Chemically Modified Nylons as Supports for Enzyme Immobilization

POLYISONITRILE-NYLON

By LEON GOLDSTEIN, AMIHAY FREEMAN and MORDECHAI SOKOLOVSKY
 Department of Biochemistry, The George S. Wise Center for Life Sciences,
 Tel Aviv University, Tel Aviv, Israel

(Received 17 April 1974)

Four-component condensations between amine, carboxyl, isocyanide and aldehyde lead to the formation of *N*-substituted amides (Ugi, 1962). The present paper describes the use of such condensations for the introduction of chemically reactive groups on to the polyamide backbone of nylon. Polyisocyanide-nylon was synthesized by partial hydrolysis of nylon-6 powder, followed by resealing of the newly formed $-\text{CO}_2 \cdots \text{NH}_2-$ pairs via a four-component condensation, by using acetaldehyde and 1,6-di-isocyanohexane. Polyisocyanide-nylon could also be converted into a diazotizable arylamino derivative, polyaminoaryl-nylon, by a four-component condensation by using a bifunctional amine, *pp'*-diaminodiphenylmethane, in the presence of an aldehyde and a carboxylate compound. The versatility of four-component condensations involving the isocyanide functional group of polyisocyanide-nylon allowed coupling of proteins, in an aqueous medium at neutral pH, through either their amino or carboxyl groups. Trypsin and papain were bound to polyisocyanide-nylon through their amino groups by a four-component condensation by using acetaldehyde and acetate; conversely, succinyl-(3-carboxypropionyl)-trypsin, pepsin and papain were coupled through their carboxyl groups in the presence of acetaldehyde and an amine (Tris). Diazotized polyaminoaryl-nylon could be utilized for the immobilization of papain, via the tyrosine residues of the enzyme.

Immobilized enzymes offer considerable advantages as re-usable reagents with, in many cases, increased life-time (Goldman *et al.*, 1971; Katchalski *et al.*, 1971; Zaborsky, 1973). The interest in nylons and related polyamides as possible supports for the covalent attachment of enzymes stems from their high stability and relatively low cost (Sweeny & Zimmerman, 1969). They suffer, however, from an intrinsic drawback, namely the chemical inertness of the polyamide backbone, leaving only the terminal carboxyl and amino residues as possible functional groups for covalent binding to proteins. Procedures for increasing the binding capacity of nylon by mild acid hydrolysis have been described by Hornby and co-workers (Sundaram & Hornby, 1970; Inman & Hornby, 1972). Such procedures would presumably produce some fragments of relatively low molecular weight, and the partially degraded nylon, particularly when coupled to proteins, might be soluble on prolonged exposure to an aqueous medium.

This paper describes a method for introducing chemically reactive groups on to the nylon backbone, which avoids a decrease in the molecular weight and thus does not impair the mechanical properties of the final product. The method is based on (a) partial hydrolysis of nylon (Sundaram & Hornby, 1970;

Inman & Hornby, 1972) to increase the number of functional groups on the polyamide backbone and (b) resealing the newly formed $-\text{CO}_2\text{H} \cdots \text{NH}_2-$ pairs by a four-component condensation reaction (Ugi, 1962; Gokel *et al.*, 1971; Axen *et al.*, 1971; Vretblad & Axen, 1971) which involves the neighbouring carboxyl and amino groups on the nylon backbone with an aldehyde and an isocyanide. In such a reaction (Scheme 1) the carboxyl and amino components (R^1 and R^2) combine to form an *N*-substituted amide, the aldehyde and isocyanide components (R^3 and R^4) appearing as the side chain

Scheme 1. Four-component condensation reaction between amine, carboxyl groups, aldehyde and isocyanide

on the amide nitrogen. These reactions allow for considerable versatility, since various functional groups can be introduced on to the *N*-alkyl side chain of the nylon derivative by proper choice of aldehyde and isocyanide. The procedure finally adopted utilized acetaldehyde and 1,6-di-isocyanohexane and led to nylon derivatives containing isocyanide (isonitrile) functional groups (Scheme 2). The polyisonitrile-nylon could be coupled to enzymes via the $-N\equiv C$ groups of the carrier, again by a four-component condensation, in an aqueous medium at neutral pH, in which the protein itself supplied either the amino or the carboxyl component.

Materials and Methods

Materials

Trypsin (three times crystallized and freeze-dried) and papain (twice crystallized) were from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Pepsin (porcine, three times crystallized, B grade) was

from Calbiochem (Los Angeles, Calif., U.S.A.). PhCOGly-Lys* (hippuryl-lysine) was from Cyclo Chemical Corp. (Los Angeles, Calif., U.S.A.). Cbz-Glu-GlyOCH₂Ph was a gift from Dr. M. Wilchek of the Weizmann Institute of Science, Rehovot, Israel. Acetaldehyde (purissimum p.a. grade) was from Fluka (Buchs, SG, Switzerland).

Nylon-6 (polyamide-6) in pellet form, was a product of Organico S.A., Paris, France. Its specifications were: m.p. 218°C; relative viscosity 2.38 (1% solution in 96% H₂SO₄, at 20°C); moisture regain: 4.2–4.4% in air of 65% relative humidity, at 20°C; 12%, in water.

Nylon powder. Commercial nylon-6 pellets were suspended in a 20% (w/v) solution of CaCl₂ in methanol (30 g/litre) and stirred at room temperature until a homogeneous, though extremely viscous, solution was obtained (about 18 h) (Fabel, 1947; Ford & Marshall, 1956). The nylon solution was added dropwise with strong stirring into a large excess of water; the powder thus obtained was separated on a suction filter, washed with water, resuspended in water and homogenized with an Ultra-Turrax homogenizer (Janke and Kunkel KG, Staufen i. BR., Germany). The powder was separated, washed again with water, ethanol and ether, and air-dried. Traces of solvent and moisture were removed in a vacuum desiccator over P₂O₅; the dry powder was ground in a mortar. The mean carboxyl content of the nylon powders, which was determined titrimetrically, was about 25 μmol/g of dry nylon powder (see Table 1). The nylon powder was examined by electron microscopy, by the shadow-casting technique. The particles were spherical, and their diameter ranged between 0.2 and 0.7 μm.

