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TECHNICAL REPORT
75-115-CEMEL

SYNTHESIS AND TESTING OF POLYMERS SUSCEPTIBLE TO DEGRADATION BY PROTEOLYTIC ENZYMES

by

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Contract DAAK-03-74-C-0231

May 1975

UNITED STATES ARMY
NATICK DEVELOPMENT CENTER
NATICK, MASSACHUSETTS 01760

Clothing, Equipment and Materials Engineering Laboratory
CEMEL-146

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: TR-75-115-CEMEL (19) (18) NDC
2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle): (6) SYNTHESIS AND TESTING OF POLYMERS SUSCEPTIBLE TO DEGRADATION BY PROTEOLYTIC ENZYMES.

5. TYPE OF REPORT & PERIOD COVERED: 1 July 1974 to 30 June 1975 FINAL REPORT

6. PERFORMING ORG. REPORT NUMBER: (14) U-136

7. AUTHOR(s): (10) J. P. BELL, S. J. HUANG, J. R. KNOX

8. CONTRACT OR GRANT NUMBER(s): (15) DAAK-03-74-C-0231

9. PERFORMING ORGANIZATION NAME AND ADDRESS: UNIVERSITY OF CONNECTICUT, STORRS, CONN. 06268 (12) 48p.

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 1T762720D048-02-013

11. CONTROLLING OFFICE NAME AND ADDRESS: US ARMY NATICK DEVELOPMENT CENTER, CLOTHING, EQUIPMENT & MATERIALS ENGINEERING LAB, ATTN: AMXNM-VMP, NATICK, MA. 01760 (11)

12. REPORT DATE: MAY 1975

13. NUMBER OF PAGES: 36

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office): (16) DA-1-T-762720-D-048

15. SECURITY CLASS. (of this report): UNCLASSIFIED

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

(9) Final rept. 1 Jul 74-30 Jun 75,

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

(17) 1-T-762720-D-04802

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

BIODETERIORATION
SYNTHESIS
PHYSICAL PROPERTIES
STRENGTH (MECHANICS)
ELONGATION
POLYMERS
FIBERS
POLYAMIDE PLASTICS
POLYESTER FIBERS
BLOCK COPOLYMERS
COPOLYMERS

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report describes synthesis, biodegradation, and property studies on new materials which have both biodegradability and good physical properties. A copolymer of polycaprolactone blocks, terminated with ethanolamine and linked by diisocyanatohexane, was biodegraded by the enzymes urease and rennin and also by two fungi. The tensile strength was greater than 10,000 psi, with high ultimate tensile elongation. Plasticized gelatin crosslinked with dilute diisocyanatohexane also has produced fibers with very high

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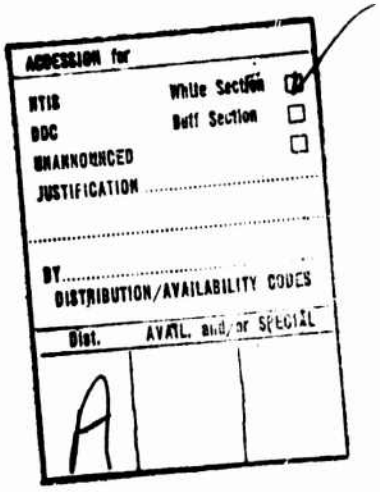
20. ABSTRACT (Continued)

biodegradability and good strength. The fibers are drawn before passing them through a bath containing the crosslinking agent. Other polymers of interest which have been found to be biodegradable include a poly(ester-urea), which is based on poly(L-phenyl-alamine), and copolymers based on mandelic acid.



FOREWORD

The investigation was begun in 1973 under the US Army Natick Laboratories' Pollution Abatement Program. The latter was established as a mission assignment from the US Army Material Command under the general guidance of Public Law 91-190 (1969) and Executive Order 11507 which established a national pollution abatement policy. The present effort was based on an unsolicited proposal from the University of Connecticut to investigate new or modified nylons and polyesters which might be susceptible to enzymatic degradation. The intent in funding the work was not only to obtain potentially biodegradable new polymers but also to provide financial support to students and thus help provide this country with manpower trained in the pollution abatement field. This report is the final report of the second year of effort and marks the completion of Contract No. DAAK-03-74-C-0231. The effort was funded under project titled "Environmental Quality Research and Development - Natick". Dr. Richard N. Macnair and Mr. John T. Stapler of the Clothing, Equipment and Materials Engineering Laboratory acted as the Project Officer and Alternate Project Officer, respectively.

The research was carried out under the direction of Professors J. P. Bell, S. J. Huang, and J. R. Knox. Students participated in the research efforts were M. Bitritto, K. W. Leong, D. Bansleben, M. Roby, N. Shoemaker, H. Atwood, K. Natarajan, W. Berghard, J. Nepumuceno and V. Malik.

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I. INTRODUCTION

The problem of disposal of plastics and synthatic fibers after use is well known. Some plastics evolve poisonous or corrosive gases during combustion, and all are very resistant to attack by microorganisms, making them almost indestructible in landfill operations and along the roadsides and trails of our country. Further, since almost all plastics and synthetic fibers are derived from petroleum or natural gas, and since our volume of plastics and fibers produced is approximately equal to the volume of metals we produce for all applications, disposal of plastics and fibers represents a significant loss of natural resources.

The biodegradable polymer research team at the University of Connecticut is conducting an interdisciplinary effort now in its second year. The three faculty members, Drs. Huang, Knox and Bell, represent the fields of polymer synthesis, polymer biodegradability, and polymer properties, respectively. There are 8-9 students involved in the program, most of whom have chosen this area for their graduate theses. The program had three overall objectives:

1) To develop polymers commercially attractive which are biodegradable by specific enzymes, such that the degradation reaction can be carried out in a controlled manner. If the biodegradation is carried out in a reactor, recycle of the monomers to form new polymers may be feasible.
2) To develop biodegradable polymers from sources other than petroleum and natural gas. Promising polymers have been produced from gelatin.
3) To train students in the field of environmental control of pollution from plastics and other polymeric materials.

Our research to date has resulted in the synthesis and preliminary testing of a number of polymers of potential interest, from which two have been chosen for concentrated development. The first, a copolymer of polycaprolactone, is susceptible to degradation by certain fungi, and has physical properties that appear from preliminary measurements to be similar to the nylons. The second polymer selected for immediate development is based on gelatin, an inexpensive protein derived from the meat industry. Here it is necessary to develop the desired mechanical properties without loss of biodegradability, and we have made substantial progress toward this goal. Both of these candidate polymers and alterations thereof are discussed in later sections of this report.

Recent literature in the field of biodegradability of synthetic polymers is mostly concerned with the problem of preventing or retarding attack on synthetic polymers and plasticizers by micro-organisms.[1-9] Potts, Clendining, Ackart, and Niegisch reported in 1973 an extensive Union Carbide Corporation study on the biodegradability of synthetic polymers by micro-organisms such as fungi and bacteria.[10] They reported that aliphatic poly(esters) were the only class of synthetic high molecular weight polymers found to be biodegradable in a study embracing a large number of synthetic polymers such as poly(esters), poly(amides), poly(ethers), and poly(olefins). Unfortunately the degradable aliphatic poly(esters) generally lack the desirable physical properties necessary for many applications. The microbial degradation of cellophane and amylose films has been studied by Carr and coworkers.[11,12]

It has been reported by several workers that low molecular weight normal paraffins and low molecular weight poly(ethylene) supported some microbial growth.[10,13-15] Efforts have been directed toward the synthesis of photodegradable polymers so that segments from photodegradation of polymers might

become biodegradable.[16-18] Attempts have also been made to improve the degradability of polymer composites by mixing biodegradable biopolymers such as starch with nondegradable synthetic polymers.[19]

Currently there are very few reported studies on truly biodegradable synthetic polymers that have the desirable physical properties necessary for the production of a wide range of biodegradable plastics, films, and fibers. Bailey and coworkers have discovered that a regular alternating copolymer of glycine and ε-aminocaproic acid (nylon 2/ nylon 6) was metabolized by micro-organism.[20] It has been reported by Gilbert and coworkers that Borax 210 (a poly(acrylonitrile) based resin) was utilized by the micro-organism Penicillium notatum.[21] Poiy(enamines) are also reported to be degradable.[22] Biodegradable polymers have been obtained from the modification of cellulose.[23] Most of these studies involved soil burial tests or culturing on agar-immersed samples. While our degradation testing includes these older methods, we have developed procedures for exposing polymer materials to concentrated enzyme solutions in order to accelerate the degradation reaction and to be able to establish the chemistry and kinetics of a single reaction in the absence of side reactions originating in the micro-organism.[24]

It became apparent from the above mentioned literature that useful biodegradable polymers will have to come from new synthetic approaches designed to incorporate biodegradable structural units into polymer chains having desirable physical properties.

The work described below is subdivided into three parts: In the first part a biodegradable copolymer of polycaprolactone is discussed. The copolymer appears to have very attractive physical properties. In the second part, the biodegradable polymers derived from gelatin are covered. The last section deals with other candidate systems which have been interesting in preliminary tests.

