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ABSTRACT

Several parameters of the thermal polymerization of L-lysine free base at 195°C were investigated over the time period from 15 minutes to 4 hours. Free lysine disappeared within one hour. The concentration of α-aminocaprolactam and of oligomers of several sizes reached a maximum after 30-45 minutes, and subsequently decreased. Molecular size, as judged by biuret color intensity and by gel filtration, increased rapidly and, in part, ultimately exceeded 100,000. Racemization was extensive and paralleled the disappearance of free lysine. Catalytic activity for the decarboxylation of oxaloacetic acid increased rapidly and reached a maximum after 2 hours. Dinitrophenylation and subsequent hydrolysis indicated that the polymers were branched, and that the linear portions contained more ε- than α-peptide bonds. A partial understanding of the method of chain growth could be inferred.

INTRODUCTION

Poly-α-amino acids that resemble proteins in many ways can be easily prepared by heating suitable mixtures of dry amino acids (1). The thermally prepared polymers can range in amino acid composition from homopolymers of glycine (2), lysine (3), or aspartic acid (4, 5) to polymers (proteinoids) containing some proportion of each of the 18 common amino acids (6); the compositional variation is both controllable and reproducible (1, 7-9). Because the thermal polymers are prepared under conditions believed to have been prevalent on the primitive (abiotic) earth, they are regarded as models for abiotic protein (1).

Although thermal poly-α-amino acids have been investigated in considerable detail, relatively little attention has been given to the time-course of the polymerization process. Previously reported studies include: the effect of time of heating on the yield and proportion of amino acids of proteinoids (6) and of copolymers containing aspartic acid, glutamic acid, and glycine (10); the effect on yield, proportion of amino acids, and molecular weight of copolymers of aspartic and glutamic acids (11); and the effect on yield of polyaspartic acid [prepared from precursors other than aspartic acid (12)]. A greater understanding of the entire polymerization process would, of course, be desirable. As an approach toward this goal, a thermal polymerization reaction has now been studied in considerably more detail. Homopolymerization offers analytical advantages; because lysine polymerizes with facility (3), giving a high molecular weight and partially characterized polymer (3, 13, 14), it was the reactant of choice. Attention was given to a variety of parameters, over a 4-hour heating period. Although earlier investigations have been concerned primarily with dialyzed, or dialytically washed (6), products, it was felt that more information could be gained by studying the total nonvolatile products; the products thus were not fractionated. This paper augments a preliminary report (15) in describing the effect of the time of heating of lysine (free base) on various properties of the unpurified products. In addition, information pertinent to the structure of the products has been obtained.

MATERIALS AND METHODS

L-Lysine free base (lot No. 2642) was obtained from K & K Laboratories, and was recrystallized by the method of Vickery and Leavenworth (16). α-Dinitrophenyl (DNP) lysine was synthesized according to the procedure of Schlossman *et al.* (17). ε-DNP-Lysine and α, ε-di-DNP-lysine were products of Mann Research Laboratories, Inc. α-Aminocaprolactam [m.p. 68-70°; lit. 68-71° (18)] was synthesized by Radiocarbon Laboratories, Inc., San Francisco; its authenticity was confirmed by mass spectrometry and by quantitative hydrolysis to lysine after 5 hours in 6 N HCl at 105°. L-Lysyl-L-lysine was a Yeda product; tri-, tetra-, and pentalysine were products of Cyclo Chemical Corp., Los Angeles.

The samples of thermal polylysine were prepared by a modification of the method of Harada (3). A 2-gram portion of L-lysine free base was firmly packed into each of nine large test tubes. A stream of prepurified nitrogen was introduced into each tube, and the tubes were placed in an oil bath maintained at 195°C (uncorr.). Most of the samples had liquefied by 15 minutes, and all had by 30 minutes. (The temperature inside a monitored tube increased rapidly to 186° after 15 minutes heating, and reached a maximum of 192° after one hour.) After being heated for various periods ranging from 15 minutes to 4 hours, the tubes were removed and cooled under nitrogen. The products were suspended in distilled water, acidified with HCl to pH about 3, and were lyophilized. (Before acidification, the products were very hygroscopic and could not be lyophilized satisfactorily.) Yields,

uncorrected for ash or moisture content, ranged from 1.8 to 2.1 grams. Unlike most earlier investigations, the products were not dialyzed prior to characterization.

Free lysine remaining in the heated samples was determined by analysis of a suitable aliquot of a Millipore-filtered aqueous solution. The 125 × 0.6 cm column of a Technicon amino acid analyzer was used, with elution carried out by means of the pH 5.00 buffer (19) only. Total lysine was determined by the same method, after hydrolysis of 4 to 10 mg of polymer in 2 ml of 6 N HCl in evacuated, sealed tubes at 110° for 40 hours. Recoveries of lysine, after sample weights were corrected for water and chloride content (from acidification with HCl), were 69% for the 1-hour polymer, 68% for the 2-hour, 60% for the 3-hour, and 55% for the 4-hour. The recovery was not improved when the hydrolysis time was increased to 72 hours. Several small ninhydrin-positive peaks were observed in addition to lysine. The procedure used gave 100% recovery with pure lysine, and 109% for chemically synthesized polylysine hydrobromide (Yeda LY 45, stated mol. wt. 40,000), assuming one HBr for each of the 193 lysine residues.

The α-aminocaprolactam content could be estimated in a similar manner on the amino acid analyzer, from a sample of 10 to 20 mg of unhydrolyzed polymer, eluted with pH 2.75 buffer containing 2.4 M Na^+ and thiodiglycol (19). At a flow rate of 1.5 ml/min., the lactam peak appeared on the chart at 140 min.; lysine appeared at 70 min. The lactam gave a ninhydrin color integration value only 3.5% of that of lysine under these conditions.

Water content was determined by drying the samples to constant weight over phosphorus pentoxide at 100° in an Abderhalden vacuum drying apparatus.

