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The Inactivation of Catalytically Active Thermal Polyanhydro-α-amino Acids*

(Inactivation of Thermal Polyamino Acids)

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ABSTRACT

Thermally prepared polyanhydro-α-amino acids containing histidine catalyze the hydrolysis of p-nitrophenyl acetate. The more active preparations (histidine equivalent basis) are almost totally inactivated by heating in buffered solution. The hydrolysis of cyclic imide bonds, in which aspartic acid residues are initially bound, accompanies the inactivation by heat. Loss of activity is also observed when such imide bonds are hydrolyzed by treatment with alkali at room temperature. Related experiments, both with proteinoids (thermal polymers containing all amino acids common to protein) and compositionally simple copolymers of aspartic acid and histidine, indicate that the simultaneous presence in the same macromolecular preparation of histidine residues and imide linkages of aspartic acid residues is essential for the higher levels of activity observed.

INTRODUCTION

Polyanhydro-α-amino acids containing the eighteen amino acids common to protein (proteinoids) are prepared by heating the proper proportions of initially dry amino acids (1 and bibliography). Among the many properties that proteinoids exhibit in common with proteins is that of catalytic activity (2-10). A previous report (2) has revealed that proteinoids catalyse the hydrolysis of p-nitrophenyl acetate (NPA), that histidine residues play a key role in the catalysis, and that some proteinoids are many times as active (up to ca. 15 times) as the equivalent amount of free histidine. Results of the present investigation show that the more active proteinoids can be almost totally inactivated by heating in buffered solution. Investigation into the nature of the inactivation, through using both proteinoids and thermal copolymers of aspartic acid and histidine, has shown that the imide linkage in which aspartic acid residues are initially bound (1) is a structural feature essential for enhanced levels of activity.

MATERIALS AND METHODS

Proteinoids were prepared as described previously (2). Some were prepared and processed under aseptic conditions and were monitored on nutrient agar to show the absence of microorganisms. Thermal polymers containing only aspartic acid and histidine were prepared by mixing and firmly packing 25.0 g. of L-aspartic acid and 1.0-8.0 g. of L-histidine hydrochloride hydrate, and heating under nitrogen at 170°C for 7-9 hrs. Polyaspartic acid was similarly prepared, with histidine omitted from the reactants.

Processing was similar to that used with proteinoids (2), except that not all of the crude product was soluble in 10% sodium bicarbonate. After dialysis and lyophilisation, experiments were conducted only with the soluble fractions.

Aspartic acid residues of thermally prepared polyanhydro-α-amino acids occur predominantly in the cyclic imide form (11). Samples of some polymers were converted to the imide-free, or imide-low, form either by hydrolysis with potassium hydroxide at pH 10 with a Radiometer Titrigraph [experiments conducted by Dr. P. D. Hoagland (12) have shown that complete opening of imide bonds can be achieved by this technique], or by momentary treatment of an aqueous solution of polymer at pH 12 with sodium hydroxide (cf. 13). In either case, the solution was neutralised, dialyzed, and lyophilised. The hydrolysis of imide was verified by UV and/or IR spectra.

Sources of amino acids have been previously indicated (2). α-N-Carbobenzoxy-L-histidine was from Nutritional Biochemicals Corporation. N-Ethylmaleimide was a gift from Dr. P. D. Hoagland. p-Nitrophenyl acetate (NPA) was prepared by the method of Chattaway (14), uncorr. m.p. 76-78°C; N-ethylmaleamic acid was prepared by the method of Liwschitz et al. (15), uncorr. m.p. 124-126.5°C [lit., 123°C (15)].

The histidine contents of polymers were determined by the method of Macpherson (16), as described previously (2), or on acid hydrolysates (sealed tubes, under nitrogen, 105-110°C, 48-72 hrs.), on a Phoenix Model K-5000 amino acid analyzer, by the method of Spackman, Stein, and Moore (17). Appreciation is expressed to

Mr. C. R. Windsor for performing some of these latter analyses.

