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Effect of Molecular Weight on Hydrogen–Deuterium Exchange in a Nonhelical Polyamide

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Abstract: Hydrogen-deuterium exchange kinetics have been measured for poly(isopropylacrylamides) over a molecular weight range from 100 to 200,000. A sharp drop in rate appears as soon as one goes from monomer to trimer. Thereafter a very slow progressive decrease is displayed. The local environment of the exchanging amide residue is largely (although not completely) established by the mutual interaction of as few as three residues in these polymers of random conformation.

The rate of exchange of the N–H hydrogen of an amide reflects its local environment, in the ground state and in the transition state. To understand exchange kinetics in proteins, one must have some knowledge of the factors governing these rates in small amides and in model polymers. Previous investigations[1-3] have demonstrated that the N–H in a nonhelical polyamide undergoes exchange at a much slower rate than in a corresponding monomeric residue. The rate constants drop by a factor of about 10^2 when the amide is transferred from a small molecule of molecular weight near 100 to a nonhelical polymer of molecular weight near 200,000. An intriguing question thus arises: at what stage in molecular weight does the transition in behavior appear? The present study had been designed to answer this question and to determine whether the change in behavior occurs gradually or appears abruptly at some critical range in macromolecular weight.

Experimental Section

Materials and Methods. Reagent grade benzoyl peroxide was obtained from commercial sources. *n*-Butyl mercaptan (1-butanethiol) was purchased from Aldrich Chemical Co. [n^{20}D 1.4437 (lit.[4] n^{25}D 1.4401)]. Deuterium chloride, 38% in D_2O (warranted to contain 99.5 mol % D), and deuterium oxide (warranted to contain 99.9 mol % D) were purchased from Bio Rad Laboratories. Raney nickel–aluminum catalyst, 42–58 pulverized ingot (Gilman Paint and Varnish Co.), was a gift from Dr. A. S. Hussey.

N-Isopropylacrylamide was obtained from American Cyanamid Co. or Eastman Chemical Products, Inc., and was recrystallized twice from a toluene–hexane mixed solvent (mp 66–67°). Dioxane was purified by the method of Fieser[5] and freshly distilled before use. Anhydrous sodium acetate (J. T. Baker Co.) was dried at 120° in vacuo for 4–12 hr before use. Benzene and hexane were purified by fractional distillation; only the fractions boiling at 80 and 69°, respectively, were used. Other solvents were purchased in reagent grade from commercial sources and used without further purification.

Bio Beads S-X2, X3, and X8, Bio-Gel A-0.5m, and AG11A8 Ion Retardation Resin were purchased from Bio-Rad Laboratories. Sephadex G-10 and G-25 were purchased from Pharmacia Fine Chemicals, Inc. Thin-layer plates (0.25 mm, Silica Gel H, Brinkmann Instruments, Inc.) were prepared according to Stahl.[6] High purity Q-Gel was obtained from Quantum Industries. Silica gel grade 950, 60–200 mesh, was purchased from Davison Chemical Division, W. R. Grace & Co., washed with boiling methanol, and activated at 120° in vacuo.

Ultrafiltration membranes were obtained from Amicon Corp. and used in their Model 401 filtration cell.

Infrared spectra in the fundamental region were scanned with a Beckman IR 10 spectrophotometer. Overtone infrared spectra were obtained with a Cary 14R spectrophotometer equipped with nitrogen-purged optical and cell compartments and a thermostated cell holder. All chromatographic columns were eluted at flow rates of 1–3 ml/min. Aqueous column eluents were monitored with a Waters Associates Model R4 differential refractometer. With organic column eluents, selected fractions were spotted on thin layers of analytical silica gel, developed in 12% (v/v) ethanol–hexane, and stained with iodine vapor. A Beckman Spinco Model E analytical ultracentrifuge was used for sedimentation equilibrium experiments. Elemental analyses were determined by Micro Tech Laboratories, Inc. Solution pH was measured with a Radiometer Model PHM4c meter in conjunction with a Corning semimicro combination electrode. The pH readings were changed to pD values with the relationship of Glasoe and Long:[7] pD = pH + 0.40.

