert, coupling of ligand to the resin occurs exclusively via cyanate esters. The coupling reaction is therefore well defined and unambiguous.

The composition of pNPC activated resins and their coupling capacity as a function of the amount of pNPC employed are shown in Fig. 3. Since the activation

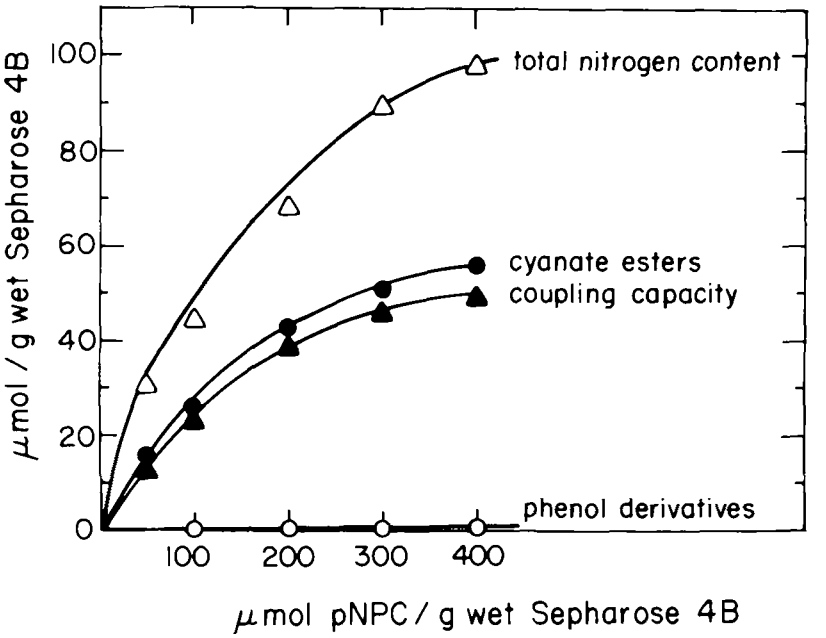

Fig. 3. Composition of pNPC activated Sepharose 4B after acid treatment as a function of the amount of pNPC employed for activation.

procedure, as well as the subsequent acid-treatment step, are reproducible, the coupling capacity of the activated resins can be readily controlled by the amount of pNPC employed for activation. Commercially available CNBr-activated Sepharose 4B (from Pharmacia, Lot FF16294) was found to contain about 15 μmol cyanate esters/g wet resin (*5*). As illustrated by Fig. 3, an activated resin with identical coupling capacity was obtained when 50–70 μmol pNPC (35–50 μL of pNPC reagent) were employed for the activation of 1 g wet Sepharose 4B.

Since pNPC- and CNBr-activated resins contain the same active cyanate esters, the reaction involved in coupling of ligand to the resin could be expected to be identical in both instances. This was confirmed by the observed similarity in the kinetics of coupling of *dl*-methionine (a monovalent ligand) to resins activated by either pNPC or CNBr. In both cases the coupling reaction was complete in 2–3 h at 23°C and in 7–8 h at 4°C. The amount of covalently linked methionine was always proportional to the amount of cyanate esters present on the resin prior to coupling. The observed coupling yield of pNPC-activated resins was found to be about 85%, identical to the coupling yield of CNBr-activated resins (*5*). During the coupling reaction of pNPC activated resins, the last traces (<1 μmol/g wet resin) of phenol derivatives present on the resin were hydrolyzed, liberating the intensely yellow *p*-nitrophenolate ion. Hence the coupling medium acquired a slightly yellowish tinge. After coupling, no further phenol derivatives could be detected on the resin.