Controlled hydrolysis of nylon powder. Nylon-6 powder (10 g) was suspended in 3 M-HCl (300 ml) and stirred at room temperature (20°C) for the desired length of time. The powder was separated on a suction filter, washed exhaustively with water, ethanol, and ether, and then air-dried. Traces of solvent and moisture were removed in a vacuum desiccator over P₂O₅. The powders were stored in closed vessels. The dependence of the carboxyl content of nylon-6 powders on the time of hydrolysis is shown in Fig. 1. The nylon powder samples used as a routine were hydrolysed for 4 h (mean carboxyl content 60–70 μmol/g; Table 1).

Carboxyl content of nylon powders. The carboxyl content of the nylon powder samples was determined by anhydrous titration with sodium methoxide essentially as described by Patchornik & Ehrlich-Rogozinski (1961). Nylon powder (50 mg) was

Scheme 2. Synthesis of polyisonitrile-nylon

* Abbreviations: PhCOGly-Lys, *N*-benzoylglycyl-L-lysine (hippuryl-lysine); CbzGlu-GlyOCH₂Ph, *N*-benzyl-oxycarbonyl-L-glutamylglycine benzyl ester; PhCO-ArgOEt, *N*-benzoyl-L-arginine ethyl ester.

suspended in dimethylformamide (2ml) and stirred magnetically in a stoppered test tube for 4 h (to achieve uniform swelling for all titrated samples). Thymol Blue indicator (2 drops of a 1% solution in ethanol) was added and the stirred sample titrated with 0.05 M-sodium methoxide (in benzene-methanol; 8:2, v/v) delivered from a micro-burette, until the appearance of blue colour which persisted for 5–10 s. Appropriate solvent blanks were determined for each titration. Care was taken to keep the titration vessels covered to prevent penetration of CO_2 .

Synthesis of isocyanides. The general synthetic procedure adopted was based on the conversion of a primary amine into the appropriate *N*-alkyl-formamide by treatment with ethyl formate, followed by dehydration to the isocyanide (Ugi *et al.*, 1965; Hoffmann *et al.*, 1971).

***N*-Formylcyclohexamine** (see Ugi, 1961). Cyclohexylamine (91 ml; 79 g, 0.8 mol) was introduced into a flask and chilled over ice. Then 56.5 ml of ethyl formate (59 g, 0.8 mol) was slowly added. After the termination of the exothermic reaction, the mixture was refluxed for 2 h and vacuum-distilled (140 mmHg pressure, i.e. 19 kPa, water pump). The fraction distilling at 196–202°C was collected; 73 g of cyclohexylformamide (58% yield) was obtained. The i.r. spectrum in chloroform had maxima at 1460 cm^{-1} (alicyclic $-\text{CH}_2-$), 1680 cm^{-1} (amide CO), 2860, 2940 cm^{-1} (aliphatic $-\text{CH}_2-$) and 3700 cm^{-1} (amide NH) (see Silverstein & Bressler, 1967).

Cyclohexyl isocyanide (see Wittman, 1961). *N*-Formylcyclohexylamine (31.75 g, 0.25 mol) was added to a beaker containing 105 g of K_2CO_3 in 500 ml of acetone. 2,4,6-Trichlorotriazine (cyanuric chloride; 53 g, 0.5 mol) in acetone (500 ml) was slowly added with stirring. The reaction was allowed to proceed with stirring at room temperature for 3 h, acetone being added to keep the total volume at about 1 litre. Water (300 ml) was then added, followed by ether (300 ml) containing pyridine (25 ml). The yellowish ether phase was separated, washed again with water and dried over anhydrous Na_2SO_4 . The ether was removed by distillation and the crude oil vacuum-distilled (140 mmHg, i.e. 19 kPa, water pump); 10 g of a colourless oil, of distinctive odour, was obtained (40% yield). The i.r. spectrum in chloroform had maxima at 1460 cm^{-1} (alicyclic CH_2), 2170 cm^{-1} ($\text{N}\equiv\text{C}$) and 2860, 2950 cm^{-1} (aliphatic CH_2) (see Silverstein & Bressler, 1967). Because of its rather penetrating smell the material was stored in tightly closed vials under a hood.

***NN'*-Di-formyl-1,6-diaminohexane.** This was prepared by a modification of the procedure of Moffat *et al.* (1962). 1,6-Diaminohexane (60 g, 0.5 mol) was dissolved in ethyl formate (170 ml, 3.3 mol) with stirring over ice. When it had completely dissolved a white precipitate formed; an additional portion of

ethyl formate (50 ml) was then added and the mixture refluxed for 2 h. In the course of the reaction, solid *NN'*-di-formyl-(1,6-diaminohexane) separated at the bottom of the flask. The liquid was decanted while still hot, and the solid transferred to a rotatory evaporator to remove residual solvent. The solid material (60 g; 70% yield; m.p. 105°C) was used in the next step without further purification. The i.r. spectrum in chloroform had maxima at 1680 cm^{-1} (amide CO) and 2840, 2920 cm^{-1} (aliphatic CH_2) (see Silverstein & Bressler, 1967).