II. POLYCAPROLACTONE COPOLYMER

Studies previously reported[10] have shown that polycaprolactone is biodegraded by certain fungi and mixtures of fungi. Degradation was also enhanced by acid protease enzyme from Rhizopus chinesis.[25] Unfortunately, the physical properties of polycaprolactone homopolymer are not suitable in a competitive sense for most applications. The strength is relatively low and the polymer melts in the 60°-70°C range. Efforts in the present research have been directed toward copolymerization of polycaprolactone blocks with another selected monomer. Polycaprolactone is first partially hydrolyzed with ethanol amine, leaving polycaprolactone blocks of the desired length, which are terminated with OH groups. These groups are then linked together by reaction with 1,6 diisocyanatohexane. Details of the synthesis, biodegradation studies, and property and process studies follow:

A. Synthesis and Property Studies

The copolymer which contains ester, amide, and methane linkages, is synthesized by the following scheme:

Scheme 1

$$\{O(CH_2)_5CO\}_n$$

polycaprolactone

Mn 15,000

$\downarrow$ $H_2NCH_2CH_2OH$

$$HO(CH_2)_5CO\{O(CH_2)_5CO\}_nO(CH_2)_5CONHCH_2CH_2OH$$

Mn 1,500

$\downarrow$ $(OCN(CH_2)_6NCO$

$$+O(CH_2)_5CO\{O(CH_2)_5CO\}_xO(CH_2)_5CONHCH_2CH_2\text{-}OCONH(CH_2)_6NHCO+$$

Poly(caprolactone-ethanol amine-1.6-diisocyanatohexane)

Tough fibers were melt-drawn from this new polymer. The molecular weight of early material was found to be about 9000, corresponding to an initial caprolactone-amine-diisocyanate ratio of approximately 10/1/1. This was verified by elemental analysis, which gave 0.9% nitrogen. The formulation was as follows:

First stage: 11.4 gm polycaprolactone, 0.61 gm ethanol amine, 10.0 gm DMF (solvent).

The mixture was heated for 7 hrs. at 80°C. Next the cleaved polymer (pre-polymer) was cooled, and 1.68 gm 1,6-diisocyanatohexane was added.

Second stage: 1.68 gm 1,6-diisocyantohexane, [illegible] gm diethylaminoethanol, with heating at 120°C for 8 hr.

The mixture was then cooled and washed with 95% ethanol, and dried in a vacuum oven.

The formulation was next changed to use chlorobenzene as the reaction medium, and the second stage reaction time was increased to 16-18 hours. Substantial crosslinking was observed, and swelling but insolubility in benzene was found. To avoid crosslinking in later runs the second stage reaction time was adjusted to 8 hours, the temperature was adjusted to 70°C, and a nitrogen purge over the mixture was instituted. After the second stage reaction a large volume (2-3 times that of reaction mixture) was added and allowed to stand for 24 hours to eliminate any residual isocyanate groups. After 24 hours no isocyanate absorption was observed by infrared analysis. The polymer was white, and films could be pressed.

Next, triethylene diamine was tested as a catalyst for the first-stage reaction; this catalyst does not introduce OH groups, and thus it was hoped that, in the following second stage, the reaction of the diisocyanate would be with the OH groups on the ends of the polycaprolactone sequences. The

polymer from these tests was thermally stable to above 200°C, but a melting peak near 60°C indicated that crystalline polycaprolactone sequences were still present. The polymer was soluble in m-cresol and hexafluoroisopropanol. The material was white, and was pressed into films for biodegradation studies. Each batch produced approximately 10 gms of product.

In order to increase the number of linkages between the caprolactone sequences, a final formula change was made:

The following were mixed and then heated to 120°C for one hour:
30 gms polycaprolactone and 1.5 gms ethanolamine, in 25 ml redistilled THF.

The mixture was cooled below 80°C and 3 gms of diisocyanate, 0.1 gm triethylenediamine, and 100 ml chlorobenzene were added. The mixture was then heated for 10 hr at 120°C, cooled and washed with 800 ml of a mixture of acetone and ethanol.

This procedure increased the nitrogen content of the finished polymer to 1.8%, indicating that one of each nineteen caprolactone units was reacted to the diisocyanate. Only a very slight melting peak at 320-330°K, due to residual crystalline polycaprolactone, was observed.

The polymer from this procedure was extruded into a monofilament, using an Instron capillary rheometer and a variable speed windup. Below 95°C the melt seemed inhomogeneous; the extrusion temperature used was 97°C. The monofilament thus produced could be drawn by hand The ultimate percent elongation of the drawn fiber was approximately 350%, and the undrawn, 1000%. The strength of the drawn fiber was in excess of 10,000 psi, although exact figures were not obtained due to non-uniformity of the cross-sectional area.

B. Biodegradation Studies

The biodegradation studies on the polycaprolactone copolymer have been of two types: 1) degradation caused by certain fungi, and 2) degradation

caused by specific enzymes. Biodegradation of the polycaprolactone homopolymer and details of the techniques used in the present work are described in our August, 1974 report.[24] These procedures are also described in the Appendix to the present report.

1. Fungal Growth

A standard technique for testing fungal growth on polymers is provided in ASTM method D1924-61T. The procedure requires three plates: polymer, filter paper, and a control, all using non-nutrient agar devoid of utilizable carbon. The filter paper control should show heavy growth indicating fungal viability, while the blank control should show no growth on the agar medium itself. We found, however, that fungi were able to grow lightly on the non-nutrient agar blank control. This is due to agar decomposition which commonly occurs during autoclaving. Therefore we used in addition a second method of testing by which polymer strips were immersed in spore suspensions contained in test tubes (see Appendix). Four fungi were used separately. These were Aspergillus niger, Aspergillus flavus, Penicillium funiculosum, and Chaetomium globosum. The samples were incubated for four weeks, and substantial growth was observed on the polymer in dishes or tubes containing Aspergillus flavus and Penicillium funiculosum. These same fungi grew well on the homopolymer. Chaetomium globosum, which also grew on the homopolymer, did not grow on the copolymer. Aspergillus niger did not grow on either.

2. Enzymatic Degradation Studies

The polycaprolactone copolymer was subjected to various enzymes for a period of ten days. The optimal pH for each enzyme was maintained by use of standard buffer solutions, and the enzymes were replenished daily. Details of the test procedure can be found in our previous annual report[24] and in the Appendix. The rate of degradation was measured by the production of free

amino groups (via ninhydrin analysis), by observation of molecular weight change of the solid polymer remaining, and by polymer weight loss.

All three methods of measuring degradation have deficiencies. The weight loss is complicated by absorption of enzyme onto the polymer, and by mechanical losses. The ninhydrin method measures the free amino group increase in the solution, but not in the remaining polymer; that is, if the degradation is not sufficient to solubilize the polymer, no amino increase will appear in solution. Then too, the molecular weight of the remaining solid polymer may actually increase, with time of enzyme exposure, if lower molecular weight chains are selectively degraded.

In the positive sense, however, if a substantial increase in amino groups is found in the solution and if substantial molecular weight reduction of the solid is observed, degradation has occurred.

Exposure of the copolymer to papain, subtilisin, chymotrypsin, and elastase did not indicate significant degradation in terms of the criteria in the previous paragraph. Bacterial contamination of the test vials was a problem in some of the tests, but in such cases the experiment was repeated with appropriate anti-bacterial agents until reproducible results were obtained with no visual or olfactory evidence of bacteria. Results with urease enzyme did indicate significant degradation, as shown by the following tables:

Table 1. Copolymer Degradation by Urease Enzyme

Sample	Relative Viscosity	Amino Content by Ninhydrin Analysis (micromoles)
Undegraded polymer	1.142	-
Polymer and buffer	1.141	0
Polymer, buffer & enzyme	1.098	308
Buffer and enzyme	-	286

Conditions: Sample weight was 0.2gm, 2 drops of toluene was added to vials as a bacteriocide, 0.1% 0.1 M phosphate buffer, pH = 7, 0.001 M EDTA. Both relative viscosity and the ninhydrin analysis indicated significant degradation, of the order of 25% of the available degradable linkages.

The run was repeated using thymersol. 0.1 gm/100 ml, as a bacteriocide. All other conditions were the same.

Sample	Intrinsic Viscosity* (dl/gm)	Ninhydrin Analysis (micromoles)
Polymer-buffer	0.161	-
Polymer-buffer-enzyme	0.132	303
Buffer-enzyme	-	286

*In formic acid at 25°C.

Another candidate enzyme, rennin, was also tested and found to be somewhat effective. Rennin is a major product of the Aspergillus bacteria strains which we found to grow on the copolymer. The conditions and results follow:

Table 2. Copolymer Degradation by Rennin Enzyme

Sample	Polymer Molecular Weight	Ninhydrin Analysis (micromoles)
Control polymer	15250	-
Polymer-buffer	16850, 18450	0
Polymer-buffer-enzyme	15700	70
Buffer-enzyme	-	35 or 35μ moles/0.2 gm

Enzyme: Rennin 1 mg/ml, stored at pH 5 in 0.2 M citrate buffer with 0.1 M NaCl, used at pH 4 in 0.2 M citrate buffer.

Bacteriocide: A small crystal of thymol was added to each vial.

A definite increase in amino content is observed. The molecular weight change is questionable, although the lower molecular weight species may possibly be extracted by the buffer solution, giving an effectively higher polymer molecular weight.

Thus, the copolymer was actively attacked by two of the four fungi tested, and was found to be susceptible to hydrolysis by urease enzyme. Rennin enzyme also indicated promise, but further studies are needed.

III. MODIFIED GELATINS

Gelatin is an animal protein byproduct which is available in quantity at relatively low cost. It already has many commercial uses, among which is its use as the main component of photographic emuslions. In this application it is cross-linked, usually with formaldehyde or glutaraldehyde.[27-38] Our goal has been to produce a material which is cross-linked, yet pliable enough to be used as fibers, ribbons, or sheets. The polymer would at the same time be easily biodegradable, since the main component is protein. We discuss below the development of processing techniques which are being used to produce cross-linked gelatin fibers containing various plasticizers. The results of our degradation studies on these materials are also presented.

