Journal of Solid-Phase Biochemistry, Vol. 1, No. 4, 1976

CHEMICALLY MODIFIED NYLONS AS SUPPORTS FOR ENZYME IMMOBILIZATION

II. Isocyanide, Dibromoisocyanide, and Acylhydrazide Derivatives of Nylon

A. FREEMAN, M. SOKOLOVSKY, and L. GOLDSTEIN

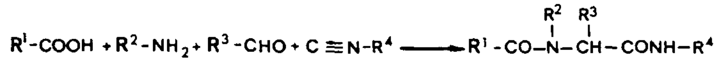
Department of Biochemistry
The George S. Wise Center for Life Sciences
Tel Aviv University
Tel Aviv, Israel

Accepted January 2, 1977

An isocyanide derivative of nylon, polyisonitrile–nylon (1,2), was used as a starting material whereby, through a series of modification reactions, different chemically reactive functional groups could be introduced on the polyamide backbone. The chemistry employed allowed for considerable flexibility in the choice of procedures for covalent fixation of proteins, all starting from the same chemically reactive parent polymer, polyisonitrile–nylon. Thus, polyisonitrile–nylon could be used directly for the immobilization of enzymes via four-component condensation reactions. The isocyanide functional groups of the parent polymer could be transformed, by treatment with bromine, into the strongly electrophilic dibromoisocyanide (—N=CBr_2) groups. The selectivity of the —N=CBr_2 group toward the various functional groups present in proteins could be regulated by appropriate control of the pH of the coupling reaction. Dibromoisocyanide–nylon was also further modified into other types of chemically reactive nylon derivatives.

INTRODUCTION

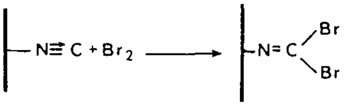
A method to introduce isocyanide (isonitrile) functional groups on the backbone of synthetic polyamides (e.g., nylon-6 and nylon-66) was described in previous communications from our laboratory (1,2). The isocyanide derivatives of nylon, polyisonitrile–nylon, could be utilized for the chemical fixation of proteins via four-component condensation reactions (4CC) involving amine, carboxyl, aldehyde, and isocyanide:



$$R^1\text{-COOH} + R^2\text{-NH}_2 + R^3\text{-CHO} + C{\equiv}N\text{-}R^4 \longrightarrow R^1\text{-CO-}\overset{\overset{R^2}{|}}{N}\text{-}\overset{\overset{R^3}{|}}{CH}\text{-CONH-}R^4 \quad (1)$$

In this reaction, carried out at neutral pH, the polymeric support supplies the isocyanide component; the protein supplies either the amine or the carboxyl components; a water-soluble aldehyde, e.g., acetaldehyde, and the missing fourth component are added to the aqueous medium.

Despite its many attractive points and considerable flexibility, this method can be used to advantage only when the biological activity of a protein is not impaired in the presence of aldehyde. Isocyanides can, however, be transformed into many other types of chemically reactive species (3,4) that could in principle be employed for the covalent fixation of proteins. In this communication we describe a method in which the isocyanide groups on a polyamide support are converted into the strongly electrophilic dibromoisocyanides:



$$-N{\equiv}C + Br_2 \longrightarrow -N{=}CBr_2 \quad (2)$$

The dibromoisocyanide–nylon derivatives could be used directly for the immobilization of proteins under relatively mild conditions. They could also serve as starting material for the preparation of other types of nylon derivatives.

MATERIALS AND METHODS

Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and papain (EC 3.4.22.2) were purchased from Worthington Biochemical Corporation (Freehold, New Jersey). Subtilisin BPN' (EC 3.4.21.14) was purchased from the Sigma Chemical Company, St. Luois, Missouri). PhCOGlyHis[1] (hippuryl-histidine) was from the Cyclo Chemical Corporation (Los Angeles, California). LeuNH$_2$, AcTyrOEt and PhCOArgOEt were from the Sigma Chemical Company. Nylon powders with a mean particle diameter of about 1 μM were prepared from commercial nylon-6 pellets as previously described(1,2); 1,6-diisocyanohexane was synthesized according to Goldstein et al. (1,2). All other reagents and buffer salts were of the purest grade available commercially.