Solutions for catalytic assay, at 30.0°C, were 10^{-3} M in NPA, 4% in dioxane, in 0.067 M phosphate buffer, pH 6.8, and contained 2.0 mg. of polymer in a total volume of 5.0 ml. Initial rates (in units of μmoles of p-nitrophenol produced from 10^{-3} M NPA per min.) were obtained from slopes of linear progress curves, were corrected for the rate of spontaneous hydrolysis, and were converted to unit concn. of polymer (1.0 mg./ml.). A linear dependency of activity upon concn. of polymer employed, over the range tested, permitted the latter treatment. The activity values so obtained are referred to as k'/c. The activities of the polymers were related to that of free histidine by dividing by the k'/c value for the equivalent amount of histidine, the latter being obtained from a linear plot of activity versus histidine concn.

Thermal polyanhydro-α-amino acids dissolved in pH 6.8 phosphate buffer were inactivated by heating in sealed or covered test tubes in a boiling water bath, usually for either 15 or 20 min. After heating, catalytic assays were conducted on both the heated sample and the unheated control.

Measurements of ultraviolet spectra in the region of 190-200 mμ were made with a Beckman DK-2A recording spectrophotometer under prepurified nitrogen purge, using 1.0 cm. cells. Base lines, with buffer in both the sample and reference cells, did not deviate from zero by greater than 0.03 O.D. units. (Results of such experiments have been duplicated with similar polymers at the Ames Research Center, Moffett Field, California, using a Turner

Model 210 recording spectrofluorimeter as a spectrophotometer.) Infrared absorption spectra were obtained with a Perkin-Elmer Infracord spectrophotometer, Nujol mulls being used.

RESULTS AND DISCUSSION

The catalytic action on NPA and the inactivation by heat of one proteinoid are depicted in Fig. 1. As evaluated from the slopes of the progress curves, the heated proteinoid has little more activity than that of the spontaneous control. Heating the buffer had no effect on the rate of spontaneous hydrolysis.

In Table I are recorded results representative of more than 100 inactivation experiments. The percentage inactivation is the quantity

$$1 - \frac{k'/c, \text{ heated}}{k'/c, \text{ unheated}} \times 100,$$

and ranges, for proteinoids, from 95 to 11. In general, the more active proteinoids (histidine equivalent basis) are the ones showing greater percentages of inactivation (cf., e.g., E-1.3, L-3.4, and B-13.5). The level of activity after heating was frequently quite similar to that of the equivalent amount of free histidine. Polymers prepared and processed under aseptic conditions (K-2.8-b, K-3.4) are catalytically active and are subject to inactivation by heating their buffered solution; these results show that activity, and inactivation by heat, is not due to the presence, and subsequent denaturation, of contaminating microbial enzymes. Prior hydrolysis of imide linkages of aspartic acid residues (I-2.8-b, L-3.4) results in a lowered level of activity

TABLE I

Catalytic Activity and Heat Inactivation of Various Thermal Poly-α-amino Acids[a]

Material[b]	k'/c Unheated	k'/c Heated	Inactivation, %	Activity of Unheated Sample Relative to Histidine
Proteinoids				
A-2.8	3.00	0.14	95	14
E-1.3	0.60	0.05	92	10
B-8.0	0.72	0.42	40	1.7
E-8.0	0.88	0.70	20	1.9
B-13.5	1.10	0.98	11	1.6
I-2.8-b	0.70	0.22	69	3.9
" , imide-free[c]	0.36	——	--	——
K-2.8-b[d]	0.39	0.12	69	——
K-3.4[d]	0.54	0.34	37	——
L-3.4	0.83	0.35	58	2.4
" , imide-free[c]	0.26	0.25	4	——
F-No Basic Amino Acids	0.01	-0.01	—	——
Copolymers of Aspartic Acid and Histidine				
N-2.8	1.10	0.50	55	1.8
" , imide-low[c]	0.50	0.38	24	——
N-5.5	0.96	0.72	25	1.5
N-7.9	1.08	0.70	35	2.5
O-2.8	0.48	0.18	62	1.3
O-12.3	2.32	1.73	25	1.7
O-18	2.77	2.25	19	1.7
Polyaspartic Acid	0.00	——	—	——
Others				
α-Histidine	3.48[e]	3.45[e]	1	——
N-Carbobensoxy-α-Histidine	3.88[e]	3.85[e]	1	

[a]Conditions for catalytic assay and for inactivation were as described in the METHODS section, except that polymer A-2.8 was evaluated at pH 6.2.