A. Gelatin Processing and Extrusion

We have succeeded in producing a cross-linked gelatin which is water-insolbule, yet free of brittleness. To obtain a pliable material it was necessary to blend plasticizers into the mix prior to extrusion and cross-linking.

Four plasticizers have been used in combination with gelatin to obtain melt spun monofilaments. The polyalcohol plasticizers (see Table 4) were introduced into the system to reduce the hygroscopicity of the gelatin by blocking reactive sites; each molecule of plasticizer solvates one or more hydrophilic amino acid residues. To obtain a homogeneous mixture, plasticizer and water are mixed thoroughly before the gelatin is added. The mixture is then heated in a hot water bath until the plasticizer diffuses into the undissolved gelatin particles. At approximately 60°C the gelatin begins to

swell, at which point stirring is stopped to avoid incorporating air into the mixture, as air bubbles would complicate later melt spinning. Extrusion of gelatin filaments, using an Instron Rheometer, was carried out at the temperature and extrusion rate necessary to obtain uniform filaments (see Figures 1-4). Depending on the plasticizer and relative amounts used, extrusion temperatures varied from 50-60°C and crosshead speeds ranged from 0.02 to 0.2 in/min.

Crosslinking of the filaments was undertaken after they had been allowed to come to equilibrium at ambient conditions. In all cases this meant a loss of some water from the system, and a corresponding rise in the softening temperature. A 3% diisocyanatohexane solution in toluene with a triethylene diamine catalyst was used for all crosslinking. The solution and filaments were kept at 60° $\pm$ 1°C throughout the reaction. Data were collected for crosslinking times of 10-50 minutes and for 17 hours. The gelatin was then placed in an ethyl alcohol bath for 10-15 minutes to deactivate the diisocyanate, and allowed to dry at ambient conditions.

An Instron Tensile Tester was used to compare the effectiveness of the crosslinking in the gelatin system, using a gage length of 1" and an elongation rate of 20% per minute. Figures 5-7 show a comparison of tensile properties of gelatin mixtures using different plasticizers. Glycerin produced a relatively weak but highly elastic fiber, whereas ethylene glycol greatly increases the modulus but gives very brittle tensile behavior. Polyethylene glycol (mol. wt. 200) incorporates the high modulus of the ethylene glycol mixture with the high elongation of the glycerin into much more acceptable tensile properties. Experiments are presently being conducted to see if triethylene glycol might reduce the excessive elongation while still maintaining the high modulus and tensile strength. All these filaments are rather

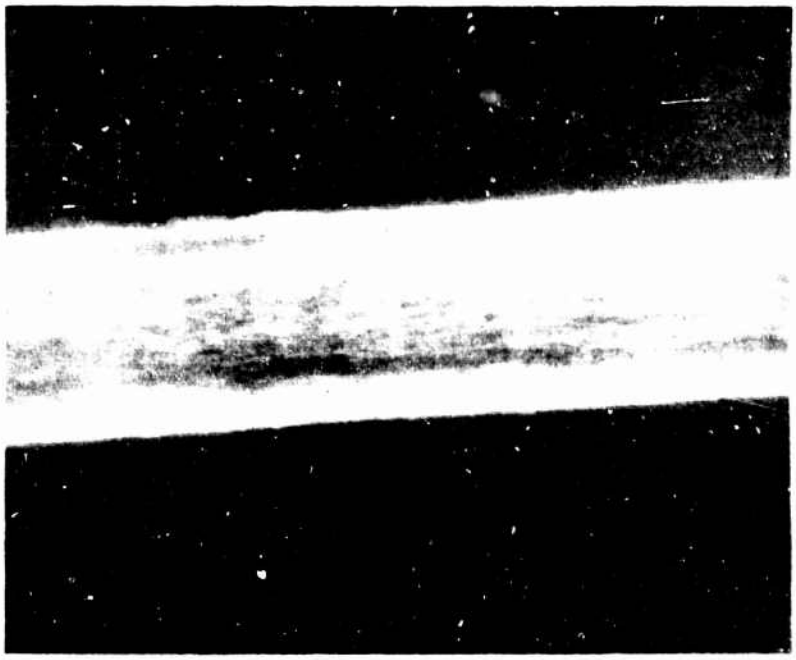


Figure 1. Crosslinked Gelatin Fiber (75x). Gelatin: Glycerin: H_2O = 20: 10: 10

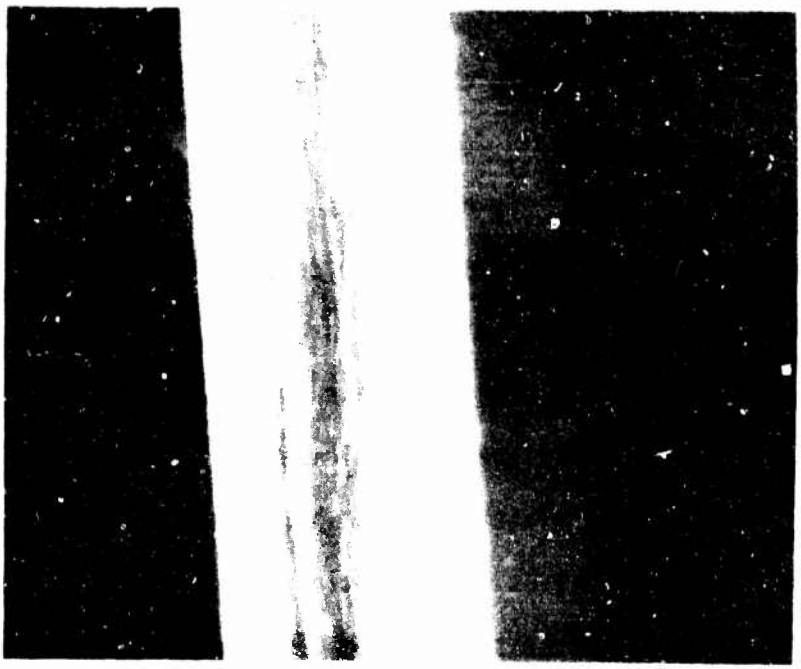


Figure 2. Crosslinked Gelatin Fiber (75x). Gelatin: Triethylene Glycol: H_2O = 20:10:10.

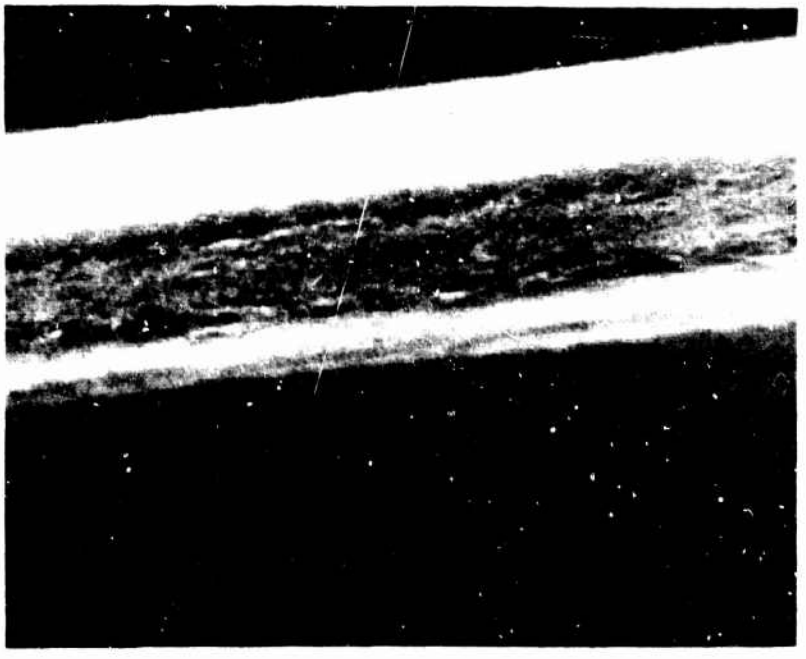


Figure 3. Crosslinked Gelatin Fiber (75x). Gelatin: Ethylene Glycol H_2O = 20:8.6:9.6.

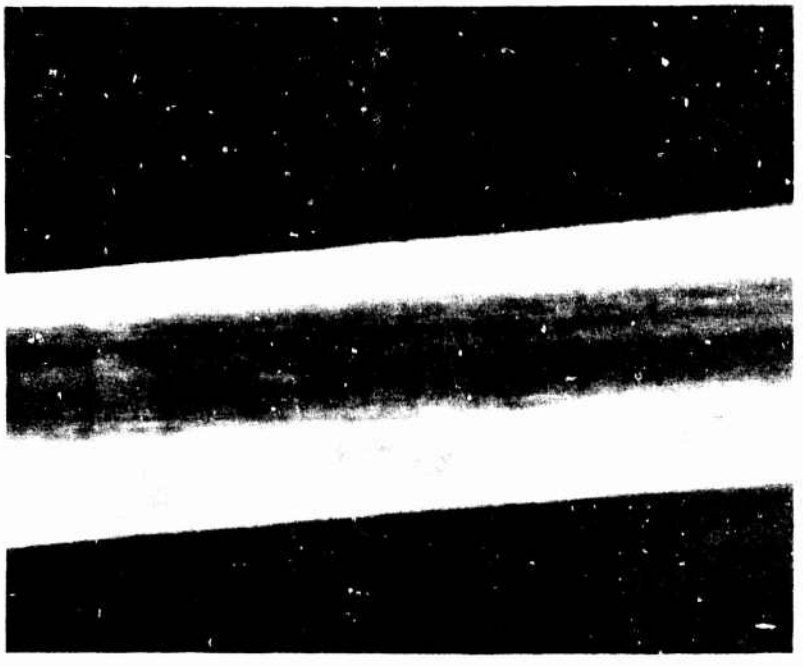


Figure 4. Crosslinked Gelatin Fiber (75x). Gelatin: Polyethylene Glycol: H_2O = 20:10:10.

