Catalysis of Hydrogen–Deuterium Exchange in Polypeptides*

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ABSTRACT: The kinetics of hydrogen–deuterium exchange of poly-L-glutamic acid have been followed in dioxane–D_2O (1:1) over a pH range from below 1 to almost 7, at each of five temperatures from 25 to 61°. Parabolic rate–pD profiles are obtained demonstrating that D^+ and OD^- catalyze the exchange of NH in polyglutamic acid even in the helical conformation. The activation energy is 27 kcal mole^{-1}, close to that for a variety of model amides and peptides. The helical polypeptide is also subject to general acid–base catalysis of exchange.

The position of the pD of minimum exchange as well as k_{min}, the rate constant at pD_{min}, can be understood in terms of inductive and environmental effects which appear in studies of simple model amides. It is apparent that hydrogen–deuterium exchange rates of a polypeptide reflect both its structure and the environment in which it is immersed.

Rates of isotopic exchange of the hydrogen atoms of peptide NH groups in proteins must reflect the nature of the environment of these groups as well as the tertiary structure of the macromolecule. Early studies (Lenormant and Blout, 1953; Hvidt *et al.*, 1954) were aimed at an elucidation of these factors. Linderstrom-Lang (1955) adopted the tempting assumption that slowly exchanging NH groups could be assigned to helical portions of the polypeptide chain and rapidly exchanging ones to unfolded segments. On this basis, exchange rates could provide a measure of helicity of a polypeptide. It seems apparent, however, that this assumption is an oversimplification of the actual state of affairs, and that a much more extensive examination of the factors that influence hydrogen–deuterium exchange rates will be necessary before this technique can give some insight into protein structure.

A reasonable model compound that might behave like the NH group of an unfolded polypeptide is a simple amide, such as *N*-methylacetamide. Hydrogen–deuterium exchange in this compound

$$CH_3\overset{\overset{O}{\|}}{C}NHCH_3 + D_2O \underset{k_r}{\overset{k_f}{\rightleftharpoons}} CH_3\overset{\overset{O}{\|}}{C}NDCH_3 + HOD \qquad (1)$$

has been studied in several laboratories (Berger *et al.*, 1959; Nielsen, 1960; Klotz and Frank, 1962, 1965). All have observed that the exchange is catalyzed by H^+ and OH^-, and one (Klotz and Frank, 1962, 1965) has also found general acid–base catalysis by carboxylic acids and by nitrogenous bases. Furthermore, the activation energy for exchange in the monomeric amide, about 20 kcal/mole, is comparable to that reported for proteins (Linderstrom-Lang, 1955).

In one important respect, however, the behavior of *N*-methylacetamide differs from peptides. Every substance so far examined shows a minimum rate at some pH (pH_{min} or pD_{min} in D_2O), as must obviously be true if catalysis by both H^+ and OH^- exists. However, pD_{min} for simple amides in water occurs at about 5–6, whereas that for small peptides and for (nonhelical) poly-DL-alanine is in the range 2–3.5 (Nielsen *et al.*, 1960; Bryan and Nielsen, 1960) as one might expect from inductive influences on the peptide group. To examine inductive effects, the kinetics of exchange were studied for acetylglycine ethyl ester and for chloroacetylglycine ethyl ester.

With this background on the factors that determine exchange rates in models of accessible peptide NH groups we have turned to an examination of the kinetics of exchange in a helical polypeptide. The effects of H^+, OH^-, general acids and bases, and temperature have been studied. In many respects the behavior of NH groups in the helical state parallels that in the unfolded conformation. However, it is evident that when the rate constants are suitably normalized they are markedly lower in the helical form. It is still not clear, nevertheless, how much of this decrease is due to the locking in of the exchangeable hydrogens in NH···O=C bonds and how much is due to inaccessibility of steric origin from side chains or solvent.

Thus there are many factors which affect exchange rates of NH groups in a helical conformation, as well as in an unfolded state. The contributions of all of these factors must be assessed before one can assign

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an observed change in rate to a helix–coil transition or to differences in stability of helices.

Experimental Section

Materials. Heavy water, warranted to contain a minimum of 99.8% D_2O, was purchased from Bio-Rad Laboratories. Its purity was confirmed by measurement of the integrated absorbance of the OH peak in nuclear magnetic resonance. The dioxane used was Fisher Certified Reagent grade, except in studies with polylysine. For the latter work a sample of dioxane was purified by refluxing overnight with calcium hydride and cuprous chloride, filtering, and distilling at atmospheric pressure. This dioxane was stored over sodium.

Solutions of NaOD were prepared by dissolving low-carbonate NaOH pellets (Mallinckrodt) which had been deuterated by repeated cycles of dissolution in D_2O and drying in a closed system. Tetramethylammonium deuterioxide was prepared by an analogous procedure starting from a 10% aqueous solution of the hydroxide (Eastman Organic Chemicals). Deuterium chloride was purchased from Bio-Rad Laboratories as a 38% aqueous solution of 99.5 atom % D.

γ-Benzyl-L-glutamic acid (Mann Laboratories) was recrystallized three times from water. Potassium salts of trichloroacetic and dichloroacetic acids, respectively, were prepared from the corresponding reagent grade acids (Fisher Scientific and Eastman Organic Chemicals) by neutralization with KOH followed by lyophilization. Potassium dichloroacetate was recrystallized from methanol–ether to remove a yellow impurity. Potassium trifluoroacetate was purchased from Peninsular Chemical Research. All other salts were reagent grade.

Sodium poly-α-glutamate was purchased from Pilot Chemicals, Inc. Lots G44, G53, and G57 had a degree of polymerization of 475–610; lot G17 a degree of polymerization of 85. Poly-α-L-lysine, also obtained from Pilot, lots L38, L55, and L57, was estimated by the manufacturer to have a molecular weight of 90,000–115,000.

Exhaustively dialyzed poly-L-glutamic acid showed some ultraviolet absorption at 257, 262, and 267 mμ owing to residual benzyl groups. Overnight treatment of the polymer at pH 12 removed these groups completely. The polymer was then dialyzed exhaustively against water and recovered by lyophilization. The original ultraviolet absorption, compared to solutions with known concentrations of benzylglutamic acid, indicated that there had been only 1 benzyl group/500 glutamate residues in the polymer. Nevertheless, every lot of polymer was treated to remove this residual impurity. To make certain that this procedure did not degrade the polymer, sedimentation velocity experiments were carried out at pH 7.3 in 0.2 M NaCl for treated and untreated solutions of polymer. At 59,780 rpm in the Spinco Model E analytical ultracentrifuge, only one peak with a sedimentation coefficient of 2.15 ± 0.1 S was observed in all samples of degree of polymerization 475–610. Furthermore, the peak areas were essentially unchanged. As a control, 10% poly-L-glutamic acid of degree of polymerization 85 was added to that of 500; extensive broadening of the 2.1S peak was apparent and a long-trailing tail was observed.

