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CHEMICALLY MODIFIED NYLONS AS SUPPORTS FOR ENZYME IMMOBILIZATION

III. Polyacrylamide–Nylon Grafts

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Linear polyacrylamide bearing 5 mol percent acylhydrazide functional groups was grafted on the surface of chemically modified spun-bonded nylon fabric sheets and nylon fibers. The grafting reaction involved dibromoisocyanide functional groups on the solid polyamide and some of the acylhydrazide groups on the acrylamide polymer, to form five membered heterocyles of the 1,3,4-oxadiazole type. The acylhydrazide groups on polyacrylamide–nylon could be utilized directly for the coupling of enzymes by the azide method or converted into other types of chemically reactive groups. Polyacrylamide–nylon derivatives exhibited higher protein binding capacity and higher specific activity and temperature stability of the bound protein than ungrafted nylon supports.

INTRODUCTION

Methods for the introduction of chemically reactive functional groups on the backbone of synthetic polyamides (nylons) have been described in several communications from our laboratory (1–4). By these methods isocyanide, aminoaryl, dibromoisocyanide, and acylhydrazide functional groups could be generated on the surface of nylon powders, the latter being utilized after appropriate activation for the immobilization of enzymes. The combination of a versatile chemistry of modification and mechanical strength of the support material suggested that nylon-bound biologically active molecules would be of interest for application in large-scale processes.

This communication describes the adaptation of the chemical techniques originally developed for nylon powders (1–3) to preformed nylon structures, nylon fabric sheets, and nylon fibers. These materials are well suited for utilization in continuous packed column reactors because of their good flow characteristics. The surface properties of nylon could be much improved, in terms of binding capacity and stability of bound protein, by the grafting of polyacrylamide.

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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). Subtilisim BPN′ (EC 3.4.21.14) and urease (EC 3.5.1.5) were purchased from Sigma Chemical Corporation (St. Louis, Missouri). Spun-bonded nylon fabric sheets (Cerex, Type 11; nylon 6,6; specific gravity 1.14) were a gift from the Monsanto Company (St. Louis). The mean diameter of the constituent filaments as determined under the microscope was 125 μm. Nylon 6,6 fibers, commercial textile treads (mean diameter, 90 μm) were a product of Rhone Poulenc Textiles, France. The 1,6 diisocyanohexane and hexanedioic acid dihydrazide (adipic dihydrazide) were synthesized as described previously (1,3). All other reagents, substrates, and buffer salts were of the purest grade available commercially.

Linear Polyacrylamide Bearing Acylhydrazide Groups

Linear polyacrylamide was prepared as follows: To a 20% aqueous solution of acrylamide monomer (30 ml), 10 ml of a stock solution of *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) (0.46 ml/100 ml) was added and the mixture brought to 40°. A prewarmed solution of ammonium persulfate (40 mg/30 ml) was then added and the polymerization allowed to proceed with magnetic stirring at 40° for 30 min. The viscous polymer solution was diluted with 150 ml water and added dropwise with vigorous stirring into six volumes of methanol. The polymer was separated by filtration, washed with methanol and ether, and used for the next step without further purification. The mean molecular weight ($\bar{M}n$) of the linear polyacrylamide sample was 1.7×10^5.

Controlled hydrazinolysis of linear polyacrylamide was carried out essentially as described by Inman and Dinitzis (5,6): 15 ml hydrazine hydrate (98%; Fluka, purum grade) was added to a 4% aqueous solution of polyacrylamide (50 ml), to bring the final concentration of $H_2N.NH_2$ to 5 M. The mixture was incubated at 50°, with stirring for 3 h. The polymer was precipitated by fast dropwise addition of the reaction mixture into vigorously stirred methanol (400 ml). The fine precipitate was filtered off and washed with methanol and ether. The polymer was dissolved in water (60 ml) and reprecipitated with methanol as described above. By this procedure 5% of the amide groups on the polymer sample were converted to acylhydrazide.

