Deuterium–Hydrogen Exchange in Amide N–H Groups[1]

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Rates of deuterium–hydrogen exchange in proteins reflect the structure and conformation of these macromolecules in ways that are still obscure. It has seemed pertinent, therefore, to examine rates of N–H → N–D exchange in a model peptide system, $CH_3CONHCH_3$. The kinetics were followed in dioxane–water and in pure water solutions, as well as in the liquid amide. Catalysis was produced not only by H^+ and OH^- but also by a number of acids (e.g., formic, acetic) and bases (e.g., imidazole, amines). Activation energies (ca. 20 kcal.) for exchange in the amide when it is definitely monomeric in water are comparable in magnitude to those reported for slowly exchanging hydrogens in proteins. All these observations make it unlikely that slowly exchanging hydrogens in proteins represent a measure of helically bonded ones.

Introduction

The rates of exchange of deuterium for hydrogen in proteins certainly must reflect the structure and conformation of these macromolecules. To find this relationship was the objective of the early investigators in this area.[3,4] When it was discovered that peptide hydrogens fall roughly into two rate classes, those that exchange very rapidly and those that exchange slowly, it seemed reasonable to assign the former to unfolded portions of the polypeptide chain and the latter to helical sections.[5] On this basis one could ascribe observed increases in exchange rates on denaturation to an unfolding of helices. Likewise one could explain the measured heat of activation of 20 kcal./mole for the slow exchange as due to the need to open three adjacent hydrogen bonds in order to unfold a helical segment, each hydrogen bond presumably requiring 6–8 kcal.

If one is to use relative rates of exchange to provide information on the number and the nature of the hydrogen-bonded groups in a protein molecule,[5] it seems desirable to examine some of the factors which might influence the kinetics in simple systems. For this purpose we have chosen N-methylacetamide, $CH_3CONHCH_3$, as a model amide,[6] and have studied the effects of solvent, temperature, and acid–base catalysts on the D–H exchange rates. These factors all have some relevance in regard to the behavior of amide N–H groups within the framework of a protein macromolecule.

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(2) Predoctoral Fellow, National Institute of General Medical Sciences, 1963–1964.

(3) H. Lenormant and E. R. Blout, *Nature*, **172**, 770 (1953).

(4) A. Hvidt, G. Johansen, K. Linderstrøm-Lang, and F. Vaslow, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **29**, 129 (1954).

(5) K. Linderstrøm-Lang, Special Publication No. 2, The Chemical Society, London, 1955, p. 1.

(6) I. M. Klotz and J. S. Franzen, *J. Am. Chem. Soc.*, **84**, 3461 (1962).

Experimental

Materials. N-Methylacetamide and N,N-dimethylacetamide, purchased from Eastman Organic Chemicals, were distilled at atmospheric pressure through a 15-in. Vigreaux column; fractions boiling at 205–206° and 164–165°, respectively, were collected. Carbon tetrachloride and *p*-dioxane were Fisher Spectroanalyzed Certified Reagent grade.

Heavy water warranted to contain a minimum of 99.8% D_2O was purchased from Bio-Rad Laboratories. Its purity was checked by measuring the absorbance of the O–H band in the near infrared. NaOD was prepared by dissolving sodium hydroxide pellets (Baker Reagent Grade) of low carbonate content in D_2O. Since the final concentration of sodium hydroxide did not exceed 0.5 *M*, the additional dilution of D_2O did not reach 0.3 mole % H_2O. DCl in D_2O was prepared from the reaction of NaCl with D_2SO_4, the latter being more than 99.5 mole % pure.

Tetramethylammonium chloride was obtained from Eastman Organic Chemicals, dried under vacuum, and stored in a desiccator. Imidazole purchased from the same source was recrystallized from benzene. Tris(hydroxymethyl)aminomethane, hydroxylamine hydrochloride, methoxylamine hydrochloride, sodium acetate trihydrate, sodium benzoate, and succinic anhydride were all used as obtained from commercial sources. N-Methylmorpholine from Jefferson Chemical Co. was distilled at atmospheric pressure through a 15-in. Vigreaux column; the fraction boiling at 114–115° was collected and stored under nitrogen. N,N,N′,N′-Tetramethylethylenediamine was also distilled through a Vigreaux column, the fraction boiling at 119–120° being collected. Sodium propionate was recrystallized twice from distilled water. This compound and sodium formate gave equivalent weights, upon titration, within 0.5% of the calculated value.

Anhydrous $NiCl_2$ was prepared from recrystallized $NiCl_2 \cdot xH_2O$ by dehydration under vacuum.

Preparation of Reaction Mixtures. (1) Dioxane–Water Solvent. A predetermined weight of N-methylacetamide was added to one glass-stoppered erlenmeyer flask containing half the dioxane needed for the final solution. To a second flask, holding the other portion of dioxane, a definite amount of D_2O containing any desired solute was added. A reference solution for the optical measurements was also prepared by adding enough CCl_4 to dioxane to dilute the latter to the point where its absorption at 1.42 μ would balance that of the dioxane in the reaction mixture. After the spectrophotometer had been adjusted with reference solution in both cells, the two flasks containing N-methylacetamide and D_2O were mixed quickly with vigorous swirling and a sample of the mixture was introduced into the optical sample cell sitting within the thermostated compartment of a Cary Model 14R or Beckman DK-2 spectrophotometer.

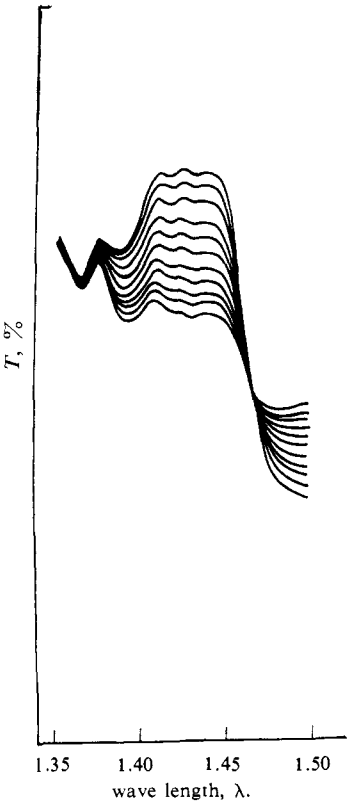


Figure 1. Absorption spectra in the near infrared at different times after mixing N-methylacetamide and D_2O in dioxane.