[b]Identical capital letters in the polymer code indicate that the polymers were prepared at the same time. The numerical values refer to the weight % histidine, expressed as the free base, present in the reactants.

[c]Converted to the imide-free (or imide-low) form by treatment with base at room temperature.

[d]Prepared and processed under aseptic conditions.

[e]These rate constants are adjusted to unit molar concn. instead of weight concn.

which is not further reduced by heating in buffer (L-3.4). The polymer devoid of basic amino acids, but containing imide linkages, is essentially inactive, and heating in buffer does not significantly affect the observed k'/c value. (The negative value obtained for the heated sample results from correcting for the rate of spontaneous hydrolysis.)

Thermal copolymers containing only aspartic acid and histidine are catalytically active (cf. 18) and are subject to inactivation (19-62%) by heating in buffer. Partial opening of imide bonds (E-2.8) by alkaline titration also results in loss of activity, although some further loss was observed upon heating in buffer. (Infrared analysis revealed that some imide was still present prior to heating, a fact which may explain the further loss of activity.) Thermal polyaspartic acid was inactive, indicating again (2) the importance of histidine to the catalytic power. As expected, L-histidine and its carbobensoxy derivative were not inactivated by heat.

The fact that imide-free polymers were less active than were the parent polymers (Table I) led to experiments that have shown that heating in buffer, in addition to causing inactivation, results in the hydrolysis of imide bonds. Such experiments, exemplified in Fig. 2, were evaluated by the decrease in strong absorption due to imide (20-22) near 200 mμ. Opening of imide by treatment with strong base (13) results in an appreciable lowering of optical density (hypochromic effect) and also a slight shift in the wavelength of maximum absorption. Heating the polymer in pH 6.8 buffer also produces both effects. The residual absorption

is attributed to amide (peptide; 23). Similar experiments have been conducted with other thermal polymers and with monomeric imides. For example, the extinction coefficient of N-ethylmaleimide was reduced some 40% when the material was heated in buffer, and the spectrum of the heated material was superposable on that of the half-amide, N-ethylmaleamic acid; the spectrum of the latter compound was unaffected by heat. These data indicate that heat in buffered solution results in the complete hydrolysis of the imide to the half-amide. Thermal polyaspartic acid also exhibited a 40% hypochromic effect when heated in buffer; ca. 30% was noted with copolymers of aspartic acid and histidine, and ca. 10% for proteinoids. (The lower values for proteinoids are undoubtedly due in part to their lower content of aspartic acid.) Opening of imide linkages with strong base resulted in a lowered specific absorptivity, as in Fig. 1, which was not further reduced by heating in buffer. All polymers showed maximum absorption near 200 mμ, N-ethylmaleimide at 218 mμ, and N-ethylmaleamic acid at 204 mμ. Infrared analyses, using the bands at 1720 and 1780 cm^{-1} due to cyclic imide (11,19) as criteria, verified the results of the ultraviolet analyses.

Heating polymers in water resulted in smaller percentages of hypochromicity and of inactivation than did heating in buffer. These results are attributed to the lowering of pH by nascent carboxyl groups; a sufficient hydroxide ion concentration is necessary to achieve hydrolysis of imide (12).