Chloride analyses were conducted on a Buchler-Cotlove Chloridometer, and were calculated as hydrochloride content. These values decreased with time of heating and ranged from 21.1 to 14.3% of polymer dry weight. Moisture content, however, was not related to time of heating, and ranged from 7.3 to 13.7%, except for 3.1% in the 15-min. sample.

To examine the distribution of oligomers, the undialyzed lysine polymers were chromatographed on carboxymethyl cellulose (CMC) columns by the method of Stewart and Stahmann (20). The columns, 37 × 1.9 cm, were eluted with a convex NaCl gradient formed by adding 1 M NaCl from a separatory funnel to a magnetically stirred 500 ml round bottom flask of water. Flow rates in the region of 0.5 ml/min. were used, and the absorbance of the effluent at 220 mμ was determined with a flow monitor, or after fractions were collected. To determine the concentration of each peptide, the fractions comprising each peak were pooled and read at 220 mμ vs. water, and the lysine equivalents were calculated from an experimentally determined molar extinction coefficient for each compound.

The biuret color of the polymers was developed by adding 0.5 ml of 0.2% $CuSO_4 \cdot 5H_2O$ in 15% NaOH to 1.0 ml of aqueous polymer solution (21). Optical densities of the resulting solutions were measured at 300 mμ with a Zeiss spectrophotometer. Corrections were made for the absorbance of controls consisting of H_2O + $CuSO_4$ (14% of the maximum uncorrected value) and of polymer + NaOH (11-29% of uncorrected values); the resulting values were converted to unit concentration of polymer (1 mg/ml).

A series of Bio-Gel columns was used to estimate the distribution of molecular sizes of the various samples of polylysine. The polymers,

at 6-7 mg/ml, were eluted from columns about 1 × 10 cm, using a solvent 0.1 N in HOAc and NaCl. Biuret color intensities of the effluents were evaluated; the reported values are the percentages of the total biuret color intensity that eluted in the same volume required for the elution of bovine serum albumin from each column. These results were corroborated by determining the optical density at 220 mμ. (Because the albumin standard was of molecular weight less than the exclusion limits of the P-100 and P-200 gels, the values obtained with these columns are considered less accurate than those from other columns.)

Specific rotations at the sodium D line were determined with a Rudolph model 80 polarimeter, equipped with an oscillating polarizer. Polymer concentrations were from 0.48 to 0.63% in N HCl. Standard deviations were from 0.5° to 0.8°. The extent of racemization of lysine was also estimated by gas chromatography, according to the method of Pollock and Oyama (22).

The ability of the samples to catalyze the decarboxylation of oxaloacetic acid (OAA) was evaluated at pH 5.0 in 0.2 N acetate buffer by a manometric technique described previously (13). The values reported are the apparent first-order rate constants, corrected for the rate of spontaneous decarboxylation and converted to unit concentration of polymer (g/l).

Aliquots of the various polymers were converted to the dinitrophenyl (DNP) derivative by a modification of the method of Sanger and Thompson (23). To about 20 mg of polymer in 2 ml of water and 5 ml of 2% aqueous trimethylamine was added 1.6 ml of ethanol containing 0.2 ml of fluorodinitrobenzene (FDNB). The mixture was shaken in the dark for 4 hours and lyophilized.

It was then placed in a vacuum desiccator over NaOH, and the desiccator was placed in a 60° oven for 3 hours to sublime the remaining FDNB. The residue was extracted with 5 ml of ethanol and 10 ml of ether, and again sublimed at 60° for a few hours. A weighed sample of about 5 mg of the dried residue was hydrolyzed in 5 ml of 6 N HCl in an evacuated, sealed tube at 110° for 24 hours. The hydrolysate was analyzed for DNP-lysines and free lysine by chromatography on IRC-50, as described recently (24). Nearly identical results were obtained on repeating the entire procedure with each polymer. Authentic ε-DNP-lysine and, α, ε-di-DNP-lysine were stable to hydrolysis for 48 hours under these conditions. In two separate determinations, the recovery of α-DNP-lysine was found to be 100% and 92% after 24 hours of hydrolysis, and about 75% after 36 hours.

RESULTS

The amount of free lysine remaining in the unhydrolyzed samples is shown in Fig. 1. Practically none remains after one hour of heating. The oligomers in the polymer preparations were separated by chromatography on CMC columns. In Fig. 2 are shown elution diagrams of the 30-min. polymer (A), where the maximum concentration of oligomers was found, and of the 60-min. polymer (B). The first peak in each diagram is due to unidentified, highly UV-absorbing material that was present in all samples of lysine examined (free base or hydrochloride). Peak 2 is the position at which both lysine and its lactam are eluted. Peak 3 was identified as lysyllysine by co-chromatography with the authentic compound; the following peaks are assumed to be due to oligomers increasing by one lysine residue

each (20). The latter peaks may be heterogeneous, if condensations between lysines can occur at the ε-amino in addition to the α-amino group.

The concentrations of the dimer through pentamer were calculated from the absorbance at 220 mμ of fractions obtained in the CMC chromatography of each polymer. The results are expressed as micromoles of lysine per mg of polymer (Fig. 3). The oligomers formed rapidly upon the heating of lysine, reaching a maximum concentration at 30 minutes (45 minutes for pentalysine) and then declining. A very similar pattern was noted for the lactam. At 30 minutes, approximately 17% of the lysine was present as the dimer through pentamer, and 15% as the lactam.

Biuret color intensities of the polymers (Fig. 1) increased rapidly during the first hour of heating, after which a plateau was reached. The 15-minute sample was biuret-negative. To the extent that color intensity is directly related to the number of peptide bonds (25, 26), these data indicate that relatively few peptide bonds are formed during the later stages of heating.