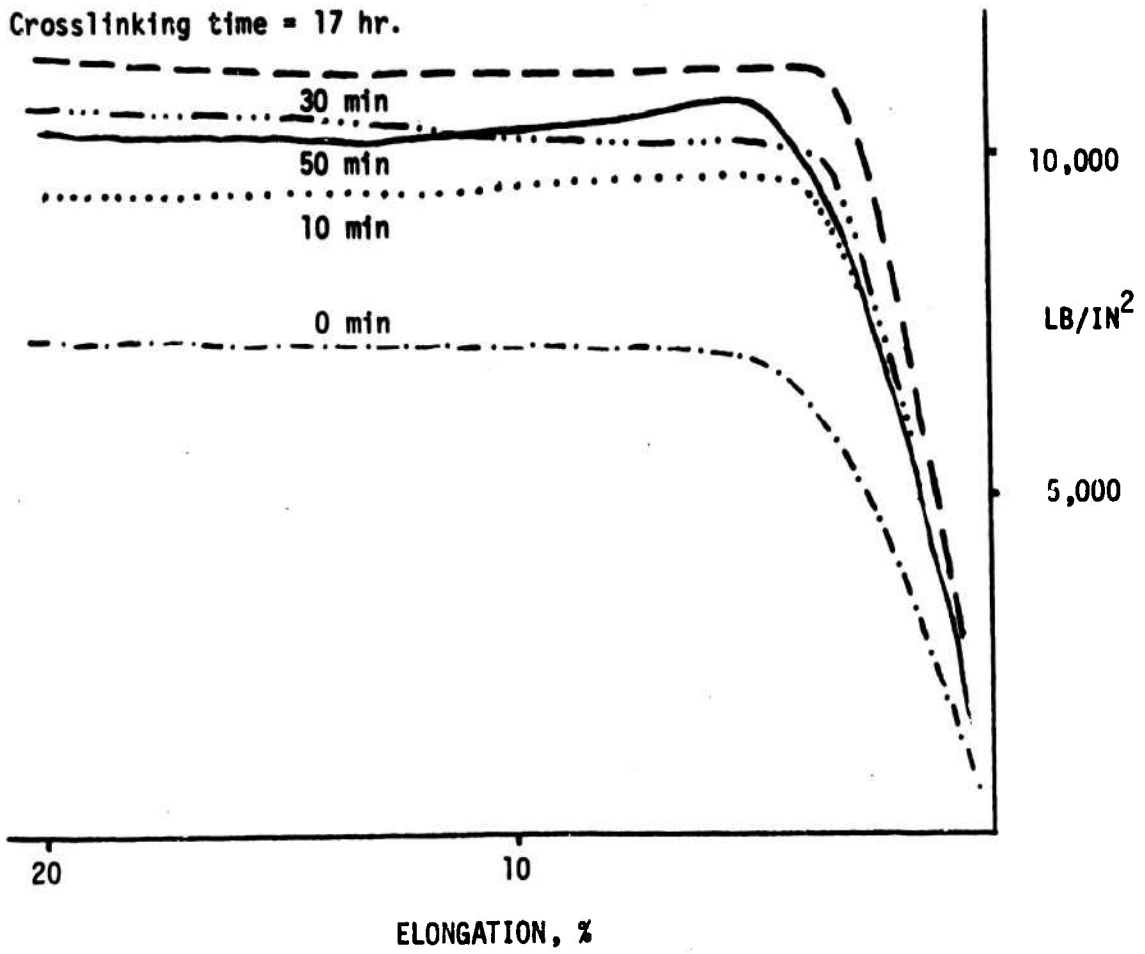


Figure 5. Stress-Strain Curves for Crosslinked Gelatin as Function of Crosslinking Time. Plasticizer: Polyethylene glycol. (MW 200).

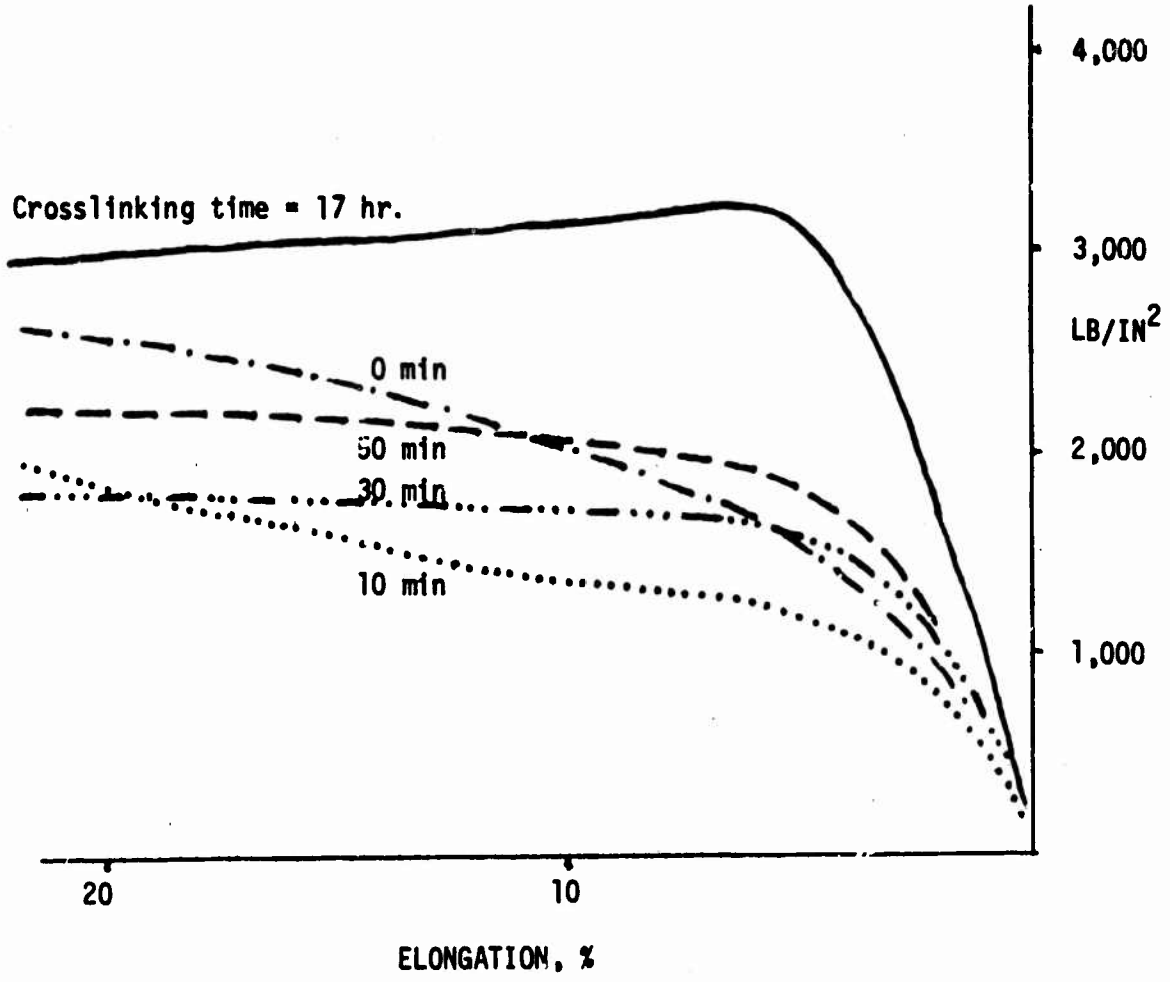


Figure 6. Stress-Strain Curves for Crosslinked Gelatin as Function of Crosslinking Time. Plasticizer: Glycerin.