Polyisonitrile–Nylon

The isocyanide derivative of nylon was prepared from partially hydrolyzed nylon powder essentially as described by Goldstein et al. (1,2). Nylon powder (2 g) was suspended in 3 N HCl (60 ml), stirred for 4 h at room temperature, and then washed with water, methanol, and ether and air-dried. The partially hydrolyzed nylon powder (1 g; 60–70 μmol

[1] Abbreviations: LeuNH$_2$, L-Leucine amide; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester; PhCOArgOEt, *N*-benzoyl-L-arginine ethyl ester; PhCoGlyHis, *N*-benzoylglycyl-L-histidine (hippuryl-histidine); —NCBr$_2$, dibromoisocyanido group; —CONH.NH$_2$, acylhydrazido group; —CON$_3$, acylazido groups; EDTA, ethylenediamine tetracetate.

COOH/g) was suspended in propan-2-ol (40 ml). Isobutyral (16 ml) was then added, followed by 1,6-diisocyanohexane (4 ml; 0.03 mol), and the reaction allowed to proceed with stirring, in a closed vessel at room temperature, for 24 h. The polyisonitrile–nylon powder was separated by filtration, washed with methanol and ether, and air-dried. The mean isocyanide content of the powder was 40–50 μmol/g (residual carboxyl content, 10–20 μmol/g; see references 1,2).

Dibromoisocyanide-Nylon Derivatives

Polyisonitrile–nylon powder (100 mg; 40–50 μmol —NC/g) was suspended in chloroform (10 ml) and stirred magnetically for 10 min over ice. A bromine solution (1 ml of a 1% solution in $CHCl_3$; 200 μmol Br_2) was added to the ice-cooled polymer suspension and stirred for 15 min. Triethylamine (0.2 ml) was then added with stirring (to remove unreacted bromine). After the color had disappeared (2–3 min) the polymeric powder was separated on a suction filter, washed with cold chloroform and then with ether, and air-dried. Due to its high reactivity, the dibromoisocyanide–nylon derivative (30–35 μmol —$NCBr_2$/g) was used immediately after preparation.

Hexanedioic Acid Dihydrazide (Adipic Dihydrazide)

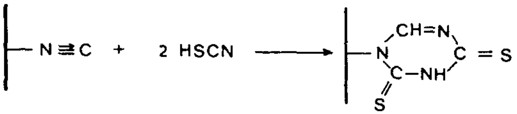
Adipic dihydrazide was prepared by the procedure of Lamed et al. (5) and recrystallized from a water–ethanol (10:65 vol/vol) mixture.

Acylhydrazide-Nylon Derivatives

Freshly prepared dibromoisocyanide–nylon powder (100 mg; 30–35 μmol —$NCBr_2$/g) was added slowly to 10 ml of a vigorously stirred, ice-cooled, 0.02 M solution of adipic dihydrazide, brought to pH 8, and the reaction allowed to proceed under the pH-stat for 1 h. The acylhydrazide–nylon powder was separated on a filter, washed with water, methanol, and ether, and air-dried. The polymer (25–30 μmol —CONH.NH_2/g) was stored in stoppered vials at room temperature.

Determination of Isocyanide Groups

The isocyanide content of polyisonitrile–nylon powders was determined according to the reaction



(3)

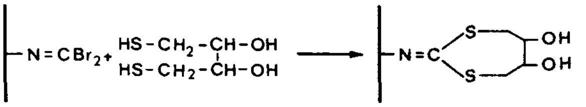
by an adaptation of the method of Arora et al. (6).

Method. Polyisonitrile–nylon powder (30 mg, 1.5–2 μmol —NC groups) was suspended in 1.4 ml ethyl acetate and cooled over ice. A fivefold molar excess of isothiocyanic acid in ethyl acetate (0.1 ml of a 0.1 N solution) was added and the cooled raction mixture stirred magnetically for 30 min. Dimethyl formamide (1.5 ml) and one drop of neutral red/methylene blue indicator were then added. The stirred sample was titrated at room temperature with a 0.05 N triethylamine in ethyl acetate. Blank titrations with all reagents except the nylon powder were carried out in parallel. The amount of isocyanide in the polyisonitrile–nylon sample was calculated from the difference between the two titrations assuming a stoichiometry of 2 : 1 for isothiocyanic acid to isocyanide.