N-Acetylglycine ethyl ester was prepared in a reaction of acetic anhydride and glycine ethyl ester hydrochloride following the procedure of Wolf and Niemann (1963); mp 44–46°, lit. (Wolf and Niemann, 1963) mp 44–46°. *Anal.* Calcd for $C_6H_{11}NO_3$: C, 49.7%; H, 7.58%; N, 9.66%. Found: C, 49.6%; H, 7.02%; N, 9.53%. The infrared spectrum showed no free amino or carboxyl groups.

N-Chloroacetylglycine ethyl ester was prepared, following the procedure of Holley and Holley (1952), in a reaction of chloroacetyl chloride and glycine ethyl ester hydrochloride; mp 61.5–62.6°, lit. (Diels and Heintzel, 1905) mp 62–63°. *Anal.* Calcd for $C_6H_{10}ClNO_3$: C, 40.2%; H, 5.57%; N, 7.81%. Found: C, 39.97%; H, 5.55%; N, 7.83%. The infrared spectrum showed no free amino or carboxyl groups.

Two mixed-bed resins were used to deionize the solutions of polyglutamate. Amberlite MB1 was washed beforehand with distilled water to remove an amine contaminant. Bio-Rad AG 501X8 was also washed with distilled water even though it seemed free of any amine impurity. Bio-Rad AG1X2 (Cl^-) was used, after washing with water, to replace Br^- in poly-L-lysine hydrobromide. Sephadex was purchased from Pharmacia Fine Chemicals and washed according to standard procedures.

pH Measurements. A Corning Model 12 meter was used with a Sargent miniature combination electrode. The pD was calculated from the equation of Glasoe and Long (1960), pD = pH reading + 0.40.

In the kinetic studies with polyglutamic acid at high temperatures direct pH measurements were not made at the temperature of the reaction. Instead the pH values were measured at 25°. These numbers were then corrected to the higher temperature by reference to calibration curves obtained from separate titrations of the polymer at each temperature. In the region of carboxyl group ionization these temperature corrections were essential.

In experiments with acid catalysts, pH values of the solutions were measured at the temperature of the exchange reaction, 61°. The pK values of the catalysts in the solvent mixture at 61° were obtained by determination of pD at the half-equivalence point.

Optical Rotation. Specific rotations $[\alpha]_D$ were measured with a Rudolph Model 80 precision polarimeter using water-jacketed 1-dm cells at the temperature of the exchange reaction. Although b_0 or $[m']_{233}$ is usually used to follow the helix–coil transition of polyglutamic acid, there is ample evidence (Wada, 1960; Yamaoka, 1964; Doty *et al.*, 1957) that changes in $[\alpha]_D$ parallel these other parameters.

Viscosity. An Ostwald viscometer with a flow time of 86 sec for water was used.

Exchange Rate Measurements. Hydrogen–deuterium exchange was followed in Cary Model 14R spectro-

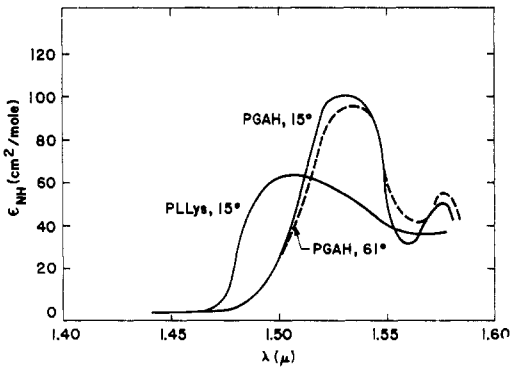


FIGURE 1: Absorption spectrum in dioxane–D_2O (1:1) of poly-L-lysine hydrochloride and poly-α-L-glutamic acid at pD 3.

photometer by measuring the absorbance (with the 0–0.1 slide wire) usually at 1.425 or 1.49 μ, the former being the band of the OH generated and the latter that of the NH disappearing. Temperature was controlled by circulation of water through the cell compartment as well as through a specially constructed cell holder. Temperature was measured with a thermistor probe (Yellow Springs Instrument Co.). Spectrophotometer cells of 2-cm path length were usually used. The reference cell contained D_2O–dioxane (1:1). In this solvent, dioxane is 5.8 M and D_2O is 27.6 M; exchangeable peptide NH was 0.1–0.3 M.

Polylysine, after dialysis to remove an aromatic odor and passage through an AG1X2 resin column, was slurried with purified dioxane. To the cooled slurry, cooled D_2O was added with vigorous stirring. A clear solution resulted within 4 min. The exchange of a 2% polymer solution was followed by scanning the near-infrared spectrum from 1.1 to 1.6 μ.

Solutions of polyglutamic acid in dioxane–D_2O (1:1) were prepared from the solid acid form of the polymer. Deionization of polyglutamate was achieved, following the technique of Nagasawa and Holtzer (1964), with aqueous solutions containing 0.45% or less polymer. The effluent from the mixed-bed resin was lyophilized to yield fluffy solid polyglutamic acid. This material was slurried with pure dioxane. Upon addition of water the slurry dissolved in less than 30 sec. The exchange of a 2% (0.16 residue molar) solution of polymer was followed by scanning in the Cary spectrophotometer in the range 1.1–1.6 μ. The solutions of polyglutamic acid were slightly turbid, but absorption in the range 1.1–1.3 μ was independent of the extent of exchange. A reading at 1.265 μ was used, therefore, to establish a base line for each solution, and absorbancy changes were measured from this base line, at 1.435 μ to follow OH increase, and at 1.538 μ to follow NH decrease. All reported rate constants for polyglutamic acid are averages of measurements at these two wavelengths.

Results

Spectra in Near Infrared. The spectrum of poly-L-lysine in the mixed dioxane–D_2O solvent at pD 3 is shown in Figure 1. This was obtained by taking absorbance readings of the polymer solution and subtracting the contributions, at any given time, of the OH and OD absorbances (ND does not absorb in this range). Due to possible cumulative errors in these computations, the molecular extinction coefficients, ϵ_{NH}, are estimated to be reliable only to ±10%.

Acetylglycine ethyl ester and chloroacetylglycine ethyl ester showed spectra similar to that of polylysine, with a single peak near 1.5 μ, but with ϵ_{NH} of about 100 cm^2 mole. In contrast, polyglutamic acid at pD 3 in the mixed solvent shows a double peak at 1.53 and 1.57 μ (Figure 1).

These observations are consistent with the presence of "free" NH groups in the glycine esters and in polylysine, *i.e.*, NH groups not bonded to C=O (Klotz and Franzen, 1962). The resemblance of polylysine to the small amides provides further evidence that this polymer at pD 3 is in a disordered nonhelical conformation. In contrast, the presence of double peaks at 1.53–1.57 μ in polyglutamic acid, similar to those found in concentrated solutions of *N*-methylacetamide (Klotz and Franzen, 1962; Hanlon and Klotz, 1965) in which NH···O=C bonds are predominant, is consistent with a helical conformation for this polypeptide at pD 3 in a mixed dioxane–water solvent. Furthermore the infrared spectra indicate that this helical conformation is retained as the temperature is raised from 15 to 61°.

