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# Thermodynamic Evidence for Internal Bonding in Serum Albumin

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Much of the fascination of protein chemistry lies in the individuality of members of the protein family. All proteins consist of chains of amino-acid residues, held together by peptide bonds. Protein molecules, however, are frequently rigid compact units, rather than extended chains. Such a structure must be maintained by secondary bonding, and it is probable that at least a part of the individuality of proteins is due to the nature of this secondary bonding. (Fibrous proteins, which do exist as extended chains of helices, and which do not show as much individuality as globular proteins, are excluded from the present discussion.)

Among these secondary, internal bonds there are certain to be disulfide links (10), and, if the recent suggestions of Pauling and Corey (9) are correct, –N–H···O=C– hydrogen bonds to hold the polypeptide chains in helical coils. In the type of reaction considered in this paper, bonds of these types are not likely to be ruptured. In any event, these bonds alone are not sufficient to account for the compactness of globular protein molecules. Other bonds which have been suggested (7) are hydrogen bonds between carboxyl and hydroxyl groups, and it is evidence for such bonds in serum albumin which are presented in this paper.

## Theory

A protein molecule usually contains a large number of a given reactive group (e.g., serum albumin contains 108 carboxyl groups). All these groups are considered equivalent, and their reaction with some ionic species is studied. If the assumption of equivalence is false, this will usually be apparent from the data. No such case is discussed here.

The standard free energy change for the reaction under consideration is then determined at constant temperature and ionic strength.

As an example, let us consider the dissociation of hydrogen ions from the carboxylic acid groups of a protein. From pH determinations on solutions containing various amounts of protein, acid, base and neutral salt (always at constant total ionic strength), it is usually possible to obtain quite accurately the ratio of the number of $COO^-$ groups to that of COOH groups at any pH (14). This ratio, multi-

plied by the measured hydrogen ion activity, will then be the apparent dissociation constant of the carboxylic acid groups. The standard free energy change is then $-RT \ln K$.

The value obtained for $\Delta F^\circ$ is not a constant, even at constant ionic strength and temperature, but depends on the pH. The reason for this is that the charge on a protein molecule varies with pH, and there will be electrostatic interaction between the hydrogen ion and the protein molecule, the extent of which depends upon the charge.

It is convenient, because of this charge effect, to divide the observed value of $\Delta F^\circ$ into two factors,

$$\Delta F^\circ = \Delta F^\circ_{int} + \Delta F^\circ_{elec} \qquad (1)$$

the first of which, the intrinsic free energy change, contains charge-independent terms only, while the second, the electrostatic contribution, represents the charge-dependent factor. It is assumed at this point that the charges present on a protein molecule are randomly distributed over its surface, so that the electrostatic interaction is a function of the total charge only, and independent of the location on the protein molecule of the reactive group under consideration. The value of the electrostatic contribution to the free energy change must then be proportional to the product of the charge on the reacting ion (in this case unity), and that on the protein molecule ($Z_p$). In order to make this present treatment correspond with previous work in this field, the proportionality constant will be designated by $2RTw$. It is easy to show that, with the assumptions made, $w$ is a constant depending only on the size and shape of the protein molecule, the ionic strength of the solution, the dielectric constant of the solvent, and the temperature. For the dissociation of a hydrogen ion from a carboxylic acid group, a positive charge will decrease $\Delta F^\circ$, so that equation (1) becomes

$$\Delta F^\circ = \Delta F^\circ_{int} - 2RTwZ_p \qquad (2)$$

The experiments used to determine the ratio of COOH to $COO^-$ groups, together with knowledge of the binding of other ions, such as chloride, will, by proper book-keeping, yield a value for $Z_p$ at any pH. It is then possible to plot $\Delta F^\circ$ *versus* $Z_p$. The intercept of such a plot where $Z_p$ is zero is then equal to $\Delta F^\circ_{int}$. Since this factor is, by definition, charge-independent, the value of $w$ at any given value of $Z_p$ can then be determined with the aid of equation (2).

If the size and shape of a protein molecule remain unchanged in a series of experiments at constant temperature and ionic strength, $w$ should be a constant, and the plot of $\Delta F^\circ$ *versus* $Z_p$ should be a straight line. Deviations from a straight line would imply either