Reagents. (a) 0.1 N isothiocyanic acid: an aqueous solution of NH_4SCN (900 mg in 2.5 ml) was mixed with 100 ml ethyl acetate. Ten percent sulfuric acid (5 ml) was added dropwise with stirring and cooling. The organic phase was separated and dried over $MgSO_4$ or Na_2SO_4. To remove traces of Fe(III), powdered potassium ferrocyanide (100 mg) was added with stirring and then filtered off. The normality was determined on an 0.1 ml aliquot by titration with 0.05 N triethylamine in ethyl acetate as described above. (b) Indicator: 0.2% solution of neutral red and methylene blue in methanol. Color change from purple to green. (c) 0.05 N triethylamine: 0.6 ml triethylamine (distilled over KOH) was dissolved in 100 ml ethyl acetate. The normality was determined by titrating 50 μl of 0.1 N aqueous HCl in 1.5 ml ethyl acetate and 1.5 ml dimethyl formamide as above.

Determination of Dibromoisocyanide Groups

Dihaloisocyanides undergo ring closure reactions with dithiols (7,8). Therefore —$NCBr_2$ groups were estimated by reacting dibromoisocyanide–nylon with excess dithioerythritol:



(4)

The unreacted sulfhydryl groups were determined spectrophotometrically by the Ellman procedure (9,10). Since the determination of —$NCBr_2$ was carried out in an aqueous medium, the values obtained represent the *effective* dibromoisocyanide content under the conditions employed for coupling nucleophiles.

Procedure. A freshly prepared solution of dithioerythritol (2 ml; 5×10^{-3} M in —SH; in 0.02 M *N*-ethylmorpholine buffer, pH 8) was added to the dibromoisocyanide–nylon test sample (50 mg; about 1.5 μmol —$NCBr_2$

groups) and the suspension stirred magnetically for 10 min at room temperature. Aliquots (0.2 ml) were transferred into 1.8 ml water and the polymer separated by filtration; 0.2 ml of filtrate was added to 2.8 ml of Ellman's reagent (3.3×10^{-4} M 5,5′-dithiobis (2-nitrobenzoic acid) in 0.1 M phosphate, pH 8) and the adsorbance at 412 nm determined using a value $\varepsilon_{412} = 14{,}600\ \text{cm}^{-1}\ \text{M}$ (9). Blank determinations were carried out in parallel using polyisonitrile–nylon. The amount of —$NCBr_2$ was calculated from the difference between the two determinations.

Determination of Acylhydrazide Groups

Acylhydrazide groups on nylon were determined by a two-step procedure consisting of succinylation followed by the titrimetric determination of carboxyls, essentially as described by Inman (11).

Coupling of Enzymes to Polyisonitrile–Nylon (2)

Polyisonitrile–nylon powder (50 mg) was suspended in 2 ml of a cold enzyme solution (10 mg protein) in 0.1 M sodium phosphate 0.5 M sodium acetate, pH 7.5. Cold acetaldehyde (0.1 ml) was then added and the reaction allowed to proceed overnight at 4°. The insoluble enzyme derivative was separated by filtration, washed with water, with 1 M KCl, and again with water, resuspended in water (4 ml), and stored at 4°.

Binding of Enzymes to Dibromoisocyanide–Nylon Derivatives

Freshly prepared dibromoisocyanide–nylon powder (50 mg) was added in the course of 10 min to a magnetically stirred, ice-cooled solution of enzyme (10 mg) in 2 ml 0.2 M *N*-ethylmorpholine buffer, pH 8.0. The reaction was allowed to proceed for 20 min. The insoluble enzyme derivative was separated by filtration, washed, and stored as described above.

Coupling of Enzymes to Acylhydrazide–Nylon Derivatives

Acylhydrazide–nylon powder (50 mg) was suspended in 5 ml cold 0.1 M HCl. Sodium nitrite (50 mg) was then added and the reaction mixture stirred for 30 min over ice. The acylazide–nylon derivative was separated by filtration, washed exhaustively with cold water, and added to a cold solution of the enzyme (10 mg/2 ml) in 0.1 M *N*-ethylmorpholine buffer, pH 9. The reaction was allowed to proceed overnight, with stirring at 4 °. The immobilized enzyme derivative was separated by filtration, washed, and stored as described above.