Thermal polymers that were converted to the imide-free form by treatment with base were subsequently neutralised, dialysed (to remove NaCl), and lyophilised prior to catalytic assay. In

Table II are recorded the results of an experiment in which the dialysis and lyophilization steps were omitted, and in which activity on NPA and specific absorptivity of the parent polymer were evaluated in simultaneous comparison with those of the heated, base-treated, and base-treated-and-heated samples. The specific absorptivities indicate that either heating in buffer or treating with alkali at room temperature results in the same degree of opening of imide; either treatment is equally detrimental to the catalytic activity. Heating the base-treated (i.e., imide-free) sample did not significantly affect either property. These results, obtained with a copolymer of aspartic acid and histidine, illustrate the importance of imide, and because thermal poly-aspartic acid (containing imide but no histidine) is inactive (Table I), one may conclude that the simultaneous presence in the same macromolecular preparation of both histidine residues and imide linkages of aspartic acid residues is essential for an enhanced level of activity.

In experiments in which samples of polymer were removed from the Titrigraph after various extents of hydrolysis of imide, it was found that the degree of catalytic activity was in part directly proportional to the amount of imide present in the polymer (I-2.8-b), with some activity remaining after total opening of imide. A similar result was obtained when imide was fractionally opened by heating for 15 min. at different temperatures (30-100°C).

Possible ways by which the imide linkage may mediate its effect may include the following: The gross conformation of the

TABLE II

Activity and Specific Absorptivity of N-2.8 after Various Treatments

Material[a]	pH	Catalytic Activity[a] k'/c	Specific Absorptivity
N-2.8 (control)	6.88	1.40 (1.00)[b]	57.0 (1.00)[b]
" , heated	6.89	0.80 (0.57)	41.5 (0.73)
" , base-treated	6.88	0.90 (0.65)	41.5 (0.73)
" , base-treated-and-heated	6.88	0.84 (0.60)	40.0 (0.70)

[a]The polymer was dissolved in water, a measured aliquot was adjusted to pH 12 with NaOH, and then neutralized with HCl. NaCl was added to the control aliquot to compensate for that formed by the base-acid treatment. Measured aliquots of each solution were then diluted with buffer, and portions of each solution were heated at 100°C for 15 min. The solutions for catalytic assay were 10^{-3} M in NPA, 4% in dioxane, approximately 0.014 M in NaCl, and contained 0.44 mg. of polymer/ml. in approximately 0.1 M phosphate buffer. Further dilutions with buffer were made prior to determining the specific absorptivities.

[b]Values in parentheses are relative to the (untreated) control.

polymer may be altered upon opening of imide, which could disrupt a favorable juxtaposition of critical residues. [An apparent 20-60% increase in weight average molecular weight (24), e.g., from 3500 to 5300 for N-2.8, is consistent with this concept.] Alternatively, the hydrolysis of imide bonds would result in a change in the ionic nature of the polymer and thereby could influence the degree of activity (25,26). A further possibility is a more direct involvement of imide linkages in the catalytic process. Examples of enhanced reactivity due to the presence (or formation) of imide bonds have been reported (27-29). Authors of these and other (13,30) reports propose that the catalytic powers of some enzymes may be due in part to aspartic acid residues in imide form. Such interest in the possible contribution of imide bonds to enzymic processes may point to the use of thermal polyanhydro-α-amino acids as enzyme models, since this structure is an inherent consequence of thermal polymerizations involving aspartic acid.

The role of water in the preparation of, and inactivation of, the thermal polymers is fundamental. Thermal condensation of amino acids requires hypohydrous conditions, whereas the presence of water as a solvent for the macromolecular products obtained leads to loss of activity when those products are heated. This can now be understood on the basis that the hypohydrous conditions of synthesis favor the formation of imide bonds, whereas heat in the presence of solvent water, is conducive to the hydrolysis of imide. Whereas a change of conformation comparable to that in the denaturation of helical protein is not indicated, some change in total conformation theoretically must result from the opening of

imide bonds.

These considerations relate to proteinoids as models of primitive abiotic protein (1,3,31). These and other results on catalysis by proteinoids suggest how primitive protein-like macromolecules with inactivatable catalytic power could have been formed abiogenically from amino acids.