From these spectra it is apparent that exchange kinetics can be followed at 1.49 (free NH) or 1.53 μ (bonded NH) as well as at 1.425 μ (OH generated).

Calculation of Rate Constants. The rate expression for the pseudo-first-order reaction

$$>NH + D_2O \xrightarrow{k} >ND + HOD \qquad (2)$$

is

$$-\frac{d(NH)}{dt} = \frac{d(OH)}{OH} = k(NH) \qquad (3)$$

Absorbance data were used to compute rate constants for the model amides by the procedure of Guggenheim (1926) and by that of Kezdy *et al.* (1959). The latter proved more convenient in showing the order of the reaction and the scatter of the data, but essentially the same rate constants were found with the two methods. For polyglutamic acid conventional logarithmic plots of optical density were used.

Catalytic constants for D^+ and OD^- were calculated from the results of the following analysis. Recognizing catalysis by deuterium and by deuterioxide ions, we may write

$$k = k_0 + k_D(D^+) + k_{OD}(OD^-) \qquad (4)$$

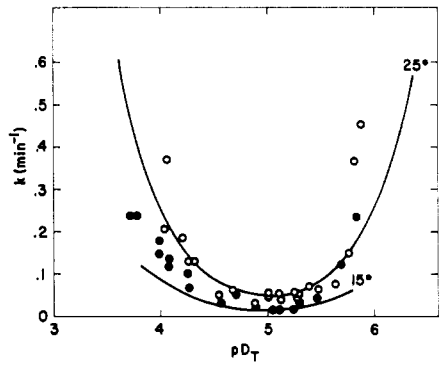


FIGURE 2: Hydrogen–deuterium exchange rate constants *vs.* pD_T at the respective temperatures shown for acetylglycine ethyl ester in D_2O–dioxane (1:1).

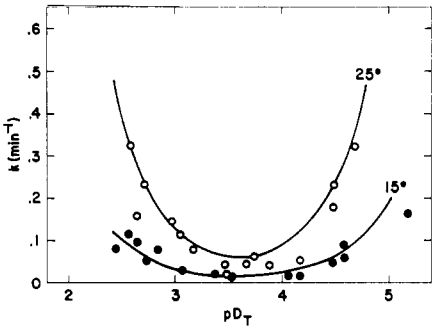


FIGURE 3: Hydrogen-deuterium exchange rate constants *vs.* pD_T at the respective temperatures shown, for chloroacetylglycine ethyl ester in D_2O–dioxane (1:1).

$$= k_0 + k_D(D^+) + k_{OD}\frac{K_w}{(D^+)} \qquad (5)$$

where k_0 is the rate constant for the spontaneous reaction, k_D that for the acid-catalyzed reaction, and k_{OD} that for the base-catalyzed reaction, and K_w is the self-dissociation constant for water in the mixed solvent being used. It is apparent from eq 5 that k must go through a minimum at some (D^+) or pD. This (D^+_{min}) can be related to the rate constants by differentiating eq 5

$$\frac{\partial k}{\partial(D^+)} = k_D - k_{OD}\frac{K_w}{(D^+_{min})^2} \qquad (6)$$

setting (6) equal to zero and solving for (D^+_{min})

$$(D^+_{min})^2 = \frac{k_{OD}}{k_D}K_w \qquad (7)$$

If we now write for the minimum exchange rate

$$k_{min} = k_0 + k_D(D^+_{min}) + \frac{k_{OD}K_w}{(D^+_{min})} \qquad (8)$$

and use the result of eq 7 to replace the third term on the right in eq 8 by $k_D(D^+_{min})$ we obtain

$$k_{min} = k_0 + 2k_D(D^+_{min}) \qquad (9)$$

Let us define

$$n = \frac{(D^+)}{(D^+_{min})} \qquad (10)$$

Then eq 5 may be written in an alternative form

$$k = k_0 + k_D n(D^+_{min}) + \frac{k_{OD}K_w}{n(D^+_{min})} \qquad (11)$$

Simple algebraic manipulations give

$$k - k_{min} = k_D(D^+_{min})\left(n - 2 + \frac{1}{n}\right) \qquad (12)$$

which in turn can be converted into

$$k - k_{min} = k_D(D^+_{min})\frac{(n-1)^2}{n} \qquad (13)$$

Obviously a graph of k *vs.* $(n-1)^2/n$ will give a straight line with a slope of $k_D(D^+_{min})$.

Although the use of this graph requires us to choose (D^+_{min}) in order to evaluate n, this choice is not difficult to make; for a graph of k *vs.* pD can be shown to be symmetric about pD_{min}, and hence, if pD_{min} is chosen correctly, all points of the branch above pD_{min} will fall on the same line as those below pD_{min}.

Having evaluated k_D from eq 13, we can obtain k_{OD} from eq 7. In turn k_0 can be computed from eq 9.

In the calculations of k_{OD} one must know K_w. For water–dioxane, the figure and tables of Harned and Owen (1958) supply the necessary K values. These K values were normalized to D_2O–dioxane by multiplying by the known value of K_{D_2O}/K_{H_2O} at 25° (Kingerley and LaMer, 1941).

In the presence of general acid–base catalysts, HA, the catalytic constant, k_{cat}, may be defined as

$$k_{cat} = k' - k \qquad (14)$$

where k' is the observed rate constant in the presence of catalyst. As has been shown previously (Klotz and Frank, 1965), the catalytic rate constant, k_a, for the acid form, DA, of the catalyst and that, k_b, for the basic form, A, may be evaluated from

$$\frac{k_{cat}}{(DA) + (A)} = k_a + \frac{k_b - k_a}{1 + (D^+)/K_a} \qquad (15)$$

where K_a is the apparent dissociation constant of the catalyst in the mixed solvent used.

TABLE I: Exchange Rate Parameters for Free NH Groups in D_2O–Dioxane (1:1).

Substance	Temp (°C)	pD_{min}	k_{min} (min^{-1})	$t_{1/2,min}$ (min)	k_D (M^{-1} min^{-1})	k_{OD} (M^{-1} min^{-1})	k_0 (min^{-1})
N-Methylacetamide[a]	25	6.20	0.078	9	4.02×10^4	13.2×10^8	0
Acetylglycine ethyl ester	15	5.00	0.016	43	8.0×10^2	1.2×10^{10}	0
Acetylglycine ethyl ester	25	5.00	0.05	14	2.4×10^3	1.6×10^{10}	0
Chloroacetylglycine ethyl ester	15	3.60	0.015	46	3.0×10^1	2.8×10^{11}	0
Chloroacetylglycine ethyl ester	25	3.60	0.06	12	1.2×10^2	4.8×10^{11}	0
Poly-L-lysine	15	2.55	0.013	53	2.0	2.9×10^{12}	0

[a] Measurements made by Mr. P. L. Feidelseit.