(*2*) *Pure Water Solvent.* Since N-methylacetamide is solid up to about 30°, it was transformed to the liquid state, to speed up mixing with solvent, by addition of a trace of H_2O. For example, to 0.73 g. of amide in a 10-ml. volumetric flask 0.02 g. of H_2O was added; this mixture remained liquid to about 20°. The D_2O solution, containing any desired solute, was then added to the volumetric flask and, after mixing, a sample of solution was transferred to the thermostated optical cell. This procedure was satisfactory for rate measurements near room temperature but was inadequate for studies at 12° or 32°. For studies above or below room temperature an alternative procedure was used. D_2O solution (3 ml., containing salt or buffer) was added to the optical cell and allowed to come to thermal equilibrium in the thermostated compartment of the spectrophotometer. Then 0.25 ml. of liquid N-methylacetamide–H_2O was pipetted into the optical cell and mixing obtained with the aid of a flat-bottom glass rod.

(*3*) *Pure N-Methylacetamide Solvent.* All studies were carried out at 32°, just above the melting point of this substance. Amide (3 ml.) was pipetted into the optical cell and allowed to come to thermal equilibrium within the spectrophotometer. Then 0.060 ml. of D_2O solution containing salt or buffer was placed on a flat-bottom glass rod, the rod was quickly introduced into the optical cell, and rapid mixing obtained with a few strokes.

pH Measurements. All pH values were measured at 25° with a Beckman Model G pH meter. In every case the meter was standardized with H_2O–buffer solutions. After standardization the electrodes were washed and wiped dry. In D_2O solutions the pH value read on the meter was changed to pD according to the recommendation of Glasoe and Long.[7]

$$\text{pD} = \text{pH (meter reading)} + 0.40 \qquad (1)$$

Optical Measurements. Absorbance or transmission readings in the near infrared were followed mostly with the Cary Model 14R spectrophotometer. Some observations were also made with the Beckman DK-2 spectrophotometer.

Temperature. A thermistor probe, with a precision of ±0.5°, was inserted into the absorption cell through the cap, and temperatures were measured with a Telethermometer Model 425C of the Yellow Springs Instruments Co.

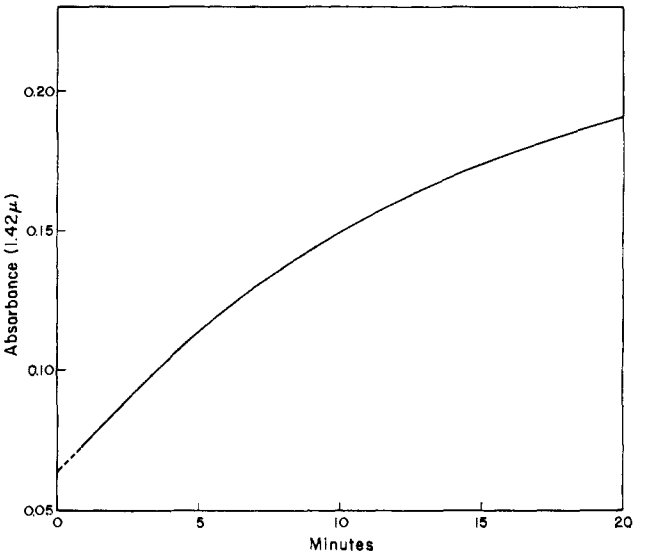


Figure 2. Change of absorbance with time for hydrogen–deuterium exchange reaction of N-methylacetamide in D_2O–dioxane solution.

Results

Calculation of Observed Rate Constants. Since the D–H isotopic exchange is a reversible reaction, it should be represented as

$$CH_3-\underset{\underset{H}{|}}{\overset{\overset{O}{\|}}{C}}-N-CH_3 + D_2O \underset{k_r}{\overset{k_f}{\rightleftharpoons}} CH_3-\underset{\underset{D}{|}}{\overset{\overset{O}{\|}}{C}}-N-CH_3 + HOD \qquad (2)$$

where k_f is the forward rate constant and k_r the reverse rate constant. The progress of a typical reaction is shown in Figure 1 in which successive tracings were taken at various time intervals over the spectroscopic range of 1.4–1.5 μ. To simplify the computations we have evaluated k_f by extrapolating the observed absorbance changes (Figure 2) to zero time and, in essence, finding the initial slope. At zero time, the reverse reaction of eq. 2 may be ignored and one may write

$$\left(\frac{\Delta(\text{O-H})}{\Delta t}\right)_0 = \text{initial rate} = k_{obsd}(CH_3CONHCH_3)_0(D_2O)_0 \qquad (3)$$

where $(\ )_0$ indicates initial values of the respective quantities, k_{obsd} is the same as k_f, and t is the time. Since $\Delta(\text{O–H})/\Delta t$ is proportional to $\Delta A_{1.42\,\mu}/\Delta t$, where A

(7) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, 64, 188 (1960).