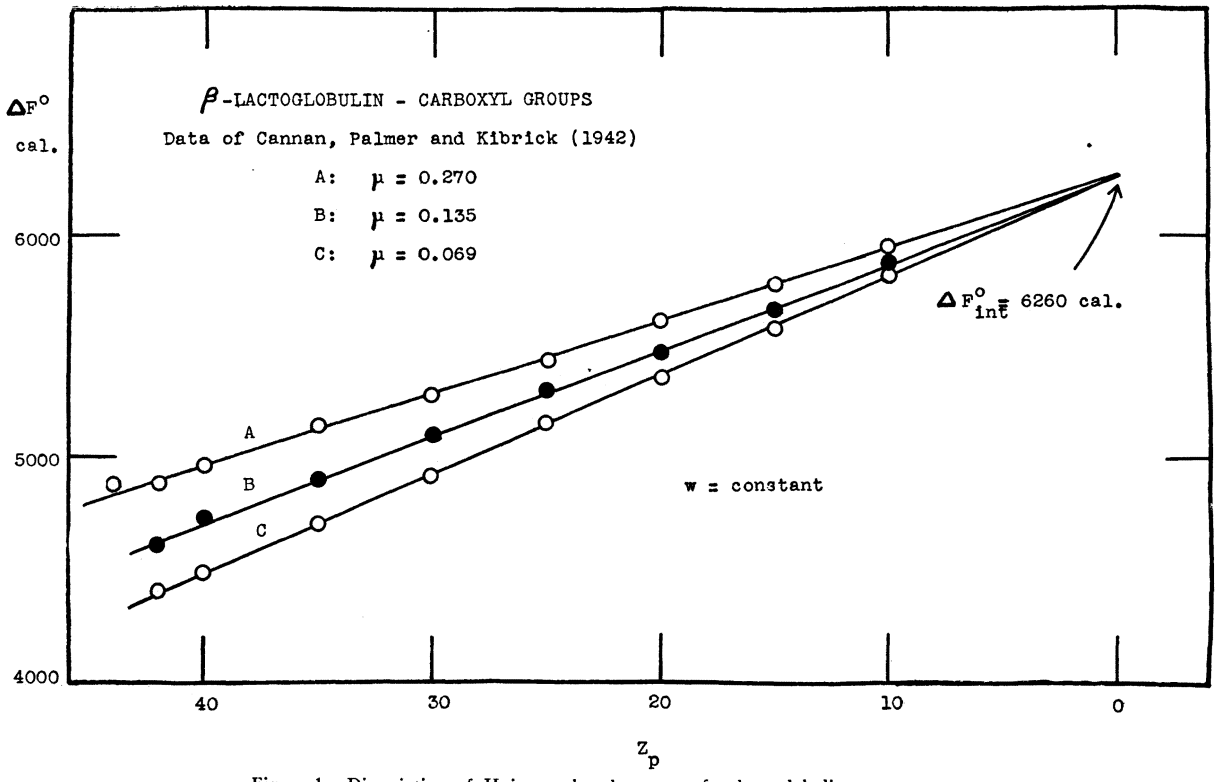


Figure 1. Dissociation of H ion carboxyl groups of β-lactoglobulin.

that a change in charge alters the size and shape of the protein molecule studied, or that all the groups under consideration are not equivalent, as assumed, so that they cannot be described by a single $\Delta F^{\circ}_{int}$. The two effects produce opposite curvature, so that they can be distinguished.

The treatment here described for the dissociation of hydrogen ions from COOH groups can be applied to any other reaction between proteins and ions. If the reactions of a number of different ions with the same protein are studied in the same solvent, at the same ionic strength and temperature, the same value of $w$ should always be found, unless the reactions studied alter the size and shape of the protein molecule.

## Calculations

*β-Lactoglobulin.* $\Delta F^{\circ}$ values for the dissociation of $H^{+}$ from the carboxyl groups of β-lactoglobulin have been calculated from the data of Cannan, Palmer and Kibrick (2). The calculations are based on a molecular weight of 40,000 and a carboxyl group content of 58 per mole. All the data are from the pH range acid to 4.6, where no groups other than carboxyl groups can contribute to hydrogen ion dissociation. The neutral electrolyte in these experiments was KCl. Probably neither of its ions are bound by lactoglobulin (15). The data are plotted in Figure 1. A straight line

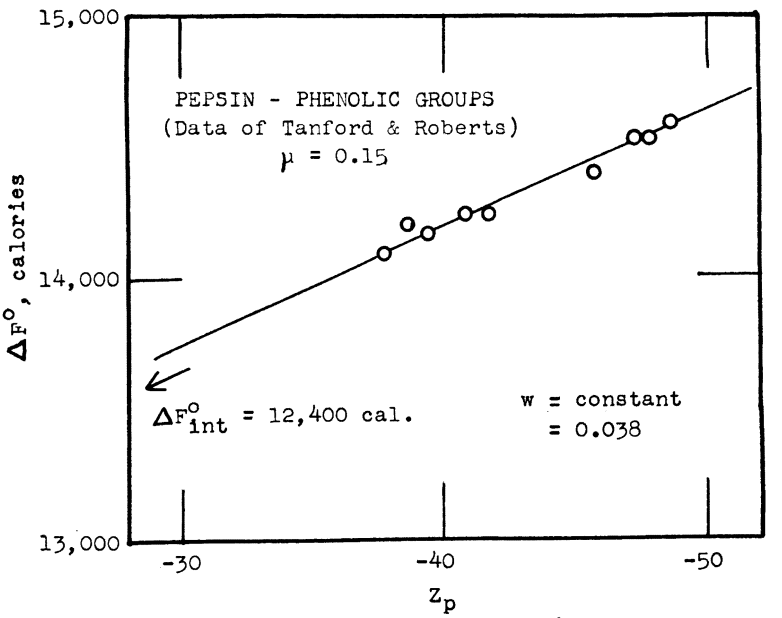


Figure 2. Dissociation of H ion from phenolic groups of pepsin.