1,6-Di-isocyanohexane. This was prepared by a modification of the procedure of Hertler & Corey (1958). Toluene-*p*-sulphonyl chloride (150 g, 0.8 mol) was dissolved with stirring in 300 ml of pyridine (KOH-dried). To the light-yellow solution, *NN'*-di-formyl-1,6-diaminohexane (30 g, 0.2 mol) was added in small portions with stirring. The dehydration reaction occurred as the formamide dissolved; the solution warmed up, and the colour darkened from yellow to brown and finally to black. After dissolution was complete the reaction mixture was stirred for 1.5 h at room temperature. Water (250 ml) was added, together with crushed ice to lower the temperature, and the solution extracted with three 150 ml portions of ether. The combined ether extract was washed with water (three 250 ml portions) and dried over anhydrous Na_2SO_4 . The ether was then removed by evaporation and the crude oil vacuum-distilled (pressure less than 0.1 mmHg). The fraction distilling at 85–92°C (9 g; 30% yield) was collected and stored in closed vials at -5°C . The i.r. spectrum in chloroform had peaks at 1440 cm^{-1} (aliphatic CH_2), 2130 cm^{-1} ($\text{N}\equiv\text{C}$) and 2840, 2929 cm^{-1} (aliphatic CH_2). N.m.r. in C^2HCl_3 : hydrogen ratio 2:1 (corresponding to 8:4); $\alpha\text{-CH}_2$: $\tau = 6.70$; $J_{\text{NH}} = 2\text{ Hz}$; $\beta\text{-CH}_2$: $\tau = 8.36$ (multiple splitting); $\gamma\text{-CH}_2$: $\tau = 8.52$ (multiple splitting) (Kurtz *et al.*, 1961).

Polyisocyanitrile-nylon. Partially hydrolysed nylon powder (2 g), prepared as above, was suspended in propan-2-ol (80 ml). Acetaldehyde (20 ml, 0.3 mol) was then added, followed by 1,6-di-isocyanohexane (8 ml, 0.06 mol) and the reaction allowed to proceed in a closed vessel for 24 h with stirring at room temperature. The polyisocyanitrile-nylon powder was separated on a suction filter, washed with propan-2-ol (50 ml) and then with ether (about 200 ml) and air-dried. Traces of solvent were removed in a vacuum desiccator over P_2O_5 . The powdered nylon derivative was stored at -5°C in a dark stoppered vial over silica gel. The mean carboxyl content of the derivative was about 20 $\mu\text{mol/g}$ (see Table 1).

Polyaminoaryl-nylon. *pp'*-Diaminodiphenylmethane (2 g, 0.01 mol) was dissolved in 160 ml of propanol, cooled over ice, and 4 ml of acetaldehyde (0.06 mol) was added. Polyisocyanitrile-nylon powder (2 g; isocyanitrile content 25–50 $\mu\text{mol/g}$) was suspended

in 160ml of propan-2-ol. The solution of *pp'*-diaminodiphenylmethane and acetaldehyde and 2ml of acetic acid (0.033 mol) were then added. The reaction was allowed to proceed in a closed vessel for 24h, with stirring at room temperature. The polyaminoaryl-nylon was separated on a suction filter, washed with ethanol and then with ether and air-dried. The diazotization capacity of polyaminoaryl-nylon was about 20 $\mu\text{mol/g}$ (see Table 2).

Succinyl- (3-carboxypropionyl-)trypsin. Trypsin (250mg) was dissolved in cold half-saturated sodium acetate (6ml) and the solution adjusted to pH 8. Succinic anhydride (600mg) was added in the course of 1h to the ice-cooled enzyme solution, the pH being maintained at 8 with an automatic titrator with 2M-NaOH as titrant. The reaction mixture was exhaustively dialysed against water at 4°C and freeze-dried (net weight of freeze-dried powder 235mg). Determination of the free amine nitrogen by the Van Slyke method (Archibald, 1957) indicated that 13 out of the 14 lysine residues of trypsin (Walsh & Neurath, 1964; Mikes *et al.*, 1966) had been succinylated. The specific activity of the succinyl-trypsin sample was 20 esterase units/mg as compared with 33 esterase units/mg for native trypsin.

Trypsin-nylon. Polyisocyanide-nylon (50mg) was suspended in 2ml of cold 0.1M-sodium phosphate, 0.5M in sodium acetate, pH 7. A cold aqueous solution of trypsin (2–10mg in 1ml) was then added, followed by 0.1ml of acetaldehyde. The acetaldehyde (b.p. 21°C) was pipetted with a precooled pipette to prevent the formation of bubbles. The reaction mixture was left stirring overnight at 4°C. The insoluble enzyme derivative was separated on a filter, washed with water, then with 1M-KCl, 0.1M in NaHCO₃ and again with water, resuspended in water (4ml) and stored at 4°C.

Succinyl-trypsin-nylon. Polyisocyanide-nylon (50mg) was suspended in 1.0ml of cold 0.1M-Tris-HCl buffer, pH 7.0. A cold solution of succinyl-trypsin in the same buffer (2–10mg in 1ml) was added, followed by 0.1ml of acetaldehyde, pipetted in the same way as before. The reaction mixture was left stirring overnight at 4°C, washed and resuspended in water as described for trypsin-nylon.

Maximum recoveries of immobilized enzymic activity (30–40% of the amount added to the reaction mixture) were obtained for both trypsin and succinyl-trypsin with 4–5mg of enzyme per 100mg of support.

Pepsin-nylon. Polyisocyanide-nylon (50mg) was suspended in 2ml of cold 0.1M-Tris-HCl, pH 5, and 2–10mg of pepsin was added. After the enzyme had dissolved 0.1ml of acetaldehyde was added in the usual way. The reaction mixture was left stirring overnight at 4°C. The insoluble pepsin derivative was washed with 10mM-HCl, resuspended in 1mM-HCl (3–4ml) and stored at 4°C.

Papain-nylon. Papain was coupled to polyisocyanide-nylon as described for trypsin-nylon (papain-nylon A) or as described for succinyl-trypsin-nylon (papain-nylon C) (see also Table 4).

