Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

Explanation of Ionic Sequences in Various Phenomena. IV. General Method for Determination of the Type of Bonding in Polymers; Bonds in Ovalbumin, Ribonuclease, Collagen, Hemoglobin, and Starch Determined

Stig R. Erlander[a]
[a] Ambassador College, Pasadena, California

To cite this Article Erlander, Stig R.(1968) 'Explanation of Ionic Sequences in Various Phenomena. IV. General Method for Determination of the Type of Bonding in Polymers; Bonds in Ovalbumin, Ribonuclease, Collagen, Hemoglobin, and Starch Determined', Journal of Macromolecular Science, Part A, 2: 6, 1195 — 1221
To link to this Article: DOI: 10.1080/10601326808051888
URL: http://dx.doi.org/10.1080/10601326808051888

J. MACROMOL. SCI.—CHEM., A2(6), pp. 1195-1221, October, 1968

Explanation of Ionic Sequences in Various Phenomena. IV. General Method for Determination of the Type of Bonding in Polymers; Bonds in Ovalbumin, Ribonuclease, Collagen, Hemoglobin, and Starch Determined

STIG R. ERLANDER

Ambassador College
Pasadena, California

SUMMARY

The salting-out or -in of hydrocarbons, uncharged nitrogen bases, and acids containing polarized hydroxyl groups give different cationic sequences. Also various ionic groups have different solubility sequences. Such neutral or electrostatically charged groups are attached to polymers and are involved as forces which hold together aggregates or helicies in inter- or intramolecular interactions. In aqueous solutions, the addition of various salts can destroy or produce these inter- or intramolecular interactions. Consequently, by applying the ionic solubility sequences, the observed salting-in and -out sequences of low molecular weight molecules, and other solubility criteria to the destruction or formation of such polymer interactions, it is possible to determine the major force or forces which are involved in holding the aggregate or helix together. Such forces involve hydrophobic bonds, hydrogen bonds between neutral bases, hydrogen bonds involving polarized hydroxyl groups, ionic bonds, and/or repulsive forces between electrostatically charged groups. The method is applied to the salting-out of ovalbumin, the destruction of the α-helix of ribonuclease, the collagen-gelatin transformation, the dissociation of hemoglobin, and the retrogradation and solubilization of starch. These five examples involve all the secondary forces considered. The method should find wide applicability because it can be applied to any method of physical measurement involving ionic solutions.

INTRODUCTION

It was previously shown [1] that the reversal of charge and ion binding phenomena are not caused by the formation of insoluble ion-ion bonds. Thus the reduction in swelling of bovine serum albumin (BSA) at pH 2 by addition of SCN^- ions is due to an association of the counterion to the charged group on the BSA (formation of an "inner sphere"). Experimental data for both phenomenon show that the greater the solubility of the counterion the greater will be the added salt's ability to reduce the electrostatic charge or to "bind" to the polyelectrolyte. On the other hand, a previous examination [2] of the salting-in and -out of amino acids showed that ion-ion interactions (salt bonds) are destroyed by the reverse of the above process. In other words, the greater the insolubility of the salt complex, the greater will be the ability of the added counterion to destroy a salt bond.

The above mechanisms have been explained by using the effective or microscopic dielectric constant ($D_\pm$) of an ion and the proposed models for the structure of various hydrated ions [3]. The models are an extension of the proposals made by Frank and Wen [4] and Samoilov [5]. It was shown previously that ions having loosely bound water (B regions [4] or negative hydration [5]) can destroy hydrophobic and hydrogen bonds if their B regions have values of $D_\pm > D_1$. Here D_1 is the dielectric constant of unassociated water [3]. It will be shown in other papers in this series that urea is a zwitterion, the positive portion resembling the guanidinium ion (G^+) and the negative portion the Cl^- ion. At high molarities urea forms head-to-tail ionic bonds with itself, and therefore its high value of $D_\pm$ is curtailed. Nevertheless, the action of urea is the same as the G^+ ion, and therefore in any cationic sequence the urea zwitterion will occupy a position near the G^+ ion.

Besides considering the value of $D_\pm$, the presence of A regions or tightly bound water on cations such as Li^+ must also be taken into account. A regions increase the cation's ability to destroy hydrogen bonds involving nitrogen bases and decrease the cation's ability to destroy hydrogen bonds having hydroxyl groups. The combination of these effects gives an important tool for determining the type of bond holding a helix or aggregate together.

Previous attempts to correlate ionic sequences, such as that made by von Hippel and Wong [6], have brought out the importance of such sequences but have failed in explaining their significance and meaning. Also, Puett et al. [7] have investigated the effects of various cations and anions, but their observed sequences have not been developed into a general theory for determining the type of secondary bonding. Their [7] "diluent" effect corresponds to ions having $D_\pm < D_1$ but no A regions, whereas their "specific" effect corresponds to ions having $D_\pm > D_1$.

By piecing together the available data given in the literature, it is possible to make specific conclusions on the bonds (hydrogen, hydrophobic, or ionic) involved in the secondary and tertiary structure of polymers. That is, it is possible to determine not only the types of bonds involved, but if other data are available it is also possible to correlate these bonds with various structures such as α-helix, random coil, or aggregation (dimerization, etc.) of the polymer molecule. The purpose of explaining the effects of various salts on specific polymers is therefore twofold: (1) to show how such data can be applied, and (2) to determine the type of bonding in extensively studied polymers.

METHOD OF DETERMINING THE TYPE OF BOND

Each particular type of bond will have a specific cationic and anionic sequence. A summary of such sequences is given in Table 1. It should be noted that the salting-out sequence is the reverse of the salting-in sequence. "Salting-out" refers to precipitation of the polymer, formation of dimers, trimers, etc., or formation of a helical conformation. Examples of the types of bonds involved in intra- or intermolecular interactions of polymers are given in Fig. 1. The sequences given in Table 1 were obtained by applying the salting-in and out sequences of low molecular weight molecules to similar groups on polymers. Thus the "hydrophobic bond" sequence was obtained from studies on the salting-in and out of hydrocarbons such as benzene [8]. The "hydrogen bond having an uncharged nitrogen base" sequence was obtained by application of salting-in and out studies on ammonia and trimethyl amine [8]. The "hydrogen bond having a hydroxyl group" sequence was obtained from salting-in and out sequences for carboxylic acids and the like [8]. And the "ionic bond" and "electrostatic repulsive forces" are applications of the solubilities of inorganic salts, as shown previously [9]. An explanation of the hydration models for the various ions and why each ion behaves as it does has already been elaborated upon [3]. The general method for determining the type of bonding involved in helix-coil or aggregation processes will now be given.

Although certain properties of a bond can be determined without obtaining the entire cationic sequence, the most conclusive evidence for the existence of a particular bond is obtained from examination of the monovalent cationic sequence. The divalent cationic sequence is not as helpful because each divalent cation contains both an A and a B region. The effects of these two regions sometimes reverse themselves [9]. As seen in Table 1, "hydrophobic," "hydrogen bonds having nitrogen bases," and "hydrogen bonds having hydroxyl groups" have different monovalent cationic sequences. Also the

Table 1 (Part I). Ionic Sequences for the Characterization of Bonds in Polymers[a]