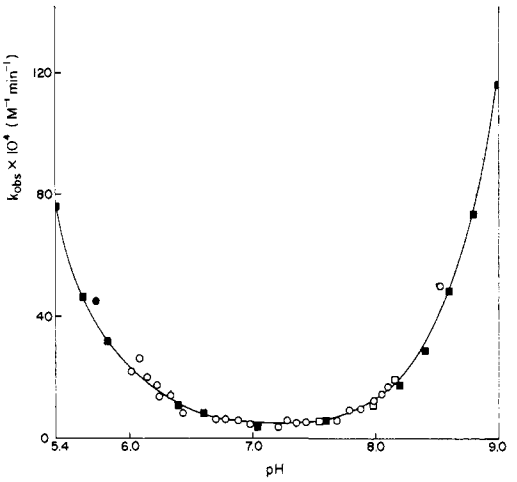


Figure 3. Rate constants for deuterium exchange in N-methylacetamide in dioxane–water at 25°: ○, no salt or 7.7×10^{-3} M $(CH_3)_4NCl$; ■, results with imidazole extrapolated to zero concentration of catalyst; □, 7.7×10^{-2} M $(CH_3)_4NCl$; ●, 1.53×10^{-2} M $NiCl_2$.

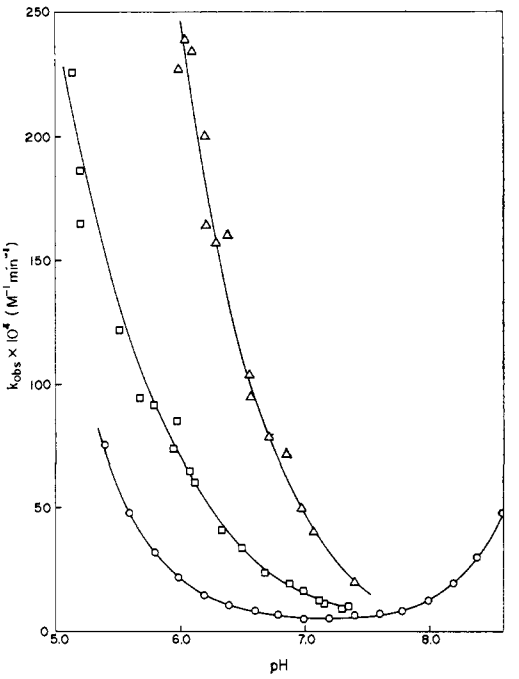


Figure 4. Catalysis by imidazole of deuterium exchange in N-methylacetamide in dioxane–water at 25°: ○, no catalyst; □, 7.7×10^{-3} M imidazole; △, 7.56×10^{-2} M imidazole.

is the *absorbance*, it is a simple matter to obtain the initial rate required in eq. 3 from a graphical differentiation of the curve in Figure 2.

An even simpler procedure was used to compute k_{obsd} in pure D_2O solvent. In this case the exchange reaction is pseudo-first-order, since the D_2O is present in overwhelming excess (55:1), and hence

$$k_{obsd} = \ln 2/t_{1/2} \qquad (4)$$

where $t_{1/2}$ is the half-life of the first-order reaction.

General Acid-Base Catalysis in Dioxane–Water Mixed Solvent. The first investigations were designed to follow rates with times of about 1 hr. so as to correspond to the slow deuterium–hydrogen exchanges in proteins. Amides in pure water at room temperature exchange protons in a few minutes.[8,9] Initial investigations were carried out, therefore, in a dioxane–water mixture which, when N-methylacetamide was added, had the following composition: 4.2 M N-methylacetamide, 4.2 M D_2O, 7.1 M dioxane.

It was observed immediately that the rate was markedly dependent upon pH (Figure 3), as indeed should have been expected since similar observations have been reported for pure water by Nielsen.[9] The shape of the curve (Figure 3) shows that both H^+ and OH^- catalyze the N–H → N–D exchange. A graph of log k_{obsd} *vs.* pH shows, furthermore, that the dependence of k_{obsd} on (H^+) or (OH^-) is first order. Consequently one may write

$$k_{obsd} = k_{H^+}(H^+) + k_{OH^-}(OH^-) + k_0 \qquad (5)$$

where k_0 is the rate constant for "spontaneous" reaction in the mixed solvent. The following values were found for these three constants[10]: $k_{H^+} = 2 \times 10^3$ M^{-2} min.$^{-1}$; $k_{OH^-} = 1 \times 10^6$ M^{-2} min.$^{-1}$; $k_0 = 2 \times 10^{-4}$ M^{-1} min.$^{-1}$.

Since H^+ and OH^- are good catalysts for the exchange, the question arose whether other acidic or basic groups might also accelerate the reaction. Such an effect would be particularly relevant in connection with proteins which contain a variety of acidic and basic side chains. As a preliminary step to such a study, however, it is necessary to see how much a simple salt effect may affect the rate. For this purpose the rates in the absence of salt and in the presence of 0.008 and 0.08 M tetramethylammonium chloride were compared. No significant difference was observed (Figure 3). Clearly salt effects are negligible in this system.

In marked contrast is the effect of imidazole on the rates of exchange.[12] A few results are illustrated in Figure 4. For example, at pH values between 6 and 7, the observed rate constant is increased 10- to 15-fold upon the addition of 7.56×10^{-2} M imidazole. Imidazole concentrations up to 0.2 M were examined and 50-fold increases in rate were observed. At a fixed pH, the observed rate constant was found to vary linearly with concentration of imidazole (Figure 5).

A catalytic constant was evaluated from the relation

$$k_{cat} = k_{obsd} - k_s \qquad (6)$$

where k_s is the observed rate constant in the catalyst-free solvent at the same pH. Since the catalytic effect was found to be first order in imidazole concentration, we may also write

$$k_{cat} = k_a(HA) + k_b(A) \qquad (7)$$

where k_a is the catalytic rate constant of the acid form of the catalyst, HA, and k_b is that of the basic form, A. It can be shown readily that

(8) A. Berger, A. Loewenstein, and S. Meiboom, *J. Am. Chem. Soc.*, 81, 62 (1959).

(9) S. O. Nielsen, *Biochim. Biophys. Acta*, 37, 146 (1960).

(10) To calculate k_{OH^-} a value must be assigned to K_w, the autoprotolysis constant of water in this mixture. This was estimated to be 10^{-17} from measurements of Harned and Owen[11] in dioxane–water solutions.