In Fig. 4 are shown the results of gel filtration on Bio-Gel columns. The ordinate is the percentage of each polymer that was eluted from each column in the same volume required for elution of albumin, i.e., the percentage of each sample with molecular weight equal to or greater than the exclusion limits indicated in parentheses. (The indicated exclusion limits are applied to the present polymers as only an approximation; relative values, however, are considered interconsistent.) Values obtained with a Bio-Gel P-30 column were nearly identical for each sample to those obtained with the P-20 column; in order to simplify the figure, the former values are not reported.

With increased time of heating, there is a regular increase in the proportion of the polymer of a size that exceeds the exclusion limit of the column (Fig. 4). Also, with any given polymer, e.g., the 3-hour sample, a regular decrease in the percentage excluded is noted as the exclusion limit of the column increases. This indicates that species occur in each size range (i.e., the difference in values between two adjacent Bio-Gel columns), connoting considerable heterogeneity in size. Inferences concerning the speed and method of polymerization are discussed later.

The results of analyzing the hydrolyzed dinitrophenylated polymers for the three DNP derivatives of lysine, and free lysine, are shown in Fig. 5. The relative amounts of the various derivatives changed during the first 60 to 80 minutes of heating, but were nearly constant from 100 minutes to 4 hours. The concentrations of α-DNP-lysine and free lysine were about three- to four-fold greater than those of ε-DNP-lysine or di-DNP-lysine.

The specific rotations and the degrees of catalytic activity for the decarboxylation of oxaloacetic acid (OAA) are shown in Fig. 6. The specific rotations of the unhydrolyzed samples decreased rapidly with respect to time of heating, and were essentially zero after about one hour. Gas chromatographic analysis (22) of a hydrolysate of the 4-hour polymer indicated at least 97% racemization of lysine. The catalytic activity increased rapidly with time of heating, and reached a plateau after about 2 hours. The maximum activity observed was nearly 10-fold greater than that shown by lysine; a value of about 16-fold was noted earlier when dialyzed polymers were used (13). α-Aminocaprolactam gave a value of 1.5×10^{-3} 1/g/minute or approximately half that shown by lysine.

DISCUSSION

Speed of the polymerization reaction. Several facts indicate that the thermal polymerization of lysine occurs rapidly. After being heated for 30 minutes only 23% of the lysine remained in free form, and only 4% remained after 45 minutes (Fig. 1). The disappearance of free lysine was accompanied by the appearance of low molecular weight oligomers of lysine (Fig. 2) and of α-aminocaprolactam. These reached a maximum concentration at 30 minutes (Fig. 3), then decreased. The percentages of species larger than the pentamer are calculated from the data of Figs. 1 and 3 to be 14, 45, 76, and 82, respectively, for the 15-, 30-, 45-, and 60-minute samples. These values, incidentally, agree closely with the biuret data of Fig. 1.

The continued rapid polymerization to yet larger species is further indicated by the rapid initial increase in biuret color intensity (Fig. 1), and by the data obtained with the Bio-Gel columns (Fig. 4). After 30 minutes of heating (Fig. 4), 69% of the sample had a molecular weight of 1600 or greater (corresponding to about 12 residues), although none was 3600 or greater. After 45 minutes of heating, nearly half the sample was of molecular weight greater than 3600, and 7% exceeded 20,000. With the 3- and 4-hour samples, appreciable percentages of the polymers exceeded a molecular weight of 100,000. This value can be compared with the value of 200,000 found by Krampitz *et al.* (14) with the ultra-centrifuge.

Method of polymerization. The elution behavior of the 2-, 3-, and 4-hour samples on a P-6 Bio-Gel column indicates little increase in the percentage of polymer of molecular weight 4600 or greater after 2 hours

(Fig. 4). Also, the patterns for the 3- and 4-hour samples on a P-20 column (and also on a P-30 column; data not shown) indicate no increase in the proportion of the sample of size 20,000. However, with these samples an increase in percentage is noted with the P-60, P-100, and P-200 columns. This indicates that continued heating produces molecules of molecular weight 60,000 or greater without appreciably changing the proportion of molecules larger than 4600 (or 20,000 during the last hour of heating). Thus, in the later stages of polymerization, chain growth is accomplished by condensation of relatively large molecules, rather than by the addition of small molecules to large ones. The lack of increase in biuret color intensity (Fig. 1) is consistent with this concept. The absence of free lysine (Fig. 1) and of low molecular weight oligomers (Fig. 3) during the later stages of heating is also consistent with this premise and, in fact, dictates polymerization in the suggested manner.

The method of chain growth during the early stages of polymerization is less readily interpreted. The amount of free lysine remaining cannot be a limiting factor (Fig. 1). α-Aminocaprolactam does not appear to be particularly reactive. It is present in larger quantity than are the oligomers of lysine (Fig. 3); it also is slower to disappear, although its precursor (lysine) is no longer present after 45 minutes of heating. Also, the free lactam does not seem to make a major contribution to the increase in molecular weight. For example, between 30 and 120 minutes of heating, the percentage of lactam in the product decreased from 15 to 6, while the biuret color intensity increased by some 65%, and the proportion of polymer excluded from Bio-Gel P-4 increased about the same amount.

The lactam was also tested directly as a polymer precursor, and was found not to polymerize appreciably when heated. In this experiment, 0.5 g samples of α-aminocaprolactam were heated for periods up to 4 hours, and chromatographed on Bio-Gel P-4 columns, by the same procedures used for the lysine polymers. The biuret reaction on the fractions representing excluded material (Vo, as determined with albumin) of the 4-hour sample indicated about 10% of the material to be of molecular weight greater than 3600. It has also been reported (27) that, under somewhat similar conditions, D-α-aminocaprolactam is converted to the D, L-lactam instead of to polymeric material. Of the possible functions for the lactam suggested by Harada (3), a role as a reactive intermediate thus seems less appropriate than a role as a solvent or catalyst.