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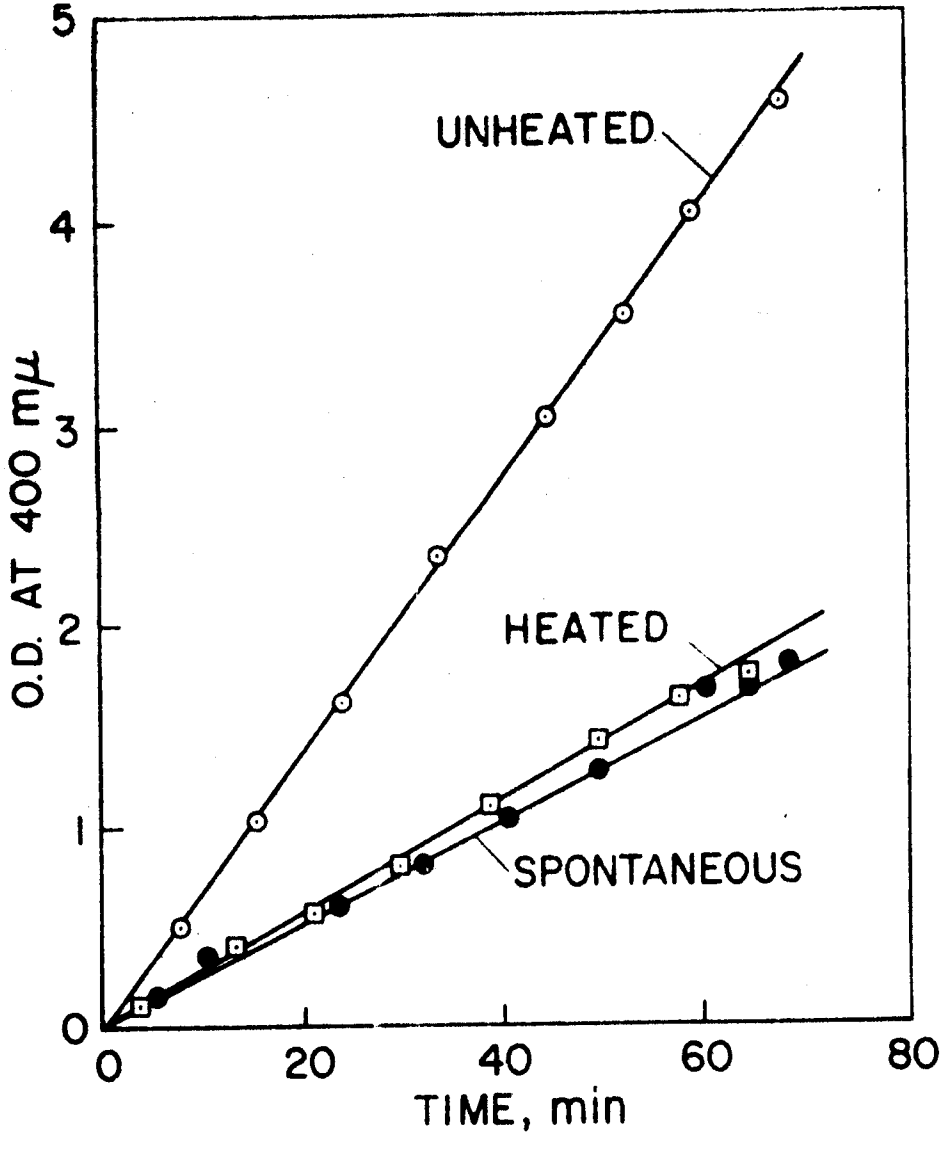

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Figure 1. Effect of heating in buffer on the activity of proteinoid A-2.8. The conditions of assay were as described in the METHODS section, except that the pH was 6.2. Heating the buffer alone had no discernible effect on the rate of spontaneous hydrolysis.