The polyacrylamide polymers were characterized as follows. The molecular weight of linear polyacrylamide, purified by reprecipitation with methanol, was estimated viscosimetrically at 25° using the relation $[\eta] =$

$6.8 \times 10^{-4} (\bar{M}n)^{0.66}$ (7). The acylhydrazide content (degree of substitution) of the acrylamide polymers following controlled hydrazinolysis was estimated by a modification of the 2,4,6-trinitrobenzene sulfonate (TNBS) method (5,8): 1 ml of the aqueous polyacrylamide hydrazide test solution (0.25 mg/ml; 0.01–0.2 μmol acylhydrazide) was added to a solution made of 1 ml TNBS 1×10^{-3} M and 3 ml 0.01 M phosphate buffer pH 7; NaOH (0.1 ml) was then added (final pH 12.7), and the reaction mixture incubated at room temperature for 30 min. The absorbance at 495 nm was read against the appropriate blank. A value of $\varepsilon_{495} = 7{,}653\ M^{-1}\ cm^{-1}$ was determined, using propionic hydrazide and adipic dihydrazide as standards.

Chemical Modification of Spun-Bonded Nylon Fabric Sheets

Disks of 30 mm diameter were cut from spun-bonded nylon fabric sheets (Cerex, Monsanto Company) and packed in a glass column (height, 50 mm; internal diameter, 30 mm) fitted with a fritted glass disk at the bottom and a ground glass adaptor at the top. Constant packing of the disks was maintained by means of an additional fritted glass disk, held in place by Teflon rings of the appropriate thickness. By this arrangement all chemical modification reactions as well as the coupling of enzymes could be carried out consecutively, by circulating the appropriate reagents through the column.

Isocyanide–Nylon Derivatives. Isocyanide functional groups were introduced on the polyamide backbone of nylon by (a) controlled acid hydrolysis, to generate neighboring COOH... NH_2 pairs, (b) resealing of the carboxyl–amine pairs by a four-component condensation reaction in the presence of 1,6 diisocyanohexane and analdehyde as described by Goldstein et al. (1). Hydrolysis was carried out as follows: 3 N HCl (100 ml) was perfused through a column containing 100 disks (about 6 g net, Cerex) to remove impurities. 3 N HCl was then circulated for 4 h at room temperature at a rate of 12–15 ml/min. The column was washed by perfusing water (300 ml), methanol (100 ml), and ether (100 ml). The second step was carried out by circulating a solution of 1,6 diisocyanohexane (3.3 ml) and isobutyral (13.2 ml) in isopropanol (33 ml) at a rate of 0.5–1 ml/min at room temperature, overnight. The column was then washed with methanol (100 ml) and ether (200 ml).

Dibromoisocyanide–Nylon Derivatives. These were prepared by an adaptation of the method of Freeman et al. (3). An ice-cooled 0.1% solution of bromine in chloroform (100 ml) was perfused through a column packed with isocyanide–nylon disks, in the course of 30 min. To remove unreacted bromine the column was washed with the following ice-cold solutions: chloroform (30 ml), 2% triethylamine in chloroform (25 ml), chloroform

(30 ml), and ether (50 ml). Due to the high reactivity of the —$NCBr_2$ group, the dibromoisocyanide–nylon discs were used immediately for the next step.

Acylhydrazide–Nylon Derivatives (3). An ice-cooled 0.05 M solution of adipic dihydrazide in 0.01 M *N*-ethylmorpholine buffer, pH 8 (50 ml), was perfused through a column packed with dibromoisocyanide–nylon disks. The effluent was collected, the pH brought back to 8, and the reagent solution recirculated through the column for 1 h at a rate of 5–10 ml/min with cooling. The column was washed with water (300 ml), methanol (100 ml), and ether (50 ml).

Aminoaryl–Nylon Derivatives. These were prepared from isocyanide–nylon utilizing the four-component reaction previously described (1, 3). Through a column packed with isocyanide–nylon disks a methanolic solution (40 ml) containing 4,4′-diaminodiphenyl-methane (400 mg; 2 mmol), isobutyral (0.1 ml; 1 mmol), and acetic acid (0.2 ml; 3.5 mmol) was circulated at room temperature for 18 h at a rate of 0.5–1 ml/min. The column was washed with dimethylformanide (100 ml), methanol (200 ml), and ether (100 ml).