Structure of the polymers. The amount of the various DNP derivatives of lysine, although variable during the early stages of polymerization, became approximately constant after about 80 min. of heating (Fig. 5). The approximate molar ratios were: α-DNP-Lys, 4; free Lys, 3; ε-DNP-Lys, 1; di-DNP-Lys, 1. These values connote the relative amounts of lysine participating in ε-, di-, and α-peptide linkages and N-terminal residues, respectively. It is difficult to suggest a structure that would account for these data. The interpretation may be influenced by non-quantitative recovery on hydrolysis of the dinitrophenylated polymers; e.g., the recovery, uncorrected for moisture or salts, for the 4-hour polymer was 57% of the lysine content of the original polymer. Similar low recoveries were experienced with dinitrophenylated Leuchs polylysine and with dinitrophenylated lysyllysine. (The time of hydrolysis is limited to about 24 hours because α-DNP-lysine begins to decompose at longer times.) Additionally,

a hindered structure could prevent quantitative dinitrophenylation; the presence of terminal lactam or diketopiperazines would also complicate the interpretation. These features could result in a value for free lysine in the hydrolysates that is not an accurate reflection of the number of branch points. In spite of these difficulties, the amount of free lysine present indicates that considerable branching occurs in the polymers, and the amount of α-DNP-lysine shows that a large proportion of the singly linked residues is linked through the ε-amino group. The latter finding is similar to that of Harada and Fox (10) with copolymers of alanine and lysine. These investigators found that the proportion of α-peptide linkages could be increased by increasing the temperature of preparation; the effect of temperature was not studied in this investigation.

Thermally prepared polyamino acids have been suggested as models for abiotic proteins (1). The large proportion of branching and ε-peptide linkages found in the present samples is, of course, not typical of present-day proteins. However, a homopolymer is less representative of models for abiotic protein than are heterocompositional polymers that contain the 18 amino acids common to present-day protein. Because of a lesser proportion of tri-functional amino acid residues, branching or non-α-peptide linkages must occur to a considerably lesser extent in the more complex thermal polymers. To what extent, for the most part, awaits investigation, but it has already been shown (10) that the conditions of synthesis influence the product. In addition, the thermal polymers have not had the advantages of the modifying action of a long evolutionary process, as current proteins have had.

Optical and catalytic activities. Lysine is either rapidly racemized during heating (Fig. 6), or is converted to an intermediate compound that a) is very vulnerable to racemization, or b) has a specific rotation of compensating, opposite sign. The decrease in optical activity closely parallels the decrease in the amount of free lysine remaining, which suggests that lysine as such is not racemized. Although L-α-aminocaprolactam has a $[\alpha]_D^{22}$ in N HCL of -24.5° (27), the samples did not show a negative rotation when, after lysine was no longer present, the lactam was present in appreciable quantities. The finding (27) that D-α-aminocaprolactam is racemized at 160° (in the presence of catalytic amounts of sodium) is consistent with the concept that, during the thermal polymerization of lysine, the lactam is vulnerable to racemization. It is of interest to note that aspartic acid is readily racemized during thermal polymerizations, whereas glutamic acid and some neutral amino acids are racemized to a lesser extent (6, 8, 28).

The increase in the degree of catalytic activity closely parallels the increase in molecular weight (Figs. 1, 4, and 6), and it is apparent that molecules of appreciable size are needed for full activity. For example, after 30 minutes of heating, when 69% of the polymer was comprised of species of molecular weight 1600 or greater but none greater than 3600 (Fig. 4), the catalytic activity was about one-third the maximum value. After 45 minutes of heating, two-thirds of the maximum activity was shown, and only 14% of the polymer was of molecular weight less than 1600; 44% was 3600 or greater. The reason for the large increase in activity with respect to molecular weight is not clear. Polylysine prepared from the N-carboxy-anhydride (Leuchs polylysine) and assumed to be a linear arrangement of α-peptide bonds was found to be more active than free lysine, but

less active than thermal polylysine (13). Krampitz *et al.* (14) have reported that thermal polylysine catalyzes an amination reaction, whereas Leuchs polylysine does not. The somewhat similar behavior in catalytic systems of thermal polylysine from the two laboratories may be due to similar structural features not found in Leuchs polylysine. Free amino groups were found to be essential for either catalytic activity (13, 14). The greater activity of the thermal polymers could, in part, be due to the presence of free α-amino groups, which would result from ε-peptide linkages and would not be present in Leuchs polylysine. On the other hand, α-aminocaprolactam (which can be considered an intramolecular analogue of the ε-peptide linkage) was only about one-twentieth as active as thermal polylysine. As has been suggested by Krampitz *et al.* (14), a combination of factors such as molecular size, shape, and number of free amino groups may be contributory to activity.

This investigation has shown that the length of time lysine free base is heated affects several properties of the resulting product; the speed at which polymerization occurs is notable. The time-course investigation of other kinds of thermally prepared poly-α-amino acids (1) would not only provide a fuller understanding of the polymerization process, but could indicate ways to exert better control over the reactions.

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FIGURE CAPTIONS

Fig. 1. Free lysine content (○) and biuret color intensity (●) as a function of time of heating lysine free base at 195°C.

Fig. 2. Separation of lysine oligomers in thermal polylysine by elution from carboxymethyl cellulose with a NaCl gradient. Fraction size: 4 ml. (A) Heated 30 minutes; 19.6 mg. (B) Heated 60 minutes; 21.1 mg. Peak 1-Unidentified; 2-Lysine; 3-Lysyl-lysine.

Fig. 3. Effect of time of heating lysine on the proportions of α-aminocaprolactam and oligomers of lysine. The theoretical maximum value is 6.85 μmoles lysine equivalent/mg of sample. See METHODS for analytical procedures. ○—○ Lactam; ●—● Dilysine; ○---○ Trilysine; ×—× Tetralysine; △---△ Pentalysine.

Fig. 4. Behavior of the samples of thermal polylysine on Bio-Gel columns. The ordinate is the percentage of each sample that was eluted in the volume required for elution of albumin. The values in parentheses are the exclusion limits of the Bio-Gels.

Fig. 5. Analysis of dinitrophenylated, hydrolyzed polymers for: ○ α-DNP-Lys; △ di-DNP-Lys; ◇ ε-DNP-Lys; □ free lysine.