Coupling of Amino Acids and Peptides to Dibromoisocyanide–Nylon Derivatives

Freshly prepared dibromoisocyanide–nylon powder (40 mg) was added in the course of 10 min to a magnetically stirred, ice-cooled, 0.01 M solution of an amino-acid derivative or peptide (10 ml), under the pH-stat, set at the appropriate pH. The reaction was allowed to proceed for 20 min and the insoluble derivative was separated by filtration, washed exhaustively with water, methanol, and ether, and air-dried.

Amino-Acid Analysis

Amino-acid analyses were carried out with a modified Beckman–Unichrom amino-acid analyzer using a micro single-column technique (2,12,13).

Assay Methods

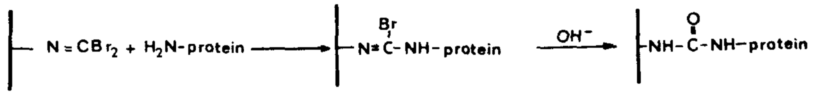
Bound protein was estimated by the Lowry method (14,15) and confirmed by total amino-acid analysis of acid hydrolyzates of the appropriate enzyme–nylon conjugate.

The enzymic activities of trypsin, chymotrypsin, subtilisin BPN′, and papain were determined at 25° by the pH-stat method (16,17). The substrate solutions (5 ml) were: 15 mM PhCOArgOEt, 50 mM in KCl for trypsin; 18 mM AcTyrOEt, 10 mM in KCl for chymotrypsin and subtilisin BPN′; 50 mM PhCOArgOEt, 5 mM cystein, 2 mM EDTA for papain. The titrant was 0.1 N NaOH. The enzyme assays were performed at the appropriate pH optima: pH 8 for trypsin and chymotrypsin; pH 8.6 for subtilisin BPN′; pH 9.0 for the nylon conjugates of trypsin and chymotrypsin and pH 9.2–9.4 for immobilized subtilisin; pH 6.3 for papain and pH 7.5 for immobilized papain (2,18–22). One unit of esterase activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of substrate/min under the specified assay conditions. The specific activities of the native enzyme samples used were as follows: trypsin, 33 esterase units/mg of protein; chymotrypsin, 350 esterase units/mg of protein; subtilisin BPN′, 280 esterase units/mg of protein; and papain, 20 esterase units/mg of protein.

RESULTS AND DISCUSSION

Dibromoisocyanide–Nylon Derivatives

Isocyanide functional groups can easily be converted into the strongly electrophilic dihaloisocyanides by treatment with the corresponding halogen (8). Dibromoisocyanide groups could thus be generated on nylon by treating polyisonitrile–nylon with a cold bromine solution [Eq. (2)]. The bromination reaction was fast and easy to carry out, and essentially quantitative (Table 1). The highly reactive, insoluble dibromoisocyanide–nylon derivative, in effect a polymeric, structural analog of phosgene, would be expected to react with the unprotonated forms of the nucleophilic groups commonly present in proteins; see for example, Means and Feeney (23); Glazer et al. (24). In the case of amino groups the reaction with $—NCBr_2$ would lead to the formation of stable, ureide-type bonds:



$$\vert\!-N{=}CBr_2 + H_2N\text{-protein} \longrightarrow \vert\!-N{=}\overset{Br}{C}\text{-NH-protein} \xrightarrow{OH^-} \vert\!-NH\text{-}\overset{O}{\overset{\|}{C}}\text{-NH-protein} \qquad (5)$$

Similar behavior would be expected for the imidazole ring of histidine. With aromatic hydroxyls or with sulfhydryl groups, the formation of urethane or thiolurethane derivatives would be anticipated (7,8,25). However, —NH—COS— structures would be rather unstable at pH values above neutrality (26–28).

Coupling of enzymes was carried out by the portionwise addition of the $NCBr_2$–nylon powder to a cold enzyme solution; the reaction (as followed pH-statistically) was complete in less than 30 min in the cold. Representative data on the immobilization of several enzymes are given in Table 2. The data show that the yield of immobilized enzyme activity increases by a factor of 3 to 5 when the pH of the binding reaction is increased from 7 to 9. This was shown to be due mainly to an increase in the specific activity rather than in the amount of protein bound (see Table 2).