Coupling of peptide ligands. PhCOGly-Lys and CbzGlu-GlyOCH₂Ph were coupled to polyisocyanide-nylon essentially as described for trypsin and succinyl-trypsin (for details see Table 2).

Diazotization of polyaminoaryl-nylon. Polyaminoaryl-nylon (100mg) was suspended in cold 0.2M-HCl (7ml), and aq. NaNO₂ (25mg in 1ml) was added dropwise. The reaction mixture was stirred for 30min, over ice; the red-brown diazotized polyaminoaryl-nylon was separated on a suction filter, washed with cold water and finally with cold 0.1M-sodium phosphate, pH 8. It was resuspended in the same buffer and used directly in the coupling experiments.

Papain-polyaminoaryl-nylon. An aqueous solution of papain (5–10ml, 1–1.3mg of protein/ml) was added to a magnetically stirred suspension of diazotized polyaminoaryl-nylon in 0.1M-phosphate buffer, pH 8 (100mg in 6ml). The reaction mixture was left stirring overnight at 4°C. The insoluble papain conjugate was separated by filtration, washed with water, 1M-KCl and again with water, resuspended in water (5ml) and stored at 4°C.

Coupling of *p*-bromophenol to polyaminoaryl-nylon. An aqueous solution of *p*-bromophenol (50mg dissolved in 3ml of water by the dropwise addition of 2M-NaOH) was added to diazotized polyaminoaryl-nylon (50mg) suspended in cold 0.1M-sodium phosphate buffer, pH 8.0 (6ml). The reaction mixture was left stirring overnight at 4°C. The nylon conjugate of *p*-bromophenol was separated on a funnel, washed with 0.05M-Na₂CO₃ buffer, pH 10.5, or water adjusted to the same pH, followed by washing with 0.02M-NaOH, then deionized water and finally methanol and ether and dried *in vacuo* over P₂O₅. The diazotization capacity of polyaminoaryl-nylon was estimated from the bromine content of the *p*-bromophenol conjugate, determined by the Schoniger combustion method (Steyermark, 1961).

Methods

Amino acid analyses. These were carried out with a modified Beckman-Unichrom amino acid analyser by using a micro, single-column technique (Hamilton, 1963, 1967). The column (0.55cm × 20cm) was packed with type C3 spherical ion-exchange resin (Technicon Corp., Terrytown, N.Y., U.S.A.). The developing solutions (pH 3.25, 4.25 and 5.55, the last 1M in NaCl) were pumped through the column at 20ml/h, 60°C. Under these conditions resolution between 5-amino-hexanoic acid and lysine was obtained.

Assay methods. Protein concentrations were estimated from their 280nm absorbance by using the following $E_{1\%}^{1\text{cm}}$ values: pepsin, 14.7 (Knowles

et al., 1969); trypsin, 15.6 (Baines *et al.*, 1964); papain, 24.8 (Glazer & Smith, 1961).

The enzymic activities of trypsin, succinyl-trypsin and papain and of their water-insoluble derivatives were determined at 25°C by the pH-stat method (Walsh & Wilcox, 1970). The substrate solutions (5 ml) were: 15 mM-PhCOArgOEt, 50 mM in KCl for trypsin and succinyl-trypsin; 50 mM-PhCOArgOEt, 5 mM-cysteine, 2 mM-EDTA for papain (Smith & Parker, 1958; Arnon, 1970). The titrant was 0.1 M-NaOH. The assays were carried out at pH 8 for trypsin, at pH 9 for succinyl-trypsin, at pH 9.5 for immobilized trypsin and succinyl-trypsin, at pH 6.5 for papain and at pH 7 for immobilized papain. One unit of esterase activity was defined as that amount of enzyme which catalysed the hydrolysis of 1 μ mol 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; succinyl-trypsin, 20 esterase units/mg of protein; papain, 12 esterase units/mg of protein.

The enzymic activity of crystalline pepsin and the immobilized pepsin derivatives was determined at 37°C by the haemoglobin digestion method (Herriott, 1955; Bohak, 1970). The reaction mixtures containing immobilized enzyme were stirred magnetically to ensure effective mixing of the reagents.

Results and Discussion

Preparation of nylon derivatives

The method chosen for the introduction of chemically reactive side chains on nylon consisted of partial hydrolysis of the polyamide backbone (Inman & Hornby, 1972), followed by resealing the pairs of neighbouring carboxyl and amino groups by a four-component condensation reaction in the presence of an aldehyde and an isocyanide (Schemes 1 and 2). The physical properties and binding capacity of such a product would thus mainly depend on (a) the number of $-\text{CO}_2\text{H} \cdots \text{NH}_2-$ pairs formed by hydrolysis and (b) the efficiency of the resealing of such pairs to form *N*-alkylated peptide bonds (see Scheme 2).

The time-course of the controlled acid hydrolysis of nylon powder, as judged from the increase in titratable carboxyl groups, is illustrated in Fig. 1 for a typical set of experiments. On prolonged hydrolysis the rate of appearance of carboxyl groups decreases; this is most probably due to leaching of fragments of low molecular weight from the particles. For all nylon powder samples used below, hydrolysis was terminated when the upper part of the linear portion of the curve was reached (4 h hydrolysis; 60–70 μ mol of carboxyl group/g).

