On the Mechanism of Action of Neutral Salt on the Collagen-Fold*

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It is shown that the effects of neutral salts on the inhibition of the collagen-fold are compatible with a mechanism involving the interaction of ions with protein, contrary to a recent claim of proof that such a mechanism is not possible.

von Hippel and Wong (1962) presented data on the effects of a variety of neutral salts on the kinetics of formation and the stability of the collagen-fold, as determined by optical rotation measurements. From these data and from data of Bello *et al.* (1956) on the melting of gelatin gels, von Hippel and Wong concluded that neutral salts affect the collagen-fold by rearrangement of the solvent (water), rather than by "direct binding" to the protein. They did not define the term "direct binding." It is our purpose to show that the arguments of von Hippel and Wong are not correct and that the data cited by them do not require the mechanism proposed. Also, von Hippel and Wong's note "Added in Proof" implies that we are in agreement with their conclusions, which is not the case. The points at issue are enumerated below.

(1) von Hippel and Wong state that, since the stability of gelatin gels may be viewed as the sum of a concentration-independent and a concentration-dependent component, and since the change in melting point per mole of added salt is independent of gelatin concentration, it follows that salts "must alter the melting temperature" by acting on the "collagen-type helix lying between cross-links rather than attacking the interchain cross-links themselves." But this conclusion is not a necessary consequence of the data. Firstly, the resolution of the melting point of a gel into two components is a mathematical artifice and not *necessarily* an accurate reflection of the physical reality. Secondly, not enough is known of the mechanism of gelation for prediction that a postulated mechanism of inhibition should show a concentration-dependence.

(2) von Hippel and Wong state that even at relatively low salt concentrations "essentially every water molecule lies within the sphere of influence of at least one ion." The same is true of the protein surface, and if ions can disturb the structure of the solvent they can disturb the structure of the protein by similar ion-dipole interactions.

(3) von Hippel and Wong combined our data (Bello *et al.*, 1956) on the effects of salts on the melting points of 5% gels with their data on transition temperatures of 0.1% solutions and found that the effect of a given concentration of salt is independent of gelatin concentration. They considered these data in terms of equation (3) (numbered to conform to von Hippel and Wong), where the concentrations without subscript are the equilibrium concentrations, and S is salt, G is binding site, and SG is the complex between S and G.

$$\frac{[SG]}{[S][G]} = K_{eq} = \frac{[SG]}{([S]_{total} - [SG])([G]_{total} - [SG])} \quad (3)$$

They concluded that the concentration-independence provides proof that salts cannot be acting by "direct binding" to the protein. The discussion of this point was continued in their note "Added in Proof." Part of the latter arose from correspondence with the present author, but we are not in agreement with part of the argument nor with the conclusions. We are in agreement on section (1) of the "Added in Proof" that, if direct binding were effective, a large value of K_{eq} would not fit the data. The biuret-type complex of copper with gelatin illustrates this point. K_{eq} for the binding of copper is very high, and the effect of a given amount of copper on the melting point of a gel depends on the gelatin concentration. In section (2) "G" is used to represent the number of effective binding sites per chain, rather than the concentration of effective sites in the solution, as is done in equation (3) and in the main text of von Hippel and Wong. However, we agree that a large value of K_{eq} would not fit the data.

(4) Sections (3) and (4) of the "Added in Proof" are at the heart of the matter. At this point some numerical values of binding are required as a guide to the discussion. In Table I of this paper are some values of interest, calculated for a variety of K_{eq} values, on the assumption that K_{eq} is constant. In section (3) of their "Added in Proof" von Hippel and Wong state that K_{eq} cannot be small when G is large because complete suppression of helix formation can be achieved "at concentrations of salt of the same order of magni-

TABLE I

COMBINATION OF SALTS WITH PEPTIDES, ACCORDING TO EQUATION (3)

$$\frac{[GS]}{[G][S]} = K_{eq} = \frac{[GS]}{([G]_{tot} - [GS])([S]_{tot} - [GS])}$$