An explanation for the low specific activity observed for trypsin, chymotrypsin, subtilisin BPN′, and papain, immobilized at pH 7, could be based on the assumption that active-site histidyl residues were preferentially modified at this pH (29,30). The chemical selectivity suggested by the data of Table 2 could be demonstrated by model binding experiments (Table 3). In these experiments $LeuNH_2$ (free amine), PhCOGlyHis (imidazole), and AcTyrOEt (phenolate) were coupled singly, in combinations of two, and all three together, in an aqueous medium, in the pH range of 6 to 9. No experiments with peptides containing sulfhydryl groups were carried out because of the intrinsic instability of esters of thiolcarbamic acid in the pH range investigated (26,27). For each of the compounds coupled separately to dibromoisocyanide–nylon, maximal binding was attained at pH values equal to or higher than the pK of the respective functional group, supporting the view that the unprotonated forms of the nucleophiles were the reactive species involved (Table 3; for pK values of the various nucleophilic groups, see footnote to Table 3).

TABLE 1. **Characterization of Chemically Modified Nylons**

Isocyanide–nylon derivative	Dibromoisocyanide–nylon derivative		Acylhydrazide–nylon derivative		
—NC content[a] (equiv × 10^6/g)	Effective —$NCBr_2$ content[b] (equiv × 10^6/g)	Efficiency of conversion —Nc → —$NCBr_2$ (%)	—$CONH.NH_2$ content[c] (equiv × 10^6/g)	Efficiency of conversion —$NCBr_2$ → —$CONH.NH_2$ (%)	Overall yield —NC → —$CONH.NH_2$ (%)
44	31	71	27	87	61

[a] Determined titrimetrically according to the reaction of Eq. (3) (see experimental section).

[b] Determined by reaction with dithioerythritol according to Eq (4) (see experimental section).

[c] Estimated from the carboxyl content of the succinylated derivative [see Materials and Methods and reference (11)].

TABLE 2. **Coupling of Enzymes to a Dibromoisocyanide Derivative of Nylon**[a]

		Bound protein		
		Total bound protein[b]	Active bound protein[c]	
Enzyme	pH of coupling reaction	(mg/g support)	mg/g support	% of total
Trypsin	7	13.1	2.0	15
	8	14.2	5.5	39
	9	21.0	14.2	68
Chymotrypsin	7	10.0	1.6	16
	9	15.3	10.0	66
Subtilisin BPN'	7	3.3	0.14	4
	9	9.3	1.14	12
Papain[d]	7	26.6	3.9	15
	9	23.4	11.4	49

[a] The coupling reactions were carried out by the portionwise addition of 50 mg of dibromoisocyanide–nylon powder to an ice-cooled enzyme solution (10 mg/2 ml) in 0.2 M *N*-ethyl morpholine buffer at the specified pH.

[b] Determined by amino-acid analysis of acid hydrolyzates of enzyme–nylon conjugate.

[c] Determined by rate assay (for details, see Materials and Methods).

[d] In the case of papain, the enzyme (10 mg) was dissolved in 5 ml of 0.04 M *N*-ethyl morpholine buffer at the specified pH.

In the experiments with mixtures, the relative amounts of each model peptide bound to the —$NCBr_2$-containing polymer at a given pH were similarly proportional to the state of ionization of the corresponding functional group. Thus, the binding of histidine peptide was predominant at pH 7, while AcTyrOEt took over at pH 9 (Table 3). The binding through amine groups, as represented by the data with leucine amide, was somewhat lower in the whole pH range investigated, probably because of competing reactions with histidyl or tyrosyl residue arising from partial overlap of pK. The total amount of bound peptide was essentially invariant in the various experiments (Table 3). The data of Tables 2 and 3 thus strongly suggest that in proteins reacting with dibromoisocyanide–nylon, histidyl residues are preferentially modified at pH 7, while amino groups and tyrosyl residues are affected at higher pH values. This pattern of behavior might change, however, in the case of proteins containing free sulfhydryl groups.