(11) H. S. Harned and B. B. Owen, "*Physical Chemistry of Electrolytic Solutions*," 3rd Ed., Reinhold Publishing Corp., New York, N. Y., 1958, p. 756.

(12) I. M. Klotz and B. H. Frank, *Science*, 138, 830 (1962).

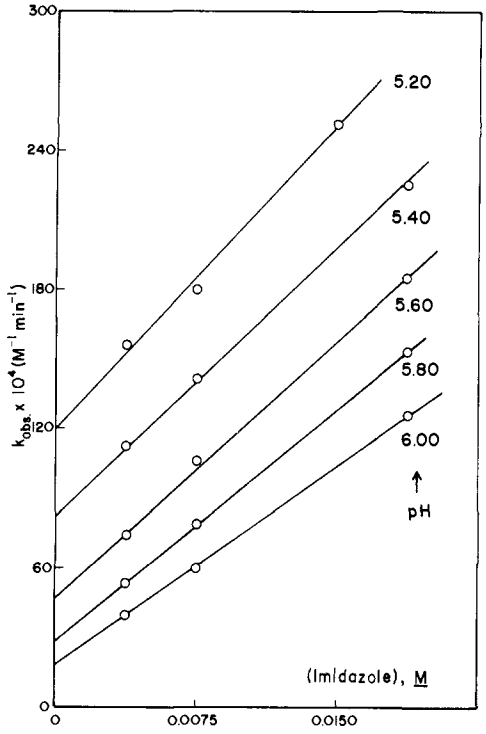


Figure 5. Observed rate constants *vs.* concentration of imidazole at each of a series of pH values.

$$\frac{k_{cat}}{(HA) + (A)} = k_a + \frac{k_b - k_a}{1 + (H^+)/K_a} \qquad (8)$$

where K_a is the apparent dissociation constant of the catalyst in the mixed solvent used. For imidazole in this mixed solvent pK_a was found to be 6.1, if pH meter readings are assumed to be $-\log (H^+)$ in this solution. With the use of eq. 8, the constants k_a and k_b were readily obtainable from a graph of $k_{cat}/(\text{catalyst})_{total}$ *vs.* $[1 + (H^+)/K_a]^{-1}$. For imidazole k_a was found to be 1.1 M^{-2} min.$^{-1}$, k_b 0 (see Table I).

Table I. Catalytic Rate Constants in Dioxane–D_2O

Catalyst	k_a, M^{-2} min.$^{-1}$	k_b, M^{-2} min.$^{-1}$	pK_a'
Imidazole	1.10	0	6.1
	0.29	0	6.5
$(CH_3)_2NCH_2CH_2N(CH_3)_2$	0.21	0	8.3
HCOOH	0.27	0.05	7.1
Succinic acid	0.20	0.04	7.7
Benzoic acid	0.17	0.11	7.8
CH_3COOH	0.12	0.22	8.5
CH_3CH_2COOH	0.11	0.64	8.8
$(CH_2OH)_3CNH_2$	0.21	0.83	8.9

Once it was apparent that imidazole catalyzes deuterium–hydrogen exchange, it became of interest to know if other acid–base groups of the type found in protein side chains might also be effective. Experiments with acetic acid showed immediately that both the –COOH and $-COO^-$ were active catalysts. The investigation was extended therefore to a number of other carboxylic acids in order to cover a range of pK_a values. The variation of k_{cat} with pH for one of these acids, formic, is shown in Figure 6. Similar experiments were carried out with tris(hydroxymethyl)-aminomethane (Tris) and glycine methyl ester to assess the effects of $-NH_2$ and $-NH_3^+$.

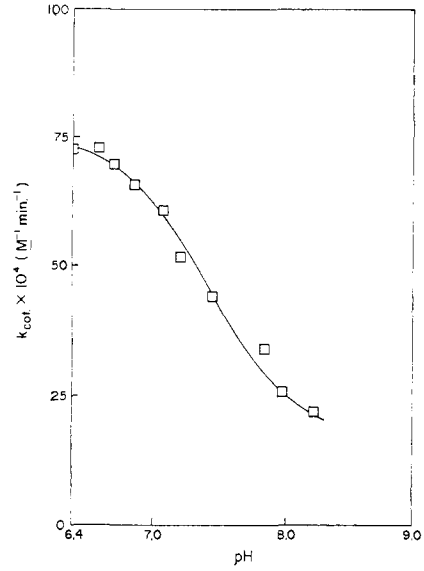


Figure 6. Catalytic constant for formic acid as a function of pH.

Two compounds, succinic acid and N,N,N′,N′-tetramethylethylenediamine, each containing two acid–base functions, were also tried in the hope of finding particularly active catalysts in a substance with a general-acid and general-base group tied together. However, no extraordinary catalysis was observed. Finally, since metal ions, particularly in the transition series, may be considered acids, one experiment was tried with 0.015 *M* Ni^{2+} (Figure 3), but no appreciable effect was seen. In all three substances described in this paragraph as well as with some phosphates and glycine methyl ester, solubility problems were encountered and hence adequate concentration ranges could not be examined.

The catalytic constants of these acid–base systems were evaluated using procedures similar to that described for imidazole. The final results for k_a and k_b are assembled in Table I.

Catalytic Effect in Pure D_2O Solvent. Since catalysis of exchange has been demonstrated in a mixed dioxane–water solvent it becomes of interest to know whether similar effects can be seen in pure aqueous solvent.

It has been shown already by Nielsen[9] that H^+ and OH^- catalyze exchange of N-methylacetamide in D_2O. Since Nielsen followed this reaction by means of absorption at 6.5 μ, whereas we have been using the 1.4 μ region, it seemed desirable to carry out first a few studies under conditions corresponding to his. A few such experiments following exchange as a function of the acidity (pD) in the aqueous solvent are shown in Figure 7, together with some of Nielsen's results; the two investigations agree very well.