Polymer Synthesis. Small-scale polymerization of *N*-isopropylacrylamide was carried out in a 1-l. round-bottom four-neck flask equipped with a stirrer, thermometer, gas dispersion tube, and reflux condenser. All reagent solutions were deaerated with, and kept under a slight positive pressure of, nitrogen throughout the reaction. To a solution of 11.3 g of *N*-isopropylacrylamide in 300 ml of benzene was added one of the following: (a) 4.31 ml of butyl mercaptan (0.1 *M* in final reaction mixture), (b) 40 ml of a 0.1 *M* solution of mercaptan in benzene (0.01 *M*), or (c) 40 μl of the solution in b (10^{-5} *M*). High mercaptan concentration led to a low-molecular-weight polymer. Benzoyl peroxide (1.2 g) was added along with enough additional benzene to bring the total volume to 400 ml. The solution was refluxed for 24 hr, during which time it gradually became yellow.

The reaction mixture was chilled in an ice bath and extracted with 500 ml of cold water. Aqueous extracts were further extracted with 25 ml of benzene and then filtered. Lyophilization of the translucent, light-yellow, aqueous solution yielded a fluffy white solid. Lyophilization from dioxane gave a more easily handled compact solid.

For a large-scale preparation the above quantities were increased by a factor of 6.25 and the reaction was carried out in a 3-l. flask. Solvent was removed by rotoevaporation at 30°. About 100 ml of product, in the form of a viscous liquid, was obtained.

The high-molecular-weight polymer was prepared as described previously.[1]

Polymers of Moderate Polydispersity[8] from Fractionation[9] of Small-Scale Synthetic Products. $\overline{P28}$.[8] The solid from a preparation containing 0.01 *M* mercaptan was dissolved in cold water and fractionated by dialysis. Lyophilized dialysate (low-molecular-weight polymer) was dissolved in cold water, applied to a bed of agarose 0.5m, and eluted with water at 5° (with reversed flow). A middle fraction was concentrated by ultrafiltration and lyophilized. Further reduction in polydispersity was achieved with chromatography on Sephadex G-25. Adsorption by the gel beads during fractionation removed most of the yellow impurities.

$\overline{P5}$.[8] To reduce polydispersity the high- and low-molecular-weight components were removed by ultrafiltration; the fraction passed by a 100,000 and retained by a 500 molecular weight cutoff membrane was lyophilized. This material was chromatographed on Sephadex G-25 under the same conditions as for $\overline{P28}$. Again impurities became adsorbed to the membranes during ultrafiltration.

Polymers of Low Polydispersity from Fractionation and Purification of Products from Large-Scale Synthesis. $\overline{P7}$, P4, P3.[8] Viscous syrup from a large-scale preparation containing 0.1 *M* mercaptan was applied to a bed of silica gel and eluted with diethyl ether to remove unreacted starting materials. Thereafter acetone was used to elute polymer fractions. A middle fraction was chromatographed on another silica gel column with 12% ethanol–hexane as the eluent. Two partially resolved fractions were obtained. A final fractionation was achieved by gel permeation chromatography on cross-linked polystyrene gels, an S-X3 bed being placed in tandem with an S-X8 bed. Eluent (10% ethanol–benzene) descended the S-X3 bed and ascended the S-X8 bed. Colored impurities appeared first in the effluent and then fractions with $\overline{P7}$, P4, and P3. Further purification of the oligomer sample was achieved by a series of four chromatographic procedures. (a) Each product was developed on a bed of S-X2, the center 75–85% of the polymer being isolated. (b) Chromatography of $\overline{P7}$ on a bed of Sephadex G-10 removed some of the color. (c) Additional decolorization of each oligomer sample was achieved by chromatography on columns of Q-gel silica in acetone. (d) Each sample was subjected to discontinuous gradient chromatography. The oligomer was dissolved in $CHCl_3$ and placed on a bed of Q-gel. Elution with CCl_4, in which the oligomers are insoluble, fixed them to the top of the bed. Addition of a small portion of 1-butanol to the CCl_4 eluting solvent separated a pair of yellow bands from the polymer. The oligomer was then recovered by elution with $CHCl_3$ and rotoevaporation.

Desulfurization of $\overline{P7}$, P4, and P3 in ethanol solution was carried out by reduction with Raney nickel catalyst (W-6).[10] The isolated products were chromatographed with water on a bed of AG11A8.