Type of bond	Salting-in sequence	Characteristics
Hydrophobic bond (nonpolar sequence)	$Na^+ < K^+ < Li^+ = Rb^+ < Cs^+ < G^+ < (CH_3)_4N^+$ $Ba^{2+} < Sr^{2+} < Ca^{2+} < Mg^{2+}$ $Cl^- < Br^- < I^- < SCN^-$ $(CH_3)_4N^+ < (C_2H_5)_4N^+ < (C_3H_7)_4N^+ < (C_4H_9)_4N^+$	All cations and anions salt-out if $D_\pm < D_1$ except aliphatic cations. The Cl^- and Br^- salts of G^+ and Mg^{2+} salt-in if concentration of salt is about 4 M even though $D_- < D_1$. Solubility of a hydrocarbon increases as the temperature is lowered from 25 to 0°C. Therefore, dissociation of hydrophobic bond should also increase with decrease in temperature.
Hydrogen bond having uncharged nitrogen base (basic sequence)	$(CH_3)_4N^+ = K^+ < Na^+ < Rb^+ < Cs^+ < Li^+ < G^+$ $Ba^{2+} < Sr^{2+} < Ca^{2+} < Mg^{2+}$ $Cl^- < Br^- < I^- < SCN^-$ $(CH_3)_4N^+ < (C_2H_5)_4N^+ < (C_3H_7)_4N^+ < (C_4H_7)_4N^+$	The Cl^- and Br^- salts of Li^+ and divalent cations should salt-in peptide hydrogen bond at sufficiently high molarity even though $D_- < D_1$ for anion and $D_+ < D_1$ for cation because of the salting-in effect of the A regions. The alkyl amine sequence given here differs from that given for the hydrophobic bond in that the latter given salts-in and the former salts-out. The monovalent cation sequence can be distinguished from that for the ionic bond because K^+ salts-in the most and all ions salt-in for the ionic bond.
Hydrogen bond having a hydroxyl group (acidic sequence)	$Li^+ < Na^+ < K^+ \leq (CH_3)_4N^+ < Rb^+ < Cs^+ < G^+$ $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$ $Cl^- < Br^- < I^- < SCN^-$ $(CH_3)_4N^+ < (C_2H_5)_4N^+ < (C_3H_7)_4N^+ < (C_4H_9)_4N^+$	All Cl^- and Br^- salts salt-out unless $D_+ \gg D_1$ and A regions on cation are absent. At extremely high concentrations of salt, e.g., 6 M LiBr, the A region is not detrimental, and salting-in will occur if $D_- > D_1$, as in Br^- at high salt concentrations. Position of divalent cation in sequence may be altered because of influence of D_+ ($D_+ > D_1$ for Mg^{2+}).

[a]The salting-in sequences refer to the destruction of polymer aggregates or helical structures. The salting-out sequence is the reverse of the salting-in sequence. All anions or cations which are underlined are capable of salting-in the particular bond. The symbol G^+ refers to the guanidinium ion.

Table 1 (Part II). Destruction of Ionic Bonds

Type of ionic group on polymer	Salting-in sequence	Characteristics
(1) A region on polymer anion (acidic sequence)	$Li^+ > Na^+ > K^+ > Rb^+ > Cs^+ > (CH_3)_4N^+$ $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$ $(CH_3)_4N^+ > (C_2H_5)_4N^+ > (C_3H_7)_4N^+ > (C_4H_9)_4N^+$	If KCl or KBr salt-in, then the bond must be ionic because $D_{\pm} < D_1$ and because there are no A regions on K^+, Cl^-, or Br^-. The cationic sequence obtained will depend on the pK of the anionic polymer group. The pK in turn determines the existence of A or B regions or intermediate hydrate forms.
(2) B region on polymer anion having $D_- < D_1$ (basic sequence)	$K^+ > Na^+ > Rb^+ > Li^+ > Cs^+ > (CH_3)_4N^+$ $Rb^+ > Na^+ > K^+ > Li^+ > Cs^+$ *(for acetate)* $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$ $(CH_3)_4N^+ > (C_2H_5)_4N^+ > (C_3H_7)_4N^+ > (C_4H_9)_4N^+$	If both (+) and (–) groups of polyelectrolyte form insoluble salts with added salt, then the polymer will be salted-out unless sufficient uncharged hydrophilic groups are present.
(3) Cation on polymer has $D_+ < D_1$	$Cl^- > Br^- > I^- > SCN^-$	
(4) Cation on polymer has $D_+ > D_1$	$SCN^- > I^- > Br^- > Cl^-$	

Table 1 (Part III). Reduction of Electrostatic Repulsive Forces

Type of ionic group on polymer	Ability of ion to reduce electrostatic force of polymer (ability of ion to reverse charge)	Characteristics
(1) A region on polymer anion (acidic sequence)	$(CH_3)_4N^+ < Li^+ < Na^+ < K^+ < Rb^+ < Cs^+$ $Ba^{2+} < Sr^{2+} < Ca^{2+} < Mg^{2+}$ $(CH_3)_4N^+ > (C_2H_5)_4N^+ > (C_3H_7)_4N^+ > (C_4H_7)_4N^+$	Repulsive forces can force the formation or destruction of other bonds. The net charge of polymer will be opposite to that of ion, which reduces repulsive force. The more soluble the salt bond between polymer ion and added salt for the monovalent sequence, the greater will be the reduction in electrostatic forces. The divalent cationic sequence is the reverse of this because formation of an insoluble salt with monovalent polymer anion does not cancel charge of insoluble salt; e.g., $R{-}CO_2Ba^+$ is formed.
(2) B region on polymer anion having $D_- < D_1$ (basic sequence)	$(CH_3)_4N^+ < K^+ < Na^+ < Rb^+ < Li^+ < Cs^+$ $Rb^+ < Na^+ < K^+ < Li^+ < Cs^+$ (for acetate) $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$ $(CH_3)_4N^+ > (C_2H_5)_4N^+ > (C_3H_7)_4N^+ > (C_4H_7)_4N^+$	
(3) Cation on polymer has $D_+ < D_1$	$Cl^- < Br^- < I^- < SCN^-$	
(4) Cation on polymer has $D_+ > D_1$	$SCN^- < I^- < Br^- < Cl^-$	

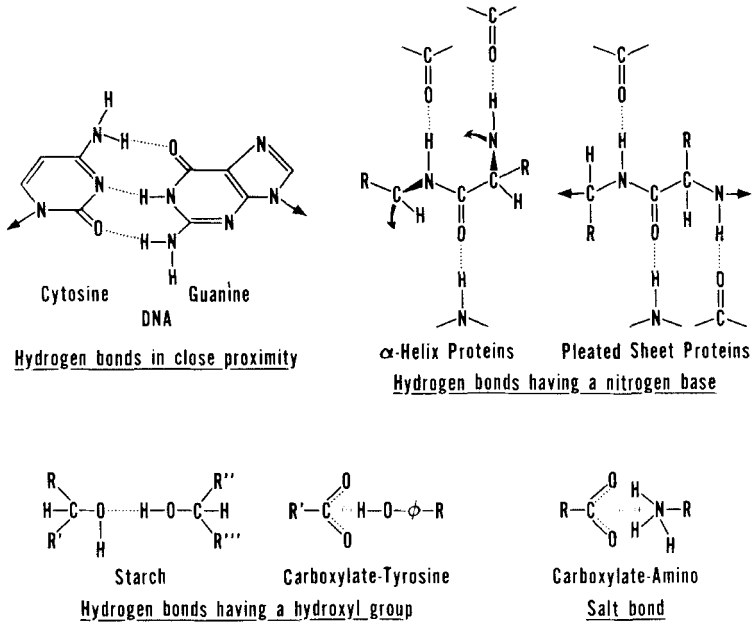


Fig. 1. A few of the types of secondary bonds involved in polymers. The highly polarized water molecules in the A region of Na^+ and Li^+ ions repel or salt-out polarized hydroxyl groups such as those depicted or such as those on neutral carboxylic acids. Conversely, these A regions are attracted to neutral nitrogen atoms.

salting-in of the ionic bond (Table 1, Part II) can give both a basic and an acidic type of sequence. However, the latter are the exact reverse of the hydrogen bond sequences. For example, in the basic sequence for destroying an ionic bond, the K^+ ion is the most effective, whereas in the basic sequence for destroying a hydrogen bond, the guanidinium ion (G^+) is the most effective. Therefore, the sequences are the same but the relative abilities to salt-in have been reversed. Negative charges where $D_- > D_1$ as in the case of I^-, SCN^-, and ClO_4^- ions are not attached to polymers and therefore can be eliminated as possibilities.

In some cases the force which holds either the helix together or the aggregated polymer is produced in part by a reduction in electrostatic repulsion forces. As shown in the reversal of charge and in the ion binding phenomena, such a reduction does not depend on the formation of an insoluble salt but rather on the formation of a soluble salt. The more soluble the ion-ion bond between the charged group and its counterion, the more effective will be the reduction in electrostatic repulsion forces. This reversal of charge phenomenon

—if it is important—may therefore obscure the detection of the type of bond involved in the helix or aggregated polymer.

