

# Protonation of Polypeptides in Helix-Breaking Solvents. Electrical Conductance\*

Marija A. Stake† and Irving M. Klotz

**ABSTRACT:** Electrical conductances have been observed and measured for solutions of poly-L-alanine, poly-L-leucine, poly-L-glutamine, and poly- $\gamma$ -benzyl-L-glutamate, as well as of polyvinylpyrrolidone, in trifluoroacetic acid. The observations show that protonation of amide groups occurs in all cases.

Conductances increased for the polymers in mixed solvents of  $\text{CHCl}_3$ - $\text{CF}_3\text{COOH}$  as the concentration

of  $\text{CF}_3\text{COOH}$  increases, particularly in the composition range for transitions in properties of the polyamino acids.

Polyvinylpyrrolidone cannot undergo a helix-coil transition since it does not contain amide hydrogen bonds. The effect of higher acid concentrations can be interpreted in terms of solvation of anions of the polymer-anion ion pairs.

Polypeptides in solutions containing increasing quantities of organic acids undergo transitions in a variety of physicochemical properties. It has been concluded recently (Hanlon and Klotz, 1965) from observations in the overtone region of the infrared that amide groups of polypeptides such as poly- $\gamma$ -benzyl-L-glutamate, poly-L-alanine, and poly-L-leucine are protonated by strong organic acids. The primary basis of this conclusion was the similarity in position of the N-H overtone band in these polymeric amides with that observed in a simple model amide, *N*-methylacetamide, in strong acids; in the latter model system a variety of physical measurements provided overwhelming evidence of protonation (Klotz *et al.*, 1964).

Although charged polymeric molecules contribute only weakly to the electrical conductance of a solution, since their mobilities are low, their presence should be detectable. Furthermore, in the system of present interest, if protonation occurs in a solution containing, e.g., trifluoroacetic acid



then small  $\text{CF}_3\text{COO}^-$  ions are generated and these may exhibit substantial conductances. It has seemed appropriate, therefore, to examine the electrical conductance of solutions of polypeptides so that the con-

clusion inferred from infrared measurements could be tested.

## Materials and Methods

Trifluoroacetic acid, purchased from Eastman Organic Chemicals, was distilled through a Vigreux column and the fraction boiling at 70.5–71.0° was collected. *N*-Methylacetamide was distilled in a like fashion, the 205–206°-fraction being collected. Chloroform was purified by first removing traces of ethanol by shaking with concentrated sulfuric acid, washing with dilute sodium hydroxide and water, drying, and distilling. Poly-L-leucine and poly-L-alanine were purchased from Pilot Chemical Co. (Lots no. 6441 and 6442) and were dried overnight at 70°; they were similar to the samples described previously (Hanlon and Klotz, 1965). Poly-L-glutamine (Lot no. 6292:81,  $M_0 \sim 35,000$ ) was kindly supplied by Dr. L. H. Krull of the Northern Utilization Research and Development Laboratory of the U. S. Department of Agriculture (Krull *et al.*, 1965). Polyvinylpyrrolidone (K-30) was a commercial product of General Aniline and Film Corp.

Resistance (*R*) measurements of the polymer solutions were made with a Model RC-1B conductivity bridge made by Industrial Instruments, Inc. Equivalent conductances were calculated from the relationship

$$\Lambda = \frac{10^3(0.348)}{RC_r} \quad (2)$$

where 0.348  $\text{cm}^{-1}$  is the conductance cell constant, and  $C_r$  is the concentration of residues in moles per liter. Resistances (*R*) were measured with a precision of at least 2% in the range of 50–250,000 ohms in a conductance cell with nonplatinized platinum electrodes. All measurements were carried out at 25°.

To ascertain whether small amounts of moisture in

\* From the Biochemistry Division, Department of Chemistry, Northwestern University, Evanston, Illinois. Received February 14, 1966. This investigation was supported in part by a grant (GM-09280) from the National Institute of General Medical Sciences, U. S. Public Health Service. It was also assisted by support made available by a U. S. Public Health Service Training Grant (5T1-GM-626) from the National Institute of General Medical Sciences.