Once again since the exchange is catalyzed by D^+ and OD^- we may write

$$k_{obsd} = k_{D^+}(D^+) + k_{OD^-}(OD^-) + k_0 \qquad (9)^{13}$$

(13) Equation 9 has been distinguished from eq. 5 because in these experiments in pure D_2O solvent essentially all of the acidity is provided

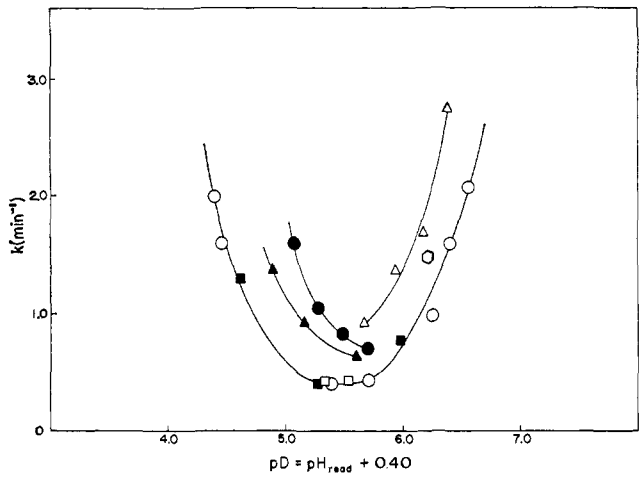


Figure 7. Rates of deuterium–hydrogen for N-methylacetamide (1 *M*) in D_2O at 24°: ○, 0.02 *M* sodium acetate buffer; ●, 0.9 *M* acetic acid–acetate; □, 0.8 *M* sodium chloride plus 0.02 *M* sodium acetate; ■, Nielsen's results[9]; △, 0.4 *M* hydroxylamine; ▲, 0.7 *M* methoxyamine; ⬡, 0.6 *M* imidazole.

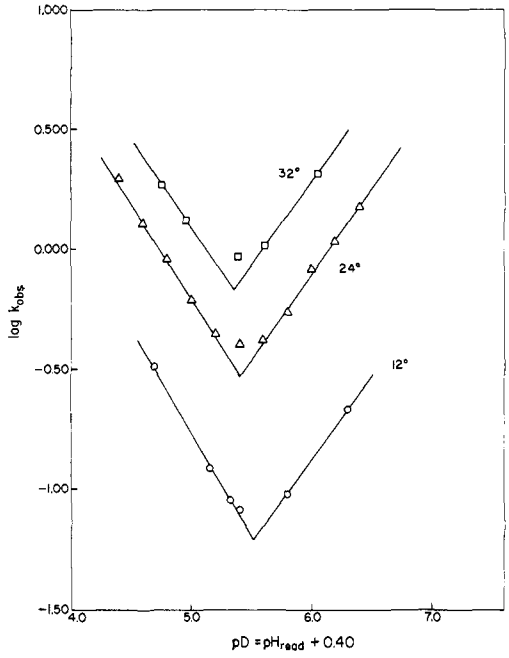


Figure 8. Logarithm of k_{obsd} *vs.* pD at 12°, 24°, and 32°. Solutions were 1 *M* N-methylacetamide in D_2O containing 0.02 *M* sodium acetate buffer.

The values found for these three constants are[14]: $k_{D^+} = 5 \times 10^4\ M^{-1}$ min.$^{-1}$; $k_{OD^-} = 5 \times 10^8\ M^{-1}$ min.$^{-1}$; $k_0 \simeq 0$.

Having confirmed the catalysis by D^+ and OD^- in pure D_2O solvent we then examined the possibility of a simple salt effect on the kinetics. Concentrations of NaCl for 0.1 to 0.8 *M* were used. Even for the highest concentration of this salt the increase in k_{obsd} was less than 10% (see Figure 7).

In proceeding to examine possible catalytic effects in this solvent system we must recognize at the outset that the rates in the absence of catalyst are very much faster in D_2O than in the D_2O–dioxane mixed solvent. Hence higher concentrations of acid or base may be necessary to produce an effect. Furthermore the pK_a values of acids and bases are different in water than in water–dioxane, and hence their catalytic effectiveness may fall into a different order.

Experiments with 0.9 *M* acetate–acetic acid showed marked catalysis of deuterium–hydrogen exchange by $CH_3CONHCH_3$ in D_2O solvent (Figure 7). Severalfold increases in rate were obtained in the pH range 5–6. The increased catalytic effect at the lower pH indicates that the acid form of acetic acid is the more effective species. In comparing the two solvent systems it is pertinent to note that the pK_a of acetic acid is near 5.0 in D_2O but approximately 8.5 in our D_2O–dioxane mixture.

A few nitrogenous bases were also examined. Imidazole at 0.6 *M* concentration (Figure 7) showed only a minor effect which was not appreciably beyond experimental error. On the other hand, hydroxylamine ($HONH_2$) and methoxylamine (CH_3ONH_2) were effective catalysts of deuterium–hydrogen exchange (Figure 7).

The catalytic constants for these substances have been assembled in Table II. It is evident that general acid–base catalysis of amide hydrogen–deuterium exchange occurs in pure aqueous solvent although it is not as striking as in mixed dioxane–water solvents.

Table II. Catalytic Constants in Aqueous Solution

Catalyst	k_a, M^{-1} min.$^{-1}$	k_b, M^{-1} min.$^{-1}$	pK_a in H_2O
Methoxylamine	2	0	4.6[a]
Acetic acid	2	0	4.8
Hydroxylamine	0.5	1	5.8[b]

[a] H. K. Hall, Jr., *J. Am. Chem. Soc.*, **79**, 5441 (1957). [b] T. C. Bissot, R. W. Parry, and D. H. Campbell, *ibid.*, **79**, 796 (1957).