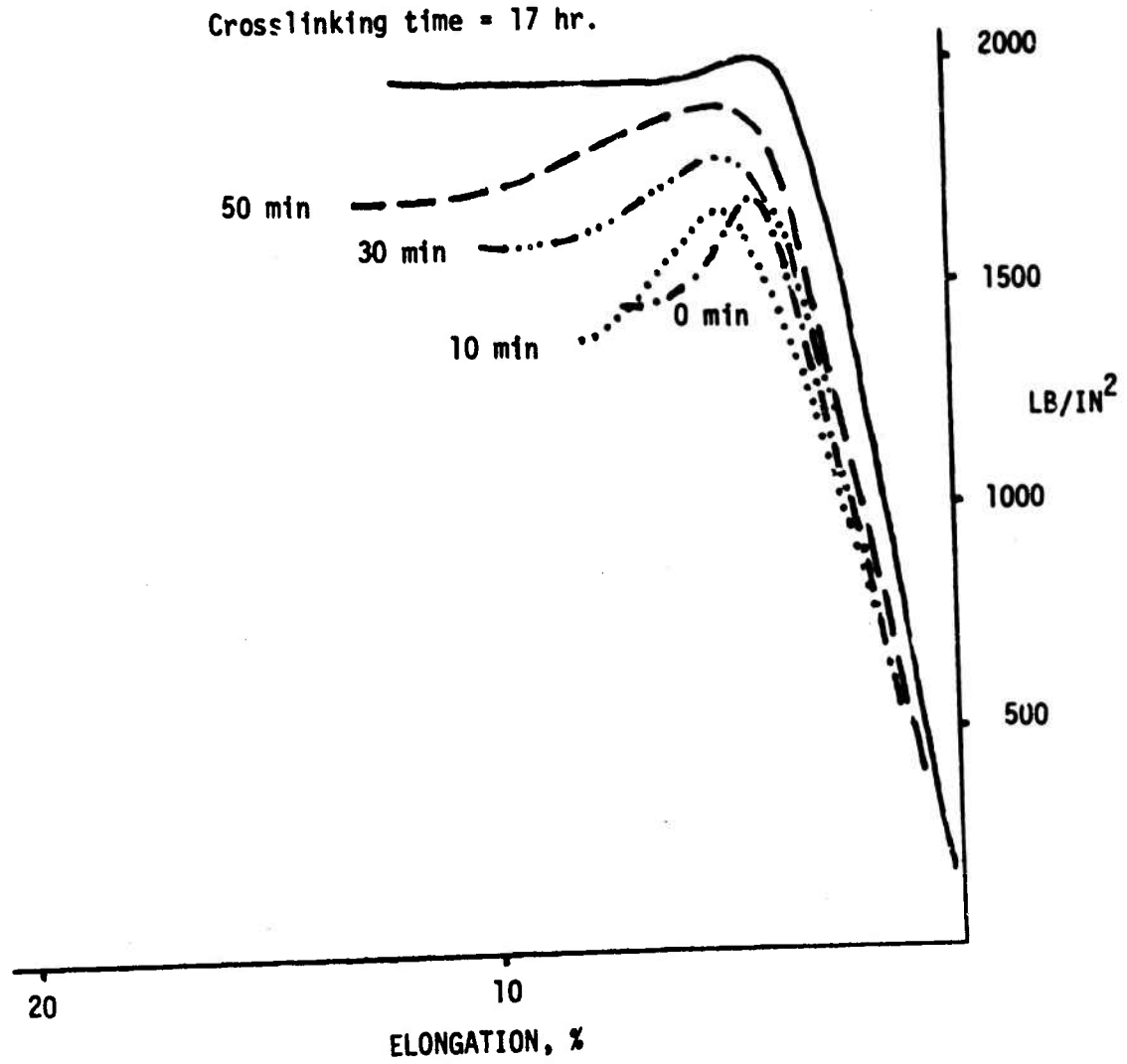


Figure 7. Stress-Strain Curves for Crosslinked Gelatin as Function of Crosslinking Time. Plasticizer: Ethylene Glycol.

sensitive to temperature and humidity changes in the laboratory. A ten degree increase in temperature and the corresponding increase in humidity reduces the tensile strength by 30%, and further study of plasticizer concentration is underway to reduce this sensitivity.

B. Gelatin Degradation Results

Tables 3 and 4 summarize the degradation of various gelatin products by selected enzymes. Procedures and analytical methods used by us for studies such as this one are given in Appendix 1. Briefly, the polymer is soaked in an aqueous enzyme solution buffered at the enzyme's optimum pH. Samples are gently agitated for 7-10 days, and the enzyme is replenished every 24-48 hours to compensate for enzyme denaturation. Degradation is analytically monitored by measuring the concentration of terminal amino groups appearing in the solution. In this way, both the kinetics and total extent of degradation may be obtained.

Undrawn bulk material was exposed to four enzyme solutions (Table 3). All materials were attacked by papain and subtilisin enzymes, of plant and bacterial origin, respectively. The lengths of the soluble fragments were estimated by the ninhydrin method to be approximately 10-15 monomer units long. The lengths of the polymer fragments in the control solution were longer in all cases than in the enzyme solution. For urease enzyme no degradation was observed, probably due to the enzyme's inability to recognize the substituted -NH-CO-NH- linkage as urea, its preferred substrate. Trypsin cleaved 2 of the 4 bulk materials. The high stereospecificity of trypsin for positively charged residues may have prevented cleavage of the other 2 materials.

Drawn gelatins were crosslinked and exposed to papain, subtilisin, and urease (Table 4). These 3 enzymes were able to solubilize all polymers. Here again, urease was not as effective in reducing chain length as were papain and subtilisin which have very broad substrate specificities.

Table 3. Degradation of Modified Gelatins: Undrawn Bulk Material

Gelatin Polymer	Enzyme Used	Length of Soluble Fragments (as number of monomer units) in Enzyme Soln	in Buffer Control
Native uncrosslinked	papain	7-9	70-80
	subtilisin	7-11	85
	urease	no degradation	
	trypsin	no degradation	
Interfacially crosslinked	papain	9-11	10-12
	subtilisin	4-6	7-9
	urease	no degradation	
	trypsin	10-12	10-12
Solid state bulk crosslinked	papain	20-22	140
	subtilisin	5-7	8-10
	urease	no degradation	
	trypsin	17-19	100
Hot-water washed bulk crosslinked	papain	7-9	550
	subtilisin	7-9	1200
	urease	no degradation	
	trypsin	no degradation	

Table 4. Degradation of Modified Gelatins: Drawn Fibers

Gelatin Fiber gel: plast: H_2O[a]	Enzyme Used	Length of Soluble Fragments (as number of monomer units) in Enzyme Soln.	in Buffer Control
Uncrosslinked	papain	5-7	6-8
20: 10: 10	subtilisin	8-10	30
polyethylene glycol	urease	66	85
Crosslinked[b]	papain	7-9	28
20: 10: 10	subtilisin	8-10	35
polyethylene glycol	urease	48	90
Uncrosslinked	papain	5-8	6-9
20: 10: 10	subtilison	7-9	30
glycerin	urease	66	72
Crosslinked	papain	5-8	32
20: 10: 10	subtilisin	7-9	35
glycerin	urease	36	68
Uncrosslinked	papain	4-6	5-7
20: 8.6: 9.6	subtilisin	6-8	30
ethylene glycol	urease	86	90
Crosslinked	papain	4-6	38
20: 8.6: 9.6	subtilisin	6-8	28
ethylene glycol	urease	50	58

[a] Ratio of gelatin: plasticizer: H_2O

[b] The crosslinking agent was 3% diisocyanatohexane in toluene, 69°C

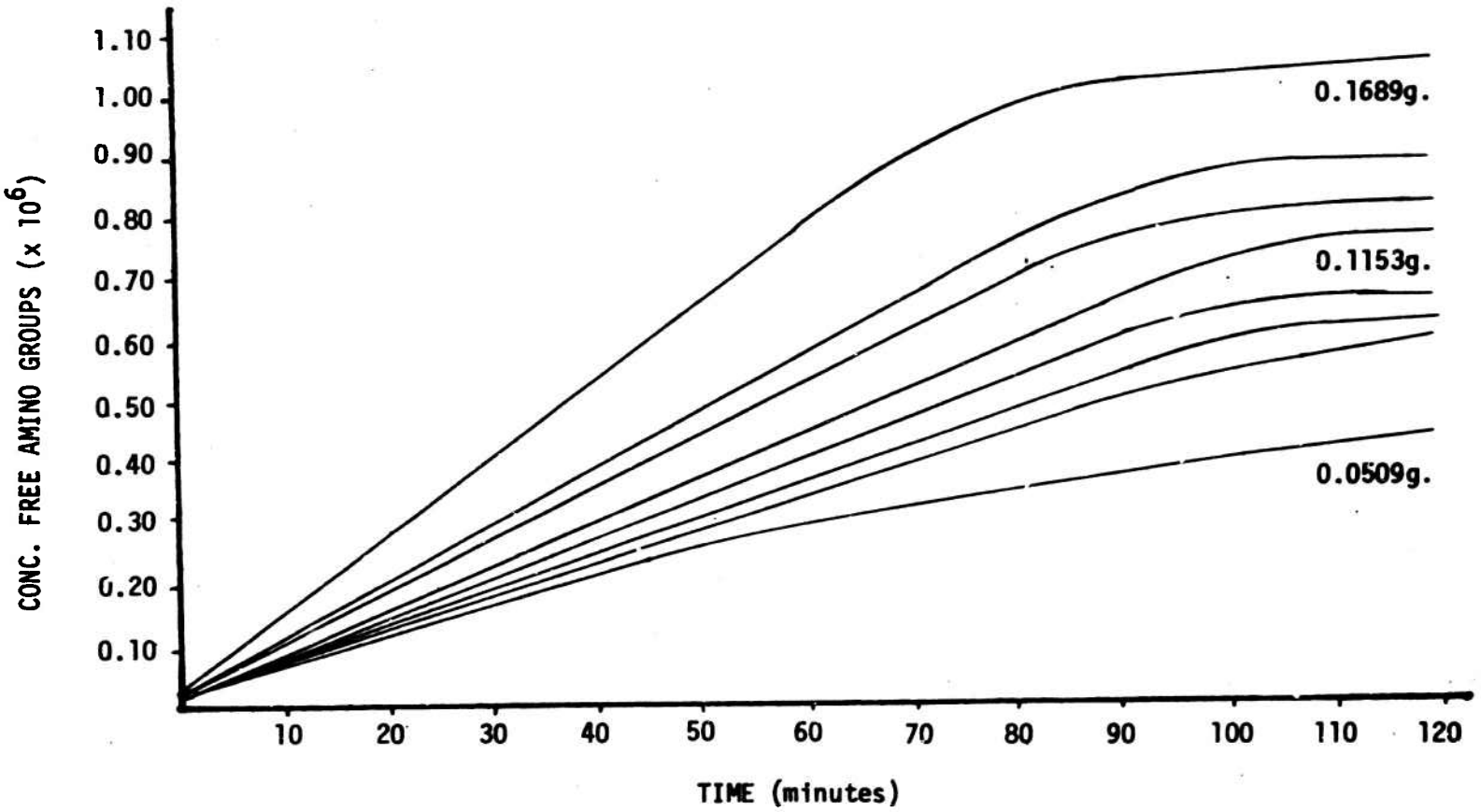


Figure 8. Degradation of Hot-Water Washed Bulk Crosslinked Gelatin by 1 mg. of Subtilisin Enzyme at pH 7.5.