The results of experiments on the resealing of $-\text{CO}_2\text{H} \cdots \text{NH}_2-$ pairs in partially hydrolysed nylon

Fig. 1. Time-course of hydrolysis of nylon powder

Nylon powder (50 mg) was suspended in 3 M-HCl (2 ml) and stirred at room temperature for the specified length of time; it was then quantitatively transferred to a suction filter, washed exhaustively with water, ethanol, ether and air-dried. Traces of moisture were removed *in vacuo* over P_2O_5 and NaOH pellets. The carboxyl content of the whole sample was determined by anhydrous titration with 0.05 M-sodium methoxide.

powder, by using various combinations of aldehyde and cyanide, are summarized in Table 1. The data show that acetaldehyde in the presence of either cyclohexyl isocyanide or 1,6-di-isocyanohexane (Expts. 3 and 4, Table 1) led to a drastic decrease in the amount of titratable carboxyl groups. The carboxyl content of these nylon preparations was even lower than that of the original non-hydrolysed nylon powder (Expt. 1). The efficiency of masking of carboxyl groups under the relatively mild conditions of the reaction suggests that $-\text{CO}_2\text{H} \cdots \text{NH}_2-$ pairs, formed on the surface of a nylon particle as a result of hydrolysis, remain in close proximity. This situation would be sterically favourable for the occurrence of a reaction requiring four different components. Further, the fact that the carboxyl content of the nylon derivatives obtained in Expts. 3 and 4 was lower than that of the starting unhydrolysed nylon powder, implies that at least a fraction of the end groups originally present were close enough to participate in four-component condensations.

When benzaldehyde or glutaraldehyde are used in combination with either cyclohexyl isocyanide or 1,6-di-isocyanohexane no significant decrease in carboxyl content is observed (Expts. 5 and 6, Table 1). This finding is in agreement with the accepted mechanism of four-component condensation reactions (Ugi, 1962, 1965; Gokel *et al.*, 1971): the protonated Schiff's base (immonium ion) formed from the

Table 1. Four-component condensation reactions with partially hydrolysed nylon powder

The reaction mixture contained 50 mg of nylon powder, 0.5 ml of aldehyde and 0.2 ml of isocyanide in 2 ml of propan-2-ol, and was stirred for 18 h at room temperature. Carboxyl-group content was determined by anhydrous titration (see the text).

Expt. no.	Reaction mixture			Carboxyl content	
	Nylon powder	Aldehyde	Isocyanide	($\mu\text{mol/g}$)	(% of reference)
1	Non-hydrolysed	—	—	24.2	—
2	Partially hydrolysed	—	—	62.5	100
3	Partially hydrolysed	Acetaldehyde	Cyclohexyl isocyanide	21.4	34.3
4	Partially hydrolysed	Acetaldehyde	1,6-Di-isocyanohexane	16.9	27.1
5	Partially hydrolysed	Benzaldehyde	Cyclohexyl isocyanide	54.0	86.5
6	Partially hydrolysed	Glutaraldehyde	Cyclohexyl isocyanide	60.0	96.0
7	Partially hydrolysed	Acetaldehyde	—	56.6	90.5
8	Partially hydrolysed	Benzaldehyde	—	55.0	88
9	Partially hydrolysed	Glutaraldehyde	—	59.2	94.2
10	Partially hydrolysed	—	Cyclohexyl isocyanide	52.3	83
11	Partially hydrolysed	—	1,6-Di-isocyanohexane	56.5	90.5

aldehyde and the amine (Scheme 3a, Step 1), adds to an $\text{N}\equiv\text{C}$ group in a concerted fashion with carboxyl group (α -addition) to produce, after intramolecular rearrangement, an *N*-substituted amide (Scheme 3a, steps 2 and 3). A bulky side chain on the aldehyde participating in the formation of immonium ion with the sterically constrained amine of a $-\text{CO}_2\text{H}\cdots\text{NH}_2-$ pair on the polymer backbone could prevent the approach of isocyanide and thus decrease the efficiency of peptide-bond resealing.

The nylon derivatives used for enzyme immobilization were prepared from partially hydrolysed nylon powder (4 h hydrolysis; 60–70 μmol of carboxyl group/g; Fig. 1) by reaction with acetaldehyde and the bifunctional isonitrile 1,6-di-isocyanohexane under the conditions of Expt. 4 of Table 1. The product, polyisonitrile-nylon, contains isocyanide functional groups (see Scheme 2).

The isocyanide residues on polyisonitrile-nylon could be roughly estimated from the decrease in titratable carboxyl groups relative to unmodified hydrolysed nylon powder; from the data of Table 1 a value of about 45 $\mu\text{mol/g}$ was obtained (see Table 2). Effective binding capacities of nylon derivatives were obtained by coupling model compounds of low molecular weight (e.g. PhCOGly-Lys and CbzGlu-GlyOCH₂Ph) in an aqueous solution under conditions analogous to those used for the binding of proteins. Details for the different procedures are given in Table 2. The effective binding capacity of polyisonitrile-nylon for PhCOGly-Lys (coupling via amino groups) was 24–26 $\mu\text{mol/g}$. The binding capacity of polyisonitrile nylon for CbzGlu-GlyOCH₂Ph (coupling via carboxyl groups) was 9–10 $\mu\text{mol/g}$. (See Table 2 and the following section for a discussion of these values.)

Polyisonitrile-nylon could serve in addition as starting material for the preparation of other types

of nylon derivatives via four-component condensations in an inert organic solvent (propan-2-ol in our case), in which polyisonitrile-nylon would supply the isocyanide component. This is illustrated in the synthesis of a diazotizable derivative of nylon, polyaminoaryl-nylon, where *pp'*-diaminodiphenylmethane (methylenedianiline) supplies the amine (Scheme 4). The effective diazotization capacity of polyaminoaryl-nylon, estimated from the bromine content of its conjugates with *p*-bromophenols, was 19–20 $\mu\text{mol/g}$ (Table 2). This value is in agreement with the effective capacity of the original polyisonitrile-nylon, calculated from the binding of PhCOGly-Lys (24 $\mu\text{mol/g}$; Table 2).