Activation Energies. Various investigators[5,16] have published estimates of the energy of activation for deuterium exchange with peptide groups in proteins. Its molecular significance cannot be ascertained, however, until the activation energy is known for a model system, such as $CH_3CONHCH_3$. A study of the temperature dependence of deuterium–hydrogen exchange was therefore undertaken with N-methylacetamide (1 *M*)[17] in D_2O. The observed rates (at different pH values) at three different temperatures are summarized in Figure 8.

The observed rates at pD values more than 0.5 unit below the pD of the minimum were more than 90% acid catalyzed. The activation energy in the acid region was calculated from the temperature coefficient at pD 5. The observed rates at pD values more than 0.5 unit above pD of the minimum were greater than 90% base catalyzed. The activation energy in the basic region was calculated from the temperature coefficient at pD 6.3. The results obtained are: $E^*_{acid} = 17$ kcal. mole^{-1}; $E^*_{base} = 23$ kcal. mole^{-1}.

by D^+ since $CH_3CONHCH_3$ contributes only a few protons. In contrast, in the D_2O–dioxane solvent the concentration of $CH_3CONHCH_3$ was comparable to that of D_2O.

(14) To evaluate k_{OD^-} the figure of 2×10^{-15} was used for the autoprotolysis constant[15] of D_2O.

(15) R. W. Kingerley and V. K. LaMer, *J. Am. Chem. Soc.*, **63**, 3256 (1941).

(16) S. J. Leach, *Rev. Pure Appl. Chem.*, **9**, 33 (1959).

(17) At this concentration in water, N-methylacetamide is entirely in the monomeric state.[6]

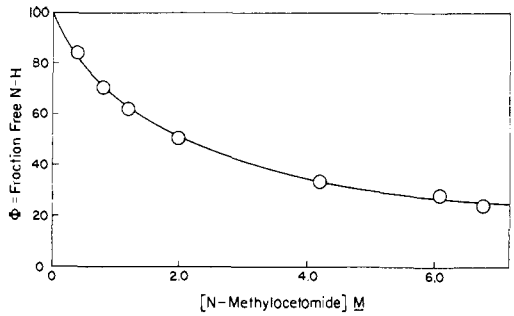


Figure 9. Fraction of free N–H groups for N-methylacetamide in D_2O–dioxane mixed solvents. Mole fraction of D_2O maintained at 0.26 ± 0.02.

From the experiments assembled in Figure 8 it was also possible to compute the catalysis constants of D^+ at different temperatures. The results are summarized in Table III.

Table III. Temperature Dependence of Catalytic Constant k_{D^+}

Temp., °C.	k_{D^+}, M^{-1} min.$^{-1}$
12	1×10^4
24	5×10^4
32	10×10^4

Effect of Degree of Association. It would be particularly pertinent in connection with exchange in proteins to determine the effect of hydrogen-bonded association, N—H· · ·O=C, on the kinetics of H–D interchange in a model amide. In an ideal experiment one would vary the fraction of hydrogen-bonded N–H while maintaining the composition of the solvent constant. This we have been unable to achieve, for substantial changes in composition in our dioxane–water mixtures were necessary to shift the amide association equilibrium. Nevertheless some interesting kinetic observations were made for a series of solutions with different contents of free N–H groups.

A series of solutions was prepared containing a constant mole fraction (0.26) of D_2O but different quantities of dioxane and N-methylacetamide so as to provide variable fractions of free N–H. From the infrared absorptions of these solutions and appropriate calibration measurements it was possible to compute the fraction of free N–H.[18] These results are summarized in Figure 9.

Rates of deuterium–hydrogen exchange were then measured for solutions containing 0.4–6 *M* N-methylacetamide. For each solution rates were measured as a function of pH. For the solutions low in concentration of N-methylacetamide the observed rates were extremely slow. Consequently the rate constants were evaluated by suitable extrapolations from sets of experiments containing different concentrations of catalyst [either imidazole or tris(hydroxymethyl)aminomethane]. These catalysts simultaneously served to maintain constant pH in the reaction system.

Table IV summarizes the results of these studies of rates as a function of degree of association of N–H for solutions at pH 7 and 25° containing 0.26 ± 0.02 mole fraction of D_2O. The range of free N–H covered is not large, but within this region no significant variation in k_{obsd} was detected.

(18) B. H. Frank, Ph.D. Dissertation, Northwestern University, Evanston, Ill., 1964.

Table IV. Rates of Exchange of N-Methylacetamide in Solutions with Varying Fraction of Free N–H Groups

N-Methylacetamide, *M*	$k_{obsd} \times 10^4$, M^{-1} min.$^{-1}$	Fraction free N–H
0.395	5.0 ± 1	0.84
1.20	4.0 ± 1	0.62
4.20	5.0 ± 1	0.34
4.84	5.5 ± 1	0.28
6.12	6.0 ± 1	0.22

A few studies were also made to compare catalytic effects in solutions with very different degrees of association of N–H groups. These included some experiments with *liquid* N-methylacetamide to which D_2O had been added, so that the respective concentrations were 12.5 and 1 *M*, respectively. The results with imidazole are summarized in Table V, together with data for the apparent pK_a of this substance. In all cases the acid species is catalytically active, the basic species is not. Furthermore, catalytic effectiveness of imidazole increases in parallel with increasing (apparent) acidity constant and with increasing fraction of free N–H in the reacting mixture. On the other hand, the catalytic effectiveness of tris(hydroxymethyl)aminomethane (Table VI) does not vary with solvent composition despite a large variation in apparent pK_a and in fraction of free N–H.