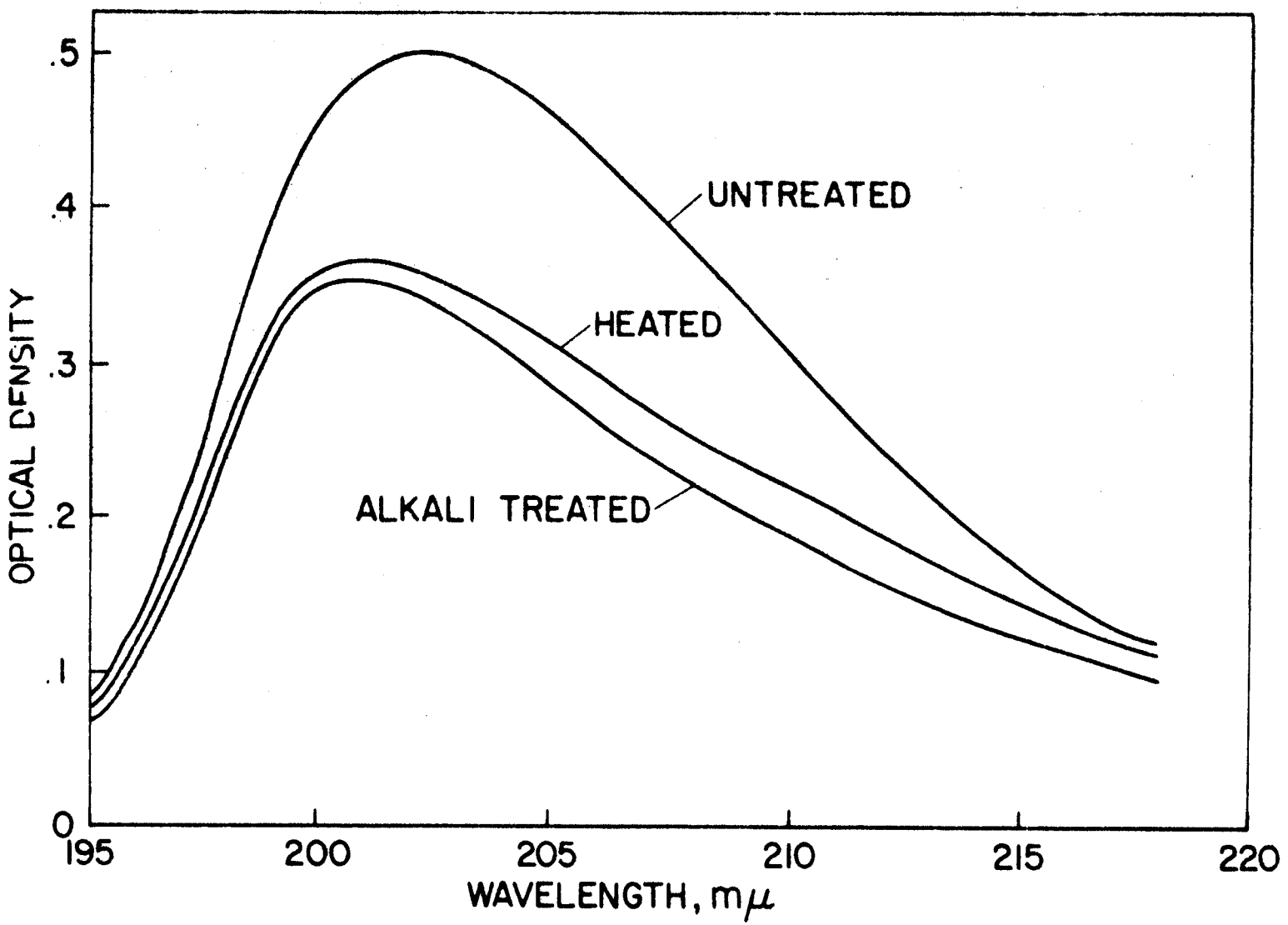

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Figure 2. Ultraviolet absorption spectra of polymer N-2.8 before and after heating in buffer or treating with alkali. Concentrations were 8.8 mg./l. in 0.067 M phosphate buffer, pH 6.8. Buffer was used as the blank. The spectrum of the alkali-treated sample was not significantly affected by heating.