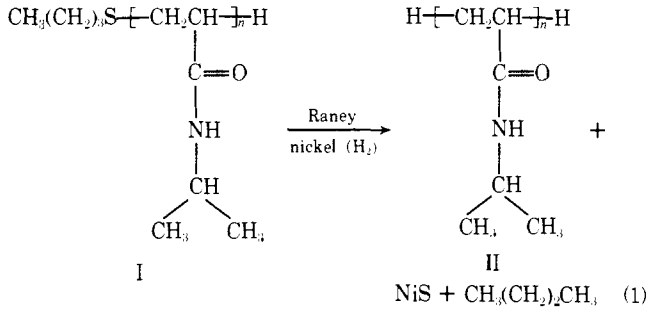
Kinetic Measurements. Buffer solutions contained 0.05 *M* sodium acetate in D_2O and were adjusted to the desired pD by addition of 0.1 *M* DCl or NaOD. Polymers $\overline{P28}$ and $\overline{P5}$, after lyophilization from dioxane, were pulverized and dissolved at 25° by vigorous mixing aided by pieces of Teflon to produce a 0.15 residue molar solution. The solution was then transferred through a glass wool plug into a cell with 5-cm path. The time between addition of buffer to the polymer and the first absorbance scan was 3–5 min. A similar procedure was used to dissolve polymers $\overline{P7}$, P4, and P3 except that samples, lyophilized from water, and buffer were precooled to 5°. A first absorbance reading could be obtained with the samples in less than 3 min.

The region from 1.55 to 1.23 μmol was scanned at 2.5-min intervals for the first hour of each run and at progressively longer intervals thereafter. Infinity readings were obtained after 10–12 half-lives, and the final pH was measured directly in the spectrophotometer cell.

Results

Properties of Polymers. The molecular weight of poly(*N*-isopropylacrylamide) was controlled by chain transfer[11] to butyl mercaptan during polymerization. Although each product contained some unwanted high polymer, the average molecular weight of the major component was inversely related to mercaptan concentration as shown in Figure 1. Comparison of the low-molecular-weight regions of profiles B, C, and D indicates a parallel decrease in polydispersity with decreasing molecular weight. Dashed lines delineate that portion isolated by preparative gel permeation chromatography on agarose.

Incorporation of (on the average) one butyl sulfide residue per polymer molecule resulted in products with structure I. The sulfur-containing end group provided a convenient measure of the number average molecular weight, $\overline{M}_n$. The effect of the terminal butyl sulfide group on the properties of the oligomers could be eliminated by removal of the group in a reductive desulfurization (eq 1) which produced II.



Elemental compositions and empirical formulas for $\overline{P7}$, P4, and P3 before reduction are shown in Table I. $\overline{M}_n$ was calculated from sulfur content assuming one sulfur per molecule.

$\overline{M}_w$ for $\overline{P28}$ was also measured by sedimentation equilibrium. A small amount of rapidly sedimenting material plus the visible turbidity of dilute (2%) aqueous solutions indicated the presence of aggregated constituents. The material in sedimentation equilibrium had an $\overline{M}_w$ of 3300.

Infrared spectra in the fundamental region of all products (in KBr pellets) were identical with that published for poly(*N*-isopropylacrylamide)[12] except for a decrease in peak width with decreasing molecular weight. In addition, P4 and P3 showed only a hydrogen-bonded NH stretching frequency typical of small amides which are usually completely hydrogen bonded in the solid state.[13,14] Aqueous solutions of all polymers showed absorbance peaks in the overtone infrared at 1.525 and 1.489 μm corresponding to NH hydrogen bonded to carbonyl and not hydrogen bonded, respectively, as previously identified in the high polymer.[1]

Solubility in organic solvents was similar to that of the high polymer[1] except that the oligomers reported here were