Fig. 6. Specific rotation in N HCl (□) and catalytic activity (○) of the samples of thermal polylysine. Rates for the catalytic decarboxylation of oxaloacetic acid at pH 5.0 are corrected for spontaneous decarboxylation.

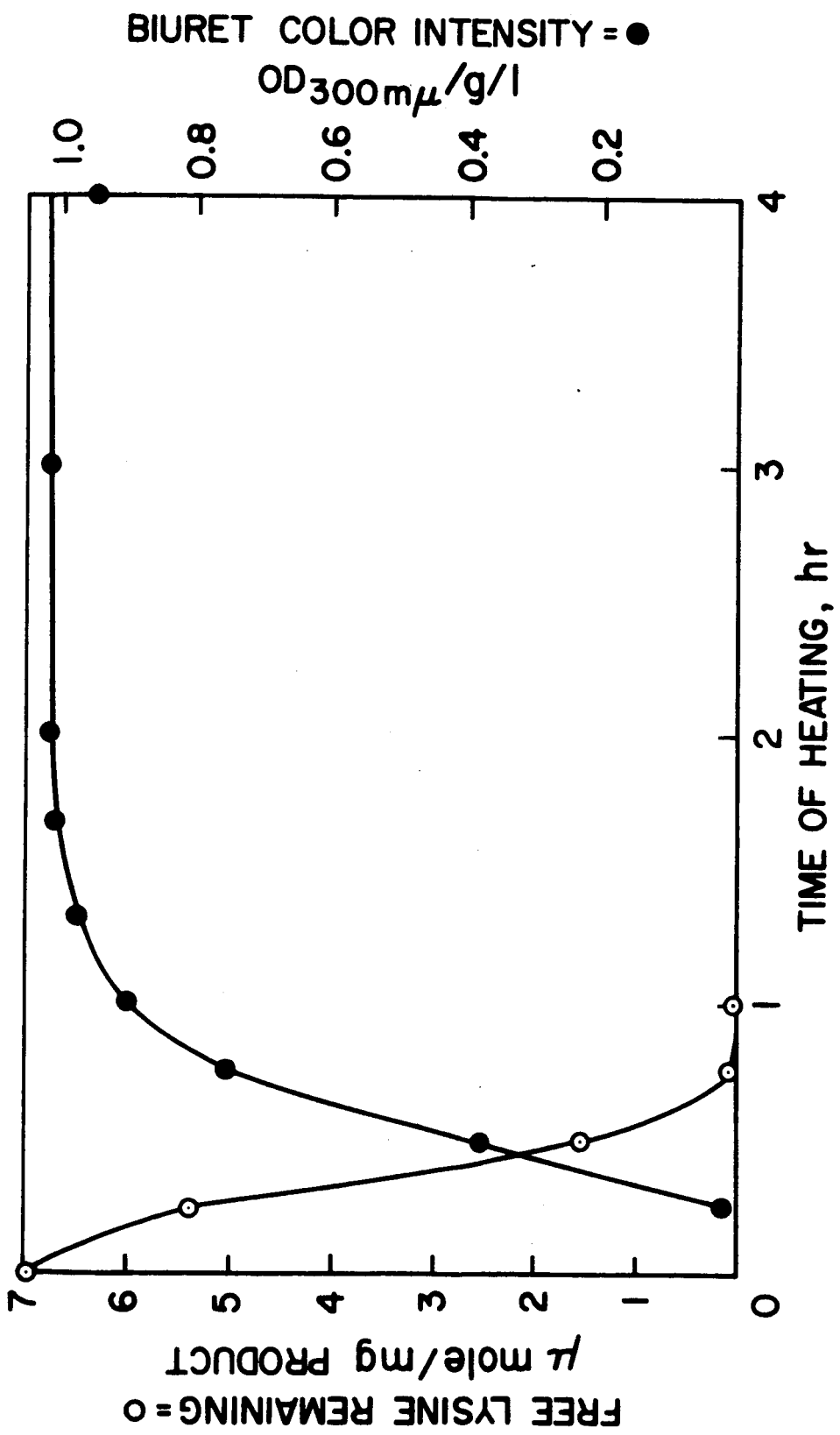

Fig. 1.