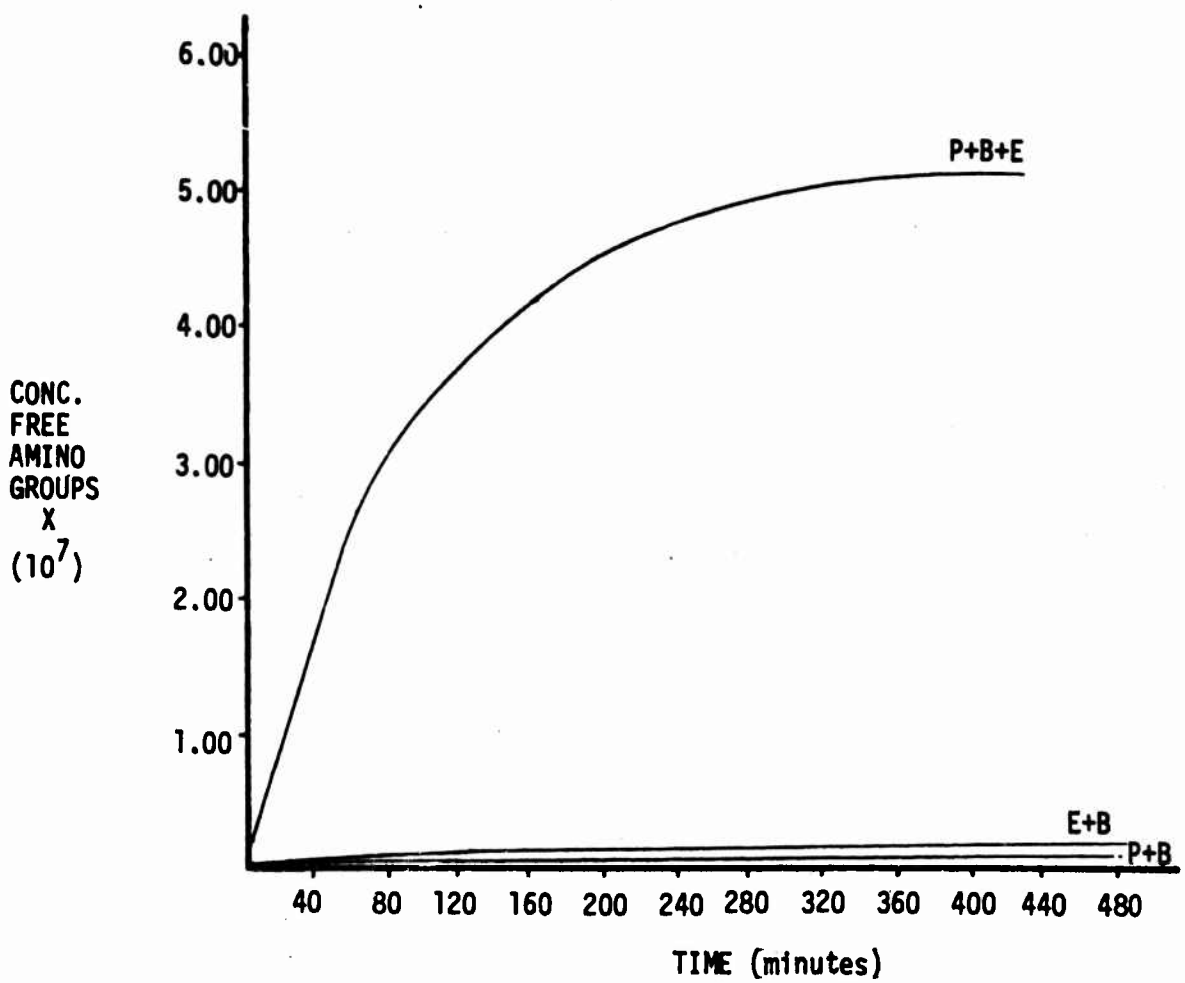


Figure 9. Degradation of Bulk Crosslinked Gelatin by Subtilisin, with Control Curves.

The kinetics of the degradation of bulk crosslinked gelatin are shown in Figures 8 and 9. It is seen that the solubilization is extremely fast even at room temperature; 1 mg of subtilisin enzyme can hydrolyse at least 170 mg of polymer within 2 hours. Limiting substrate concentration has not yet been reached, as seen in Figure 8.

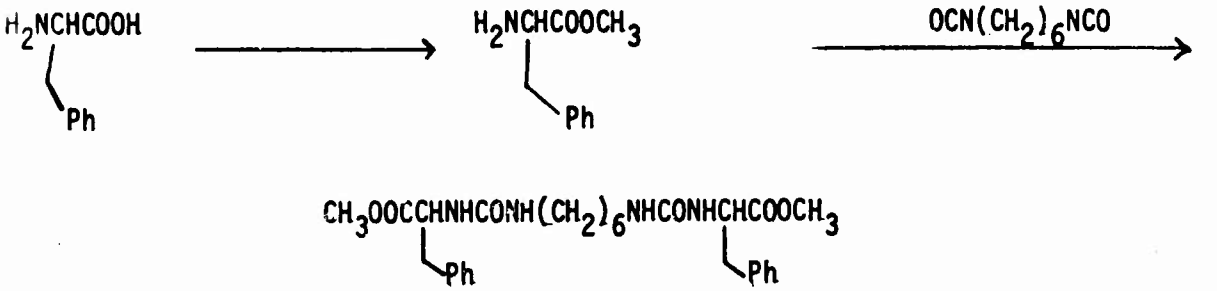
IV. OTHER POLYMERS OF INTEREST

The third area of our research has been directed toward the design, synthesis, and enzyme degradability testing of other new polymers. Since most known biopolymers can be hydrolyzed by specific enzymes normally present in living organisms, proper design of a polymer to more closely resemble the essential molecular architectural ingredients of biopolymers is expected to increase the susceptibility of the polymer to enzyme hydrolysis. Polymers degraded by enzymes would have an added advantage in that the degradation fragments are of a narrow molecular weight distribution and potentially reclaimable. Much of the specificity associated with proteases and esterases results from a preference for recognizable polar or hydrophobic groups adjacent to the amide or ester linkages at which the enzymes attack. Chymotrypsin and subtilisin enzymes preferentially attack amide and ester linkages adjacent to hydrophobic aromatic substituents. Since the structures and mechanism of the catalytic activities of these easily available enzymes are reasonably well understood we chose to design, synthesize, and test the degradability of new polymers containing phenyl group bearing acids, anticipating that these polymers will be degradable by selected enzymes. An additional advantage of using phenyl group bearing acids is that the degradation fragments can be easily detected by spectroscopic methods. Our results are discussed in the following sections.

A. Phenylalanine Containing Poly(ester-urea)

Since chymotrypsin is known to preferentially cleave proteins at a phenylalanine site and also since polyesters are known to be utilized by micro-organisms[10,39] we decided to explore the possibility of preparing a poly(ester-urea) molecule containing phenylalanine residues. We first prepared a model di-ester-diurea, C_1-EsPheUa-C_6-UaPheEs-C_1, according to Scheme 1.

Scheme 1



Di-ester-di-urea

Yield: L,L- 92%; D,L- 95%

A degradation study on the model di-ester-diurea was carried out using various enzymes. The procedure for testing has been reported previously.[24] Results of these studies are summarized in Table 5. Chymotrypsin clearly emerged as the enzyme of choice for the hydrolytic activity toward the model compound in agreement with the known specificity of the enzyme to cleavage at the phenylalanine site. It was concluded that the ester-urea combination should indeed be susceptible to degradation if incorporated into a polymer chain.

To prepare a poly(ester-urea) it was necessary to design and synthesize a suitable difunctional monomer of L-phenylalanine. Scheme 2 outlines the pathway which yielded the desired symmetrical monomer M-1.

Table 5. Enzyme Degradation Studies on Di-ester-di-urea[a]

Isomer	Enzyme Used	Buffer, pH	% weight loss[b]	Ninhydrin $-NH_2$ Group Increase[c]
D,L	chymotrypsin	imidazole, 7.8	27	d.
L,L-	chymotrypsin	imidazole, 7.8	79.9	d.
D,L-	elastase	tris, 8.8	18.3	1
L,L-	elastase	tris, 8.8	12.1	3.7
D,L-	papain	PO_4, 6.5	24.7	2.7
L,L-	papain	PO_4, 6.5	1.2	4.5
D,L-	acid protease	gly-HCl, 3.1	0.5	-
L,L-	acid protease	phthalate-HCl, 3.1	1.5	-
L,L-	pepsin	HCl, 2.2	0.0	-
L,L-	urease	PO_4, 7.0	3.4	1.8

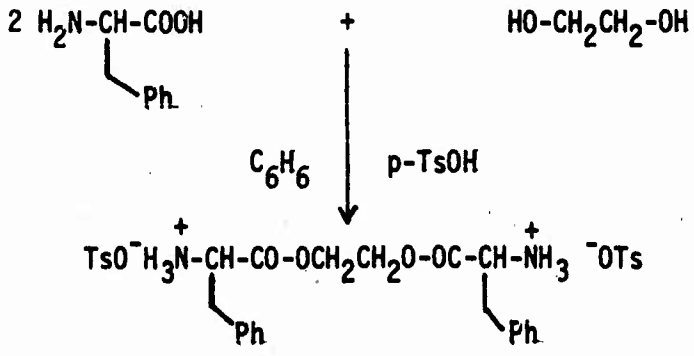
[a] Data are averages for duplicates or triplicates

[b] Weight loss differences from exposure to buffered enzyme solution compared with that of exposure to buffer only with ± 0.2% accuracy.