Example No.	K_{eq}	$[G]_{tot}$	$[S]_{tot}$	$\frac{[G]}{[GS]}$
1	0.1	3 M (30% protein)	1 M	12.8
2	0.1	1 (10%)	1	10.7
3	0.1	0.1 (1%)	1	10
4	0.1	0.01 (0.1%)	1	10
5	0.1	0.001 (0.01%)	1	10
6	0.1	1	0.2	55
7	0.1	0.1	0.2	49
8	0.1	0.01	0.2	49
9	0.1	1	2	5.9
10	0.1	0.01	2	4.9
11	1	1	0.1	19
12	1	0.1	0.1	11
13	1	0.01	0.1	10
14	0.03	1	1	35
15	0.03	0.1	1	32
16	0.03	0.01	1	32
17	0.2	1	1	5.9
18	0.2	0.01	1	5.0
19	0.3	1	1	4.1
20	0.3	0.01	1	3.4
21	0.3	0.01	0.5	4
22	0.3	1	0.5	5.7
23	0.4	0.01	1	2.5

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tude as the assumed concentration of. . . binding sites (at least for 5% gels)." Assuming the binding sites to be the peptide groups, their molar concentrations at 5% and 0.1% weight concentration are about 0.5 and 0.01 M, respectively. These are the concentrations used in studying the melting points (Bello *et al.*, 1956) and molecular transition temperatures (von Hippel and Wong, 1962), respectively. What proportion of the peptide groups need be bound to ions to prevent helix formation? Some indication may be obtained from the fact that binding of copper (II) ion to gelatin, in the form of a biuret-type complex, completely prevents the gelation of 5% gelatin at 0° when the ratio of peptide groups to copper is about 18 (Bello and Vinograd, 1958; Bello *et al.*, 1962). Suppression of helix formation takes place at a peptide-to-copper ratio of 20 at *p*H 12. We assume that one copper is bound to four peptide groups. (The ratio is not known with certainty. Copper is usually four-coordinated, but Cooper *et al.* [1962] have found by x-ray diffraction that copper in the crystalline biuret-type complex with glycylglycylglycine is five-coordinated to four nitrogens and one carboxyl. They cite evidence that in solution the situation may be different. The proline and hydroxyproline content of mammalian gelatin is about one-fourth of the total residues. These imino acid residues do not form the biuret-type complex, as was shown by J. Vinograd and M. Kiefer [private communication, confirmed by us] for poly-L-proline. It is probable that many of the copper atoms are bound to three peptide groups with an imino acid residue as the fourth, unbound, group. We consider all four to be bound insofar as inhibition of helix formation is concerned. We are investigating the spectral and optical properties of copper complexes of proteins and model peptides in order to learn more of the nature of the complexes.) On this basis, one out of four to five peptide groups is occupied by copper when the peptide-to-copper ratio is 18–20. Therefore, we shall assume that when one-fourth to one-fifth of the peptide groups are bound to salt, helix formation or gelation will not occur. Obviously this line of reasoning must be applied with great caution, since the nature of the binding of copper (II) to peptide is not the same as for ordinary ions. Also, the bending of the protein chain around the copper will be different than in the cases of other ions. The value of one-fourth to one-fifth which we arrived at above is in good agreement with the finding of Yaron and Berger (1961) that pentaproline side-chains grafted to poly-DL-lysine form helices, while lower peptides do not. Full helix development was reached at the hexapeptide stage. (Since about one-fourth of the residues in collagen are proline and hydroxyproline, the minimum number of residues required to form a helix may be different. The number is not likely to be less, but may be greater. In the latter case, a given amount of ion binding would have a greater effect, require a lower value of K_{eq}, and strengthen the argument being developed here.) Each bound ion may prevent helix participation, not only on the part of the peptide group with which it is associated but by several adjacent groups, say two on each side, making a total of five. Thus, binding of one copper may inactivate the four peptide residues to which it is attached, as well as four adjacent residues, making a total of eight (perhaps more, owing to the bending of the chain required by the copper), while binding of ions to each of four widely separated peptide groups may inactivate 20 residues. It is also possible that one simple ion may interact with several peptide groups, especially at high salt concentrations. It has been shown (D. Harker and H. Haas, private communication) that in a crystal of the adduct of *N*-methylacetamide, water, and lithium bromide, each lithium is surrounded by four carbonyl oxygens and each bromide by four amide hydrogens. Thus, it is possible that binding of four peptides to four ions may be as effective as, or more effective than, the binding of as many peptide groups to one copper.