Polyacrylamide–Nylon Acylhydrazide Derivative. An ice-cooled solution of polyacrylamide hydrazide (5 mg/ml; acylhydrazide content about 5%) in 0.01 M *N*-ethylmorpholine buffer, pH 8 (40 ml), was perfused through a column packed with dibromoisocyanide–nylon disks.The effluent was collected, the pH brought back to 8, and the cooled polyacrylamide solution recirculated through the column for 2 h at a rate of 12 ml/min. The column was washed with water (300 ml), methanol (50 ml), and ether (50 ml).

Polyacrylamide–Nylon Aminoaryl Derivative. The aminoaryl derivative of PAA–nylon was prepared from the corresponding acylhydrazide derivative via the acylazide method.

An ice-cooled 1% solution of sodium nitrite in 0.1 M HCl (50 ml) was perfused through a column packed with polyacrylamide–nylon acylhydrazide disks at a rate of 2–3 ml/min. The activated column was washed with cold water (100 ml) and reacted immediately with a 4,4-diaminodiphenyl methane solution (50 ml) circulated through the column at 4° for 18 h. The reagent solution was prepared by dissolving 0.5 g. 4,4′-diaminodiphenyl methane in 25 ml dimethylformamide, followed by the addition of 25 ml water with stirring, the pH being adjusted to 9.0. The column was washed with dimethylformamide (100 ml), water (200 ml), methanol (100 ml), and ether (50 ml).

Chemical Modification of Nylon Fibers

Commercial nylon 6,6 fibers (3 g overall weight) were packed in a glass tube (6 mm internal diameter, 40 cm length), in parallel to the tube's axis.

The fibers were washed by perfusing 300 ml ether in the course of 2 h to remove lubricants. All further manipulations were carried out as described for nylon fabric disks.

Coupling of Enzymes to Acylhydrazide Derivatives of Nylon and Polyacrylamide–Nylon Disks

An ice-cooled 1% solution of sodium nitrite in 0.1 N HCl (100 ml) was prefused through a column packed with nylon- or polyacrylamide–nylon–acylhydrazide disks at a rate of 2–3 ml/min. The activated column was washed with cold water (100 ml). An enzyme solution (5 mg/ml; 40 ml) in 0.1 M *N*-ethylmorpholine buffer, pH 9.0, was then circulated through the column at 4° for 18 h. The column was washed with cold water (500 ml), 1 M KCl (200 ml), and water (100 ml). The enzyme–nylon disks were stored under water at 4°.

Coupling of Enzymes to Aminoaryl Derivatives of Nylon and Polyacrylamide–Nylon Disks

A column packed with aminoaryl–, nylon, or polyacrylamide–nylon disks was activated by circulating 1% sodium nitrite as described above. The activated column was washed with cold water (100 ml). An enzyme solution (5 mg/ml; 40 ml) in 0.1 M phosphate buffer, pH 8, was circulated through the column at 4° for 18 h. The enzyme–nylon disks were washed and stored as described above.

Analytical and Assay Methods

Bound protein was estimated by the Lowry method (9, 10) and confirmed by total amino-acid analysis of acid hydrolyzates of the appropriate enzyme–nylon conjugate as previously described (1–3). The enzymic activities of the trypsin–chymotrypsin, subtilisin BPN′–, and papain–nylon conjugates were determined at 25° by the pH-stat method, essentially as described in the preceding communications (1–3). The enzyme–nylon disks or fibers were cut into small pieces to ensure efficient mixing.

The enzymic activity of urease–nylon was determined by the nitroprusside method according to Chaney and Marbach (11, 12) using a whole urease–nylon disk. Continuous enzyme-column assays are described elsewhere (13).