Table V. Comparison of Catalytic Efficiency of Imidazole in Deuterium–Hydrogen Exchange of N-Methylacetamide

Reaction system	Fraction free N–H	k_a, M^{-2} min.$^{-1}$	k_b, M^{-2} min^{-1}	Imidazole pK_a
0.395 *M* amide 4.0 *M* D_2O 10.6 *M* dioxane	0.84	2.5	0	5.0
4.2 *M* amide 4.2 *M* D_2O 7.1 *M* dioxane	0.34	1.1	0	6.1
12.5 *M* amide 1.1	0.1	0.29	0	6.7

Table VI. Comparison of Catalytic Efficiency of Trishydroxymethylaminomethane in Deuterium–Hydrogen Exchange of N-Methylacetamide

Reaction system	Fraction free N–H	k_a, M^{-2} min.$^{-1}$	k_b, M^{-2} min.$^{-1}$	Tris pK_a
0.395 *M* amide 4.0 *M* D_2O 10.6 *M* dioxane	0.84	0.23	0.85	7.7
1.2 *M* amide 4.0 *M* D_2O 9.8 *M* dioxane	0.62	0.25	0.88	8.2
4.2 *M* amide 4.2 *M* D_2O 7.1 *M* Dioxane	0.34	0.21	0.83	8.9

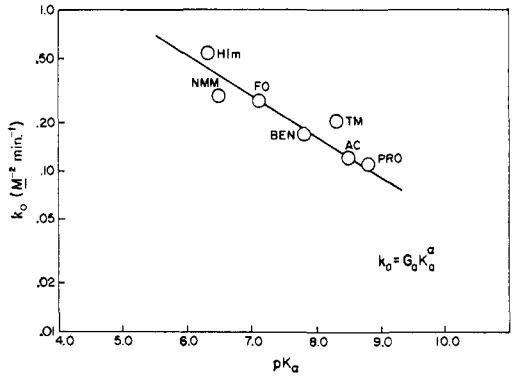


Figure 10. Brønsted plot for catalytic constants of acids in solution of 4.2 *M* N-methylacetamide, 4.2 *M* D_2O, and 7.1 *M* dioxane: HIm, imidazole; NMM, N-methylmorpholine; FO, formic acid; BEN, benzoic acid; TM, tetramethylethylenediamine; AC, acetic acid; PRO, propionic acid. For imidazolium ion, k_a was corrected for the statistical effect due to the presence of two equivalent protons.

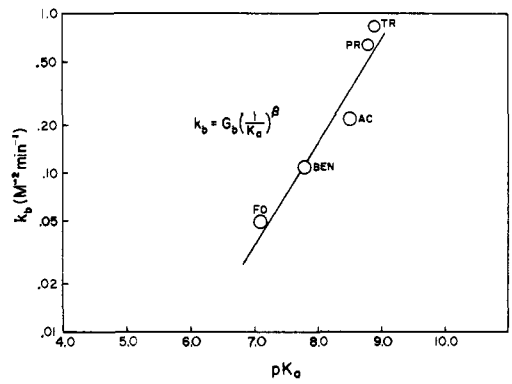
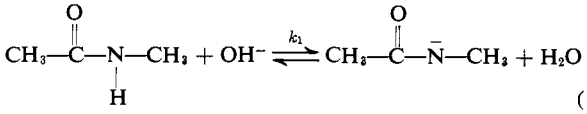


Figure 11. Brønsted plot for catalytic constants of bases in solution of 4.2 *M* N-methylacetamide, 4.2 *M* D_2O, and 7.1 *M* dioxane: TR, trishydroxymethylaminomethane; AC, acetate ion; BEN, benzoate ion; FO, formate ion; PR, propionate ion.

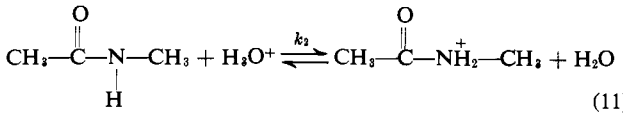
Discussion

It seems to be generally agreed[8,9] that the hydroxide ion catalyzed exchange reaction of amides may be represented by



(10)

and the hydrogen ion catalyzed reaction by



(11)

The observations reported here are consistent with this formulation. Furthermore, the observed first-order catalytic effects of D^+ and OD^- and analogous salt effects in a variety of mixed D_2O–dioxane solvents and even in liquid N-methylacetamide indicate that the same mechanism applies to these systems also.

In addition we have found that the exchange reaction is subject to general acid–base catalysis. The most extensive observations have been made in one D_2O–dioxane mixed solvent. The relative effectiveness of the acid catalysts follows the Brønsted relationship[19] (Figure 10)

$$k_a = G_a K_a^{\alpha} \qquad (12)$$

where G_a and α are constants characteristic of the system and k_a and K_a are the catalyst rate and acid-dissociation constants, respectively. The value of α of 0.25 found in this system shows that the dependence of catalytic effectiveness on acid strength is not strong. The base-catalyzed reactions fit the corresponding Brønsted equation (Figure 11)

$$k_b = G_b(1/K_a)^{\beta} \qquad (13)$$

and β turns out to be 0.52. Thus the base-catalyzed reaction has a higher sensitivity to base strength.

It is also apparent that general acid–base catalysis occurs in purely aqueous solvents also. Carboxylic acids such as acetic, as well as nitrogen-containing bases such as hydroxylamine and methoxylamine, are effective, albeit at higher concentrations than in dioxane–water. The higher concentration required is primarily a consequence of the much faster rates in pure water (55 *M*) than in dioxane–water mixtures in which the water concentration is about 4 *M*.

The temperature studies reveal a surprisingly high activation energy for exchange in N-methylacetamide in pure aqueous solvent, both in the acid-catalyzed and base-catalyzed range. At the amide concentration studied, N-methylacetamide is completely in the monomeric form. There is no possibility, therefore, that the 20 kcal./mole activation energy is essential for the disruption of N—H· · ·O=C hydrogen bonds. Rather that much energy is necessary to place a D^+ on the amide (eq. 11), in an orbital comparable to that of the H already on, presumably because of the orbital rearrangements required. A similar situation holds for the proton removal by OH^- (eq. 10).