Coupling of proteins to the nylon derivatives

The versatility of a four-component reaction based on the isocyanide functional group of polyisonitrile-nylon makes possible, in principle, the coupling of proteins through either their amino or carboxyl groups (Scheme 1); in the presence of aldehyde and an excess of carboxyl groups in an aqueous reaction medium, coupling of a protein would be expected to occur mainly via its amino groups; conversely in the presence of aldehyde and an amine, coupling would most probably occur via the protein carboxyl groups.

These assumptions were tested with trypsin as the donor of amino groups and with succinyl-trypsin (a sample of trypsin in which 13 of the 14 lysine residues of the enzyme had been succinylated) and pepsin (containing 42 free carboxyl groups and only one lysine residue; Blumenfeld & Perlmann, 1959) as carboxyl donors. Trypsin was bound to polyisonitrile-nylon in an aqueous medium in the presence of acetaldehyde and an excess of acetate (0.5 M). Binding of succinyl-trypsin and pepsin to

Scheme 3. Mechanism of four-component reactions

(a) The main reaction: 1, formation of protonated imine; 2, formation of the first adduct; 3, intramolecular O → N acyl shift. (b) Possible competing side reaction.

Table 2. Effective binding capacity of nylon derivatives under different conditions

For Expt. 1, the reaction mixture contained 20mg of polyisocyanide-nylon, 5mg of PhCOGly-Lys, 0.1ml of acetaldehyde in 2ml of 0.1M-sodium phosphate, 1M in sodium acetate, pH7. Glycine content of acid hydrolysate of PhCOGly-Lys-nylon conjugate was determined in an amino acid analyser. For Expt. 2, the reaction mixture contained CbzGlu-GlyOCH₂Ph (5mg dissolved in 0.5ml of methanol) and acetaldehyde (0.1 ml) added to 20mg of polyisocyanide-nylon in 2ml of 0.1M-Tris-HCl, pH7. Glycine and glutamic acid content of acid hydrolysate of CbzGlu-GlyOCH₂Ph-nylon conjugate was determined in the amino acid analyser. For Expts. 3 and 6, saturated trypsin-nylon and papain-polyaminoaryl-nylon samples were prepared as described in the legend to Table 4. For Expts. 4 and 5 see the Materials and Methods section.

Expt. no.	Support	Methods	Capacity (μmol/g)
1	Polyisocyanide-nylon	Four-component coupling of PhCOGly-Lys via ε-amino group of lysine	24.1
2	Polyisocyanide-nylon	Four-component coupling of CbzGlu-GlyOCH ₂ Ph via γ-carboxyl group of glutamic acid	9.1
3	Polyisocyanide-nylon	Four-component coupling of trypsin via the amino groups on the protein*	39
4	Polyisocyanide-nylon	Anhydrous titration of carboxyl groups†	46
5	Polyaminoaryl-nylon	Coupling of p-bromophenol to diazotized polyaminoaryl-nylon	19
6	Polyaminoaryl-nylon	Coupling of papain to diazotized polyaminoaryl-nylon‡	18.4

* Calculated on the basis of disappearance of six lysine residues per molecule (see Table 5).

† Estimated from the disappearance of titratable carboxyl groups after 'four-component resealing', relative to untreated hydrolysed nylon powder (see Table 1).

‡ Calculated on the basis of disappearance of four tyrosine and one lysine residues (see Table 5).

Scheme 4. Synthesis of polyaminoaryl-nylon

polyisocyanide-nylon was affected in an aqueous solution of acetaldehyde and an amine, 2-amino-2-hydroxymethylpropane-1,3-diol (Tris). Both trypsin and succinyl-trypsin gave maximal yields of immobilized enzymic activity, when the condensation took place in the neutral pH region (Table 3). The omission of any of the components required for coupling, in the pH range 5–8, resulted in negligible immobilized activity. (All experiments were carried out with an excess of enzyme, to attain maximal binding.) With pepsin, which inactivates irreversibly at neutral and alkaline pH values, optimal recovery of immobilized activity was recorded between pH 3.5 and 5.

The binding capacities of polyisocyanide-nylon for the various proteins were determined from coupling experiments with increasing amounts of enzyme (2–20 mg of enzyme/100 mg of support). The maximal binding capacities (150 mg of trypsin/g of support, 44 mg of succinyl-trypsin/g of support and 78 mg of pepsin/g of support; Table 4) were estimated from the regions where the binding curves reached a plateau (Goldstein *et al.*, 1970). The values were confirmed by amino acid analysis of acid hydrolysates of samples prepared under saturating conditions. Table 4 shows that polyisocyanide-nylon binds less protein when coupling occurs via carboxyl groups on the ligand. The retention of activity in the immobilized enzyme derivatives calculated on the basis of total bound protein was about 60% for trypsin- and succinyl-trypsin-nylon and about 7% for pepsin-nylon (Table 4). The low specific activity of immobilized pepsin is probably due to partial inactivation of the enzyme, resulting from binding via active-site carboxyl groups (Fruton, 1971), or from prolonged exposure to the relatively high pH (pH 5) of the coupling reaction (see also Fig. 2).