† Postdoctoral Fellow of the U. S. Public Health Service, 1963–1965.

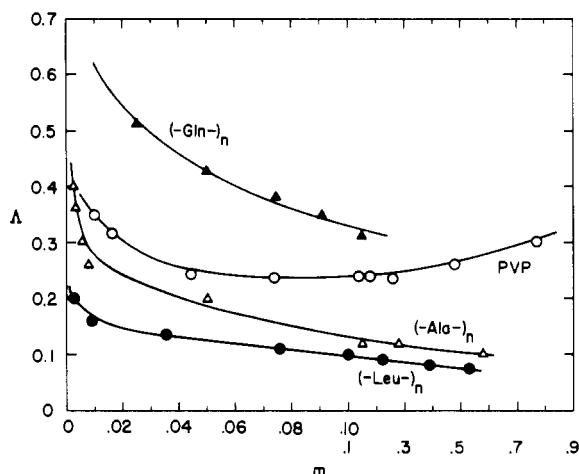


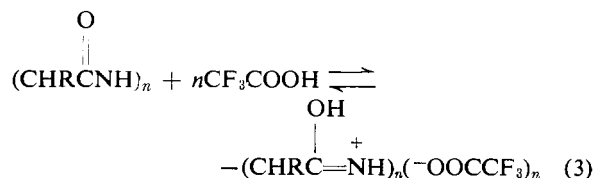
FIGURE 1: Equivalent conductances,  $\Delta$ , in 100% trifluoroacetic acid, as a function of residue molar concentration,  $m$ , for poly-L-glutamine (Gln) $_n$ , polyvinylpyrrolidone (PVP), poly-L-alanine (Ala) $_n$ , and poly-L-leucine (Leu) $_n$ .

the polypeptide samples would affect the conductances appreciably, we added portions of water purposely. Up to 0.5% water in trifluoroacetic acid produced no perceptible change in conductance.

## Results

The equivalent conductances of poly-L-alanine, poly-L-leucine, and poly-L-glutamine in trifluoroacetic acid are shown in Figure 1 as a function of polymer concentration. The steep rise in conductance as the polymer approaches infinite dilution is similar to that observed for a simple amide, *N*-methylacetamide, in trifluoroacetic acid (Klotz *et al.*, 1964). With the poly-amino acids, however, the observed conductances continuously drop with increasing concentration, whereas *N*-methylacetamide shows a minimum at about 0.1 M concentration and then the conductance increases again. It is necessary to keep in mind in this connection that the polypeptide solutions increase in viscosity with increasing concentration of solute. If equivalent conductances are corrected for this viscosity increase, then a minimum is also observed, at about 0.05 residue M for poly-L-leucine and near 0.1 residue M for poly-L-alanine. Furthermore for poly-L-leucine, *e.g.*, the corrected conductance at 0.35 residue M is more than four times as high as the minimum value. However, in view of the uncertainty in the appropriateness of correction based on a *macroscopic* viscosity, the corrected conductances are not shown. For poly- $\gamma$ -benzyl-L-glutamate the dependence of equivalent conductance on concentration follows closely that shown for polyleucine in Figure 1.

Since these polypeptide solutions conduct current, ions must be present. Charged species could be expected from the following reaction



The charged polypeptide should have only a small electrical mobility, as judged from transference numbers of other polymeric ions. Trifluoroacetate ions could show strong conductance, but in a solvent of low dielectric constant and in a solution containing a polymeric cation of high charge density, the mobility of the small anions would be severely restricted. Dissociation of ion pairs would be favored, however, at low concentrations and hence the observed conductance increases as infinite dilution is approached. The conductance at high concentrations, if corrected for increased macroscopic viscosity of the solution, also increases and presumably reflects the formation of triple ions in which two cationic segments of the polymer became associated with a common  $\text{CF}_3\text{COO}^-$  ion.