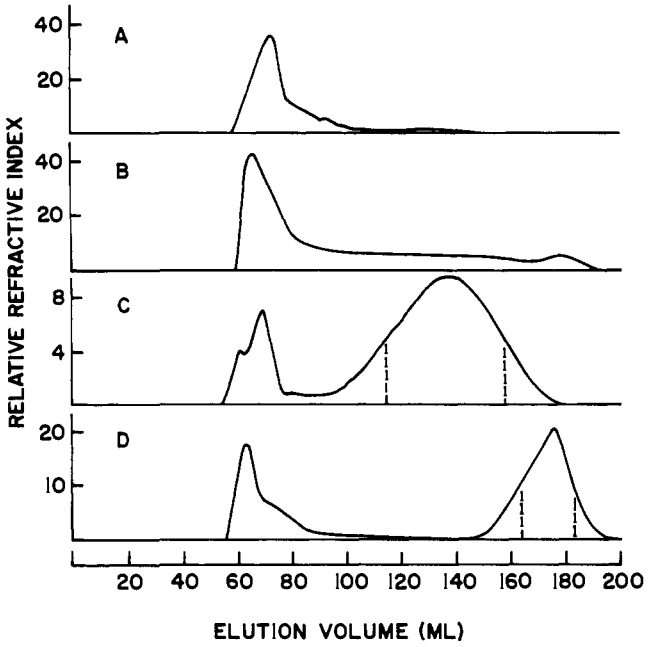


Figure 1. Molecular weight distribution of polymers synthesized with added butyl mercaptan: Bio-Gel agarose 0.5m (200–400 mesh) in a column of 1.9 × 69 cm at 25°; water was eluent; 1 ml/min flow rate; 0.5-ml samples of 0.5–1% aqueous polymer solutions placed on column: (A) high polymer ($\overline{P1700}$); (B) product from polymerization with 10^{-5} *M* mercaptan; (C) product from polymerization with 0.01 *M* mercaptan after dialysis; (D) product from polymerization with 0.1 *M* mercaptan. Fractions between dashed lines were isolated by preparative fractionation: $\overline{P28}$ from C, $\overline{P5}$ from D.

Table I. Properties of Poly(*N*-isopropylacrylamides)

Polymer	% S found	$\overline{M_n}$
P_3	7.23	444
P4	5.85	548
$\overline{P5}$		650
$\overline{P7}$	3.49	919
$\overline{P28}$		3300

soluble in aromatic solvents. Properties in aqueous solution depended on (1) degree of polymerization, (2) presence or absence of terminal butyl sulfide, and (3) choice of solvent (water or dioxane) from which the polymer was lyophilized. The turbidity of aqueous 2% solutions of $\overline{P28}$, $\overline{P7}$, $\overline{P5}$, and $\overline{P4}$ increased with decreased size as the hydrophobic contribution from the terminal alkyl sulfide residue increased. P3 was insoluble in water. Reduced polymers exhibited greater solubility and much less turbidity in water than their unreduced homologs. If lyophilized from water the oligomers were readily soluble in water and gave slightly translucent solutions that could be clarified by ultrafiltration. All polymers precipitated from aqueous solution when heated above room temperature but not as sharply as previously described for the high polymer. Reproducible hydrogen-deuterium exchange rates were obtained for each polymer.

Rate Constants for Hydrogen–Deuterium Exchange. The exchange reaction

$$NH + D_2O = ND + HOD \quad (2)$$

was followed in a large excess of D_2O; hence, only the forward reaction was kinetically significant. The pseudo-first-order rate process may be formulated as

$$-d[NH]/dt = d[OH]/dt = k_{obsd}[NH] \quad (3)$$

In each experiment, spectra were repeatedly scanned from 1.55 to 1.23 μm. The increase in absorbance by [OH] at 1.407 μm and decrease by [NH] at 1.489 and 1.525 μm were monitored for kinetic analysis. To compensate for any light scattering, absorbances were normalized to that of a reference point at 1.24 μm in a transparent region of the spectrum. Rate constants were calculated as previously described.[1,15]

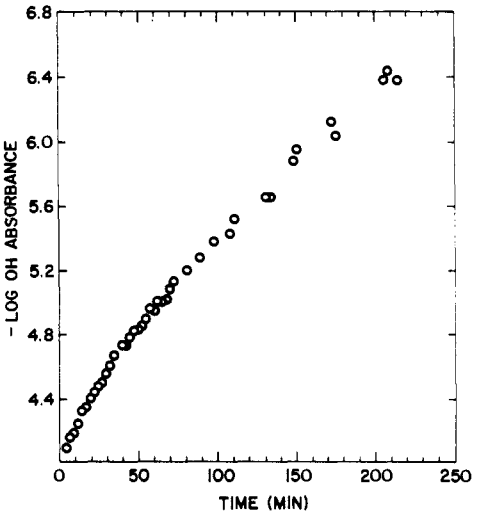


Figure 2. Exchange data for oligomer P4R at pD 5.0 and 25°. [OH] monitored at 1.407 μm.

A plot of log $[A_\infty - A_t]$ vs. time for the OH absorbance of P4R at pD 5.0 is shown in Figure 2. Departures from linearity at early times, indicative of a fast exchange process, were clearly observed for oligomers P4R and P3R. $\overline{P7}$ and higher polymers exhibited a single first-order exchange rate. Table II lists values for k_{obsd} at several pD values. Similar values of k were obtained whether calculated from OH or NH absorbance data. For oligomers P4R and P3R even the fast exchange rate is much slower than that of the monomer at the same pD.