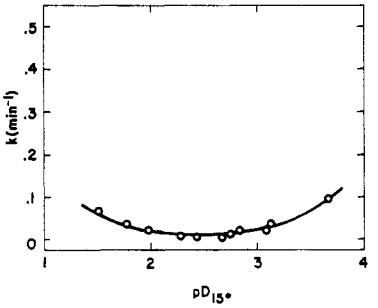


FIGURE 4: Hydrogen–deuterium exchange rate constants *vs.* pD for poly-L-lysine in D_2O–dioxane (1:1) at 15°.

TABLE II: Activation Energies for NH–ND Exchange.

Substance	E_D* (kcal/mole)
N-Methylacetamide[a]	17
Acetylglycine ethyl ester	19 ± 5
Chloroacetylglycine ethyl ester	23 ± 5
Insulin[b]	20
Poly-DL-alanine[c]	17
Poly-L-glutamic acid	27 ± 5

[a] Klotz and Frank (1965). [b] Linderstrom-Lang (1955). [c] Hvidt and Nielsen (1966).

Activation energies, E^*, were computed from the familiar Arrhenius equation applied to k_D data. As will be pointed out in the discussion, this procedure is equivalent to using observed rate constants at the extremes of pH, where only acid or only base catalysis is effective.

Exchange Rates of Glycine Esters. Rate constants for the exchange of the free peptide NH groups in acetylglycine ethyl ester and in chloroacetylglycine ethyl ester are summarized in Figures 2 and 3, respectively. Various parameters for exchange rates in these and related compounds with free NH groups are assembled in Table I. The reactions all followed first-order kinetics. Total absorbancy changes corresponded with those expected for one exchangeable hydrogen per molecule.

Particularly noteworthy is the relative constancy of k_{min}, at a given temperature, for the different substances listed, despite large shifts in pD_{min}. Activation energies in the acid-catalyzed region, calculated from the rates at 15 and 25°, are listed in Table II.

Exchange Rates of Polylysine. Rate constants for the NH group in poly-L-lysine are shown in Figure 4, and associated parameters are listed in Table I. In all experiments the reaction followed first-order kinetics. The initial very rapid absorbancy change corresponded to three hydrogens per residue, which we attribute to very fast exchange of the hydrogens from the NH_3^+ group of each lysyl residue. The total absorbancy change of the measurably slow reaction corresponded to one H per residue and we assume this represents the peptide NH.

It is noteworthy that k_{min} for polylysine is close to that of the small model compounds despite the difference in pD_{min}. The near-infrared spectrum of polylysine (Figure 1) is that of a "free" NH, that is, not NH···O=C bonded. Optical rotations were also measured with our sample of polymer. Between pD 1.07 and 8.54, $[\alpha]_D^{25}$ was 73.9 ± 2.6. The lack of dependence of $[\alpha]_D$ on pH as well as the magnitude of the optical rotation indicate that polylysine is in a nonhelical conformation (Doty *et al.*, 1958).

Region of Helix–Coil Transition of Polyglutamic Acid. To interpret the exchange kinetics we must know the pD below which this polypeptide exists in a helical conformation. Four methods were used to delineate this region.

Titration curves for the COOH groups were determined at each temperature from 16 to 61°. Graphs of $pD - \log(\alpha/1 - \alpha)$ *vs.* α, the degree of dissociation, were similar to those of previous investigators (Wada, 1960; Doty *et al.*, 1957; Nagasawa and Holtzer, 1964; Jacobsen, 1964; Miller and Nylund, 1965). These were used to determine pK_{app}, and the pD and α at the onset of the helix–coil transition in the mixed solvent (dioxane–water) and at the polymer concentration (2%)

TABLE III: Helix–Coil Transition from Titration Data.

Temp (°C)	pK_{app}	pD at Onset of Transition	α at Onset of Transition
16	6.83	6.4	0.20
25	6.75	6.3	0.18
40	6.60	6.2	0.17
52	6.45	6.1	0.17
61	6.35	5.9	0.15

used in our exchange studies. These parameters are listed in Table III.

Titrations were also carried out at 25 and 52° with DCl to see if the amide groups became protonated at low pD. No such protonation was observed down to a pD of 0.7. (At 25° the polymer precipitated below pH 4 but the titration curve remained smooth.) From these results plus those obtained in the titration of COOH groups, we conclude that poly-L-glutamic acid in dioxane–water remains helical in conformation between pD 0.7 and 6.

In addition to acid–base titrations, optical rotations were measured in the dioxane–water solvent at 25, 40, 52, and 61°. Although solvent does affect the rotation, our results are in good agreement with those of earlier investigations (Doty *et al.*, 1957). The rotation data confirm the conclusions from titrations that poly-L-glutamic acid is helical in the pD range 0.7–6.

Likewise viscosity measurements showed no important changes in the region below pD 6. Finally the near-infrared spectra, at all temperatures and at all pD values at which exchange was slow, showed the doublet at 1.53 and 1.57 μ (Figure 1), as would be expected for an NH···O=C bond.

Thus all four methods of examination give results that are consistent with a helical conformation for poly-L-glutamic acid in dioxane–water (1:1) at pD values below 6.

Exchange Rates of Poly-L-Glutamic Acid. In all experiments first-order kinetics was observed throughout the period of observation (2–4 half-lives). Furthermore in all cases complete exchange was reached eventually, although in the slowest reactions this state

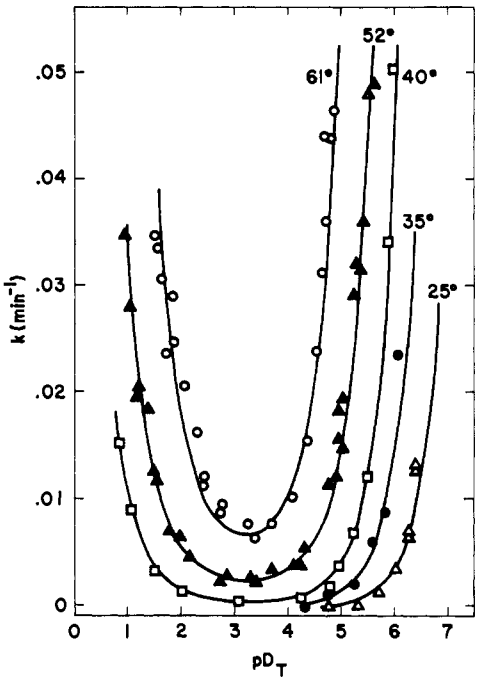


FIGURE 5: Hydrogen–deuterium exchange rate constants *vs.* pD_T at the respective temperatures shown for 2% poly-L-glutamic acid in D_2O–dioxane (1:1).

was attainable in a reasonable time only by raising the temperature. The amount of slowly exchanged hydrogen always corresponded to one H per residue.

The rate constants for all temperatures and pD values examined are shown in Figure 5. Appropriate parameters are summarized in Table IV. At 25 and 35° exchange could not be followed below pD 4 because the polymer came out of solution. Therefore, k_0 was calculated from observations at higher temperatures by extrapolating linearly in a graph of log k_0 *vs.* $1/T$. Also we assumed pD_{min} is 3.20 at 25 and 35° in view of its constancy in the measurable region 40–61°. With this value of pD_{min} graphs of k *vs.* $(n-1)^2/n$ were linear, indicating that the rate constants were linearly dependent on (D^+) and on (OD^-).