After the ionic sequences have been obtained, the way in which an aqueous salt solution interacts with the polymer should be examined. For example, does the particular salt salt-in or salt-out the polymer? Also, is the same sequence obtained at both high and low ionic strengths? One particular bond may be salted-in (destroyed) by a specific salt, whereas another type of bond may not. In Table 1 those ions which are capable of salting-in a particular bond have been underlined. Any combination of these cations and anions should salt-in the particular bond. All ions which do not salt-in because of A regions on the ion or because of adverse values of $D_{\pm}$ are not underlined. If, for example, it is observed that KCl salts-in the polyelectrolyte, then one of the main stabilizing forces holding the aggregate or helix together must be ionic in character, because KCl salts-out all other types of bonds. However, ionic forces may be either repulsive or attractive. In order to distinguish between these two possible effects, it is therefore helpful to know the structure of the ionic group (sulfate, carboxylate, etc.) and its solubility sequence. The solubility sequence can be obtained from a comparison of the pK value of the group with that of known salts having the same group [1, 2]. In this respect it should be noted that the acetate ion gives an intermediate sequence. The pK of this group is similar to that of the γ-carboxylate ion of aspartate (or glutamate). Consequently, if the pK of such carboxylate groups are maintained at around pK = 4.8, then ionic bonds involving them should be readily detectable (see the collagen example given below).

Thus considering only ionic bonds or repulsive forces, if the cation or anion which salts-in the most is also the most soluble cation, then the forces are repulsive, since such a sequence is the same as the reversal of charge phenomenon described previously [1]. If, on the other hand, the salting-in sequence is the same as the insolubility sequence of the charged group, then salt bonds between anionic and cationic groups of the polymer or polymers are one of the major bonding forces, just as in the case of salting-in of amino acids [2]. In other words, a salt bond is broken by insolubilizing one of the ionic groups, but repulsive forces are destroyed more effectively with the most soluble cation or anion. Because the guanidinium group of arginine has a value of D_+ greater than that of water (D_1) whereas the amino group of lysine has a value of D_- less than that of D_1, the two groups can be distinguished as shown in Table 1 and previously [1, 2, 9].

If the bonds are between carboxyl and hydroxyl groups, then the sequence will be acidic. The same is true for hydrogen bonds between hydroxyl groups of polysaccharide molecules. However, the salting-in properties for such polymers may be quite different at

extremely high concentrations of salts. For example, starch granules can be solubilized by 8.5 to 9.5 M LiCl solutions even though concentrations of LiCl less than 8.5 M do not solubilize the starch. At such high concentrations of salt, the Cl^- ion becomes unhydrated and attaches itself to the hydroxyl groups of the starch, thus solubilizing it. Further discussion of this and related matters is given in another paper [10].

Besides the salting-in and -out behavior of various ions, the position of the tetramethyl amine ion in the monovalent cationic sequence is extremely important. As observed previously [3], in calculating values of D_+ for the $(CH_3)_4N^+$ ion from benzene solubility studies, the value of D_+ is too large with respect to the expected charge per unit surface area and is most likely equal to that of the K^+ ion. Consequently, the method of obtaining $D_\pm$ fails if the ion has hydrophobic groups attached to it. In other words, the hydrophobic groups on $(CH_3)_4N^+$ increase the solubility of benzene and thereby falsely increase the effective dielectric constant. If hydrophobic bonds are not involved, then the position of $(CH_3)_4N^+$ in the cationic sequence should be close to that of the K^+ ion. Thus, if $(CH_3)_4N^+$ has a salting-in property greater or equal to that of the guanidinium ion (G^+), then the bond must be hydrophobic. In this case, an increase in the length of the hydrocarbon chain of R_4N^+ also increases the solubilization effect. For hydrogen bonds, however, the effect of the $(CH_3)_4N^+$ ion will be approximately equal to that of the K^+ ion. Nevertheless, the salting-in effect will increase with an increase in the size of the aliphatic group on the R_4N^+ ion, just as in the case of the hydrophobic sequence. This effect is due to a decrease in charge per unit surface area and hence an increase in the value of D_+ as the size of R increases. The important differences are (1) the $(CH_3)_4N^+$ and analogous ions salt-in hydrocarbons and salt-out (produce) hydrogen bonds, and (2) the position of $(CH_3)_4N^+$ is altered in going from the cationic sequence for the destruction hydrogen bonds to that of hydrophobic bonds, i.e., $G^+ < (CH_3)_4N^+$ in its ability to destroy hydrophobic bonds (Table 1).

The $(CH_3)_4N^+$ ion is also important in distinguishing between ionic bonds and repulsive forces. Because of its bulkiness, there is a limit to the number of $(CH_3)_4N^+$ ions that can become associated as counterions around a negatively charged polymer group. For the same reason, the bulkiness of the $(CH_3)_4N^+$ ion prevents it from effectively reducing (or reversing) the electrostatic charge by ion association. Thus even though $(CH_3)_4N^+$ ion has a value of D_+ equal to or slightly greater than that of the K^+ ion, the $(CH_3)_4N^+$ ion will, because of this bulkiness, always behave as a soluble salt in the destruction of ionic bonds and as an insoluble salt in the reduction of electrostatic forces. In other words, the position of the $(CH_3)_4N^+$ ion can be used to distinguish between ionic bonds and repulsive forces.

The "ionic bond" and "repulsive forces" sequences for the R_4N^+ ions are also influenced by the bulkiness of these ions. Thus for repulsive forces the inability of the R_4N^+ ion to reduce the charge will increase with an increase in the size of the aliphatic group in the amine. Consequently, if the negatively charged groups on a polymer have A regions, then the cationic sequence for the ability to reduce or reverse the electrostatic charge of the polymer will be

$$(C_4H_9)_4N^+ < (C_3H_7)_4N^+ < (C_2H_5)_4N^+ < (CH_3)_4N^+ < Li^+ < Na^+ < K^+ < Cs^+$$

In the case of the ionic bond, the bulkiness of the R_4N^+ groups together with their aliphatic nature will inhibit them from destroying the ionic bond. Consequently, the positions of the R_4N^+ ion will again be unequal to that of the K^+ ion, even though both have about the same value of D_+. However, unlike the reversal of charge phenomenon, the position of the R_4N^+ ion will now be shifted to those cations which have a higher value of D_+. Thus the sequence for the ability of a cation to salt-in an ion-ion bond where the anion is positively hydrated, i.e., has an A region, is

$$Li^+ > Na^+ > K^+ > Cs^+ > (CH_3)_4N^+ > (C_2H_5)_4N^+ > (C_3H_7)_4N^+ > (C_4H_9)_4N^+$$

These results on the tetraaliphatic amines are summarized in Table 1. As seen, the larger R_4N^+ ions emphasize the discrepancy in the ionic and repulsive types of sequences. However, the sequences for R_4N^+ ions will be the same for both types of forces. Nevertheless, examination of Table 1 shows that the R_4N^+ ions give unique information regarding the type of bond.

Other tests can also be used to determine the presence of hydrophobic or hydrogen bonds. For example, concentrated solutions of LiCl or $CaCl_2$ (1 to 4 M) should destroy those hydrogen bonds which have a nitrogen base because of the strong interaction of the A regions of the cation with the base. Also, according to the data of von Hippel and Wong [6], a 1 M solution of $(C_3H_7)_4NBr$ or $(C_4H_9)_4NBr$ should destroy hydrophobic bonds. In addition, it should be noted that the urea zwitterion (see the introduction) can destroy both hydrophobic and hydrogen bonds if concentrated ($\geq$4 M) solution of urea are used. However, because of the urea zwitterion structure, concentrated urea solutions cannot destroy ionic bonds. Hence urea can be used as a tool to distinguish between ionic bonds and hydrogen or hydrophobic bonds.

In summary, the type of bond involved in coil to helix transitions, aggregation (dimerization, tetramerization, etc.) of polymers in

solution, or the precipitation of polymers (or the reversal of these phenomena) can be obtained as follows:

1. Determining the ionic sequences at both extremely low and moderate ionic strengths.
2. Observing whether the polymer is salted-out or -in; "salted-out" structures are helices, soluble aggregates, or precipitates. An example would be the fact that KCl should only salt-in ionic bonds and LiCl should salt-in hydrogen (nitrogen base) but not hydrophobic bonds in the cationic sequence.
3. Determining the position of the $(CH_3)_4N^+$ ion.
4. Distinguishing between ionic and hydrogen (or hydrophobic) bonds by using concentrated urea solutions.
5. Correlating the types of charged or uncharged groups on the polymer with the experimental data.

Various polymers will now be examined to show how these points are applied and to determine the types of bonds involved in some extensively studied polymers.