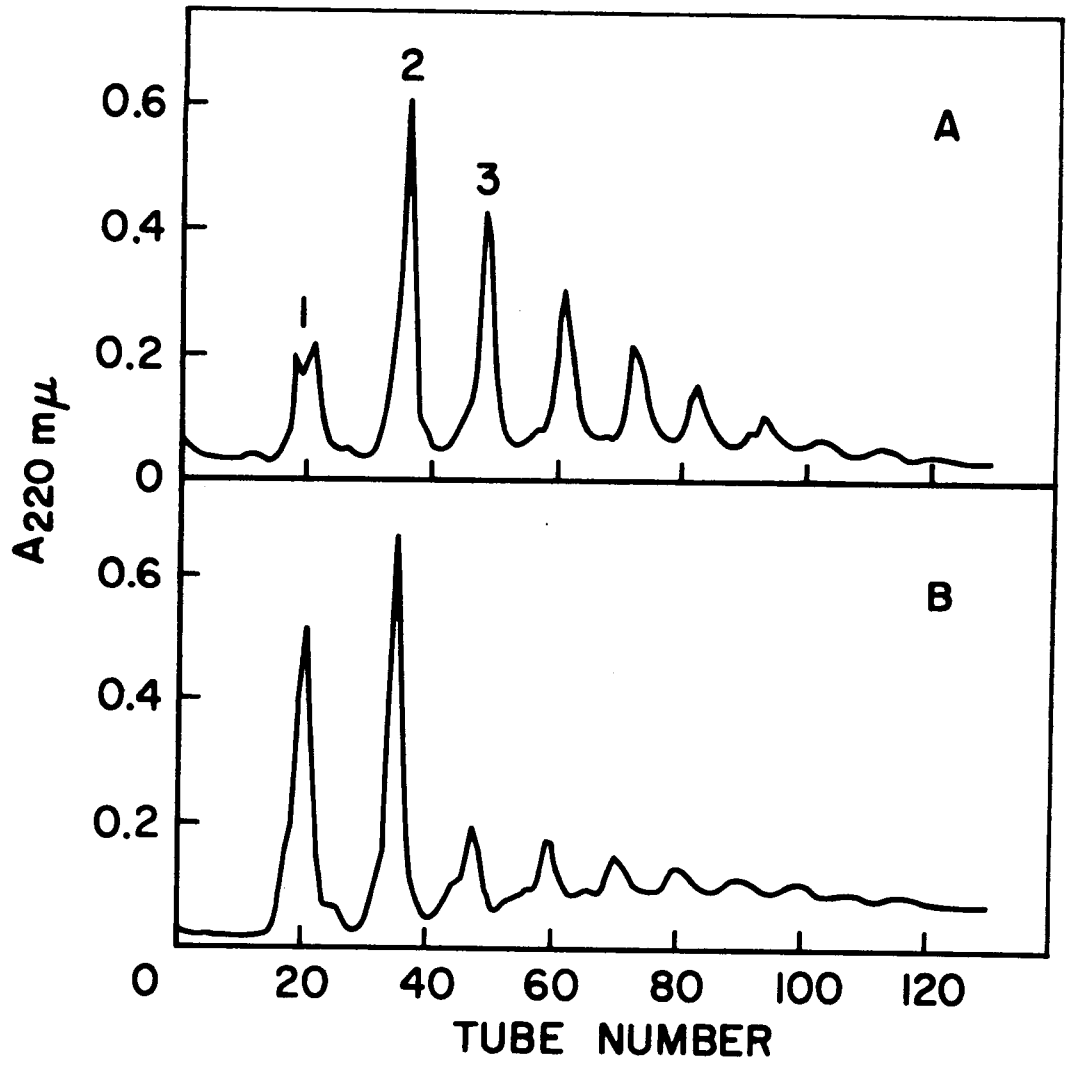

Fig. 2.

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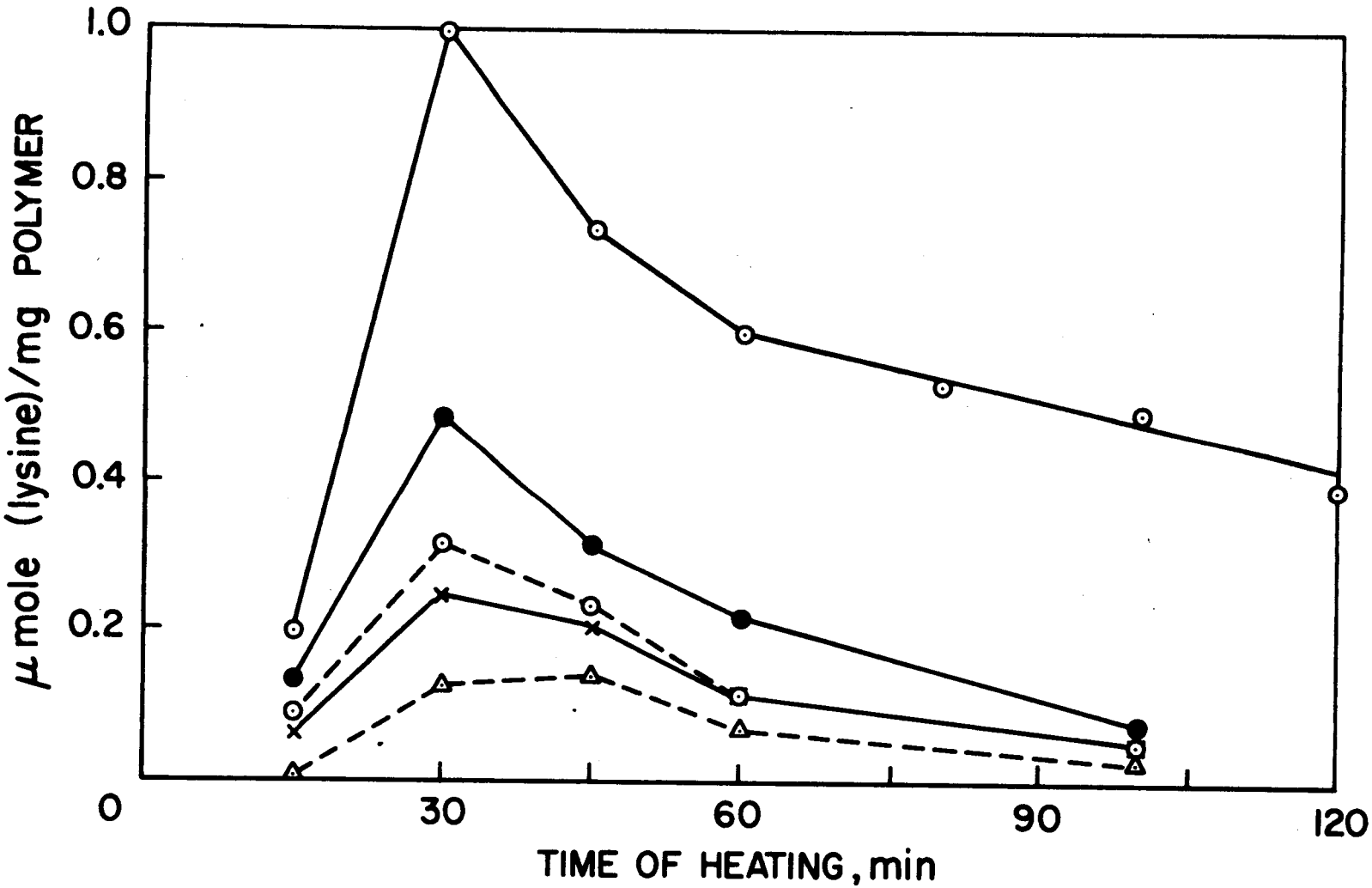

Fig. 3.