We shall now see what values of K_{eq} could account for occupancy of about one-fourth to one-fifth of the peptide groups. From Table I, we see that a salt that can occupy one-fifth to one-fourth of the binding sites at a concentration of 1 M need have a K_{eq} value of only about 0.2–0.3, or 0.4 at most, *e.g.*, Examples 19, 20, and 23. This applies at both high and low protein concentrations. Such a salt, at 1 M concentration, would prevent helix formation at 0°, as our argument was based on nongelation of the biuret complex at 0°. None of the salts used by von Hippel and Wong had this level of effectiveness. According to their Table I, the most effective salts studied lowered T_m only about halfway to 0° at 1 M concentration. (von Hippel and Wong state that tetrapropyl and tetrabutyl ammonium bromides completely inhibit collagen-fold formation above 7°, but they do not give the concentration involved, possibly 2 M from the context of their Table II, nor did they study the effect of this salt as a function of gelatin content.) It appears, then, that for the most effective salts studied by von Hippel and Wong, a K_{eq} of not over 0.2 and, perhaps, as low as 0.1 would account for the data (Examples 17, 18, and 2–5). This is contrary to what von Hippel and Wong imply in section (3) of their "Added in Proof," although they do not use any numerical values for K_{eq}.

Table I also shows (Examples 2–5, 6–8, 14–18) that $[G]/[SG]$ is nearly independent of protein concentration for the salts and protein concentrations studied. If the effective binding site is not the peptide group but a less abundant group, the value of $[G]_{to}$ might be, say, 0.05–0.001 M in the experiments of Bello *et al.* and von Hippel and Wong. Table I shows that the argument above would apply even more forcefully to this situation (Examples 3 and 5 *vs.* 2 and 4). Therefore, the observation that the effect produced by salt is independent of protein concentration may be a consequence of a low K_{eq} value rather than a consequence of the salt's acting on the solvent.

Table I shows the conditions under which a protein concentration dependence might be found. With the salts and protein concentrations used by von Hippel and Wong the variation in $[G]/[SG]$ between 0.5 M and 0.01 M peptide would be so small that one could not expect to detect it by their method, *i.e.*, by comparing different types of gelatins by different methods of measurement, *viz.*, melting points of concentrated gels *vs.* optical rotation of dilute solutions. When K_{eq} is small, one must compare widely varying protein concentrations, *e.g.*, Examples 1 and 3. There are difficulties in working with such concentrated solutions, partly experimental and partly due to the increasing intermolecular stabilization of the helix, which complicates the situation. Alternatively, one may attempt to use a salt of high K_{eq} value as in Examples 11–13. In attempting such experiments with diiodosalicylate, which is a very effective gelation inhibitor (Bello *et al.*, 1956), we found that the salt precipitated the gelatin at the concentrations required. We are looking for better salts.

(5) The small values of K_{eq} for the salts used by von Kippel and Wong are pertinent to the discussion of section (4) of their "Added in Proof." By equation (3), they state that "one would expect the effect of successive aliquots on the stability of the helix (meas-

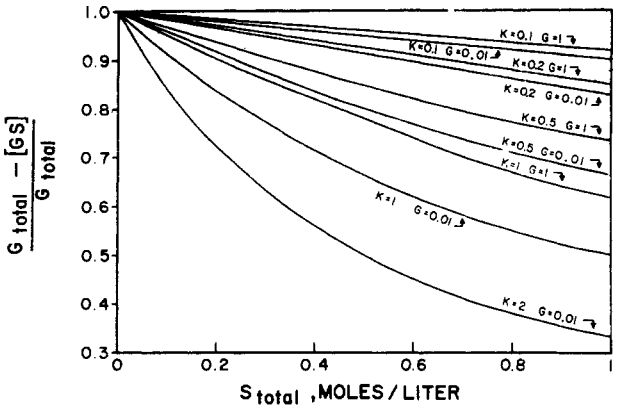

Fig. 1.—Calculated values proportional to the fraction of helix remaining as a function of salt concentration.