APPLICATION OF METHOD

Ovalbumin

Ovalbumin, i.e., egg albumin, was the first protein studied with respect to ionic sequences. In 1888 Hofmeister [11, 12] observed the acidic sequence given in Table 2 for the salting-out of this protein. This sequence is generally termed the Hofmeister series. However, examination of the literature shows that any type of series may be indiscriminately labeled the "Hofmeister series." In 1903 Pauli [12], in addition to examining the effect of more anions, found that the cationic sequence observed by Hofmeister is reversed (acidic to basic sequence) if the ovalbumin solution is acidified. To the author's knowledge the cationic sequences involved in the salting-out of ovalbumin have not been studied further. However, the more recent work of Harrington et al. [15, 16] has shown that ovalbumin contains eight carboxylate and eight tyrosine groups which are hydrogen-bonded together, although this work has not been confirmed [17]. Alkali or acid titration of either group destroys the hydrogen bond between these two groups [15].

The position of the NH_4^+ ion in the sequence further proves that the sequence is acidic at neutral pH and not basic, since the value of D_+ for NH_4^+ is greater than for K^+. Consequently, $K^+ > NH_4^+$ in salting-out. The position of the NH_4^+ ion may vary because of chelation effects [9]. However, the value of D_+ for the NH_4^+ ion is always greater than that for the K^+ ion. Thus the above sequence for salting-out ($K^+ > NH_4^+$) is always valid. Hence the relative positions of the Na^+, Li^+, and NH_4^+ ions with respect to that of the K^+ ion show

Table 2. Ionic Sequences for Various Polymers

Polymer	Mechanism	Sequences	Bonds involved
Ovalbumin [11, 12]	Salting-out at neutral pH	$Li^+ > Na^+ > K^+ > NH_4^+$ $F^- > Cl^- > Br^- > I^- > SCN^-$	Tyrosine-carboxylate bonds (acidic sequence)
Ovalbumin [12]	Salting-out at acid pH	$K^+ > Na^+ > Li^+$ $Cl^- > Br^- > I^-$	Hydrogen bonds, using neutral nitrogen base (basic sequence)
Ribonuclease [6]	Destruction of hydrogen bonds (α helix)	$(CH_3)_4N^+ \leq K^+ < Na^+ < Li^+ < Ca^{2+} < G^+$ $Cl^- < Br^- < ClO_4^- < SCN^-$ $(CH_3)_4N^+ < (C_2H_5)_4N^+ < (C_3H_7)_4N^+ < (C_4H_5)_4N^+$	Hydrogen bonds, using neutral nitrogen base (basic sequence)
Collagen-gelatin [6, 13, 14]	Destruction of collagen helix	No salt added (Carpenter and Lovelace [13]): $K^+ < Rb^+ < Cs^+ < Na^+ < Li^+$ Salt added to medium: k_0 values (von Hippel and Wong [14]): $Rb^+ < Na^+ < K^+ < Cs^+ < Li^+$ $Mg^{2+} < Ca^{2+} < Ba^{2+}$ $(CH_3)_4N^+ < (C_2H_5)_4N^+ \ll (C_3H_7)_4N^+, (C_4H_9)_4N^+$ K values (von Hippel and Wong [14]): $K^+ < Na^+ < Cs^+ < Li^+$ Optical rotation (Carpenter and Lovelace [13]): $Na^+ < Rb^+ < Cs^+ < Li^+$ All methods: $Cl^- < Br^- < I^- < SCN^-$	Hydrogen bonds and ionic repulsive forces stabilize helix; hydrophobic bonds stabilize gelatin coil for helix formation

Hemoglobin [29, 30]	Destruction of tetramer to dimer	KCl destroys tetramer $Ca^{2+} > G^{+} > K^{+} > Na^{+}$; 8 M urea and hydrophobic agents destroy tetramer	Ionic bonds exist between $\alpha\alpha$ and $\beta\beta$ units; hydrophobic bonds exist between $\alpha\beta$ units
Starch [31, 32]	Inhibition of retrogradation	Iodine affinity (Loewus and Briggs [38]): $Li^{+} < Na^{+} < K^{+}$ $F^{-} < Cl^{-} < Br^{-} < I^{-}$ Solubility (Morsi and Sterling [37]): $Li^{+} < Na^{+} < K^{+}$ $F^{-} < Cl^{-} < Br^{-} < I^{-} < SCN^{-}$	Destruction of hydrogen bonds (acidic sequence)
Starch [9]	Dissolving of starch granules	$Li^{+} < K^{+} < G^{+}$	Destruction of hydrogen bonds (acidic sequence)

that the sequence is acidic. Further verification that the sequence is acidic can be made by realizing that the acidic sequence is the only monovalent cation sequence where the Li^+ ion salts-out the most. Consequently, the sequence is acidic at neutral pH and basic at acid pH, as shown in Table 2.

The acidic cationic sequence observed by Hofmeister at neutral pH eliminates the possibility that hydrophobic bonds are being destroyed or formed during the salting-out of ovalbumin. From Table 1 we see that (a) reduction in repulsive forces, (b) the ionic bond, and (c) the hydrogen bond which has a hydroxyl group will give the acidic sequence. Ionic bonds cannot be involved because addition of salt will only salt-in and will not salt-out such bonds (unless the added ions form insoluble salt bonds with both the polymer anion and polymer cation). Furthermore, if the pK's of the carboxylate groups of the amino acids glutamate and aspartate in ovalbumin are approximately equal to 4.7 as observed for ribonuclease [18], then these groups must be negatively hydrated (no A regions), and thus the salting-out sequence should be basic rather than acidic. Thus ionic bonds must be absent. In addition, the reduction in repulsive forces by ion association (reversal of charge) can be ruled out on the basis that the most soluble salt will give the most effective reduction in charge. Consequently, the observed acidic sequence shows that the salting-out cannot be due to the insolubilization of the carboxylate ions on ovalbumin, to the reversal of charge phenomenon, or to the formation of inter- or intramolecular hydrogen or hydrophobic bonds. The acidic sequence for salting-out must be due to the presence of an acid-type group such as a titrated carboxylic acid. Since the pH is neutral, the group must be tyrosine. However, tyrosine alone will not give an acidic sequence, because its —OH group is not sufficiently polar to compensate for the hydrophobic nature of the benzene ring [8]. But the tyrosine-carboxylate bond should produce a sufficiently polar —OH group. It is therefore concluded that the ovalbumin must have tyrosine-carboxylate bonds, as observed by Harrington [15] and that the resulting acidic salting-out sequence is due to the salting-out of these tyrosine groups.

As the pH is lowered these and the other carboxylate ions are titrated to form the carboxylic acid group. These groups should also give an acidic sequence. The experimental result of Pedersen [19] show that at low pH the sedimentation coefficient of bovine serum albumin (BSA) increases according to the acidic cationic sequence [1]. The BSA data, therefore, illustrate the salting-out of carboxylic acid groups. The reversal of charge phenomenon on the fatty acid oleate also changes from a basic sequence to an acidic sequence if part of the carboxylate groups is titrated [1]. It is, therefore, quite surprising that the titration of ovalbumin with acid does not maintain the acidic sequence but rather changes it to a

basic sequence (Table 2). This basic sequence cannot be due to the insolubilization of carboxylate ions because all have been titrated. That is, the pK of the carboxylate group in the tyrosine-carboxylate bond is much lower than any other present [15, 16], and therefore a change in salting-out sequence shows that all carboxylate groups must have been titrated. Hydrophobic bonding is also eliminated because of its sequence. The only possible explanation is that hydrogen bonds between peptide groups are formed during the salting-out of ovalbumin at low pH. The titration of the carboxylate groups and the consequent destruction of the tyrosine-carboxylate bond must change the conformation of the ovalbumin molecules. This change allows the neutral (titrated) carboxylate groups to become hidden and exposes the peptide bonds to adjacent ovalbumin molecules. Hence such a change in conformation would allow the formation of intermolecular hydrogen bonds. Thus the salting-out of the tyrosine-carboxylate group at neutral pH produces an acidic sequence and the salting-out of hydrogen bonds at acid pH produces a basic sequence.

The above conclusions are summarized as follows:

For neutral pH:

1. The monovalent cationic sequence is acidic.
2. The salting-out is not due to insolubilization of the carboxyl group or reversal of charge phenomenon because the free carboxylate ions on glutamate and aspartate have a solubility sequence that is basic and not acidic. Reduction in repulsive forces (reversal of charge phenomenon) does not occur because the most insoluble rather than the most soluble salt produces the greatest effect.
3. The observed acidic sequence eliminates hydrophobic and hydrogen bonds between bases.
4. The only alternative is acidic bonds involving tyrosine hydroxyl groups. The sequence is therefore due to the salting-out of the eight carboxyl-tyrosine bonds.