Acylhydrazide Derivatives of Nylon

In addition to its use for the direct binding of proteins, dibromoisocyanide–nylon can serve as starting material for further chemical modification by reaction with multifunctional reagents containing at least one nucleophilic group capable of reacting with —$NCBr_2$. Such an approach

TABLE 3. **Coupling of Model Peptides to Dibromoisocyanide–Nylon**[a]

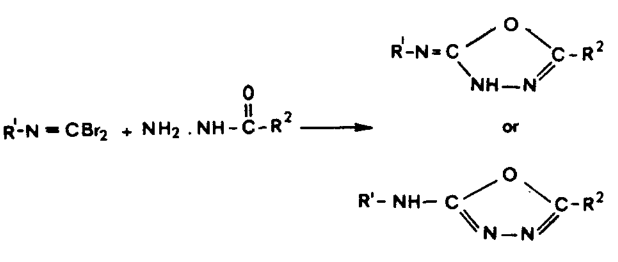
Peptides in reaction mixture[b]	pH	Amino-acid bound (μmol/g support)[c]			
		Leu	His	Tyr	Total
Leu NH_2	7	2.2	—	—	2.2
	8	9.5	—	—	9.5
	9	9.5	—	—	9.5
PhCO.Gly.His	6	—	8.4	—	8.4
	7	—	11.8	—	11.8
	8	—	11.5	—	11.5
	9	—	11.3	—	11.3
AcTyrOEt	8	—	—	9.1	9.1
	9	—	—	12.8	12.8
Leu NH_2 + PhCO.Gly.His	7	1.2	10.1	—	11.3
	9	4.3	6.7	—	11.0
Leu NH_2 + AcTyrOEt	8	3.8	—	4.9	8.7
	9	3.3	—	6.5	9.8
PhCO.Gly.His + AcTyrOEt	6	—	7.2	0.8	8.0
	7	—	10.1	1.1	11.2
	8	—	8.9	2.4	11.3
	9	—	3.6	7.6	11.2
Leu NH_2 + PhCO.Gly.His + AcTyrOEt	7	1.2	9.6	1.0	11.8
	9	2.1	3.6	6.2	11.9

[a] The dissociation constants assumed for the ionizing groups present in the model peptides used were as follows (33): Imidazole, pK = 6.8; α-amine, pK = 7.8; phenolic hydroxyl, pK = 9.8.

[b] The reaction mixtures contained 40 mg freshly prepared dibromoisocyanide–nylon and 10 ml of 0.01 M solution of the amino-acid derivative, peptide, or mixture thereof, brought to the appropriate pH.

[c] Determined by amino-acid analysis of the acid hydrolyzate of the appropriate nylon conjugate.

would allow the introduction of a variety of chemically reactive groups on the nylon support, by simple and rapid procedures. Thus, the reaction of the isocyanide dihalides with acylhydrazides (8,31,32) to form five-membered heterocycles of the 1,3,4-oxadiazole type [Eq. (6)] was utilized for the preparation of acylhydrazide derivatives of nylon:



(6)

TABLE 4. **Binding of Enzymes to Nylon Supports Carrying Different Functional Groups**[a]

	Modified nylon[b]								
	Isocyanide derivative[c]			Dibromoisocyanide derivative[d]			Acylhydrazide derivative[e]		
	Total bound protein[g] (mg/g support)	Active bound protein[h] mg/g support	Active bound protein[h] % of total	Total bound protein[g] (mg/g support)	Active bound protein[h] mg/g support	Active bound protein[h] % of total	Total bound protein[g] (mg/g support)	Active bound protein[h] mg/g support	Active bound protein[h] % of total
Trypsin	48	30	63	21	14.2	68	16.2	14.4	89
Chymotrypsin	45	10	22	15.2	10	66	13.6	10	74
Subtilisin BPN′	14.4	5	35	9.2	1.2	13	8.6	2.8	33
Papain[f]	70	32	46	23.4	11.4	49	29.2	12.2	42

[a] All coupling mixtures contained 10 mg protein and 50 mg derivatized nylon powder in 2 ml of the appropriate buffer (for details see Materials and Methods).

[b] Details on chemical characteristics of derivatized nylon powders are given in Table 1.

[c] Coupling of proteins carried out by four-component condensation at pH 7.5 (see Materials and Methods).

[d] Coupling of protein carried out at pH 9 as described in footnote to Table 2.

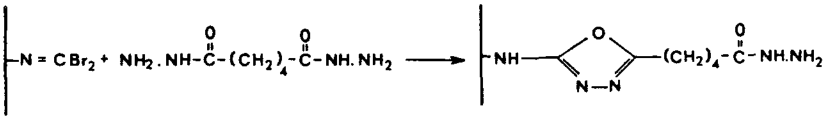
[e] Coupling of protein carried out at pH 9 (for details see Materials and Methods).