To ascertain the chemical nature of the amino acid residues participating in the coupling of the various enzymes to polyisocyanide-nylon, use could be made of the fact that four-component condensations lead to the *N*-alkylation of the amino com-

Table 3. Coupling of trypsin and succinyl-trypsin to polyisocyanide-nylon by four-component condensations

Coupling was carried out in the presence of an excess of protein to obtain saturated enzyme-nylon conjugates. The reaction mixtures contained 50 mg of polyisocyanide-nylon, 10 mg of enzyme, 0.1 ml of acetaldehyde and 2 ml of buffer (for trypsin, 0.1 M-sodium phosphate–0.5 M-sodium acetate; for succinyl-trypsin, 0.1 M-Tris-HCl). Active bound protein was measured by a rate assay, assuming that the specific activity of the enzyme does not change on binding. The specific activity of native trypsin was 33 esterase units/mg, and that of succinyl-trypsin 20 esterase units/mg.

pH	Active bound protein (mg/g of support)	
	Trypsin	Succinyl-trypsin
4.0	—	28
5.0	—	27.8
5.5	45.5	—
6.5	86	27.2
7.0	86	27.2
7.5	86	—
8.0	78.5	9.4
8.5	65	—

ponent (Scheme 1). The lysine residues on a protein participating in the formation of the covalent link with polyisocyanide-nylon would therefore be expected to appear, after total acid hydrolysis, in the form of an *N*-alkylated derivative, *N*°-(1-carboxyethyl)lysine (Scheme 5). In such a case the recovery of lysine from the hydrolysis would be lower. A carboxyl group that participates in a condensation reaction would, on the other hand, be fully recovered after acid hydrolysis. Amino acid analysis of acid hydrolysates of trypsin-nylon shows the disappearance of six to seven lysine residues per molecule (Table 5), but with pepsin-nylon and succinyl-trypsin-nylon all amino acids were recovered. These findings are in accord with the assumption that trypsin binds to

Table 4. *Coupling of proteins to nylon derivatives*

Coupling was carried out in all cases in the presence of an excess of protein (estimated from appropriate binding curves) to obtain saturated enzyme-nylon conjugates. Total bound protein was calculated from the amino acid content of acid hydrolysates of the saturated enzyme-nylon conjugates. Active bound protein was measured by rate assay (see the Materials and Methods section), assuming that the specific activity of the enzyme does not change on binding.

Enzyme	Support	Coupling methods	Total bound protein (mg/g of support)	Active bound protein	
				(mg/g of support)	(% of total)
Trypsin	Polyisocyanate-nylon	Coupling via amino groups of protein*	153	86	56
Succinyl-trypsin	Polyisocyanate-nylon	Coupling via carboxyl groups of protein†	43.5	28.6	66
Pepsin	Polyisocyanate-nylon	Coupling via carboxyl groups of protein (pH 5, 0.1 M-Tris)†	78	5.1	6.5
Papain (papain-nylon A)	Polyisocyanate-nylon	Coupling via amino groups of protein*	69.2	32	46.5
Papain (papain-nylon C)	Polyisocyanate-nylon	Coupling via carboxyl groups of protein†	47.2	14	30
Papain	Polyaminoaryl-nylon	Diazonium coupling‡	86	30	35

* Reaction mixture contained 50mg of polyisocyanate-nylon, 10mg of enzyme, 0.1 ml of acetaldehyde and 2ml of 0.1 M-phosphate-0.5M-sodium acetate, pH 7.

† Reaction mixture contained 50mg of polyisocyanate-nylon, 10mg of enzyme, 0.1 ml of acetaldehyde and 2ml of 0.1 M-Tris, pH 7.

‡ Reaction mixture contained 100mg of diazotized polyaminoaryl-nylon, 15mg of protein and 6ml of 0.1 M-phosphate, pH 8.

Fig. 2. *Effect of pH on the stability of nylon conjugates of (a) papain, (b) pepsin and (c) trypsin*

○, Native enzyme; ●, immobilized enzyme. The test solutions (0.5ml) were incubated at the appropriate pH at 37°C for 30min; 0.1ml samples were removed and the residual activity was determined by the standard procedure.

polyisocyanate-nylon via its amino groups and pepsin and succinyl-trypsin bind via carboxyl groups.

Further supporting evidence for this view was obtained from the binding of amine- and carboxyl-containing model peptides to polyisocyanate-nylon: PhCOGly-Lys (hippuryl-lysine) was bound in the presence of acetaldehyde and a large excess of acetate (to suppress the possible participation of the unblocked carboxyl group on the ligand) and CbzGlu-GlyOCH₂Ph was bound in the presence of acetaldehyde and Tris (Table 2). Amino acid analysis of an acid hydrolysate of the hippuryl-lysine-nylon conjugate showed the complete absence of a lysine peak in parallel with the appearance of a new peak [presumably N⁶-(1-carboxyethyl)lysine] between valine and methionine (cf. Scheme 5), in essentially 1:1 ratio to the glycine peak (the area of the new ninhydrin-positive peak was 96% of the glycine-peak area). On the other hand, glutamic acid and glycine were recovered in equivalent amounts from acid hydrolysates of CbzGlu-GlyOCH₂Ph-nylon. The binding capacity of polyisocyanate-nylon for this peptide (9 μmol/g) was considerably lower than its capacity for the amine-containing peptide, PhCO-Gly-Lys (24 μmol/g) (Table 2). The lower effective binding capacity of polyisocyanate-nylon for carboxyl-containing ligands (proteins as well as peptides; see Table 2 and 4) could possibly be ascribed to the competition for the isocyanide groups between two nucleophiles in the α-addition stage of the coupling,

Scheme 5. Formation of N^6 -(1-carboxymethyl)lysine in acid hydrolysates of protein-nylon conjugates

Table 5. Amino acid composition of trypsin- and papain-nylon conjugates

The results are expressed as mol of amino acid per mol* of enzyme, calculated on the basis of the mean values obtained for alanine, glycine, valine, aspartic acid glutamic acid and leucine.