Discussion

In an earlier study (*2*) on the use of aromatic cyanates as activating agents for polysaccharide resins, different aromatic cyanates were screened, but only the reaction of phenylcyanate with Sephadex G-25 was investigated in any detail. Therefore, it was not realized that aromatic cyanates may react with polysaccharides by at least two different pathways, yielding either cyanate esters (Fig. 1, III) or aliphatic–aromatic imidocarbonates (Fig. 1, II). The present investigation confirms that the reaction of phenylcyanate with polysaccharides does result in the formation of aliphatic–aromatic imidocarbonates. In contrast, the action of pNPC on polysaccharides leads to the formation of cyanate esters on the resin. Consequently pNPC-treated resins are virtually devoid of phenol derivatives. This dramatic difference in the behavior of phenylcyanate and pNPC can be explained by the "leaving group properties" of the respective phenol constituents: the initially formed aliphatic–aromatic imidocarbonate seems to be stable only if it is the derivative of a comparably weak leaving group such as phenolate. In the case of a strong leaving group, such as *p*-nitrophenolate, the aliphatic–aromatic imidocarbonate decomposes rapidly with concomitant transfer of the cyano-moiety to the resin (Fig. 4). In general, all aromatic cyanates bearing very strong electron-withdrawing substitutents would be expected to yield mainly cyanate esters, whereas phenylcyanate itself and aromatic cyanates, bearing electron-donating substituents, lead to the formation of aliphatic–aromatic imidocarbonates.

Activations with aromatic cyanates were previously performed under conditions closely resembling the usual CNBr procedure (*2*). Under those conditions all aro-

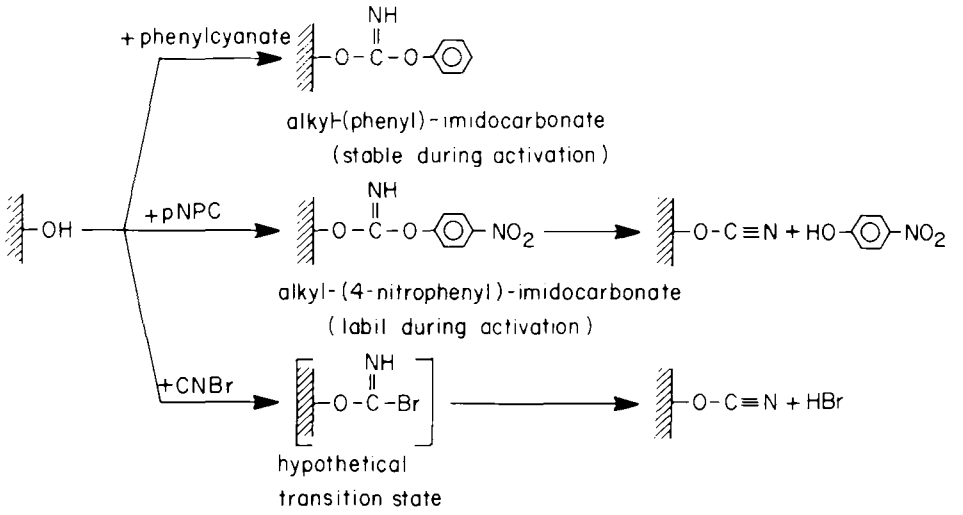

Fig. 4. Comparison of the reaction mechanism of phenylcyanate, pNPC, and CNBr. Phenylcyanate yields aliphatic–aromatic imidocarbonates as active species, whereas pNPC and CNBr yield cyanate esters as active species.

matic cyanates screened were found to be inferior to CNBr, as indicated by the coupling capacity of the obtained activated resins. According to the reported data, aromatic cyanates did not seem to offer any significant advantage over CNBr; it is therefore not surprising that they have not been employed routinely for the activation of polysaccharide resins.

Recently we reported that the use of TEA, instead of commonly employed strong bases such as NaOH or K_2CO_3, resulted in a dramatic improvement of the CNBr-activation reaction (*10*). The same effect was observed also when aromatic cyanates were employed as activating agents: In the case of pNPC (Table 1) the overall reaction yield increased by more than 10-fold, and the resin purity factor by neraly fivefold compared to conventional CNBr-activation. Therefore, activation by pNPC under the conditions described in Materials and Methods is vastly superior to conventional CNBr activation, and compares favorably with the improved TEA-mediated CNBr activation procedure reported by us earlier (*10*). The most significant advantage of using pNPC instead of CNBr as activating agent is the fact that pNPC is a stable, nontoxic (*11*) and nonvolatile solid that can be stored and handled without danger. The described pNPC activation procedure, which is a safe, reliable, and efficient means of activating agarose, may be especially valuable when the use of the volatile and highly poisonous CNBr has to be avoided.

Acknowledgments

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