At 25°, solutions at pD 5.27 and 5.95 and one of each pair at 6.18 and 6.33 (see Figure 5) were prepared with our specially purified dioxane. The exchange

TABLE IV: Exchange Rate Constants for Helical Poly-L-glutamic Acid (2%) in D_2O–Dioxane (1:1).

Temp (°C)	pD_{min}	k_{min} (min^{-1})	$t_{1/2,min}$ (hr)	k_D (M^{-1} min^{-1})	k_{OD} (M^{-1} min^{-1})	k_0 (min^{-1})
25	(3.20)	0.54×10^{-4}	213	0.0114	2.63×10^{8}	0.04×10^{-3}
35	(3.20)	1.96×10^{-4}	59	0.0412	8.98×10^{8}	0.14×10^{-3}
40	3.20	5.0×10^{-4}	23	0.108	9.83×10^{8}	0.36×10^{-3}
52	3.19	2.5×10^{-3}	4.6	0.332	15.2×10^{8}	2.1×10^{-3}
61	3.19	6.7×10^{-3}	1.7	1.30	39.8×10^{8}	5.0×10^{-3}

TABLE V: Effect of Added Salts on Exchange Rates of Poly-L-glutamic Acid.

40°	pD	3.02	3.08				
	Salt concn (M)	0.00	0.1				
	k (min^{-1})	0.00514	0.00583				
52°	pD	2.83	2.83	2.84	2.98	3.30	3.37
	Salt concn (M)	0.00	0.1	0.1	0.1	0.05	0.00
	k (min^{-1})	0.00263	0.00246	0.00272	0.00276	0.00272	0.00244
61°	pD	2.71	2.75				
	Salt concn (M)	0.00	0.5				
	k (min^{-1})	0.00869	0.00892				

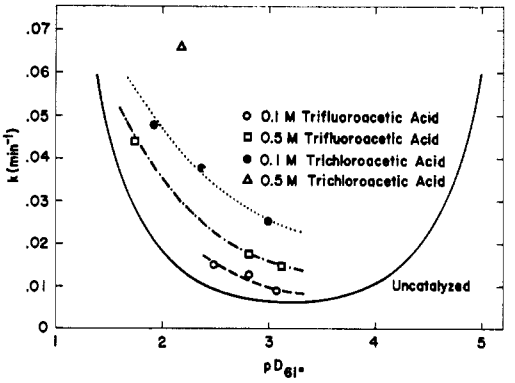


FIGURE 6: Catalysis of hydrogen–deuterium exchange in 2% poly-L-glutamic acid in D_2O–dioxane (1:1) at 61° by trifluoroacetic acid and by trichloroacetic acid.

rates were not significantly different from those observed in solutions made up with unpurified, but reagent grade, dioxane.

There are no significant salt effects on the rates of exchange. Some relevant data are summarized in Table V. These observations parallel those reported for *N*-methylacetamide (Klotz and Frank, 1965). The concentration of salt in solutions without added salt did not exceed 0.001 M. Clearly the extremely large effect of pD on the exchange rates cannot be ascribed to changes in salt concentration due to added base or acid. Studies of added salt effects were all carried out in the region of pD_{min}, where the k values are in a relatively gentle valley; outside this region apparent changes in rate constant with added salt may be due to shifts in reading of the pH electrode, and in solutions of polyglutamic acid to shifts in pK_{app} of the γ-carboxyl group. In fact salt added in the helix–coil transition region produced great increases in rate.

From the rate constants k_D and k_{OD} at various temperatures, activation energies can be calculated in different pD regions. In the acid range E_D* is 27 ± 5 kcal/mole (Table II).

General Acid–Base Catalysis of Exchange Rates of Polyglutamic Acid. These studies were carried out at 61° where the rates are experimentally most convenient. The effects of trifluoroacetic acid and of trichloroacetic acid are shown in Figure 6 and those of dichloroacetic acid are summarized in Figure 7. Marked increases in rate are observed particularly with 0.5 M additive. It should be noted that 0.5 M salt (Table V) did not produce catalysis of exchange. The catalysts used did not alter the optical rotation of polyglutamic acid at 61°. The helix–coil transition region was avoided because interpretation of observations would be complicated by the effects of the catalysts on the COOH group ionization.

Catalytic parameters are summarized in Table VI.

TABLE VI: Catalytic Parameters for Acid–Base Catalysts.

Substance	Concn (M)	pK_{app}	k_a (M^{-1} min^{-1})	k_b (M^{-1} min^{-1})
CF_3COOH	0.1	1.92	0.24	0.02
CF_3COOH	0.5	1.65	0.06	0.02
CCl_3COOH	0.1	2.18	0.38	0.17
$CHCl_2COOH$	0.1	3.37	0.03	0.15
$CHCl_2COOH$	0.5	3.17	0.03	0.03

It is evident that for trifluoroacetic acid and trichloroacetic acid, which have pK_{app} values below pD_{min} uncatalyzed, the acid form is the more effective catalyst. With dichloroacetic acid, at 0.1 M, where pK_{app} is slightly above pD_{min} uncatalyzed, the anionic form is the more effective catalyst, whereas at 0.5 M where $pK_{app} \simeq pD_{min}$ acid and base forms are equally effective. These relationships for exchange in the helical polypeptide parallel those reported previously (Klotz and Frank, 1965) with the model amide, *N*-methylacetamide.

Exchange Rates in the Helix–Coil Transition Region. Figure 8 summarizes some exchange rates at 25°

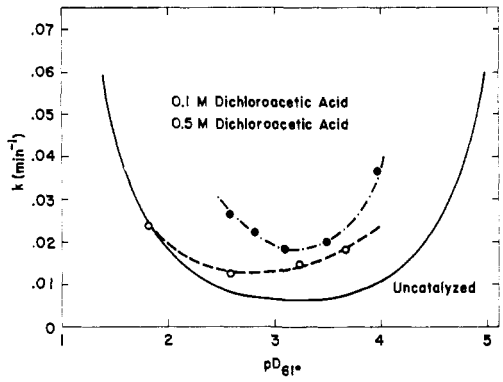


FIGURE 7: Catalysis of hydrogen–deuterium exchange in 2% poly-L-glutamic acid in D_2O–dioxane (1:1) at 61° by dichloroacetic acid.

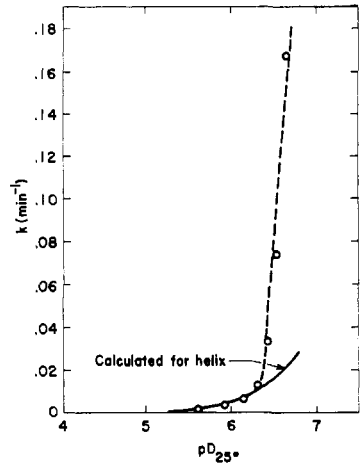


FIGURE 8: Hydrogen–deuterium exchange rate constants *vs.* pD for (2%) poly-L-glutamic acid in D_2O–dioxane (1:1) at 25° in region of helix–coil transition.