[c] This result is expressed as a percentage of possible new groups which could have appeared if all -CO-NH- bonds in the model compound were hydrolyzed.

[d] Enzyme shown to absorb onto solid sample surfaces, thus removing amine end groups from solution.

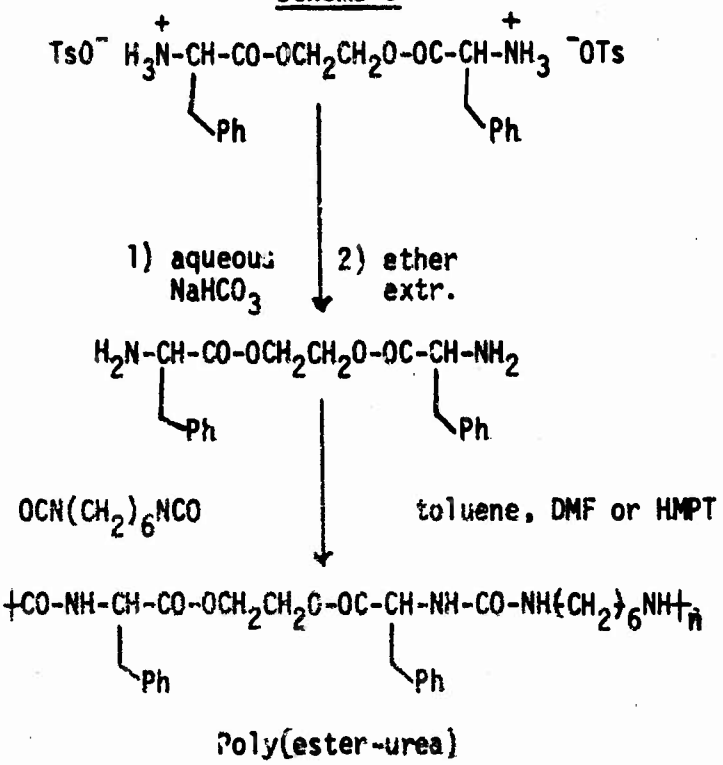
Scheme 2



It was decided to prepare a monomer with chemically equivalent reactive terminals because polymer composition would be well defined, a homopolymer would result as opposed to the possibility of obtaining a random block copolymer.

Low molecular weight poly(ester-urea), copoly(L-phenylalanine/ethylene glycol/1,6-diisocyanatohexane), was prepared from the monomer according to Scheme 3 in 73-88% yields.

Scheme 3



Poly(ester-urea)

The polymer thus prepared has $\bar{M}n$ between 1930-2640 as determined by vapor-phase osmometry, with mp 194-198°C. Substantial degradation was observed after the polymer ($\bar{M}n$ 1930) was exposed to buffered chymotrypsin at room temperature for 10 days, Table 6. A 19.3% weight loss was observed together with the detection of 23.7% phenylalanine residues in the soluble fragments. The fact that 87% of the added enzyme was found to be absorbed

Table 6. Degradation of Poly(urea-ester) by Chymotrypsin[a]

Run	1	2	3	Aver.
% Wt. loss[b]	19.2	19.4	19.2	19.3 ± 0.1
% enzyme[c] absorbed on solid polymer	85.8	87.0	88.2	87 ± 1.2
% Phe in soluble[d] fragments	23.1	22.2	24.8	23.7 ± 1.5
Increase in $-NH_2$, 10^{-4}m mol.	7.78	7.77	7.80	7.78 ± 0.3

[a] After exposure to buffered chymotrypsin at room temperature for ten days. Data corrected over controls (polymer and buffer).

[b] Weight loss differences from exposure to buffered enzyme solution compared with that of exposure to buffer only with ± 0.2% accuracy.

[c] This result is expressed as a percentage of possible new groups which could have appeared if all -CO-NH-bonds in the model compound were hydrolyzed.

[d] Enzyme shown to absorb onto solid sample surfaces, thus removing amine end groups from solution.

on the remaining insoluble polymer (which can be washed out by saline solution) provided proof that there was interaction between enzyme and polymer. The fact that little amino end group increase was observed is in agreement with the known specific acyl-cleavage activity of chymotrypsin - only ester linkages in the polymer should be hydrolyzed, generating no new amino groups.

It is interesting to compare the degradation of the polymer with that of the model di-ester-diurea, Table 7. Under the same conditions 79.9% weight loss was observed for the model compound whereas 19.3% weight loss was observed for the polymer, which contains four times as many ester linkages per molecule as the model compounds. This suggests that the polymer shows the same degradability as the model compound.

Table 7. Comparison of Chymotrypsin Degradation of L,L-Diester-diurea and L-Poly(ester-urea)

Substrate	$\bar{M}_n$	No. ester linkages	Degrad. Span (days)	% Wt. Loss[b]
L,L-4	526[a]	2	10	79.9 ± 0.2%
Polymer	1930	8	10	19.3 ± 0.1%

[a] $\bar{M}_n = \bar{M}_w$

[b] Weight loss differences from exposure to buffered enzyme solution compared with that of exposure to buffer only

The use of this poly(ester-urea) and similar polymers derived from other α-aminoacids in various applications, especially in surgery, should be explored in the future.

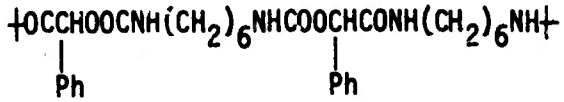
B. Benzylated Nylons

As we reported earlier[24] benzylated nylon-6,6 was found to be essentially nondegradable by most proteolytic enzymes. We have prepared benzylated nylon-6,3 (nylon-6, 3Bz) of $\bar{M}n$ 2500 from hexamethylenediamine and benzylmalonic acid by conventional interfacial and melting polymerization methods. Polymer samples exposed to buffered (at pH 7.8) chymotrypsin at room temperature for 10 days showed 6% degradation of the total amide linkages in the polymer. Efforts to prepare high molecular polymers by conventional methods were not successful due to the ease of decarboxylation of the benzylmalonic acid. In order to obtain high molecular weight polymers, methods similar to those used in peptide syntheses will have to be developed in the future.

C. Mandelic Acid Containing Poly(amide-urethane)

Aliphatic polyesters are generally degradable by micro-organisms.[10,25] Poly(glycolate) is used as sutures.[39] Polyurethanes derived from polyesters are found to be more degradable by micro-organisms than those derived from polyethers.[6] It was reasoned that homopolymer and copolymers derived from

mandelic acid (phenylglycolic acid) should be degradable by enzymes. We reported earlier[24] that poly(mandelate) is degraded at a very fast rate by the commercial detergent Axion (containing subtilisin). In order to obtain polymers of different rates for various applications we explored the possibility of preparing copolymers containing mandelic acid residues. We have prepared a poly(amide-urethane) from mandelic acid and 1,6 diisocyanatohexane with $\bar{M}n$ 7500 and mp 110-115°C.



Poly(amide-urethane)

Samples of the polymer became soluble after one day exposure to buffered (pH 8.8) elastase at room temperature. At the end of a 14-day exposure period 36% amino end group increase (by ninhydrin analysis) was observed, indicating extensive degradation. Samples of the polymers were converted into viscous gels after 14-day exposure to subtilisin (buffered at pH 8), again indicating degradation. Quantitative analyses of the subtilisin degradation were not possible because of extensive mold growth. Mold growth was much faster in the enzyme-buffer control system than in the enzyme-buffer-polymer system, indicating a strong interaction between enzyme and polymer which prevents mold from using the system protein as nutrient.

V. SUMMARY

We have prepared several new copolymers from hydroxyacids and phenylalanine and tested their degradabilities. All these new polymers are enzyme degradable. Our results show that it is feasible to design and synthesize new enzyme degradable polymers having a variety of structural

units and properties from easily available starting materials. Gelatin has been converted into degradable fibers. Future research in this area should be very fruitful.

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APPENDIX

A. DEGRADATION TESTING PROCEDURE

Although soil burial and plate culturing have been the standard methods for polymer degradation testing, we primarily use a technique involving the direct contact of polymer with concentrated enzyme solutions. This method allows us to measure the effect of a specific enzyme (usually a peptidase or esterase) on a polymer made to contain a bond especially susceptible to hydrolysis by the chosen enzyme. Because the solution is free of micro-organismic debris and complicating cellular reactions, we are able to monitor the supernatant liquid in contact with the polymer for degradation fragments. The kinetics of the breakdown can easily be determined and the reaction products can be recovered for analysis to determine where along the chain the enzyme is attacking.