[f] In the case of papain the enzyme (10 mg) was dissolved in 5 ml of appropriate 0.05 M buffer.

[g] Determined by amino-acid analysis of acid hydrolyzate of protein–nylon conjugate.

[h] Determined by rate assay (for details see Materials and Methods).

Dibromoisocyanide–nylon was reacted with the bifunctional acylhydrazide hexanedioic acid dihydrazide (adipic dihydrazide), as shown in Eq. (7).



(7)

The conversion of polymeric —NCBr$_2$ to acylhydrazide groups was nearly quantitative (Table 1). Acylhydrazide–nylon, following activation to the corresponding azide by treatment with nitrous acid, could be used for the covalent fixation of proteins.

Representative data on the binding of several enzymes to acylhydrazide–, dibromoisocyanide–, and polyisonitrile–nylon derivatives are summarized in Table 4. The data show that the protein-binding capacities of dibromoisocyanide– and acylhydrazide–nylon derivatives were comparable. However, the specific activity of the bound protein was higher in three of the four enzymes investigated when the —CONH.NH$_2$ derivative of nylon was used as support. This was probably due to the somewhat higher selectivity of the acylazide moiety toward amino groups.

CONCLUSIONS

The data of Tables 2 and 3 suggest that the selectivity of the strongly electrophilic —NCBr$_2$ group toward the nucleophiles commonly present in proteins could be regulated to a degree by proper adjustment of the pH of the coupling reaction. Moreover, the NCBr$_2$ groups of dibromoisocyanide–nylon, easily prepared from the corresponding polymeric isocyanide, could be converted, by further chemical modification, into other types of functional groups with concomitant change in reactivity and specificity. The chemical versatility inherent in isocyanides allows in principle a wide choice of coupling methods, all starting from the same chemically reactive parent polymer, polyisonitrile–nylon. The data of Table 4, which summarizes the relative merits of several binding procedures in terms of bound protein and immobilized enzymic activity, can serve to illustrate this approach.

The variations in the protein-binding capacity of the three types of nylon supports listed in Table 4 can be attributed to differences in their sensitivity to competing hydrolytic reactions. Isocyanide groups are comparatively insensitive to hydrolysis in the pH range usually employed for four-component reactions with proteins (pH 7–8). Dibromoisocyanide and acylazide groups, on the other hand, require unprotonated nucleophiles and hence higher pH values for coupling to proteins (~pH 9). Nylon supports containing —$NCBr_2$ and —CON_3 groups would thus undergo considerable hydrolysis and exhibit lower overall binding capacities for proteins than the parent polymer, polyisonitrile–nylon.

However, several of the enzymes listed in Table 4 retained a higher proportion of their activity when immobilized to nylon via the dibromoisocyanide and acylazide derivatives. Thus, enzymes sensitive to aldehyde yielded relatively inactive conjugates by four-component coupling to nylon viz. chymotrypsin (Table 4). In these cases it was expedient to use the alternative routes to immobilization on nylon offered by the —$NCBr_2$ and —CON_3 groups, particularly in view of the fact that the high reactivity and innate broad specificity of dibromoisocyanides could in principle be restricted by appropriate pH control.

Further insight into the potential of this approach could be gained from binding studies with urease, an —SH enzyme (34). Immobilized urease preparations of very low activity were obtained when the enzyme was coupled to polyisonitrile–nylon by four-component condensation (in the presence of aldehyde) or to dibromoisocyanide–nylon. The loss of activity accompanying the coupling of urease by these procedures was most probably caused by the chemical modification of active-site sulfhydryl groups. Preparations of low protein content and hence of low total immobilized enzymic activity were also obtained when acylhydrazide–nylon was used as the carrier. This finding was in accord with the low availability of modifiable amino groups reported for urease (34), although highly active urease–nylon preparations could be obtained by diazo coupling to aminoaryl–nylon derivatives (1,2,35). In this procedure tyrosyl and histidyl residues on the protein were most probably involved in forming covalent bonds with the support.

The combined methods, all based on polyisonitrile–nylon as the starting material, allow considerable flexibility in selection and optimization of coupling procedures (in terms of bound protein vs. immobilized enzyme activity), as well as in their adaptation to different preformed polyamide structures, e.g., powders, pellets, fibers, and sheets.

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