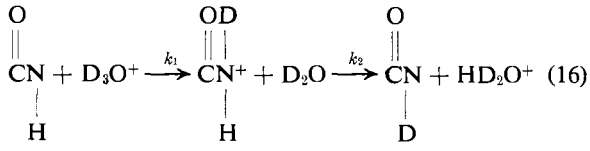
as one enters the pD range of the helix–coil transition. A reference line is also shown in this figure which is the calculated extrapolation, using eq 4, of the rate constants observed for polyglutamic acid in the helical pD region. Similar experiments were carried out at 16 and 5° in the hope that the slowing down of the rates would permit us to penetrate deeper into the coil region. Although decreasing the temperature did slow down the rates, it also shifted the helix–coil transition to a higher pD. Hence the relative increases in rate at 16 and 5° were not much different from those shown in Figure 8 for 25°.

In all experiments the reactions were first order. Furthermore the near-infrared spectrum was that of a bonded NH···O=C. The sharp increase in exchange is observed at a pD near, or slightly below, that estimated for the onset of the helix–coil transition from optical rotation and titration data. The increase may be due to a loosening of the helix, but it may also reflect the increasing local concentration of γ-glutamyl COO^- anions which may produce catalysis or may modify the structure of neighboring solvent.

Discussion

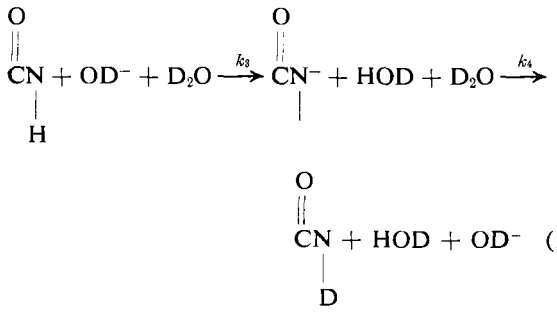
The parabolic curves for the rate constants *vs.* pD for helical poly-L-glutamic acid (Figure 5) seem very similar to those reported previously (Klotz and Frank, 1965) for a simple model amide *N*-methylacetamide. To appreciate the significance of the differences as well as the similarities in deuterium–hydrogen exchange in these two very different environments of the NH group we must first examine three features in detail: (1) pD_{min}, (2) k_{min}, and (3) E_D^*.

pD of Minimum Exchange. The mechanism of the deuterium–hydrogen exchange reaction in amides and peptides may be depicted (Berger *et al.*, 1959; Nielsen, 1960; Klotz and Frank, 1965) in acid solution by the equations



(16)

Correspondingly in basic solution we may write



(17)

In view of the weak basicity and weak acidity of the amide NH, one would expect k_1 to be rate controlling in the acid-catalyzed exchange, eq 16, and k_3 to be rate limiting in the base-catalyzed exchange, eq 17, and the kinetic behavior fits this expectation (Berger *et al.*, 1959; Nielsen, 1960; Klotz and Frank, 1965).

On the basis of these rate-controlling steps, and keeping in mind that k_0 is generally negligible, we conclude from a comparison of eq 16 and 17, respectively, with eq 4 and 3 that

$$k_1 = k_D \quad (18)$$

$$k_3 = k_{OD} \quad (19)$$

If we combine the results of eq 18 and 19 with (7), we can write in logarithmic form

TABLE VII: Effect of D_2O Concentration on Kinetic Parameters for Exchange Reaction of *N*-Methylacetamide[a] at 25°.

Solvent	pD_{min}	k_{min} (min^{-1})	$pK_{w_{D_2O}}$	k_D (M^{-1} min^{-1})	k_{OD} (M^{-1} min^{-1})
D_2O	5.45	0.48	14.8	5.9×10^4	6.3×10^8
D_2O–dioxane (1:1) (27.6 M D_2O–5.8 M dioxane)	6.20	0.078	16.8	4.0×10^4	13×10^8
4.2 M D_2O–7 M dioxane–4.2 M *N*-methylacetamide	7.53	0.0019		2.1×10^4	14×10^8

[a] Feidelseit (1967).

$$pD_{min} = {}^1/_2 pK_w - {}^1/_2 \log \frac{k_3}{k_1} \qquad (20)$$

We are now in a position to understand inductive effects on pD_{min}. An electron-withdrawing substituent attached to the CONH group will weaken the basicity of the nitrogen, and hence should *decrease* k_1, the rate of protonation. The same substituent, being electron withdrawing, should increase the acidity of NH group and hence *increase* k_3. The net result, as is apparent from eq 20, would be a decrease in pD_{min}, *i.e.*, a shift of the minimum in the rate–pH profile to lower pD. This is exactly what is observed in a comparison of pD_{min} (in dioxane–water) for $CH_3CONHCH_3$, $CH_3CONHCH_2COOC_2H_5$, and $ClCH_2CONHCH_2COOC_2H_5$ (Table I), the values shifting from 6.2 to 5.0 to 3.6. With this background it is also easy to see why Gly-Gly, Ala-Gly-Gly, and poly-DL-alanine have pD_{min} values in the range 2.2–3.2 in pure D_2O solvent (Bryan and Nielsen, 1960; Nielsen *et al.*, 1960).

If the electron-withdrawing effect, Z, is equal, but reciprocal, on k_D and k_{OD}, we may state that

$$k'_D = \frac{1}{Z} k_D \qquad (21)$$

$$k'_{OD} = Z k_{OD} \qquad (22)$$

where k' refers to the appropriate rate constant of the substance with the added electron-withdrawing substituent. Then one can combine these statements with eq 7 to write

$$(D^{+\prime}_{min})^2 = \frac{Z k_{OD} K_w}{\frac{1}{Z} k_D} \qquad (23)$$

or

$$(D^{+\prime}_{min}) = Z(D^+_{min}) \qquad (24)$$

The pD of minimum exchange is also affected by the nature of the solvent environment. It is apparent from eq 20 that even if addition of a relatively apolar substance to water does not affect k_3/k_1, it must shift pD_{min} since it will change pK_w. For example, if dioxane is added to water, pK_w rises and hence pD_{min} should be shifted to higher pH values. This indeed is observed (Table VII) for *N*-methylacetamide, although the shift is not quite equal to ${}^1/_2 \Delta pK_w$, as one would expect if only the first term on the right of eq 20 needs to be considered.