1. Exposure Of Materials To Enzymes

After the synthesis and purification of a new material, a degradation study is initiated as follows: Into 24 ml corrosion resistant borosilicate glass vials is added 50-200 mg of powdered polymer (depending on the availability of the polymer). Samples are run in duplicate for each condition. The vials are filled with 14 ml of 0.2M buffer solution at the appropriate pH for the enzyme used. A bacteriocide is added to prevent contamination. Then 1 ml of enzyme solution at about 1 mg/ml concentration is added. Two controls are used for each sample: The first contains the polymer plus buffer (15 ml) and is used to determine the amount of

polymer leached out or hydrolyzed by the aqueous buffer alone. The second contains buffer (14 ml) and enzyme (1 ml) to serve as a blank for the amine end-group determination on the supernatant liquid of the treated sample.

The sample plus controls are placed on a variable speed shaker table (30 rev/min) at ambient temperature for six to ten days. During this period, 1 ml of fresh enzyme solution is added to the sample and the enzyme-buffer control every 24 to 48 hours, depending on the stability of the enzyme. Aliquots of liquid from the sample and controls are withdrawn at timed intervals in order to follow the kinetics of the release of breakdown fragments.

At the end of the last day of reaction, the remaining solids (if present) are filtered in the predried, tared, sintered glass or cellulose filters. The filtrate is saved and the remaining solid washed with distilled water. The solids are air dried for 24 hours, then oven dried for 2 hrs. at 60°C. The filters and solids are weighed and the weight loss for the sample determined. Solids are kept for viscosity and molecular weight measurement. The filtrates are analyzed for amine end groups using either a colorimetric ninhydrin assay or a titrimetric assay with acid standards.

2. Enzyme Assays

It is necessary to monitor enzyme activity as a function of time and temperature to determine how frequently enzyme must be replenished during the 10-day runs. Standard methods are used to assay activities using known substrates. We also verify that the enzymes are not inactivated by physical adsorption to our polymer samples. For example, a 6 day test with thermolysin, chymotrypsin, and subtilitin showed that the mere presence

of the polymer caused little loss in enzyme activity over the controls without polymer.

3. Analytical Procedures For Measuring Degradation

(a) Weight loss

By weighing the solid polymer before and after exposure to enzyme, a simple measure of degradation is possible. The method assumes that all degradation fragments are soluble, regardless of molecular weight. We have found, however, that the method is difficult to use because of several problems:

i) Soluble charged fragments may adsorb to cellulose or glass, thereby interfering with both weight loss and end group detection methods.

ii) Filtration rates on filter paper or on small (3 ml) glass frits are very slow, because fine pore filters must be used.

iii) Centrifugation avoids problems i and ii, and it has been used to separate solids. However, fine particles often remain suspended because of surface or density effects. It is difficult to remove all the pellet from the tube for weighing (A 15 ml centrifuge tube is too heavy for tareing against the 100 mg. sample).

iv) It is difficult to detect degradation below 10% in small 100 mg. samples, and therefore the weight-loss method is less reliable than amino end group analysis and molecular weight viscosity measurement.

For the above reasons we feel that weight loss provides only a rough and sometimes inaccurate indication of degradation, and that only large consistent differences between the samples and controls have meaning.

(b) Amino End-Group Analysis

The NH_2 groups generated by enzyme cleavage of amide, urea, or urethane bonds (NH-CO,-NH-CO-NH- or -NH-CO-O-) can be detected in filtrates or even in the remaining solids if a suitable solvent is available. Two standard procedures have been used: titration with acid and colorimetric analysis with ninhydrin reagent. Newly generated COOH groups can also be detected by titration.

i) Acid titration. Solid fragments left after enzyme degradation are solubilized in a suitable solvent and are titrated with 0.05N HCl in the presence of thymol blue indicator (pH 1.2-2.8). The amount of titrant per gram solids is converted to number of end groups per gram, and is compared with the value obtained for the original sample. This method, however, is less accurate than the ninhydrin analysis for very small concentrations.

ii) Ninhydrin analysis. This assay is very sensitive and can detect primary amino groups in the micromolar range. For this reason, it is necessary to run control measurements on the buffer-enzyme and buffer-polymer mixtures. It is often necessary to deamminate the distilled water, for which a Durrum DC-30 cation exchange column can be used.

The following analytical procedure is used for appearance or disappearance of primary amino groups: Two gm of recrystallized ninhydrin is added to 50 ml of 2-methoxyethanol. 0.08 gm of tin chloride dihydrate is added to 50 ml of 0.2M citrate buffer at pH 5.0. Just prior to use the ninhydrin solution and the tin chloride solution are mixed. Due to the instability of this solution it is made up fresh for every assay. A solution of 50% (V/V) iso-propyl alcohol in water is prepared as a diluent.

One ml of the ninhydrin solution is then added to a test tube. To this is added 0.1 ml of the solution to be tested. This volume must be measured with a micropipet or microsyringe. The resulting mixture is shaken and covered with aluminum foil caps. The tubes are then immersed in boiling water for 20 minutes. Care must be taken to suspend the tubes so that even heating is maintained. After 20 minutes the tubes are immersed in cold water and 5 ml of the alcohol diluent are added. The optical density of the samples and controls is read at 570 mμ on a spectrophotometer against a blank of distilled water treated the same way as the sample. Sample readings should be within 3% of each other.

(c) Intrinsic Viscosity

Polymer degradation has been followed by viscosity changes, which are related to molecular weight by the Mark-Houwink equation: $\eta = KM^a$. The material must be dissolved in a suitable solvent for the measurement, and the Mark-Houwink constants for a related polymer often have to be used to obtain molecular weights for the new material. For the caprolactone copolymer samples, propionic acid was found as solvent after considerable searching, and Mark-Houwink constants for commercial polycaprolactone were used.

An automatic Fica viscometer has been used for routine viscosity measurements. This instrument has a photocell to sense menicus movement and to initiate a timer accurate to milliseconds. It is equipped with a dilution device (±0.01 ml) and temperature control. To utilize this accuracy, computer programs have been written by us to analyze the data. One program is used on batch time on an IBM 360/65 computer. By adding the viscometry data to the program deck, a complete analysis is available

in minutes. This program does a least-squares curve fit of the Huggins, Kraemer, and Maron-Reznik equations. It gives the viscosity calculated from each equation together with the constants. A less-expensive CPS terminal-time program is in the PSC library of our IBM 360/65 computer. This program is rapid (5 minutes) and inexpensive ($0.22). It produces the viscosity as determined by a least-squares curve fit of the Maron-Reznik equation.

A plotting program has also been written for the Hewlett-Packard 9820 calculator-plotter which plots the data points, fits a curve to these points, and calculates the viscosity. This is done for all three viscosity equations. Bad data points can be deleted, if necessary, and the plots can be redrawn. These computer programs together with the Fica viscometer have made our polymer molecular weight analysis quick, simple, and accurate. An error analysis of the data will soon be added to the programs.

(d) Osmometry

The number average molecular weights of polymers and their degraded segments are determined, when applicable, with vapor and/or membrane osmometry. The changes in molecular weight distributions reflect the extent of degradation.

(e) Dye Release

The release of entrapped dye molecules during degradation of dyed materials can, in principle, be used to measure the rate of breakdown. Test experiments were run on nylon 6 dyed with edicol red. The enzyme used was elastase at pH 9. Final results were inconclusive because the dye easily leached out in the basic solutions. Later trials indicated congo-red in tris buffer at pH 8.4 is a better dye for basic media.

After 6 days of enzyme treatment, nagligible degradation of nylon 6 was found. Further experiments using this procedure have not been tried. The method should, however, be useful for polymers devoid of amino linkages, for which the ninhydrin end-groups analysis could not be used, and especially when small amounts of available material would not be sufficient for weight loss analysis.

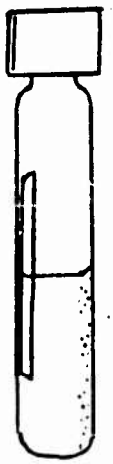
4. Fungal Growth

(a) Agar Plates

A standard technique for testing growth on polymers is provided in ASTM method D1924-61T. The procedure requires three plates: polymer, filter paper, and a control, all using non-nutrient agar devoid of utilizable carbon. The filter paper control should show heavy growth indicating fungal viability, while the blank control should show no growth on the agar medium itself. We found, however, that fungi were able to grow lightly on the non-nutrient agar blank control. This is due to agar decomposition which commonly occurs during autoclaving.

(b) Spore Suspensions

To further confirm that these modes are assimilating the polymer and not just the residual sugars of the agar, another experiment was initiated. Polymer strips, cut from polymer plates pressed by ram compression, were immersed half way into a spore suspension. No agar was used, but basal salts were in the suspension. See Diagram:



Two controls were used: a piece of sterile filter paper for a spore viability control, and a polymer strip in basal salts to show whether the salt medium alone caused degradation. Samples were done in duplicate at 300° ± 2°C.

B. PROCESSING AND PHYSICAL TESTING

Samples are provided in the form of powders by the individuals working on polymer synthesis. These powders will then be converted into specimens suitable for physical testing by either compression molding or by extrusion into a 1.8" diameter strand, cutting, and molding. Fiber formability was investigated by extrusion through a suitable spinneret. In all cases observations were made as to the ease of processing and optimum processing conditions. Tensile strength, ultimate elongation and tensile modulus were measured according to ASTM method D-638 when possible, and accepted methods were used for fibers. Melting behavior was measured by differential scanning calorimetry and crystallinity was measured by x-ray diffraction, where applicable. The tensile, melting, and crystallinity data were fed back to the polymer synthesis group to provide guidance for further improvements.