Addition of chloroform to trifluoroacetic acid would be expected to decrease the conductances of the polymeric solutes since the dielectric constant is lowered further and hence ion association is favored even more. This is indeed clearly seen for polyalanine and polyglutamine (Figure 2), the observed conductance dropping continuously as the chloroform content is increased<sup>1</sup> (or that of  $\text{CF}_3\text{COOH}$  decreased). The data for polybenzylglutamate, which are not shown on the graph, follow closely those shown for polyalanine. The observed results for polyleucine show a drop as the  $\text{CF}_3\text{COOH}$  concentration is lowered from 100 to 80%, and an apparent small rise thereafter. This behavior merely reflects viscosity changes in the solutions, however. The solutions with increasing chloroform content have lower viscosities, partly a specific reflection of solvent composition and partly because of dilution of polymer solute as chloroform is added to the original solution in trifluoroacetic acid. If corrections are made for these viscosity effects, the conductance curve for polyleucine is similar to that for the other polypeptides.

Equivalent conductances of polyvinylpyrrolidone in 100% trifluoroacetic acid as a function of polymer concentration are also summarized in Figure 1. As usual one finds a rise in conductance as polymer solute approaches infinite dilution. At higher concentrations the observed equivalent conductance tends to remain level despite the increase in viscosity of the solution. If

<sup>1</sup> These experiments were carried out in two different ways. In the first procedure solutions of polymer in 100% trifluoroacetic acid were diluted by addition of chloroform, in which case both polymer and acid concentration were decreased. In the second procedure a series of solvent mixtures was made first and weighed amounts of the polymer then dissolved in each mixture so that its concentration was essentially the same in each solution. Measured conductances were essentially the same, for corresponding solvent mixtures, as one might have expected from the flatness of the curve of  $\Delta$  vs.  $m$  (Figure 1) in the range of 0.1–0.8 residue M concentrations.

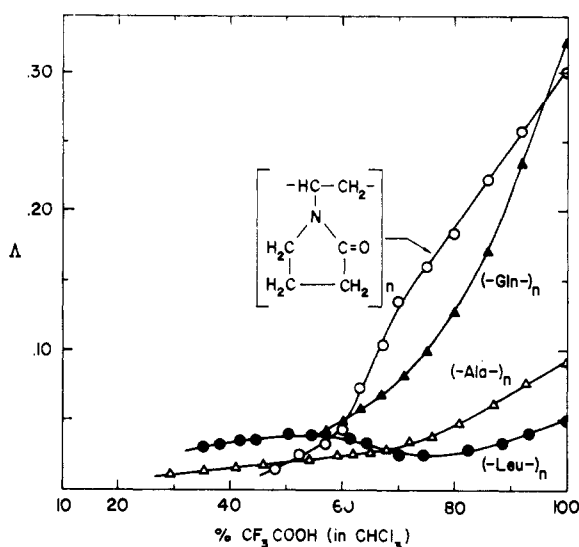
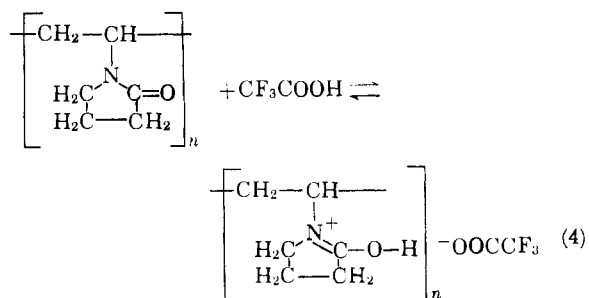


FIGURE 2: Variation of equivalent conductance,  $\Lambda$ , of polymers with content of  $\text{CF}_3\text{COOH}$  in  $\text{CHCl}_3$ - $\text{CF}_3\text{COOH}$  mixtures. Initial concentrations (residue molar) in pure  $\text{CF}_3\text{COOH}$ , before dilution with  $\text{CHCl}_3$ : PVP, 0.77;  $(\text{Gln})_n$ , 0.14;  $(\text{Ala})_n$ , 0.68;  $(\text{Leu})_n$ , 0.62.

corrections are applied for the rise in viscosity, the equivalent conductance rises gradually until at 0.9 residue M the conductance is more than eight times that at 0.1 residue M. Such behavior is similar to that observed with a small amide, *N*-methyl acetamide (Klotz *et al.*, 1964), and hence may also be a reflection of triple ion formation.