For acidic pH:

1. All negative charges on ovalbumin have been neutralized with acid. Therefore, the cationic sequence cannot be due to formation of ionic bonds or reduction of negative repulsive forces.
2. The salting-out of neutral carboxylic acid groups or of hydrophobic groups does not occur because a basic sequence is obtained.
3. The basic sequence shows that hydrogen bonds using neutral nitrogen atoms (hydrogen bonds between peptide linkages) must be involved in the salting-out of ovalbumin in acidic solutions.

Ribonuclease

It was previously thought that ribonuclease did not contain the α-helix structure. However, the recent studies of Kartha et al. [20]

have shown that ribonuclease contains two turns of the helix at positions 5 to 12 near the amino terminus and two more turns near the region of residues 28 to 35. Denaturation studies can thus involve a destruction of the hydrogen bonds in these helicies as well as destruction of any possible folded structures. In the study of ionic sequenced, it is impossible to know the position of the bonds which are destroyed. Only the type of bond determines the ionic sequence. In the study of ribonuclease it will be seen that the bonds destroyed by salts are hydrogen bonds between peptide linkages. Hence it is quite probable that the melting-temperature (Tm) observations made by von Hippel and Wong [6] involve the destruction of these α helices. However, even though this destruction will be referred to as a helix-to-coil transition, it must be emphasized that the change in Tm may only involve a change in the folding of ribonuclease.

The change in Tm as a function of salt concentration and as measured by the optical rotary dispersion method showed that some salts increase (stabilize bonds) whereas others decrease the value of Tm (destroy bonds). From Fig. 2 of von Hippel and Wong [6] it is seen that KCl stabilizes the helix (increases the value of Tm). Both K^+ and Cl^- ions have $D_\pm < D_1$. Therefore, this observation shows that ionic bonds do not maintain the structure of the helix. That is, the K^+ ion and the $R{-}CO_2^-$ ion of the protein both have $D_\pm < D_1$. Also the Cl^- ion and the $R{-}NH_3^+$ ion of the protein have $D_\pm < D_1$. Consequently, both the K^+ and Cl^- ions are capable of destroying ionic bonds on the protein, because in both cases the counterion (K^+ or Cl^-) has about the same value of $D_\pm$ as the protein group [9]. The observed basic sequence for the destruction of the α-helix ($K^+ < Na^+ < Li^+ < G^+$) substantiates the absence of ionic bonds. In other words, for ionic bonds the K^+ ion would be the most effective in destroying the α-helix, whereas for either hydrogen bonds or reduction in repulsive forces the G^+ ion would be the most effective. Consequently, ionic bonds are not involved, since the G^+ ion is the most effective in destroying the helix.

The data of von Hippel and Wong [6] show that the Ca^{2+} ion is more effective than the Li^+ ion in destroying the α helix. Consequently, the sequence $K^+ < Na^+ < Li^+ < Ca^{2+} < G^+$ shows that reduction in electrostatic repulsive forces does not cause the destruction of the α helix. In other words, if a reduction in repulsive forces were involved, then the Ca^{2+} ion would be less effective than the K^+ ion, because of the greater insolubility of the Ca^{2+} ion. On the other hand, if hydrogen bonds existed, then the highly polarized water molecules of the Ca^{2+} ion's A region would be effective in destroying them.

Further proof that repulsive forces do not stabilize the helix is obtained in examining the sequence for the tetraaliphatic amines. The bulkier the aliphatic chain, the greater will be the inability of

several R_4N^+ ions to gather around a single charged group. This steric hindrance will hinder the ability of the R_4N^+ ion to reduce the electrostatic charge of an anionic polyelectrolyte. Consequently, for effectiveness in reducing replusive forces of a polyelectrolyte, the cationic sequence $(CH_3)_4N^+ > (C_2H_5)_4N^+ > (C_3H_7)_4N^+ > (C_4H_9)_4N^+$ is obtained. However, the observed sequence as given in Table 2 is opposite to this one and corresponds to the value of the effective dielectric constant. Thus the observed sequence is the same as that for the destruction of a hydrogen or hydrophobic bond.

In addition to ionic bonds and ion-ion repulsions the observed basic cationic sequence also eliminates hydrophobic bonds and hydrogen bonds involving hydroxyl groups as forces which maintain the α helix structure. Consequently, even though it has been proposed that ribonuclease contains two or three tyrosine-carboxylate hydrogen bonds [21-23], the above data show that these bonds are not the important factor in stabilizing the helix. Rather, the above discussion and the ionic sequences show that hydrogen bonding involving a neutral nitrogen base must be the main force which holds the helix together. Because the nitrogen groups of arginine, lysine, and histidine of ribonuclease would be positively charged under conditions (low pH) where the helix is stable, it is concluded that the uncharged nitrogen in the peptide linkage forms hydrogen bonds to stabilize the helix.

The above results can be summarized as follows:

1. Ionic bonds are not involved in the formation of the ribonuclease helix because KCl does not salt-in and because G^+ and not K^+ is the most effective in destroying the helix.
2. The R_4N^+ sequence is the reverse of that expected for cancellation of repulsive forces.
3. The position of Ca^{2+} ion in the sequence also indicates the absence of repulsive forces and the presence of hydrogen bonds.
4. The observed basic sequence for the destruction of the helix eliminates hydrophobic and carboxylate-tyrosine bonds as stabilizing forces for the helix.
5. The basic sequence shows, therefore, that hydrogen bonding between peptide linkages is the main force holding the helix together.

Before leaving the discussion on ribonuclease, it should be noted that the binding of various polyatomic anions to ribonuclease occurs by the chelation of these anions to the tyrosine groups on ribonuclease. The anionic sequence [6] for the destruction of the hydrogen bonds in ribonuclease is $SO_4^{2-} < Cl^- < Br^- < ClO_4^- < SCN^-$. On the other hand, the anionic sequence for the solvent perturbation [24] of the tyrosine group of ribonuclease at pH 1.0 is $SO_4^{2-} < H_2PO_4^- < ClO_4^- < Cl^-$. The differences between these two sequences again show that the tyrosin-carboxylate bond does not stabilize the helix. Otherwise, the ability of the anion to interact with the tyrosine group

would be the same as the ability of the anion to destroy the helix. At this pH (pH 1.0) the carboxylate group of the carboxylate-tyrosine complex should be titrated, and hence these three tyrosine groups should be liberated. Examination of the perturbation sequence shows that the position of the ClO_4^- ion does not fall according to its value of D_-, since $D_- > D_1$ for ClO_4^- and $D_- < D_1$ for SO_4^{2-}, $H_2PO_4^-$, and Cl^- [1, 3]. The position of the ClO_4^- must therefore be due to its ability to chelate with the hydroxyl group of tyrosine. Hence the anionic sequence reported by Laskowski [24] in his Fig. 3 must be due to the chelating ability of the anion.

It is interesting to note that at pH 3.0 and above the solvent perturbation of the Cl^- and SO_4^{2-} ions on ribonuclease are the same and are equal to about one-half of the total possible shift. Consequently, the remaining three tyrosine groups which are not involved in the carboxylate-tyrosine bond are not capable of chelating with ClO_4^-, $H_2PO_4^-$ or SO_4^{2-}. If they were, the perturbation would be much greater than it is. The remaining three which are not complexed to carboxylate ions must therefore be inaccessible to such ions.

It should also be noted that the ion binding studies of Ginsburg and Carroll [25] and Saroff and Carroll [26] show that at pH 2.1 ribonuclease binds ions according to the sequence $SO_4^{2-} > H_2PO_4^- > Cl^-$. They conclude that the binding sites must be clusters containing two imidazolium groups and one amino (or guanidinium) group. However, SO_4^- should bind less than Cl^- according to the observed [9] solubilities of such ions because the sulfate is the least soluble. That is, the greater the soluability of the ion-ion interaction, the greater will be the degree of "ion binding" or reversal of charge, as shown previously [1]. Moreover, their [25, 26] sequence is the same as that obtained by Herskovits and by Laskowski [24] for solvent perturbation of the tyrosine groups at low pH. Also, both the ion binding and solvent perturbation studies show that the chloride ion has little effect. Because of these similarities and because of the discrepancies with respect to ion-ion interactions or "binding," it is concluded that the "ion binding" studies of Ginsburg and Carroll [25] and Saroff and Carroll [26] involve chelation of the polyatomic anion to the tyrosine group, just as in the case of the solvent perturbation studies.