The full dependence of k on pD for each oligomer is shown in Figure 3 in comparison with that for the high polymer.[1] The oligomers, like higher polymer, display a k_{min} at pD 5.0. In contrast, that for the monomer appears at pD 5.5.[1]

Figure 4 illustrates the dependence of k_{min} on molecular weight.

Discussion

Polymers $\overline{P7}$, $\overline{P28}$, and $\overline{P1700}$, that is, those of molecular weight about 800 or above, exhibit a single first-order exchange process. The dependence of k on pD is similar to that previously observed for many small amides and polymeric amides.[16,17] Evidently for the new polyisopropylacrylamides also, the exchange reaction is catalyzed by D^+ and OD^- and follows the generally accepted mechanism first proposed by Berger et al.[18]

Polymers smaller than $\overline{P7}$ display biphasic kinetics (for example, see Figure 2). In addition to a dominant slow exchange, with a rate near that of higher oligomers, a parallel exchange at a rate five-tenfold faster is also observed. For the P4R and P3R oligomers only, the data were treated to resolve the two rate constants (Table II). The slower rate was found to be about threefold faster than that for the high polymer. The faster exchange was still 2–15-fold slower than that for the monomer at the same pD.

About one-third of the amide hydrogens in the trimer and tetramer exhibit fast exchange. The different rates for a particular oligomer could be attributed to (1) individual stereoisomers or (2) specific amide residues. If we examine the polymer structure, we find that the presence of an

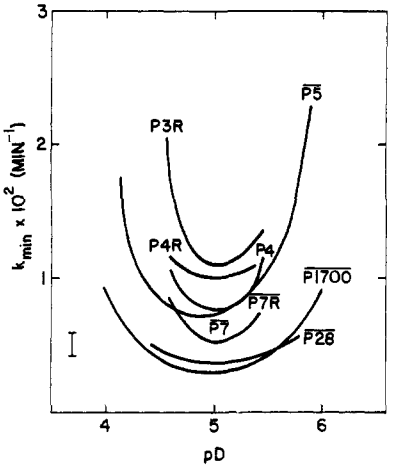


Figure 3. pD dependence of k_{min} from [OH] absorbance data. Vertical error bar represents ±10%. High polymer and monomer data from Scarpa et al.[1]

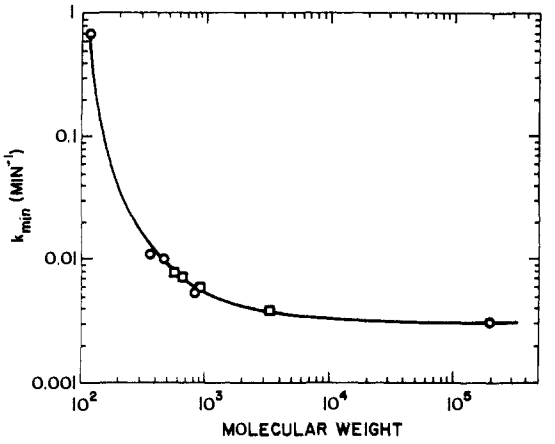


Figure 4. Dependence of log k_{min} on log molecular weight: □, products with a butyl sulfide terminal group; ○, products with no alkyl sulfide residue. High polymer and monomer data from Scarpa et al.[1]

Table II. First-Order Rate Constants

Polymer	pD	k, min^{-1} OH	NH unbonded	NH bonded
$\overline{P1700}$	5.0	3.0×10^{-3}		
$\overline{P28}$	5.8	5.8×10^{-3}	6.5×10^{-3}	6.1×10^{-3}
	5.4	4.3	6.3	7.4
	4.9	3.8	5.1	5.2
	4.4	5.0	7.6	8.7
$\overline{P7}$	5.0[a]	6.3×10^{-3}	5.3×10^{-3}	6.5×10^{-3}
	5.0[a]	5.3	5.5	7.9
	5.0[a]	5.6	6.5	8.8
	5.0[a]	6.0	6.8	9.6
$\overline{P5}$	5.9[b]	2.4×10^{-2}	2.3×10^{-2}	3.1×10^{-2}
	5.9[b]	2.6	1.7	2.0
	5.9[b]	2.8	2.1	2.6
	5.7	1.3	1.3	1.1
	5.4	1.0	0.9	1.3
	4.9	0.7	0.7	0.7
	4.1	1.8	1.8	1.9
P4	5.4	12×10^{-3}	27×10^{-3}	22×10^{-3}
	5.0	11	8.0	8.7
	5.0	7.7	7.6	9.3
	4.6	11	14	16
$\overline{P7R}$	5.4	7.3×10^{-3}	9.2×10^{-3}	15×10^{-3}
	5.0	5.3	6.6	7.7
	4.6	8.4	9.2	12
P4R	5.4	1.1×10^{-2}	1.2×10^{-2}	1.4×10^{-2}
		(1.0, 6.0)[c]	(1.1, 7.1)[c]	(1.3, 4.9)[c]
	5.0	1.0	9.5×10^{-3}	1.1
	4.6	1.1	1.4×10^{-2}	1.3
		(1.0, 4.9)[c]	(1.2, 8.3)[c]	(1.1, 4.3)[c]
P3R	5.5	1.3×10^{-2}	1.9×10^{-2}	1.8×10^{-2}
		(1.2, 8.6)[c]	(1.7, 9.8)[c]	(1.7, 10)[c]
	5.0	1.1	9.7×10^{-3}	1.0
	4.6	2.0	2.8×10^{-2}	2.5
		(1.8, 22)[c]	(2.5, 23)[c]	(2.1, 17)[c]
Monomer	5.4	17×10^{-2}		
	4.6	75		