Polyvinylpyrrolidone is a lactam type structure. Presumably, therefore, the charged species observed in the conductance measurements arise from the following reaction



Again the macromolecule probably has a small electrical mobility. Furthermore the high charge density along the polymer chain keeps the small anions largely immobilized in ion pairs. Consequently observed conductances are small; but they are of the same magnitude as found for polypeptides.

Polyvinylpyrrolidone is very soluble in pure chloroform as well as in trifluoroacetic acid. If  $\text{CHCl}_3$  is added to solutions of this polymer in  $\text{CF}_3\text{COOH}$ , the equivalent conductance drops rapidly (Figure 2). Similar

behavior has been observed with 10% solutions of polymer as with 2%. (If corrections were made for decreases in viscosity with decreasing proportion of  $\text{CF}_3\text{COOH}$ , the drop in  $\Lambda$  would be even greater.) Of particular interest in a comparison of this polymer with polyamino acids is the similarity in behavior in solutions in the region of 65–100%  $\text{CF}_3\text{COOH}$ ; in all cases this is the range in which conductance shows a marked rise with increasing proportion of  $\text{CF}_3\text{COOH}$ .

Below 65%  $\text{CF}_3\text{COOH}$ , chloroform-trifluoroacetic solutions of polyvinylpyrrolidone on standing separate into two phases, and appear cloudy. Conductances of these cloudy mixtures are included in Figure 2, and are low.

Although polyvinylpyrrolidone is very soluble in pure chloroform (where it shows zero conductance) as well as in pure trifluoroacetic acid, addition of small amounts of trifluoroacetic acid to a chloroform solution causes immediate precipitation. Such behavior would be expected if  $\text{CF}_3\text{COOH}$  protonates the lactam group and forms polymeric salt.

## Discussion

It has been shown previously (Hanlon and Klotz, 1965) that the spectra of polypeptides in  $\text{CHCl}_3$ - $\text{CF}_3\text{COOH}$  mixtures are those of protonated  $\text{COHNH}^+$  groups even at very low concentrations (<1%) of the strong acid. As the concentration of acid is increased, spectroscopic changes indicate that the polypeptide remains protonated throughout the solvent composition range (60–80%  $\text{CF}_3\text{COOH}$ ) in which optical rotatory properties show a transition in values.

The suggestion was made, therefore, that protonation, with disruption of hydrogen bonds involved in the  $\alpha$ -helix, occurs as soon as  $\text{CF}_3\text{COOH}$  is introduced into the chloroform solution of polypeptide. In solutions with little  $\text{CF}_3\text{COOH}$  the cation and anion produced by protonation would form ion pairs, since the dielectric constant of the medium is low. Hence electrical conductance should be low, as has now been observed. As the content of  $\text{CF}_3\text{COOH}$  is increased, this component being a good solvating substance could facilitate dissociation of the ion pairs. In fact, increased conductance is observed with increasing content of  $\text{CF}_3\text{COOH}$ . A particularly sharp upturn occurs at about 60%  $\text{CF}_3\text{COOH}$ . This is the range for the transition in optical rotatory properties.

The most striking aspect of these conductance data is provided by polyvinylpyrrolidone. This unprotonated polymer has no N-H groups (see eq 4) to bond with the  $\text{C}=\text{O}$ . It cannot form an intramolecularly hydrogen-bonded helix. Nevertheless, it shows a transition in electrical conductance in  $\text{CHCl}_3$ - $\text{CF}_3\text{COOH}$  mixtures at about the same composition of trifluoroacetic acid as is observed with the polyamino acids. It seems, therefore, that the solvent mixtures containing 60–100% trifluoroacetic acid exert their effect by means other than breaking  $\text{N}-\text{H} \cdots \text{O}=\text{C}$  bonds of an  $\alpha$ -helix. The explanation offered in connection with infrared observations (Hanlon and Klotz, 1965) is that the

solvent of higher  $\text{CF}_3\text{COOH}$  concentrations is a better solvating agent. In particular it can form solvated complexes with the small anion,  $\text{CF}_3\text{COOH} \cdot ^-\text{OOC}\text{CF}_3$ . This solvation of anions would increase the dissociation of ion pairs and hence produce a rise in electrical conductance.