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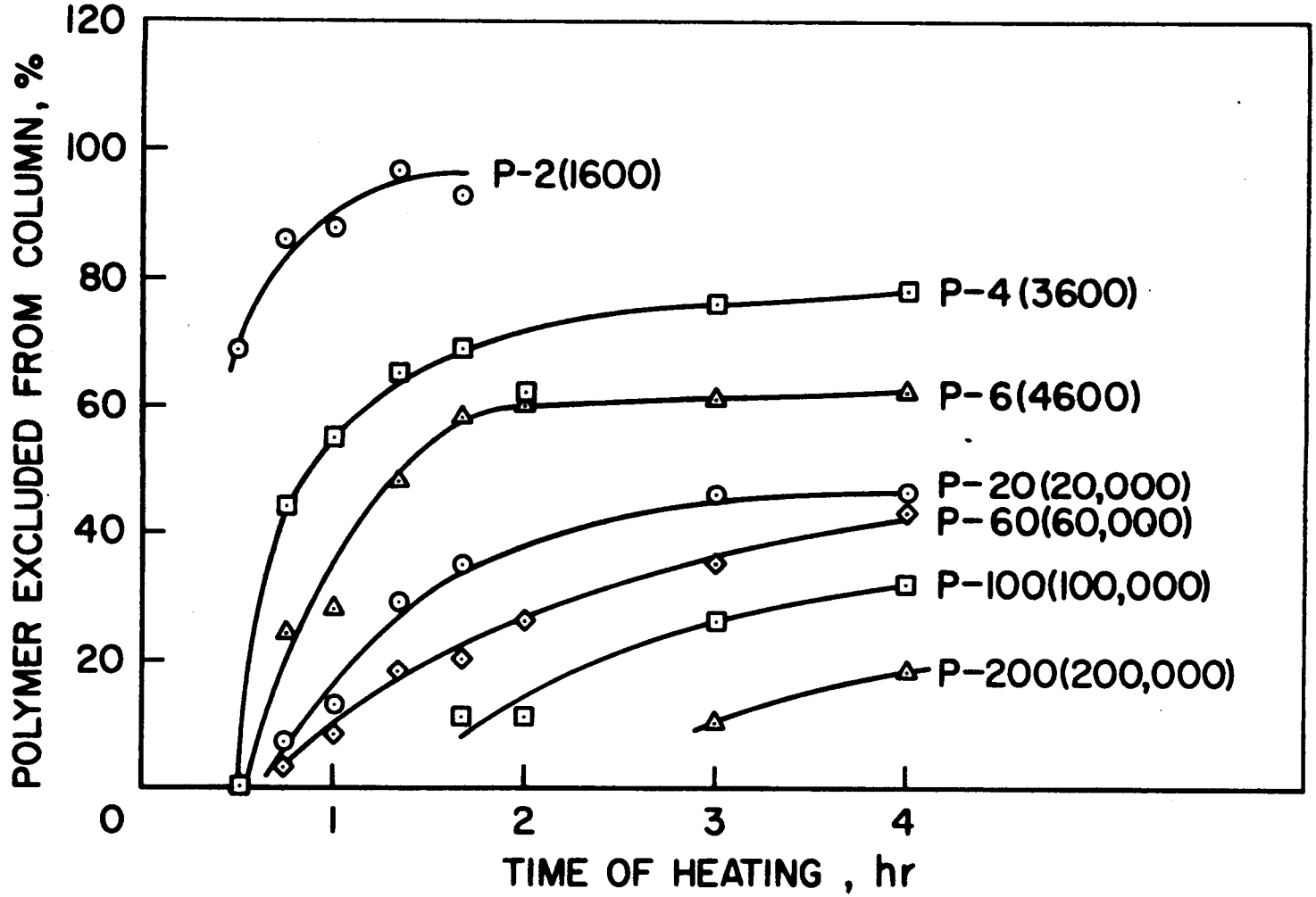

Fig. 4.