The number of lysyl residues on the protein involved in amide bond formation with the polymeric acylazide supports was determined by amino-acid analysis of acid hydrolyzates of the appropriate immobilized enzyme samples, previously deaminated with nitrous acid, by a modification of the Van Slyke procedure (14–16).

RESULTS AND DISCUSSION

Chemically modified nylons in the form of fine powders (of mean diameter about 1 μm) are well suited for the covalent fixation of enzymes, due to the favorable area-to-weight ratio of these particulate supports. The use of such powders for large-scale conversions in packed-bed reactors is limited by the high resistance to flow exhibited by the latter. Nylon structures of low hydrodynamic resistance are available commercially, however. Two types of such nylon products, spun-bonded nylon fabric sheets (Cerex, Monsanto) and nylon fibers, were chosen for this investigation.

Spun-bonded nylon is a nonwoven nylon fabric manufactured by spinning continuous filaments of nylon 6,6 in a random web formation and bonding the filaments at the crossover points without adhesives. The material has high mechanical strength. The web structure of spun-bonded nylon allows high flow rates accompanied by low pressure drops. The stirred-batch methods employed with nylon powders (1–3) could therefore easily be converted to continuous procedures. All chemical modification steps (in organic solvents) as well as the coupling of enzymes (in aqueous media) were performed consecutively by circulating or perfusing the appropriate reagent solutions through a column packed with nylon fabric disks. A similar technique was adopted for the modification of nylon fibers. Acylhydrazide and aminoaryl functional groups were introduced on spun-bonded nylon fabric disks and nylon fibers as described in the experimental section. These derivatives, following activation with nitrous acid, were used for the immobilization of several enzymes.

The amount of bound protein per unit weight of nylon fabric was low as compared with that of similarly derivatized nylon powders; e.g., 10–30 mg protein was bound per gram of acylhydrazide–nylon powder (3) as compared with 0.2–0.4 mg protein per gram of acylhydrazide–nylon fabric (Table 1).

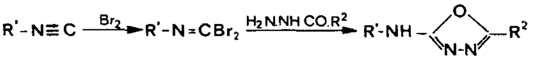
The difference in binding capacity that accompanies the change in support geometry from fine spherical particles (mean diameter, 1 μm) to fibrous filaments of mean diameter 125 μm can be related to the decrease in available surface area. Rough calculation shows that the estimated difference in surface area, two orders of magnitude, is in reasonably good agreement with the experimentally determined difference in protein-binding capacity (see Table 1).

TABLE 1. **Binding of Enzymes to Nylon Fabric Sheets**[a]

Enzyme	Type of support	Total bound protein (mg/g)	Active bound protein	
			mg/g	% of total
A. Acylhydrazide Derivatives				
Trypsin	Nylon	0.29	0.10	35
	PAA–nylon	1.56	0.72	46
Chymotrypsin	Nylon	0.24	0.09	37
	PAA-nylon	1.10	0.54	49
Subtilisin BPN′	Nylon	0.16	0.04	26
	PAA-nylon	0.48	0.25	53
Papain	Nylon	0.35	0,15	43
	PAA-nylon	2.64	2.16	82
B. Aminoaryl Derivatives				
Trypsin	Nylon	0.38	0.14	36
	PAA–nylon	0.50	0.38	77
Papain	Nylon	0.52	0.21	40
	PAA–nylon	2.16	1.53	71
Urease	Nylon	1.00	0.82	82
	PAA–nylon	8.16	7.33	90

[a] Coupling of enzyme was carried out by circulating 40 ml of a cold enzyme solution (5 mg/ml) through a column loaded with nylon or polyacrylamide–nylon fabric disks (for details see Materials and Methods). PAA–nylon, polyacrylamide–nylon graft.