[a] Mean and standard deviation for the four experiments: OH, 5.8 ± 0.4 (±7%); NH unbonded, 6.0 ± 2.0 (±17%); NH bonded, 8.2 ± 1.3 (±16%) × 10^{-3}. [b] Mean and standard deviation: OH, 2.3 ± 0.4 (±17%); NH unbonded, 2.0 ± 0.3 (±15%); NH bonded, 2.6 ± 0.6 (±23%) × 10^{-2}. [c] Numbers in parentheses are rate constants × 10^2 when the exchange reaction is resolved into two parallel first-order processes.[21]

asymmetric carbon from each residue in the carbon backbone permits the existence of diastereoisomers. The tetramer, with $n = 4$, should consist of eight isomers: two isotactic, two syndiotactic, and four atactic.[10] If syndiotactic tetramer, like syndiotactic high polymer, is insoluble in all common solvents,[19] then the presumably water-soluble remainder would be one-third isotactic and two-thirds atactic. The isotactic component could display fast exchange, the atactic remainder slow exchange. A similar analysis for the trimer shows that half should be isotactic and half syndiotactic. Thin-layer chromatography, however, indicated that the trimer contained primarily one component.[20] A single isomer of the trimer could still manifest two rates if one amide residue exchanges faster than the other two. Indeed, space-filling models of the trimer indicate that the two terminal amide side chains can fold away from the central residue and form an NH···O=C bond. The environment of the central residue in this conformer is similar to that of the monomer. Hence the two terminal residues could be undergoing slow exchanges and the central residue fast exchange. It is also possible that in the tetramer the two rates reflect the different environments of the terminal and interior residues in the chain.

When the terminal butyl sulfide group is present in the oligomers, it could affect exchange rates and vitiate any comparison of behavior of oligomers with that of monomer or high polymer, neither of which has such a terminal group. In practice it seems that the perturbation in exchange kinetics is minor. For oligomer $\overline{P28}$ there is only one alkyl sulfide group per 28 isopropylacrylamide units. Any effect on the exchange rate of the terminal residue would be difficult to sort out from the overwhelming contribution of the other residues. In oligomer P4, the terminal C_4H_9S– group may be viewed as equivalent to a residue and contributing about 20% to the mass of an oligomer. As Table II shows the presence of the butyl sulfide in the tetramer decreases k_{min} by about 30%, not much more than the uncertainty in the rates in these systems. Evidently the sum of the effects of the apolar bulky isopropyl substituent on each of the amide nitrogens overshadows the contribution of the terminal butyl sulfide group.

The variation in exchange rate with molecular weight (Figure 4) shows a sharp drop in the range from 10^2 to 10^3 followed by a shallow plateau that extends beyond 10^5. In fact, the most precipitous region for fall of k_{min} occurs between the monomer and trimer. Examination of space-filling models of the trimer shows that the environment of the pendant amide groups with juxtaposed bulky isopropyl side chains from each residue approximates more nearly that of a residue imbedded in the high polymer than that of a free monomer immersed in fully aqueous surroundings. Interestingly enough the trimer shows a negative temperature

coefficient of solubility in water, as does the high polymer.

Thus a major change in local environment of an amide residue appears with the mutual interaction of only three residues. Smaller effects are manifested slowly and progressively as the degree of polymerization is further increased in these polyamides of random conformation.

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