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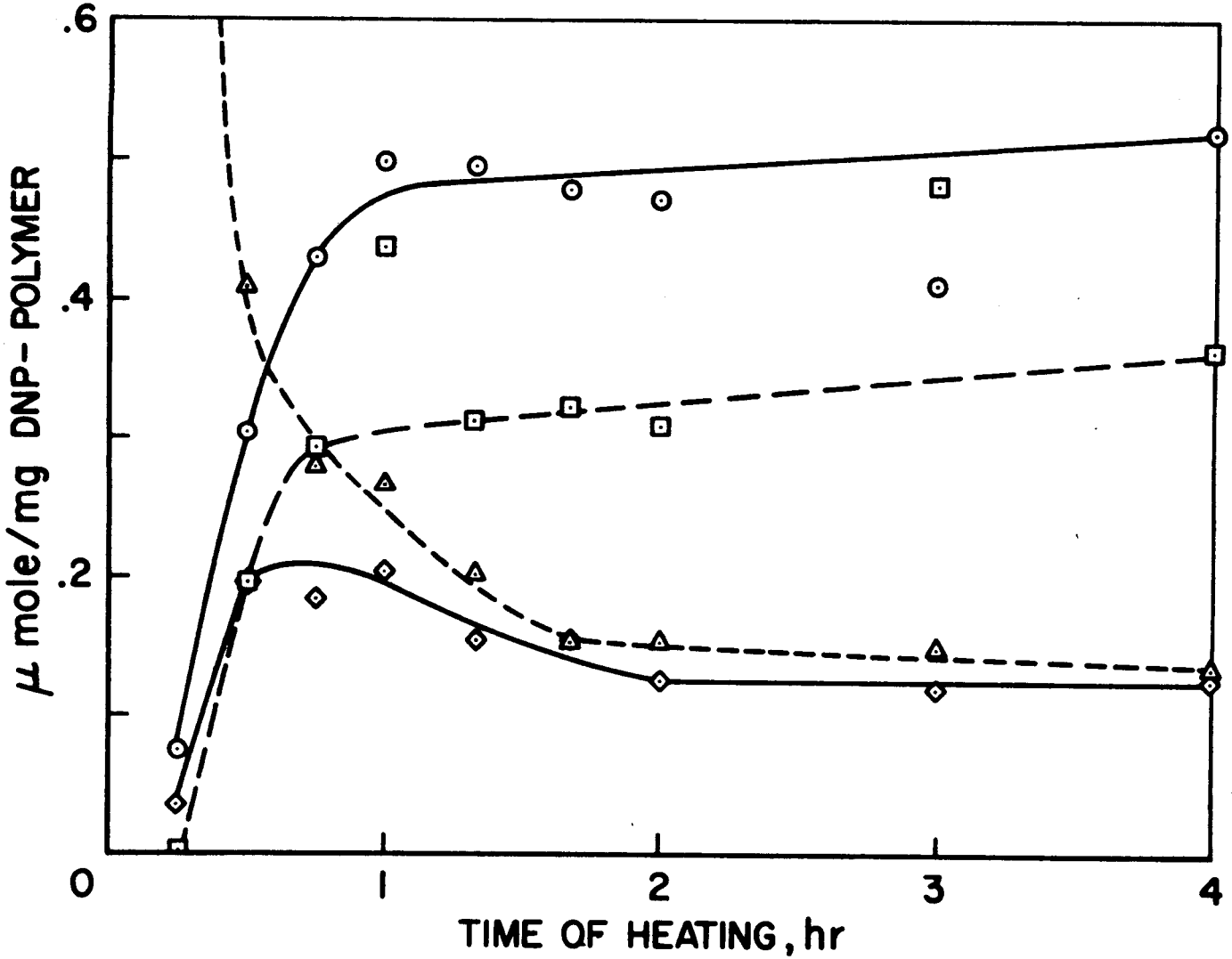

Fig. 5.

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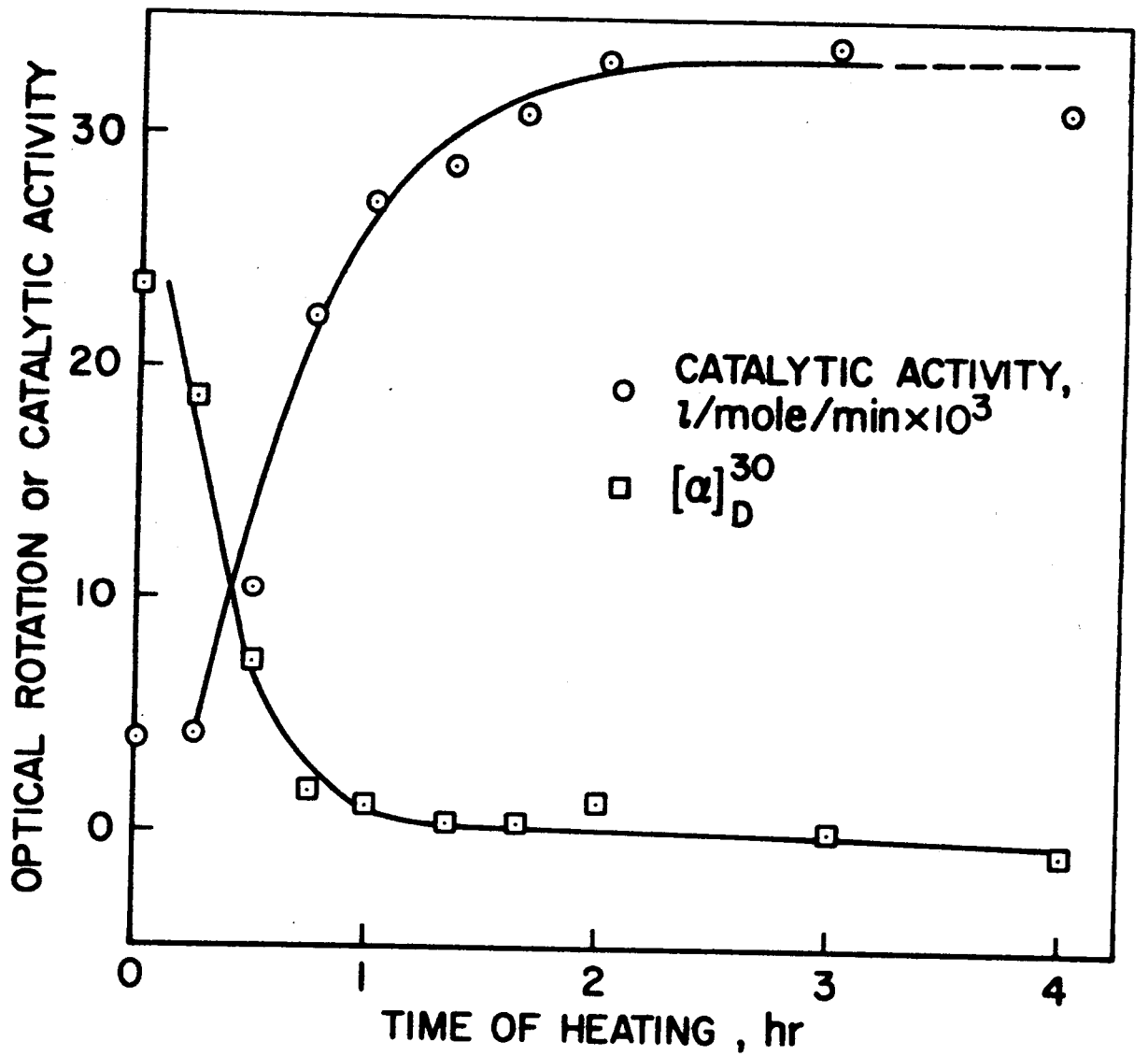

Fig. 6.