The observed pD_{min} of 3.20 for poly-L-glutamic acid in D_2O–dioxane (1:1) is a reasonable one, in the range to be expected from an inductive effect of amino acid residues on both sides of each CONH group. The value reported for poly-DL-alanine (Bryan and Nielsen, 1960) in pure D_2O is 3.2; corrected to D_2O–dioxane (1:1) on the basis of the experiments (Table VII) with a model amide, one obtains a pD_{min} of 3.9. A third polypeptide, poly-L-lysine has been found to have a pD_{min} of 2.55 in D_2O–dioxane (Table I). The difference between 2.55 and 3.9 very likely reflects electrostatic and catalytic effects of the charged ϵ-NH_3^+ side chains of polylysine the charge in itself favoring OD^- over D^+ catalysis. The smaller difference between 3.2 for polyglutamic acid and 3.9 for polyalanine may also be an indication of catalytic effects by the carboxylic acid side chains of each residue.

Minimum Exchange Rate, k_{min}. Let us consider first the effect of electron-withdrawing substituents on k_{min}. From eq 9 we may say that

$$k'_{min} = k_0 + 2k'_D(D^{+\prime}_{min}) \qquad (25)$$

assuming, as our data justify, that k_0 is essentially unchanged or negligible. Inserting the information contained in eq 21–24, we obtain

$$k'_{min} = k_0 + 2\frac{1}{Z} k_D \left(\frac{Z k_{OD} K_w}{\frac{1}{Z} k_D} \right)^{1/2} = k_{min} \qquad (26)$$

Thus we arrive at the very interesting conclusion that (to a first approximation) inductive effects should not change k_{min}, although as we have seen, they should markedly shift D_{min}. Again the data for amides

in Table I are in accord with this expectation, k_{min} (at 25°) varying only from 0.05 to 0.078 for compounds whose D_{min} is shifted by $10^{2.6}$.

A comparison of exchange rates at a single pH for two different substances is thus not meaningful in conformation discussions, for curves with different pD_{min} values will cross each other. A conclusion as to which is the faster exchanging substance would thus depend on the accidental pD chosen to make the comparison, and a different conclusion could be reached at some other pD. On the other hand, a comparison of k_{min} values should be significant, for inductive effects do not perturb this rate parameter appreciably and any observed variations in k_{min} can be attributed to environmental and conformational influences. It is thus apparent why a full k–pD profile is essential.

Although inductive effects do not perturb k_{min}, changes in the nature of the solvent should and do affect the minimum rate. According to eq 9 k_{min} will be changed in a mixed solvent if k_D and (D^+_{min}) change in any but a compensating manner. Comparison of k_D in H_2O and D_2O–dioxane (1:1) (Table VII) shows only a small change. On the other hand, D_{min} changes by a factor of $10^{0.7}$ or 5. Interestingly, k_{min} drops by almost a corresponding factor 0.48/0.078 or 6.1. It seems clear, therefore, that k_{min} will always drop when K_w becomes smaller since (D^+_{min}) must always respond to a change in K_w as is evident from eq 7.

Although pD_{min} for helical polyglutamic acid is in the same region as that for simple model amides and disordered polypeptides, k_{min}, 0.54×10^{-4} at 25° (Table IV), is 10^{-3} times as large. Thus there can be no doubt that exchange is slowed down in the helical confomation.

Contributions to this decrease in rate may come from several sources, however. In the past the decrease has been attributed entirely to the locking in of NH···O=C hydrogen bonds. It is apparent from the factors described above that if K_w in the vicinity of the helix is different from that in bulk water then k_{min} will be changed. We have no direct method of checking this point but it seems likely that K_w might be appreciably smaller in the neighborhood of CH_2CH_2COOH side chains since these provide a high local concentration of apolar, noncharged groups in the vicinity of the polypeptide macromolecule.[1] It is known that the pK values of groups conjugated to proteins and apolar synthetic polymers show a shift in the direction favoring the noncharged species (Klotz and Ayers, 1957; Klotz and Stryker, 1960). In addition to these factors bulky side chains may provide some steric interference with exchange.

One method of trying to assess the relative importance of these different factors slowing down k_{min} is to examine exchange in a polymeric amide unable to form helical hydrogen bonds but containing fairly large pendant apolar groups. Dr. J. Scarpa in this laboratory has carried out detailed studies with one such polymer, polyisopropylacrylamide, and has, indeed, found hundredfold decreases (compared to simple amides) in rates of deuterium–hydrogen exchange. The studies will be reported in a separate paper.

There is also evidence that steric factors affect k_{min} even in small amides. In addition to those reported (Klotz and Feidelseit, 1966), *N*-alkyl-substituted acetamides have been examined up to alkyl = butyl, and k_{min} has been found to decrease progressively but pD_{min} to remain virtually unchanged. Such behavior can be readily rationalized in terms of eq 7 and 9. If k_0, k_D, and k_{OD} are affected in the same direction by steric factors (as seems reasonable from an accessibility viewpoint) so that each one is changed by the same factor Z, then it follows from eq 7 that the new pD_{min} would not be different from the original. On the other hand, eq 9 gives for the new minimum rate constant

$$k'_{min} = Zk_{min} \qquad (27)$$

Thus if $Z < 1$, as would be expected for steric interference, k_{min} will decrease but pD_{min} will remain unchanged.

Since pD_{min} for polyglutamic acid is close to that for model amides but k_{min} is much lower, it is clear that the slowing down of deuterium–hydrogen in this polypeptide is in line with expectations from steric effects. It is not possible to tell, however, whether these steric affects arise directly from protruding sides chains or from the effects of these side chains on the structure of the aqueous solvent.

Activation Energies. From eq 5 and the Arrhenius relation, it follows that in the acid region of the exchange reaction

$$E_{\text{acid region}}^* = E^*_D \qquad (28)$$

where E^*_D refers to the activation energy of the D^+-catalyzed reaction. In the basic region it can be shown from eq 5 and 7 that if (D^+_{min}) is independent of temperature, as seems to be true in the experiments with the compounds reported here, then

$$E^*_{\text{basic region}} = E^*_{\text{acid region}} = E^*_{OD} + \Delta H_w \qquad (29)$$

where ΔH_w is the enthalpy of ionization of water. Direct experimental evaluation of E^* in the acid or basic limbs of the rate–pD profile (*e.g.*, Figure 5) should thus both give E^*_D and hence only this activation energy is listed in Table II.

For all the simple amides studied and for insulin and polyalanine E^*_D is about 20 kcal/mole. The value observed for polyglutamic acid is slightly higher, 27 kcal/mole, but the uncertainty is large enough to bring it in the range of E^*_D (23 kcal/mole) for chloroacetylglycine ethyl ester. In any event it is clear that the

[1] In contrast polylysine in the vicinity of pD_{min} is a highly charged, extended, or swollen macromolecule. The local concentration of side chains would thus be much smaller than for helical polyglutamic acid. Furthermore the residues would be charged and hence have a structure breaking (rather than structure making) effect on water.