The space available for the random close packing of spheres around a fibrous filament could in principle be enlarged by growing polymeric side chains out of the surface. This would allow three-dimensional packing. Increase of the protein-binding capacity of the nylon fabric through the grafting of linear polyacrylamide was attempted. The grafting procedure was based on the reaction between dibromoisocyanides and acylhydrazides to form five-membered heterocycles of the 1,3,4-oxadiazole type (17–19). Eq. 1. High-molecular-weight linear polyacrylamide ($\bar{M}n = 170{,}000$) in



which 5% of the amide groups were converted to acylhydrazide by controlled hydrazinolysis (5, 6) was reacted with the dibromoisocyanide derivative of the nylon fabric (see reference 3). The new polyacrylamide–nylon multi-functional support containing acylhydrazide functional groups (Fig. 1) could be used to immobilize enzymes by the azide method. Alternatively, it could be converted to the arylamino derivative, for the coupling of enzymes via azo bonds (see Materials and Methods).

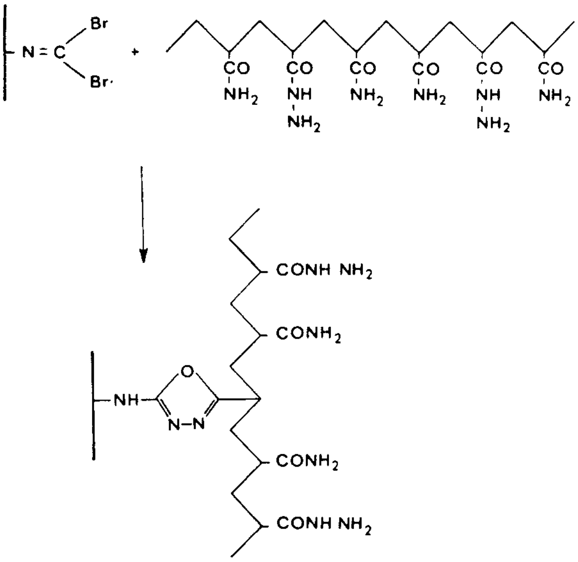


FIG. 1. Grafting of linear polyacrylamide-bearing acylhydrazide functional groups on a dibromoisocyanide derivative of nylon.

Table 1 summarizes data on the coupling of several enzymes to polyacrylamide–nylon and ungrafted nylon disks carrying acylhydrazide or aminoaryl functional groups. The data of Table 1 show that for most cases investigated, both the protein-binding capacity and the specific activity of immobilized protein were considerably higher for the polyacrylamide–nylon grafts than for the ungrafted nylon fabric supports. Similar results were obtained with fibers.

Table 2 summarizes the enrichment factors (calculated from the data of Table 1) for bound protein, specific activity, and total activity of polyacrylamide–nylon enzyme conjugates relative to the corresponding ungrafted nylon derivatives. Table 2 shows that the fivefold to fifteenfold increase in total immobilized enzymic activity observed for the polyacrylamide–nylon conjugates stems from the combination of two factors—increase in protein-binding capacity and higher retention of biological activity by the bound protein. In addition, enzymes bound to polyacrylamide–nylon also exhibited enhanced thermal stabilities. This is illustrated in Fig. 2 for chymotrypsin and subtilisin BPN′.

The increase in the protein-binding capacity of a nylon fabric following grafting of linear polyacrylamide could be attributed to the transition from

TABLE 2. **Enrichment Factors for Total Protein and Specific Activity of Enzymes Bound to Polyacrylamide–Nylon Fabric Sheets**[a]

Enzyme	Coupling method	Total protein (A)	Specific activity (B)	Total activity[b] (C)
Trypsin	acylazide	5.44	1.31	7.12
	diazo	1.29	2.13	2.75
Papain	acylazide	7.54	1.92	14.5
	diazo	4.12	1.77	7.31
Chymotrypsin	acylazide	4.59	1.33	6.12
	diazo	—	—	—
Subtilisin BPN′	acylazide	2.96	2.00	5.86
	diazo	—	—	—
Urease	acylazide	—	—	—
	diazo	8.16	1.10	8.98

[a] Enrichment factors calculated from data of Table I: (value for polyacrylamide–nylon enzyme conjugate)/(value for ungrafted nylon–enzyme conjugate).
[b] Calculated from C = A × B.

two-dimensional packing of protein molecules (essentially in a monolayer) to three-dimensional close packing within the volume encompassed by the polyacrylamide side chains. This effect can be estimated as follows: For nylon 6,6 filaments of mean diameter 125 μm and specific gravity 1.14 g/cm^3 the available area per gram of fabric is about 2.8×10^2 cm^2. This area can accommodate 2.2×10^{15} spherical trypsin molecules [mean diameter, 19.2 Å (20)] arranged in hexagonal two dimensional close packing (for this array, the fraction of surface occupied by spheres is 0.91; see references 21–24).