Collagen-Gelatin Transformation

As stated by Vies [27], Harrington and von Hippel [28], von Hippel and Wong [6], and McBride and Harrington [29], the rigid collagen macromolecule consists of three intertwined, helical units. As brought out by Sund and Weber [30] in their review, these three subunits consist of two α_1 subunits and one α_2 subunit. There are at least four polypeptide chains on each subunit, and these chains are joined by carbohydrate components by means of ester or ester-type linkages. Destruction of the bonds holding these

three subunits or chains together will produce the random-coiled gelatin molecules. It will be shown that ionic sequences and salt effects concerning this helix-coil transition suggest (1) that the gelatin molecules must be stabilized by hydrophobic bonds in order to form the helix, and (2) that the three-stranded collagen molecule is stabilized both by ionic repulsion forces and hydrogen bonds.

Carpenter and Lovelace [13] were the first ones to study the effect of anions and cations on the optical rotation of gelatin. Later it was discovered that their studies involved the examination of the collagen-helix to gelatin-coil transition [6]. Their results are given in Table 2 together with the results obtained by von Hippel and Wong [6]. Carpenter obtained two sequences: one sequence pertains to the change in optical rotation in the presence of added salt, whereas the other sequence pertains to that change occurring in the presence of only the byion but no added salt. Von Hippel and Wong obtained their sequences by measuring the effect of added salts on either the lowering of melting-point temperatures ($\overline{Tm}$) or the changes in the rate of mutarotation ($d[\alpha]/dt$). In Table 2 one sequence of theirs was obtained from values of K using the equation $Tm = Tm^0 + Km$, where Tm^0 = melting temperature (Tm) at zero salt concentration ($m = 0$). The other, similar sequence was obtained from $\log r = \log r_0 + k_0 m$, where $r_0 = (d[\alpha]/dt)_0$ extrapolated to zero salt concentration ($m = 0$).

In all the cases where salt was added, the sequence for the destruction of collagen is always $Na^+ < Cs^+ < Li^+$. If the helix is due to the presence of salt bonds, then as seen in Table 1 (Part II), the Na^+ ion should be more effective than the Cs^+ ion in destroying such bonds. But the above sequences show that it is not. In addition, the Cl^- ion of $(CH_3)_4NCl$ is also incapable of destroying the helix [14]. Moreover, the anionic sequence $Cl^- < Br^- < I^- < SCN^-$ indicates that ionic bonds are not being destroyed, since $D_+ < D_1$ for the amino groups. That is, Cl^- would form the most insoluble salt. Also the absence of unusual pK values for the carboxylate and positively charged groups on gelatin [31] shows that interactions between these two groups to form salt bonds does not occur. Consequently, the pK values and the position and behavior of Na^+ and Cl^- ions in the sequences show that ionic bonds are not involved in the stabilization of the collagen helix.

Repulsive forces in the collagen helix must be considered because the absence of such forces may allow the three-stranded collagen helix to unwind. The possibility that such forces play a part in maintaining the helix can be seen by examining the cationic sequences obtained with added salt. The monovalent sequence $Rb^+ < Na^+ < K^+ < Cs^+ < Li^+$ as obtained by von Hippel and Wong [6] (see Table 2) has the same position of the Rb^+ ion as in the case of the acetate solubility sequence ($Rb^+ < Na^+ < K^+ < Li^+ < Cs^+$) as

given in a previous paper [9]. The position of the Li^+ ion indicates, however, that the carboxylate ions of collagen have a slightly greater pK value than that of acetate. Nevertheless, close comparison of these two sequences shows that the carboxylate ion of collagen helps to stabilize the collagen. The most insoluble cation (Rb^+) has the least effect in destroying the helix. Therefore, as concluded above, salt bonds are not involved. However, as in the case of the reversal of charge phenomenon [1], the greater the solubility of the counterion, the greater will be its effect on reducing the electrostatic charge. This comparison of sequences therefore shows definitely that repulsive forces are involved in stabilizing the helix.

Further proof that repulsive forces are involved can be seen by examining the divalent cation sequence. As pointed out in Table 1 (Part III), this effect of divalent cations is opposite to that observed for the monovalent cation sequence. In the divalent sequence, the more insoluble the salt, the greater will be its effectiveness in destroying repulsive forces. This result is because insolubilization of the divalent cation imparts a positive charge to the polymer by forming $R\text{–}CO_2Ba^+$, whereas insolubilization of a monovalent cation with the carboxylate groups on the polymer does not. From Table 1 of von Hippel and Wong [14], the ability of the divalent cation to destroy the helix is $Mg^{2+} < Ca^{2+} < Ba^{2+}$. The insolubility sequence for carboxylate ions is $Mg^{2+} < Ca^{2+} < Ba^{2+}$. Thus Ba^{2+} is the most insoluble cation and also the most effective in destroying ionic repulsive forces. Ionic bonds and hydrogen bonds between hydroxyl groups also have the same divalent cationic sequence. Ionic bonds were eliminated above as possible factors. The position of Li^+ in the monovalent cationic sequence eliminates the possibility that hydrogen bonds between hydroxyl groups are a major factor in stabilizing the helix. Hence it can be concluded that the divalent sequence supports the conclusion that repulsive ionic forces stabilize the collagen helix.

Examination of Table 2 shows that an entirely different cationic sequence is obtained if only the effect of the byion is examined instead of the effect of added salt. The sequence $K^+ < Rb^+ < Cs^+ < Na^+ < Li^+$ was obtained by Carpenter and Lovelace [13] in the absence of added salt. But in this sequence the Na^+ ion has been shifted from one of the most ineffective cations to one of the most effective cations in destroying the helix. Moreover, in this sequence the position of the Rb^+ ion has been shifted back to where one would expect it to be based on its value of D_+. These shifts in the cationic sequence cannot be due to a dilution effect. Consequently, by examining Table 1 it is seen that the only possible effect for this shift to a basic sequence is a destruction of hydrogen bonds between uncharged nitrogen bases. Therefore, the results in the absence of added salt illustrate that hydrogen bonds hold the collagen helix together.

As seen in Table 2, Carpenter and Lovelace's [13] sequence obtained with added salt appears to be intermediate between the sequence observed by von Hippel and Wong [14] and their sequence observed in the absence of added salt. The reason for this discrepancy is now apparent: There are two effects which influence the cationic sequence. Consequently, this apparent discrepancy is added proof that both hydrogen bonding and ionic repulsive forces stabilize the helix.

The above basic sequences obtained in the presence or absence of added salt show that hydrophobic bonds as well as ionic bonds and tyrosine-carboxylate (or serine-carboxylate) bonds are not the major forces which hold the helix together. The odd behaviour of the R_4N^+ sequence suggests, however, that hydrophobic bonds must be involved indirectly in stabilizing the helix. The sequence obtained by von Hippel and Wong [14] for the destruction of the collagen helix is $(CH_3)_4N^+ < (C_2H_5)_4N^+ < (C_3H_7)_4N^+, (C_4H_9)_4N^+$. However, according to Table 1 the $(CH_3)_4N^+$ ion should be more effective than the $(C_3H_7)_4N^+$ ion in reducing electrostatic repulsion because of less steric hindrance. Moreover, the value of D_+ for the R_4N^+ ions should be approximately equal to that of the K^+ and Rb^+ ions. And with such a low value of D_+, the R_4N^+ ions should not be able to destroy hydrogen bonds. But the R_4N^+ ions can destroy the collagen helix [14]. Thus the observed sequence of R_4N^+ ions contradicts the above experimental evidence for the presence of both hydrogen bonds and ionic repulsive forces.

The only possible explanation for the observed action of R_4N^+ ions is that hydrophobic bonds are present. As seen in Tables 1 and 2 the observed sequence for R_4N^+ ions and the sequence predicted for the destruction of hydrophobic bonds are the same. Also the $(C_3H_7)_4N^+$ and $(C_4H_9)_4N^+$ ions inhibit the ability of the collagen helix from forming if the temperature is above 7°C [14]. Thus hydrophobic bonds, as well as hydrogen bonds and ionic repulsive forces, hold the collagen helix together. But the ionic sequences show that hydrogen bonds and ionic repulsive forces are more important factors. Hence the hydrophobic bonds most likely have a minor role. It is possible that the hydrophobic bonds are required to give the correct structure to either the α_1 or α_2 subunits before the helix can be formed. In other words, the hydrophobic bonds stabilize the subunits and thus are only indirectly involved in stabilizing the helix. Further experimentation would be necessary to substantiate this proposal. Nevertheless, the results show that three types of bonds stabilize the collagen helix: (a) hydrogen bonds, (b) ionic repulsive forces, and (c) hydrophobic bonds.