Thus it is apparent that conductance data confirm infrared observations in regard to the formation of protonated peptide groups in solvents containing

the strong acid  $\text{CF}_3\text{COOH}$ .

#### References

- Hanlon, S., and Klotz, I. M. (1965), *Biochemistry* 4, 37.  
 Klotz, I. M., Russo, S. F., Hanlon, S., and Stake, M. A. (1964), *J. Am. Chem. Soc.* 86, 4774.  
 Krull, L. H., Wall, J. S., Zobel, H., and Dimler, R. J. (1965), *Biochemistry* 4, 626.

## Antibiotics As Tools for Metabolic Studies. V. Effect of Nonactin, Monactin, Dinactin, and Trinactin on Oxidative Phosphorylation and Adenosine Triphosphatase Induction\*

Stanley N. Graven, Henry A. Lardy, Diane Johnson, and Anthony Rutter

**ABSTRACT:** Four homologous antibiotics, nonactin, monactin, dinactin, and trinactin, were studied for their effect on oxidative phosphorylation and adenosine triphosphatase (ATPase) induction. Nonactin, monactin, dinactin, and trinactin uncoupled oxidative phosphorylation at a concentration of  $1 \times 10^{-7}$  M and partially uncoupled at  $1 \times 10^{-8}$  M. All four homologs induced ATP hydrolysis. Monactin, dinactin, and trinactin were more potent inducers of ATPase activity than nonactin.

A monovalent cation,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Rb}^+$ , or  $\text{Cs}^+$ , was required for the action of the antibiotics. None of the nonactin homologs was active in the presence of  $\text{Li}^+$  as the cation. At low antibiotic concentrations

( $1 \times 10^{-7}$  M and below), less ATPase activity was induced in the presence of  $\text{Na}^+$  than in the presence of  $\text{K}^+$ ,  $\text{Rb}^+$ , or  $\text{Cs}^+$ . At low monovalent cation concentrations (7.5 mM), greatest activity was observed in the presence of  $\text{Rb}^+$ . The antibiotics are potent uncouplers of oxidative phosphorylation and inducers of ATPase activity with their activity possibly mediated through an effect on monovalent cation transport. The nonactin homologs were comparable in activity to valinomycin and less active than gramicidins A-D as measured by ATPase induction. The monovalent cation requirement of the nonactin homologs differentiated their activity from that of valinomycin, tyrocidine, and gramicidins A-D.

Corbaz *et al.* (1955) reported the isolation of an Actinomycete metabolite which they named nonactin because it lacked antimicrobial activity toward their test organisms. A similar product was isolated independently by Dutcher (1962) and designated SQ 15859. The compounds were noted to be cytotoxic for tumor and certain bacterial cells (Arnow *et al.*, 1962; Meyers *et al.*, 1965) and to uncouple oxidative phosphorylation (Lardy, 1961). In addition to nonactin, three homologs named monactin, dinactin, and trinactin

were subsequently isolated from the same fermentation (Beck *et al.*, 1962; Dominguez *et al.*, 1962). The basic structure is that of a cyclic macrotetralide (see Figure 1) composed of four nonactinic or homononactinic acid subunits. The homologs differ in that methyl substituents ( $\text{R} = \text{H}$  in Figure 1) are replaced by one, two, or three ethyl groups, and the stereoconfiguration of the constituent acids varies as well. The structure and absolute configuration of the nonactin and its homologs were determined by Gerlach and Prelog (1963). This report presents data on the effects of nonactin, monactin, dinactin, and trinactin on oxidative phosphorylation and the induction of ATPase in rat liver mitochondria.

#### Materials and Methods

Mitochondria were prepared from livers of male, 150–220-g rats according to the method of Lardy and

\* From the Department of Biochemistry and Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin. Received February 11, 1966. Supported by grants from the National Science Foundation, the Life Insurance Medical Research Fund, and the National Institutes of Health. Some aspects of this work were presented at the 140th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1961 (Lardy, 1961).

<sup>1</sup> Abbreviation used: ATPase, adenosine triphosphatase.