The space around a nylon filament, enclosed by linear polyacrylamide of molecular weight 170,000 and mean end-to-end distance $\langle \bar{R}^2 \rangle^{1/2} = 1.43 \times 10^{-6}$ cm, is about 4×10^{-4} cm^3 per gram of nylon fabric ($\langle \bar{R}^2 \rangle^{1/2} = 2\sqrt{N}l$; where N is the degree of polymerization and l is the length of monomer unit, 1.53 Å; see references 25, 26). For three-dimensional hexagonal close packing, this volume can accommodate 1.4×10^{16} spherical trypsin molecules (volume fraction occupied by spheres 0.74; see references 21–24). The ratio of the two values, 6:1, is in good agreement with the experimentally determined enrichment factors for total bound protein (Table 2).

Two observations lend support to the model described above: (a) The number of acylhydrazide functional groups as estimated from glycineamide-binding experiments was essentially the same (about 3 μmol/g support) for both polyacrylamide–nylon and ungrafted nylon fabric; (b) out of the 14

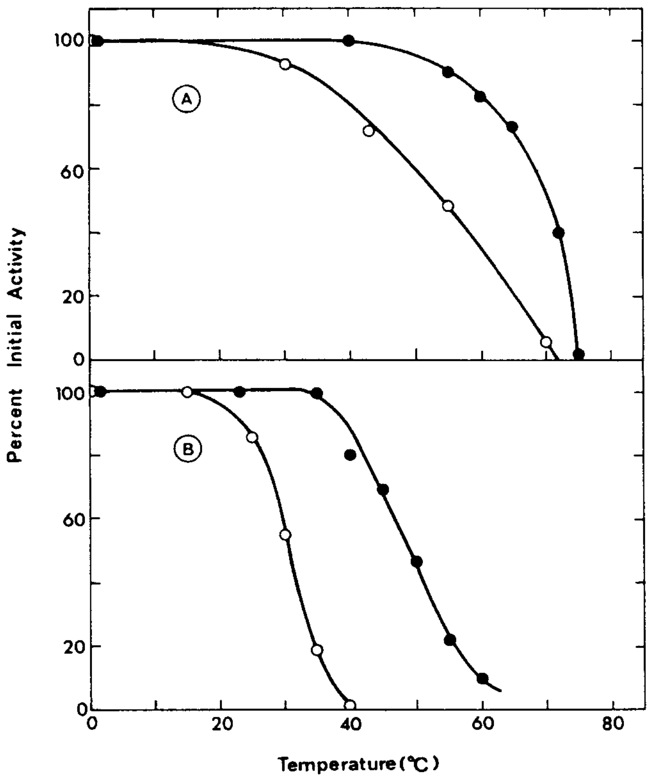


FIG. 2. Temperature stability of immobilized derivatives of subtilisin BPN′ (A) and chymotrypsin (B). Open circles denote enzyme bound to ungrafted nylon. Filled circles denote enzyme bound to polyacrylamide–nylon graft. The test sample, containing the appropriate immobilized enzyme derivative (about 1.5 esterase units) suspended in distilled water, was incubated for 15 min at the specified temperature and the residual activity determined by the standard procedures at 25°.

lysyl residues of native trypsin, nine lysyl residues participated in amide bond formation with the acylazide derivative of ungrafted nylon fabric, while only four lysyl residues participated in peptide bond formation with polyacrylamide–nylon.

These findings are in accord with the view that the higher protein binding capacity of polyacrylamide–nylon can be related to an increase in available space rather than an increase in the number of chemically reactive functional groups.