Amino acid	Native trypsin†	Trypsin-nylon	Succinyl-trypsin-nylon	Native papain‡	Papain-poly-aminoaryl-nylon	Papain-nylon A	Papain-nylon C
Asp	22	19.5	21.1	19	18.5	18.7	19.1
Thr	10	8.9	8.1	8	7.2	7.4	7.8
Ser	34	32	31	13	10.1	11.8	12.2
Glu	14	13.6	13.8	20	19.4	20.3	20.3
Pro	8	7	7.5	10	9.3	9.5	10.5
Gly	25	24.8	25.2	28	27.7	27.2	27.1
Ala	14	14.4	14.7	14	14.1	14.3	13.8
Val	17	17.3	16.1	18	16.9	16.5	18.5
Ile	15	14.7	14.5	12	11.3	11.5	11.3
Leu	14	13.7	15.3	11	10.9	11.2	11.3
Tyr	10	10.5	9.4	19	15.2	19.3	19.6
Phe	3	3.6	3.4	4	3.7	3.6	4.1
Lys	14	7.5	13.6	10	9.1	5.9	9.5
His	3	2.5	2.3	2	1.8	1.7	1.6
Arg	2	2.5	1.9	12	11.6	11.5	11.8

* The molecular weights used were 23 300 for trypsin (Mikes *et al.*, 1966) and 23 350 for papain (Drenth *et al.*, 1970).

† Walsh & Neurath (1964), Mikes *et al.* (1966).

‡ Drenth *et al.* (1970).

namely the carboxyl groups on the ligand and the OH^- ions present in the aqueous medium (see Scheme 3b).

The extent to which coupling of polyisocyanate-nylon could be selectively directed to either amino or carboxyl groups on a protein, when both types of

groups are present in significant amounts, could be demonstrated with papain. Papain contains almost equal numbers of carboxyl and amino groups (14 free carboxyl and 10 ϵ -amino groups; Drenth *et al.*, 1970) and most of the carboxyl and amino groups on the enzyme are available for chemical modification. Further, since papain contains 19 tyrosine residues the enzyme could be bound to diazotized polyaminoaryl-nylon via azo linkages involving the tyrosine phenol ring. Immobilized papain-nylon derivatives were therefore prepared by three different procedures. (1) Papain was coupled to polyisocyanide-nylon in the presence of acetaldehyde and an excess of acetate, to effect binding via the protein amino groups. The material was called papain-nylon A. (2) Papain was coupled to polyisocyanide-nylon in the presence of acetaldehyde and Tris, to effect binding through carboxyl groups. This material was called papain-nylon C. (3) A polyaminoaryl-nylon conjugate of papain was obtained by coupling the enzyme to diazotized polyaminoaryl-nylon. The protein content and enzymic activity of saturated samples of papain-nylon A, papain-nylon C and papain-polyaminoaryl-nylon are given in Table 4. The amino acid composition of the three types of papain-nylon derivatives are summarized in Table 5. In hydrolysates of papain-nylon A, about five lysine residues had disappeared as compared with the native enzyme; the coupling reaction thus occurred most probably mainly via the lysine residues of the protein. In papain-nylon C, essentially stoichiometric recovery of all amino acids was observed. The recovery of all lysine residues lends support to the assumption that carboxyl groups on the protein were mainly involved in coupling to the carrier. In hydrolysates of papain-polyaminoaryl-nylon four tyrosine and one lysine residues per molecule are modified (Table 5) suggesting that the binding of polyaminoaryl-nylon is indeed mainly through azo bonds with tyrosine residues (Sokolovsky & Vallee, 1966, 1967; Goldstein *et al.*, 1970; Goldstein, 1973a).

Some properties of nylon-bound enzymes

Aqueous suspensions of the nylon derivatives of trypsin, succinyl-trypsin and papain could be stored at 4°C for 2 months without significant loss of activity. Pepsin-nylon similarly stored in 1 mM-HCl retained 40% of its initial activity. The native enzymes when stored under identical conditions lost all their activity after a few days. On freeze-drying, about 80% of the activity of the enzyme-nylon conjugates was retained. Freeze-dried trypsin-nylon powder kept at 37°C for 2 months retained about 70% of its activity. Pepsin-nylon powder kept under the same conditions retained 10% of its activity.

The activity retained by trypsin-, pepsin- and papain-nylon after incubation for 30 min at 37°C at various pH values is shown in Fig. 2. The temperature stability of the three immobilized enzymes is shown in Fig. 3. The higher stability exhibited by nylon-bound trypsin relative to the native enzyme could be attributed mainly to the prevention of auto-digestion. The pH-activity profiles of trypsin-, pepsin- and papain-nylon were distorted and displaced toward more alkaline pH values relative to the native enzymes (Fig. 4). Similar effects have been reported for many immobilized enzyme systems (Goldman *et al.*, 1971; Zaborsky, 1973; Goldstein, 1973a,b).

Concluding remarks

The potential of four-component condensations has not been fully explored; by appropriate choice

Fig. 3. Temperature stability of nylon conjugates of (a) papain, (b) pepsin and (c) trypsin

○, Native enzyme; ●, immobilized enzyme. The test samples (0.5 ml) at the pH of maximum stability were incubated at the specified temperature for 15 min; 0.1 ml portions were withdrawn and the residual activity was determined by the standard procedures.

Fig. 4. pH-activity profiles for nylon conjugates of (a) papain, (b) pepsin and (c) trypsin

For details see the Materials and Methods section.

of aldehyde and isocyanide, a wide range of nylon derivatives of different physical properties and with different functional groups could be synthesized. Such modified polyamides should prove useful as supports for the immobilization of high- and low-molecular-weight ligands of biological and industrial importance. The use of such polyamide derivatives, tested in our work on nylon powder, can be extended to material formed into fibres and membranes. Moreover, the fact that the chemical selectivity of the isocyanide group can be controlled, demonstrated here for a few simple cases, suggests the possibility of directing the chemical reaction solely toward one type of functional group by changing the composition of the reaction mixture. Such an approach may serve as an alternative to the use of blocking groups, often laborious and complicated. The extension of this approach may lead to methods for the chemical modification of specific groups on proteins, where the site of the reaction is controlled by the composition of the medium.

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