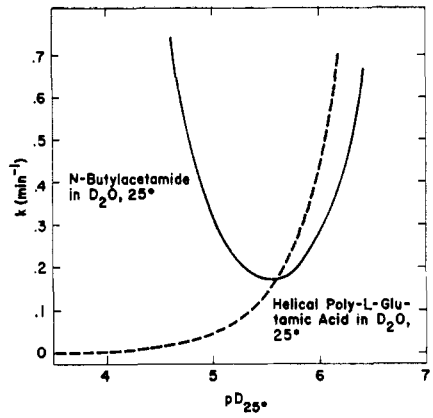


FIGURE 9: Hydrogen–deuterium exchange rate constants in D_2O at 25°. The dashed curve for polyglutamic acid has been calculated from the observed results in D_2O–dioxane corrected to pure D_2O.

helical polypeptide does not show an activation energy for deuterium–hydrogen exchange which is markedly different from that of small amides.

Conclusion

This survey of the hydrogen–deuterium exchange kinetics of helical polyglutamic acid shows how remarkedly similar the fundamental features are to the corresponding reaction in simple amides and nonhelical peptides. In all cases, the exchange is catalyzed by H^+, OH^-, and general acids and bases; and the activation energies are comparable.

Only a detailed rate–pH profile will show the essential difference in exchange behavior of the helical polypeptide, the much smaller k_{min}. Without such a detailed analysis one could be faced with some perplexing observations. For example, using the k_D and k_{OD} that fit the curve for 25° in Figure 5, one can compute that at pD 7, helical polyglutamic acid in dioxane–D_2O would exchange its amide NH with a half-life of 13 min. In pure D_2O the half-life would be about 3 min even if the polymer were totally in the helical conformation. In fact helical polyglutamic acid, if it could be maintained in that conformation above pD 5.7, would exchange more rapidly than the nonbonded NH of the simple model compound *N*-butylacetamide (Figure 9). Thus slowly exchanging NH hydrogens at neutral pH cannot be ascribed *per se* to those in a helical conformation.

There is no doubt, nevertheless, that k_{min} at pD_{min} for helical polyglutamic acid is much smaller than that for any simple amide or for nonhelical polylysine. There are a number of factors that may contribute to this reduction in rate of exchange. Bonding of NH··· O=C is the one most commonly fastened upon. The present studies show that even a nonbonded NH in a simple amide experiences a decreased exchange rate in an environment in which K_w is lowered. A change in K_w at the surface of a polypeptide is also possible, particularly if noncharged apolar side chains are present. Furthermore there may be steric effects which decrease accessibility to the amide group. These may arise from the side chains themselves or from their effects on the structure of the neighboring solvent. It is thus clear that hydrogen–deuterium exchange rates of a polypeptide reflect both the conformational structure of the macromolecule and the environment in which it is immersed.

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Metabolism of Triacetic Acid and Triacetic Acid Lactone*

Robley J. Light, Thomas M. Harris, and Constance M. Harris

ABSTRACT: Triacetic acid lactone (TAL) has been identified as a metabolite of two species of *Penicillium*. [^{14}C]Carboxyl-labeled triacetic acid (TAA) and TAL were prepared.

The *in vivo* conversion of [^{14}C]TAL and [^{14}C]TAA to other acetate metabolites was investigated. Low levels of incorporation of both compounds into 6-methylsalicylic acid (MSA), patulin, and fatty acids were observed. The radioactive MSA was degraded to CO_2 and 2,4,6-tribromo-*m*-cresol. The observed distribution of radioactivity indicated that TAL and TAA both had undergone degradation to C_2 units prior to incorporation rather than being directly utilized as C_6 units. Similar levels of incorporation into fatty acids supported this conclusion. TAL probably arises by cyclization of a thiol ester of TAA. Chromatographic and spectral evidence suggest that TAL is the "275 compound" of Bressler and Wakil [Bressler, R., and Wakil, S. J. (1962), *J. Biol. Chem. 237*, 1441] formed by the avian liver fatty acid synthetase in the absence of reduced nicotinamide–adenine dinucleotide phosphate (NADPH), thus supporting other published evidence that TAL is a by-product of fatty acid synthesis.

An increasing body of evidence indicates that many fungal aromatic metabolites are derived by condensation of acetate and malonate units (Richards and Hendrickson, 1964). Recently, Gatenbeck and Hermodsson (1965) obtained a cell-free system from *Alternaria tenuis* which converted both acetyl-CoA[1] and malonyl-CoA into alternariol. Earlier Bassett and Tanenbaum (1960) reported synthesis of patulin from acetyl-CoA by a cell-free extract from *Penicillium patulum;* in 1962, Tanenbaum and Bassett found that cell-free extracts from *Penicillium stipitatum* incorporated both acetyl-CoA and malonyl-CoA into the tropolone, stipitatic acid. Lynen (1961) reported that incorporation of [^{14}C]acetyl-CoA into MSA by extracts of *Penicillium patulum* required NADPH and malonyl-CoA. Lynen postulated by analogy with fatty acid synthesis that protein-bound polyketo chains were intermediates. At least a 6-carbon polyketo chain can be formed by the fatty acid complex. Brodie *et al.* (1964) identified the product produced by purified pigeon liver fatty acid synthetase in the absence of NADPH as 3,5-dioxohexanoic acid (TAA). Labeling evidence indicated that the product was produced from one acetyl-CoA and two malonyl-CoA units. However, they could not have distinguished TAA from its lactone (TAL) in their paper chromatographic system (Harris *et al.*, 1966). Brock and Bloch (1966) isolated TAL from incubations of the crude *Escherichia coli* fatty acid synthetase containing acyl carrier protein, acetyl-CoA, malonyl-CoA, NADPH, and thiol compounds. They were not certain whether the metabolic product was TAA or TAL, but our experiment described below with [^{14}C]TAA indicates that it is unlikely that TAA would have been converted to TAL under the conditions of their isolation.

Ehrensvärd (1955) observed that TAL stimulated the formation of aromatic compounds in *Penicillium urticae*. Harris *et al.* (1966) showed that small quantities of TAL are produced from [1-^{14}C]acetate by *P. patulum*. Bentley *et al.* (1966) found that TAL, its 6-acetyl derivative (4-hydroxy-6-(2-oxopropyl)-(2H)-pyran-2-one), and orsellinic acid accumulated in cultures of *P. stipitatum* in which the production of tropolones was inhibited by the addition of ethionine. Tanenbaum and coworkers (Brenneisen *et al.*, 1964; Acker *et al.*, 1966) isolated the methyl derivative of TAL, 3,6-dimethyl-4-hydroxy-(2H)-pyran-2-one, from another strain of the

* From the Departments of Chemistry, Florida State University, Tallahassee, Florida, and Vanderbilt University, Nashville, Tennessee. *Received Ausgust 8, 1966.* A preliminary report of these results has been made (Light *et al.*, 1966). This work has been supported by Grants AM-07536 and GM-12848 from the U. S. Public Health Service.

[1] Abbreviations: MSA, 6-methylsalicylic acid (2,6-cresotic acid; 2-hydroxy-6-methylbenzoic acid); tlc, thin layer chromatography; TAL, triacetic acid lactone (4-hydroxy-6-methyl-(2H)-pyran-2-one); TAA, triacetic acid (3,5-dioxohexanoic acid); CoA, coenzyme A; NADPH, reduced nicotinamide–adenine dinucleotide phosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.