A summary of the above conclusions is as follows:

1. Salt bonds do not stabilize the helix because the monovalent cationic sequence is the reverse of the insolubility sequence for

anions which have $D_- < D_1$, as in the case of the carboxylate group.

2. Both the monovalent and divalent cationic sequences observed when salt is added to the collagen solution suggest that electrostatic repulsive forces stabilize helix. The monovalent cationic sequence is the same as the solubility sequence for the acetate ion.

3. The monovalent cationic sequence at low ionic strength (no salt added) shifts to a more basic sequence, signifying that stability of helix is due to the presence of hydrogen bonds.

4. The basic sequence at low ionic strength eliminates either the possibility of tyrosine-carboxylate (or serine-carboxylate) bonds or hydrophobic bonds as the main force that holds the collagen helix together. Absence of abnormal pK values for carboxylate of phenoxy groups also substantiates this conclusion.

5. The R_4N^+ sequence is the reverse of that for reduction of electrostatic forces. The $(C_3H_7)_4N^+$ ion has considerable ability to destroy the helix, indicating hydrophobic bonds. Yet the basic sequence shows that hydrogen bonds and ionic repulsive forces are the main forces which hold together the three-stranded helix. Therefore, the sequence for R_4N^+ ions together with the behavior of $(C_3H_7)_4N^+$ ions suggests that hydrophobic bonds in the gelatin monomer are necessary to produce the three-stranded helix.

Hemoglobin

Hemoglobin consists of two subunits, the α and β. These subunits are aggregated together to form the tetramer $\alpha\beta\alpha\beta$. Information as to what type of bonds hold this tetramer together can be obtained from the results of Perutz [32], Chiancone et al. [33], Kawahara et al. [34], and their cited references. The results show that KCl will dissociate the tetramer to a dimer. Moreover, its ability to do so is greater than that of NaCl ($K^+ > Na^+$). Yet KCl cannot dissociate the resulting dimer. The results also show that $CaCl_2$ can dissociate the tetramer to the dimer with greater ability than GCl or $Ca^{2+} > G^+$. Again, as in the case of NaCl or KCl, the salt $CaCl_2$ cannot destroy the resulting dimer. Moreover, the GCl dissociates the hemoglobin in two discrete steps: The dimer is formed at about 1 M GCl and the monomer at about 4.5 M GCl. The results indicate that two types of forces hold the different sets of dimers together.

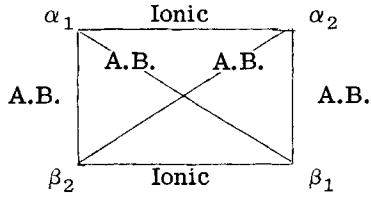
The above results show that the sequence for converting the tetramer hemoglobin into a dimer goes according to the sequence $Ca^{2+} > G^+ > K^+ > Na^+$. The sequence $K^+ > Na^+$ holds for (a) hydrogen bonds having a hydroxyl group, (b) hydrophobic bonds, (c) ionic bonds for negatively hydrated groups such as γ-carboxylate ions (no A regions), and (d) repulsive forces. The fact that KCl

can destroy the bond shows that the bond cannot be either (a) or (b). Also NaCl would aid in forming, instead of destroying, hydrophobic bonds. Moreover, repulsive forces would not be important in holding together an aggregate. Consequently, the sequence shows that the main forces destroyed upon addition of KCl are ionic bonds between carboxylate ions and positively charged histidine, amino, or guanidinium groups. Moreover, the fact that Ca^{2+} ions have such a large ability to dissociate the tetramer again shows that the bonds must be ionic.

The results given by Chiancone et al. [33, 35] show that the undissociated acetic acid can destroy the tetramer. And according to the conclusions of Chiancone et al. [33], the acetic acid solvent acts on different sites than the NaCl solvent. Moreover, the studies made by Kawahara et al. [34] illustrate that dilute hydrophobic agents such as 10 or 20% dioxane or 1-propanol can partially disrupt the tetramer. These results strongly suggest that the acetic acid and dilute hydrophobic agents are destroying hydrophobic bonds.

The results on urea confirm this. As noted, the urea zwitterion, if present in concentrated solutions, can destroy both hydrophobic and hydrogen bonds, but its zwitterion structure prevents it from destroying ionic bonds. Consequently, the ability of 8 M urea to convert the tetramer to only the dimer (not the monomer) illustrates that a combination of hydrogen and hydrophobic bonds do not hold the hemoglobin together. Otherwise, the concentrated urea solutions would convert the tetramer into the monomer. Rather, the hemoglobin must be held together by hydrophobic and ionic bonds. Thus urea converts the tetramer to the dimer by destroying hydrophobic bonds, and salt solutions such as KCl convert the tetramer to the dimer by destroying ionic bonds. Furthermore, the GCl must first produce the dimer by destroying ionic bonds (at 1 M GCl) and then convert this dimer into the monomer by destroying hydrophobic bonds, since a 3 or 4 M solution would be necessary [10] to destroy the hydrophobic but not the ionic bonds.

From the above results it can be seen that the model proposed by Perutz [32] must be modified. Perutz concluded that both hydrogen and ionic bonds stabilized the dimers $\alpha_1\alpha_2$ and $\beta_1\beta_2$. However, the above results show that if hydrogen bonds are present in these interactions, they are not the main force which holds the above units together. The model of Perutz [32] can thus be pictured as



where "A.B." designates aliphatic (hydrophobic) bonds and where "ionic" designates ionic bonds. Thus for the dissociation of hemoglobin we have:

Destruction of hydrophobic bonds:

$$\alpha\beta\alpha\beta + \text{urea} \longrightarrow \alpha_2 + \beta_2 \quad (1)$$

Destruction of ionic bonds:

$$\alpha\beta\alpha\beta + CaCl_2 \longrightarrow \alpha\beta + \alpha\beta \quad (2)$$

Consequently, in 1 M GCl the ionic bonds are destroyed [reaction (2)]. At 4.5 M GCl the remaining $\alpha\beta$ dimer is dissociated into the monomer units [reaction (1)] because of a destruction of hydrophobic bonds.

It should be mentioned that both Perutz [32] and De Santis et al. [36] suggest that van der Waals' forces contribute to a great extent in holding helicies or aggregates together. If two groups are polar, such as in the peptide hydrogen bond, then the strong dipoles will unquestionably be the force which attracts and holds them together. If they are not polar but rather are nonpolar in nature, then the hydrophobic bond will hold these molecules together. The stabilizing force for the hydrophobic bond may be van der Waals' forces, which, as is well known, are caused by temporary dipoles produced by variations in the position of the electrons on these atoms. However, it may also be caused by the exchange of photons (or phonons) due to infrared (black-body) radiation. That is, the absorption of a photon by a neighboring outer electron would cause the excited electron to jump into a more distant orbit. This, in turn, would cause the nucleus to jump in a similar manner because of a shift in the electrical field. The jump or repositioning would be in the direction of the oncoming photon and consequently would produce an attractive force. Nevertheless, whether the bond is by the van der Waals forces or by the continual exchange of photons, the attraction of one nonpolar group to another represents a "hydrophobic bond" in the above mechanism.

Starch

Morsi and Sterling [37] have examined the retrogradation of amylose and starch in the presence of various salt solutions. The ability of an anion to retain the polysaccharide in solution goes according to the sequence $Cl^- < Br^- < I^- < SCN^-$. This sequence agrees with that obtained by Loewus and Briggs [38]. They observed that the iodine binding capacity of amylose decreased more in the presence of F^- and Cl^- ions than in the presence of I^-. But a decrease in the amylose's binding capacity of iodine means that amy-

lose is retrograding from solution. Hence their sequence $F^- > Cl^- > Br^- > I^-$ for the ability of an anion to lower the binding capacity of amylose is related to the anion's ability to retain the amylose in solution. Consequently, the ability of an anion to retain amylose in solution as observed by Morsi and Sterling [37] and by Loewus and Briggs [38] is directly proportional to the anions effective dielectric constant, D_-.