The enhanced thermal stability and higher specific activity recorded for enzymes bound to polyacrylamide–nylon grafts can be ascribed to the highly hydrophilic nature of polyacrylamide.

All these effects, taken together with the good flow properties of the starting material, fabric or fiber, suggest that polyacrylamide-grafted filamentous nylon structures might serve as supports suitable for the immobilization of enzymes aimed at large-scale continuous processes.

REFERENCES

1. Goldstein, L., Freeman, A., and Sokolovsky, M. (1974) *Biochem. J.* 143 : 497.
2. Goldstein, L., Freeman, A., and Sokolovsky, M. (1974) "Derivatized Nylon: A New Support for the Immobilization of Enzymes." *In* Enzyme Engineering, Vol. 2, Pye, E. K., and Wingard, L. B. (eds.), Plenum Press, New York, pp. 97–104.
3. Freeman, A., Sokolovsky, M., and Goldstein, L. (1977) J. Solid-Phase Biochem. 1 : 261.
4. Goldstein, L., Freeman, A., Blassberger, D., Granot, R., and Sokolovsky, M. (1977) "Chemically Modified Polymers Containing Isocyanide Functional Groups as Supports for Enzyme Immobilization." *In* Proceedings Symposium on Biotechnological Applications of Proteins and Enzymes, Bohak, Z., and Sharon, N. (eds.), Academic Press, New York, in press.
5. Inman, J. K., and Dintzis, H. M. (1969) Biochemistry 8 : 4074.
6. Inman, J. K. (1974) Methods Enzymol. 34 : 30.
7. Collinson, E., Dainton, F. S., and McNaughton, G. S. (1957) Trans. Faraday Soc. 53 : 489.
8. Wilchek, M., and Miron, T. (1974) Mol. Cell. Biochem. 4 : 181
9. Lowry, D. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193 : 265.
10. Layne, E. (1957) Methods Enzymol. 3 : 447.
11. Chaney, A. L., and Marbach, E. P. (1962) Clin. Chem. 8 : 130.
12. Weatherburn, M. W. (1967) Anal Chem. 39 : 971.
13. Granot, R., Freeman, A., Sokolov, M., Segal, L., Sokolovsky, M., and Goldstein, L. In preparation.
14. Van Slyke. D. D. (1929) J. Biol. Chem. 83 : 425.
15. Archibald, R. M. (1957) Methods Enzymol. 3 : 458.
16. Emi, S., Myers, D. V., and Iacobucci, G. A. (1976) Biochim. Biophys. Acta 445 : 672.
17. Kühle, E., Anders, B., Klauke, E., Tarnow, H., and Zumach, G. (1962) Angew. Chem. Int. Ed. Engl. 1 : 8.
18. Neidlen, R., and Hanssman, W. (1967) Arch. Pharm. (Weinheim, Ger.) 300 : 180.
19. Möckel, K., and Gehlen, H. (1964) Z. Chem. 4 : 388.
20. Keil, B. (1971) "Trypsin." *In* The Enzymes, 3rd ed., Vol. III, Boyer, P. D. (ed.) Academic Press, New York, pp. 249–275.
21. Stillinger, F. H., DiMarzio, E. A., and Kornegay, R. L. (1964) J. Chem. Phys. 40 : 1564.
22. Kittel, C. (1971) Introduction to Solid State Physics, 4th Ed., John Wiley & Sons, New York.
23. Coxeter, H. S. M. (1969) Introduction to Geometry, John Wiley & Sons, New York.

24. HILBERT, D., and COHN-VOSSEN, S. (1952) Geometry and the Imagination, Chelsea, New York.
25. FLORY, P. J. (1953) Principles of Polymer Structure, Cornell University Press, Ithaca, New York.
26. CANTERINO, P. J. (1967) "Ethylene Polymers." *In* Encyclopedia of Polymer Science and Technology, (Vol. 6, MARK, H. F., and GAYLORD, N. G. (eds.), Interscience, New York, pp. 275–454.