These observations with a simple model amide should provide guideposts in the interpretation of the kinetic results in exchange with proteins.

In this regard it should be pointed out first that changes in exchange rates for proteins as pH is varied have usually been ascribed to a change in configuration of the macromolecule, increases in helical structure being claimed[20] if the exchange rate is lowered. It is apparent from studies with N-methylacetamide, however, that H^+ and OH^- are both strong catalysts of exchange and any variation in their concentration should have a profound effect on the kinetics.

Likewise, the observed activation energy of 20 kcal./mole for the slowly exchanging protons in proteins has been interpreted as the result of these protons being bridges in helical segments of the macromolecule. The value of 20 kcal. has been attributed[5] to the necessity to provide 3 × (6–8) kcal. to unfold three hydrogen bonds within one turn of the helix. It was assumed in this connection that unfolded sections of the polypeptide chain exchanged hydrogens with a greatly reduced activation energy. However, the present studies show unequivocally that an activation energy of 17–23 kcal./mole is essential for exchange with deu-

(19) J. N. Brønsted and K. J. Pedersen, *Z. physik. Chem.*, **108**, 185 (1923).

(20) It has also been recognized (see, *e.g.*, E. R. Blout, C. de Lozé, and A. Asadourian, *J. Am. Chem. Soc.*, **83**, 1895 (1961)) that amide hydrogens in apolar regions of a protein molecule might exchange slowly.

terium even when N–H is *not* hydrogen bonded to O=C.

In addition the present investigations show that, besides H^+ and OH^-, acids and bases generally catalyze deuterium–hydrogen exchange of amides. The catalytic constants in general acid–base catalysis are much smaller than those for H^+ and OH^-. On the other hand, H^+ and OH^- are present in solutions between pH 4 and 10 at very low concentrations, and their net catalytic effectiveness is a product of concentration and catalytic constant. Within the domain of a protein macromolecule, acidic and basic side chains are present in large quantities, corresponding to local concentrations near 1 *M*. Side chains of course are limited in mobility compared to corresponding small molecules such as imidazole or acetic acid. On the other hand, within the region where they reside they provide a very high local concentration of general acid or base. One need only recall how many orders of magnitude more effective an imidazole side chain is within a proteolytic enzyme macromolecule compared to a solution of imidazole. In regard to catalysis of exchange, all types of groups, carboxylic, imidazole, and amino, have been shown to be effective with the model amide (whether it is largely hydrogen bonded or not), and it seems reasonable to expect that they would be catalysts when present as side chains.

In conclusion, then, it seems unlikely that deuterium–hydrogen exchange is a reliable measure of percentage of amide hydrogens in a helical configuration. On the other hand, there seems to be no alternative to the presumption that very slowly exchanging amide hydrogens must be in regions of difficult accessibility. Regions in which solvent can penetrate to various extents should show rates of exchange which reflect the effective local concentration of solvent and of all potentially catalytic acids and bases as well as interamide hydrogen bonding. All of these factors of course reflect the conformation of a protein macromolecule, but it is not a simple matter to assess their relative contributions to the resultant experimental observation.

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Kinetics of Papain-Catalyzed Hydrolysis of α-N-Benzoyl-L-arginine Ethyl Ester and α-N-Benzoyl-L-argininamide[1]

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Kinetic data for papain-catalyzed hydrolyses are presented which support the mechanism indicated by eq. 1, in which ES is the enzyme-substrate complex, ES′ is the acyl-enzyme, and P_1 and P_2 are the alcohol (or amine) and acid portions of the substrate, respectively. Identical pH–k_{cat}/K_m(app) profiles but different pH–k_{cat} profiles are found in the papain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-argininamide. These results are not consistent with a one-step catalytic process but have been successfully analyzed in terms of the above two-step catalytic process. Although k_{cat} (the turnover rate constant) for both ester and amide hydrolysis is determined by both k_2 (acylation) and k_3 (deacylation), the predominately rate-determining step for the ester is k_3 while it is k_2 for the amide. K_S for the ester and amide are nearly identical; k_2(lim) for the ester and amide differ by only six- to sevenfold, significantly different from the result found with α-chymotrypsin. The pH–rate profiles of k_2/K_S and k_2 are bell-shaped curves determined by two prototropic equilibria for both ester and amide hydrolysis, while the pH–rate profile of k_3 is a sigmoid curve determined by a single prototropic equilibrium for both ester and amide. A carboxylic acid group and a sulfhydryl group appear to be involved as an acid–base pair in the acylation process while a carboxylate ion appears to be involved as a base in the deacylation process. Deuterium oxide decreases k_3 2.75-fold but decreases k_2 only 1.35-fold.

Recent work on the serine proteolytic enzymes, particularly α-chymotrypsin, leaves little doubt that the hydrolysis of a substrate proceeds *via* the formation of an acyl-enzyme intermediate (eq. 1),[3,4] where ES is the

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (1)$$
$$+ P_1$$

enzyme–substrate complex, K_s its equilibrium dissociation constant, ES′ the acyl-enzyme, and P_1 and P_2 are the alcohol and acid portion of an ester substrate, respectively. It has been shown[3-6] that comparison of eq. 1, which involves an acyl-enzyme intermediate, with

(1) This research was supported by grants from the National Institutes of Health and the U. S. Atomic Energy Commission.

(2) National Institutes of Health Special Postdoctoral Fellow on leave from the Department of Food Science and Technology, University of California, Davis.

(3) B. S. Hartley and B. A. Kilby, *Biochem J.*, **50**, 672 (1952); **56**, 288 (1954); H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U. S.*, **42**, 719 (1956).

(4) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3669 (1964), and references therein; M. L. Bender and F. J. Kézdy, *ibid.*, **86**, 3704 (1964), and references therein.

(5) I. B. Wilson and E. Cabib, *ibid.*, **78**, 202 (1956).

(6) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *ibid.*, **86**, 3680 (1964).