ured as a change in T_m) to follow a simple adsorption isotherm, the effect per aliquot decreasing progressively with increasing concentration of salt. However, the demonstrated linearity of T_m with respect to added salt indicates that this is not the case." This is not necessarily true, as we show in Figure 1, which presents theoretical curves of $([G]_{tot} - [GS])/[G]_{tot}$ *i.e.*, the fraction of helix remaining *vs.* $[S]_{tot}$, for various K_{eq} values, at high and low protein concentrations. (In Table I we have already shown that $[G]/[GS]$, *i.e.*, the ratio of helix to nonhelix, is linear with $[S]$, *e.g.*, Examples 3 and 7, and 4, 8, and 10.) Figure 1 shows that for K_{eq} of 0.1 or 0.2 the lines are quite straight out to 1 M salt. Even for 2 M salt at K_{eq} of 0.1, the deviation from linearity is so small as to be undetectable by the experimental measurements of T_m. Only at a K_{eq} value of about 0.5 or greater does the curvature become marked. However, salts with these values of K_{eq} would not be studied at high concentration. $K_{eq} = 1$ can be studied only below 0.2 M (above that no helix would form; Examples 12, 13); indeed, accurate T_m determinations by optical rotation, which require the observation of good low temperature plateaus, limit the concentration of such a salt to less than 0.1 M (Example 13). At such concentrations, the departure of the T_m *vs.* $[S]$ curve from linearity might not be detectable, according to Figure 1.

Also, equation (3) is an oversimplification, and the absorption isotherm concept may not be applicable to denaturation of a protein by ion interactions, because the process of denaturation may expose new interaction sites of equal or different K_{eq} values. Also, partial denaturation by whatever mechanism may destabilize the protein toward additional denaturation, leading to a greater effect when additional salt is added. Such cases are known (*e.g.*, Bello *et al.*, 1956; Harrington *et al.*, 1957).

(6) von Hippel and Wong compare the effects of neutral salts on gelatin with salting out of proteins, which "has generally been interpreted in terms of a competitive removal of water molecules from their solvent role." von Hippel and Wong show that the k_0 values for the rate of collagen-fold formation are of the same magnitude as k_s values for salting out. However, these constants apply to different types of data, kinetic and equilibrium, respectively. The mechanism of helix disruption or of inhibition of helix formation may be quite different from the mechanism of salting out. For example, the electrolytes that cause salting out are generally nondenaturants—indeed antidenaturants (Bello *et al.*, 1956; Simpson *et al.*, 1953). Of special interest is the finding that crystallization of myoglobin from sulfate solution, a type of salting out, causes little change in the helix content of this protein (Urnes *et al.*, 1961). This raises some doubt that the value of k_s for salting out can be taken as evidence for the mechanism of helix inhibition by quite different salts. Also, von Hippel and Wong cite the *p*H-independence of k_0 and of the melting points of gelatin gels in the presence of a given amount of salt, on the one hand, and the *p*H independence of k_s on the other. There is, however, a difference between these two classes of data. The salting-out curves have different intercepts, but the same slopes (k_s) at different *p*H values (Green, 1931), while the log $(d\alpha/dt)$ curves (Fig. 3 of von Hippel and Wong) for helix formation have both the slope (k_0) and the intercepts constant [except for the lag observed in log $(d\alpha/dt)$ at *p*H 10.5]. While it is true that von Hippel and Wong do observe that the salts which salted out gelatin have low k_0 values, they did state that both suppression of helix formation and salting out "appear to be based on a competitive reorganization of the solvent by the ions," with details that "differ somewhat." The points we have adduced raise the possibility that the processes differ markedly.

It is not our purpose to claim that salts suppress collagen-fold formation (or denature other proteins) by interaction with the protein, whether at the peptide groups or elsewhere. It appears to us that the mechanism of neutral salt action is still undetermined. It is possible that some ions may act by both mechanisms simultaneously, with one mechanism or the other dominant for different proteins or at different stages of the denaturation; that different ions may act by different mechanisms, and especially that cations may act differently from anions; and that large ions with hydrophobic surfaces, such as tetraalkyl ammonium, may act differently from simple ions, such as lithium, or highly polarizable ions differently from relatively nonpolarizable.

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Added in Proof

Equation (3) is not clearly applicable to the question of the linearity of T_m *vs.* $[S]$. Equation (3) can be used to calculate values of the amounts of helix and nonhelix at equilibrium. (In this connection nonhelix means that portion of the chain which would be helical in absence of salt, and not that portion which by its nature cannot be helical even in the absence of salt.) The relation between the amount of helix remaining and the T_m of that helix is not known, although we presume that as the helical segments become sufficiently short their stability will decrease.

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