Examination of the cationic sequence [37] shows that the ability of a monovalent cation to retain starch in solution is given by the sequence $Li^+ < Na^+ < K^+$. Moreover, the ability of concentrated bromide salts to dissolve starch granules was observed [10] to be $Li^+ < Na^+ < K^+ < G^+$. Also, the ability of cations to decrease the pasting temperature of starch granules increases according to the same sequence [38] ($Li^+ < Na^+ < K^+$). Consequently, in all cases the acidic cationic sequence is obtained. Moreover, the divalent cationic sequence $Ca^{2+} < Sr^{2+} < Ba^{2+}$ is obtained [37] for the cations ability to retain starch in solution (destroy hydrogen bonds). This sequence is again acidic [8]. The acidic sequence is obtained because of repulsion forces between the hydroxyl groups on starch and the highly polarized water molecules in the A regions of cation such as Li^+ and Na^+. Consequently, the more polarized are the water molecules of the A regions, the greater will be the salting-out properties of the positively hydrated cation. The observed sequences for retrogradation and dissolving of starch show that hydrogen bonds between hydroxyl groups exist.

But from previous results [39] both the amylose and amylopectin exist in aqueous solutions as a helix because of hydrogen bonding between the 3OH on one glucose residue and the 2OH on an adjacent glucose residue in the α-1, 4-linked chain. Consequently, hydrogen bonds already exist even before retrogradation. Or in the case of dissolving the starch granule, hydrogen bonds are formed after the granule is dissolved. Consequently, these sequences coupled with the previous results show that the hydrogen bonds in retrograded amylose and in starch granules must be between adjacent chains rather than adjacent glucose units. The salts therefore are capable of destroying those between adjacent chains but not those between adjacent glucose units.

CONCLUSIONS

The above method should be an important tool for determining the types of bonds in various polymers. Moreover, the method is not limited to a specific type of physical measurement. Rather, a number of different types of measurements, such as sedimentation, viscosity, optical rotation, solubility, nuclear magnetic resonance, electrophoresis, density, and other physical studies can be used.

In the examples given it is seen that all types of bonds cited in Table 1 were investigated. Thus hydrophobic bonds were observed to be present in gelatin and hemoglobin; hydrogen bonds between peptide linkages are present in collagen and are formed in the acid precipitation of ovalbumin; hydrogen bonds between hydroxyl groups exist in starch granules and retrograded amylose; ionic bonds exist in hemoglobin; and ionic repulsive forces are important for the stabilization of the three-stranded collagen helix.

In addition to the application of the method to other protein molecules, the method may be a tool in the investigation of cellular structure. For example, the uptake of glucose by isolated rat diaphragms is highest in the presence of Li^+ ions and follows the order [40] $Li^+ > Na^+ > K^+ = Rb^+ > Cs^+$. This sequence is the same as that for destruction of hydrogen bonds involving hydroxyl groups and destruction of ionic bonds involving positively hydrated anions such as sulfate or carboxylate groups which are attached to sugar units. Similar results were obtained by Kipnis and Parrish [41], where it was seen that Na^+ ions activated while K^+ ions inactivated the carrier mechanism across cell membranes. Further application of the method would therefore yield valuable information into the cell's mechanism for transportation of sugar, amino acids, etc., across cellular membranes. The method will also be applied to the structure of DNA and RNA in a subsequent paper [42]. It will be shown that ionic bonds between phosphate groups and the C_1' nitrogen base stabilize the double-stranded DNA helix.

REFERENCES

[1] S. R. Erlander, *J. Macromol. Sci.*, **A2**, 1066 (1968).
[2] S. R. Erlander, *J. Macromol. Sci.*, **A2**, 1181 (1968).
[3] S. R. Erlander, *J. Macromol. Sci.*, **A2**, 833 (1968).
[4] H. S. Frank and W. Y. Wen, *Trans. Faraday Soc.*, **24**, 133 (1957).
[5] O. Ya. Samoilov, *Trans. Faraday Soc.*, **24**, 171 (1957).
[6] P. H. von Hippel and K.-Y. Wong, *Science*, **145**, 577 (1964).
[7] D. Puett, L. V. Rajagh, and A. Ciferri, *Biopolymers*, **4**, 957 (1966).
[8] S. R. Erlander, *J. Macromol. Sci.*, **A2**, 1058 (1968).
[9] S. R. Erlander, *J. Macromol. Sci.*, **A2**, 623 (1968).
[10] S. R. Erlander and R. Tobin, *Makromol. Chem.*, **107**, 204 (1957).
[11] Hofmeister, *Arch. Exptl. Path. Pharmakol*, **24**, 247 (1888).
[12] A. W. Thomas, *Colloid Chemistry*, McGraw-Hill, New York, 1st ed., 1934, p. 344.
[13] D. C. Carpenter and F. E. Lovelace, *J. Am. Chem. Soc.*, **57**, 2337 (1935).
[14] P. H. von Hippel and K.-Y. Wong, *Biochemistry*, **1**, 664 (1962).
[15] W. F. Harrington, *Biochem. Biophys. Acta*, **18**, 450 (1955).

[16] W. F. Harrington, P. Johnson, and R. H. Ottesville, *Biochem. J.*, **62**, 569 (1956).
[17] J. Steinhardt and S. Beychok, in *The Proteins*, Vol. II (H. Neurath, ed.), Academic Press, New York, 1964, p. 203.
[18] C. Tanford, *Physical Chemistry of Macromolecules*, Wiley, New York, 1964, p. 556; C. Tanford and J. D. Havenstein, *J. Am. Chem. Soc.*, **78**, 5287 (1956).
[19] K. O. Pedersen, *J. Phys. Chem.*, **62**, 1282 (1958).
[20] G. Kartha, J. Bello, and D. Harker, *Nature*, **213**, 862 (1967).
[21] J. P. Riehm and H. A. Scheraga, *Biochemistry*, **4**, 751, 772 (1965).
[22] J. P. Riehm, C. A. Broomfield, and H. A. Scheraga, *Biochemistry*, **4**, 760 (1965).
[23] J. P. Riehm and H. A. Scheraga, *Biochemistry*, **5**, 93, 99 (1966).
[24] M. Laskowski, Jr., *Federation Proc.*, **25** (1), 20 (1966).
[25] A. Ginsberg and W. R. Carroll, *Biochemistry*, **4**, 2159 (1965).
[26] H. A. Saroff and W. R. Carroll, *J. Biol. Chem.*, **237**, 3384 (1962).
[27] A. Veis, *The Macromolecular Chemistry of Gelatin*, New York 1964.
[28] W. F. Harrington and P. H. von Hippel, *Advan. Protein Chem.*, **16**, 1 (1961).
[29] O. W. McBride and W. F. Harrington, *Biochemistry*, **6**, 1499 (1967).
[30] H. Sund and K. Weber, *Angew. Chem. Intern. Ed. Engl.*, **5**, 231 (1966).
[31] J. Steinhardt and S. Beychok, in *The Proteins*, Vol. II (H. Neurath, ed.), Academic Press, New York, 2nd ed., 1964.
[32] M. F. Perutz, *J. Mol. Biol.*, **13**, 646 (1965).
[33] E. Chiancone, P. Vecchini, L. Forlani, E. Antonini, and J. Wyman, *Biochem. Biophys. Acta*, **127**, 549 (1966).
[34] K. Kawahara, A. G. Kirshner, and C. Tanford, *Biochemistry*, **4** 1203 (1965).
[35] E. Chiancone and G. A. Gilbert, *J. Biol. Chem.*, **240**, 3866 (1965).
[36] P. De Santis, E. Giglio, A. M. Liquori, and A. Ripamonti, *Nature*, **206**, 456 (1965).
[37] M. K. S. Morsi and C. Sterling, *J. Polymer Sci.*, **A1**, 3547 (1963).
[38] F. A. Loewus and D. R. Briggs, *J. Am. Chem. Soc.*, **79**, 1494 (1957).
[39] S. R. Erlander, R. M. Purvinas, and H. L. Griffin, *Cereal Chem.*, **45**, 140 (1968); *Staerke*, **20**, 37 (1968).
[40] G. Bhattacharya, *Biochim. Biophys. Acta*, **93**, 644 (1964).
[41] D. M. Kipnis and J. E. Parrish, *Federation Proc.*, **24** (5), 1051 (1965).
[42] S. R. Erlander, *J. Macromol. Sci.*, accepted for publication (1968).

Accepted by editor November 17, 1967
Received for publication June 3, 1968