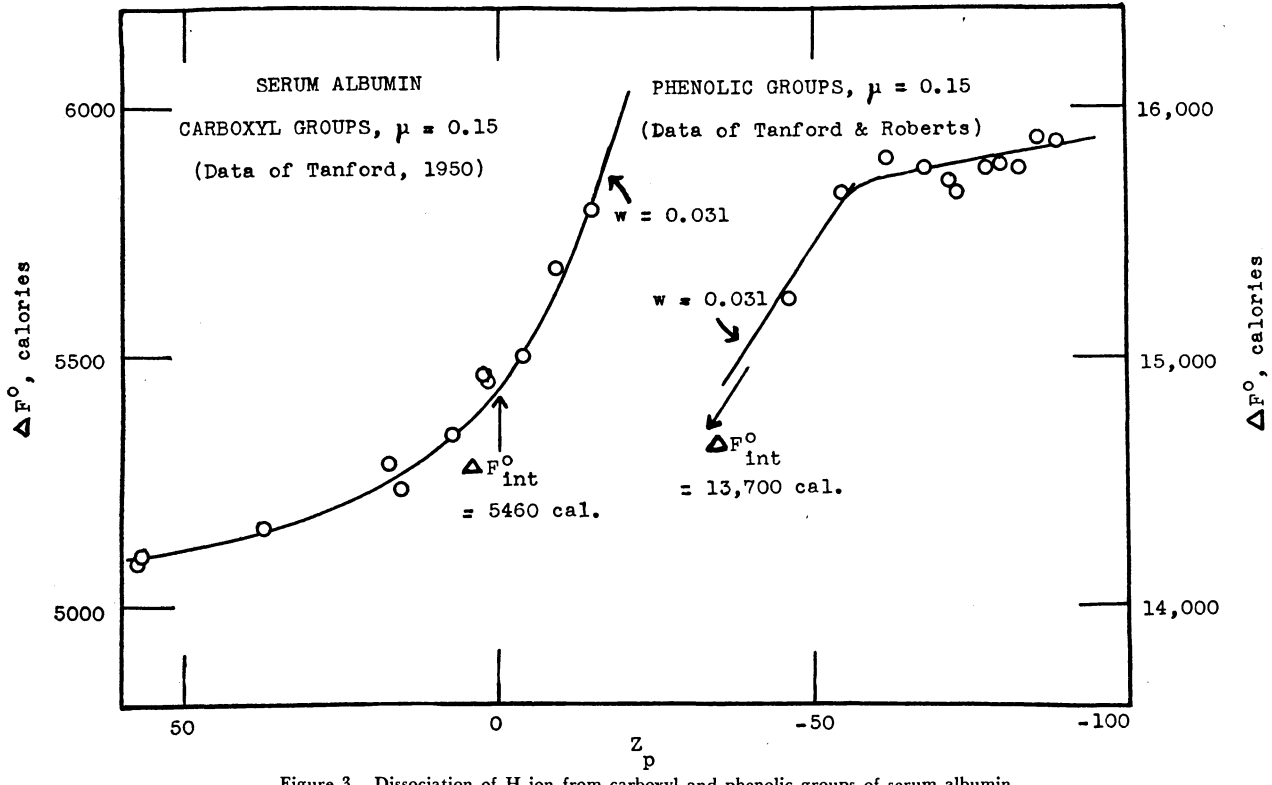


Figure 3. Dissociation of H ion from carboxyl and phenolic groups of serum albumin.

is obtained at each of three ionic strengths, indicating constant value of $w$. The value of $\Delta F^{\circ}_{int}$ is 6260 calories.

*Mysosin.* Similar calculations have been made for myosin, based on the work of Mihalyi (8). The calculations were based on a molecular weight of 1,500,000 with 2475 carboxyl groups per mole (8). No correction has been made for possible binding of chloride or potassium ion, but Mihalyi's data indicate this to be small. The calculations again indicate a constant value of $w$ (0.00075 at ionic strength 0.15), and a $\Delta F^{\circ}_{int}$ of 6320 calories.

*Pepsin.* No precise data exist for the titration of the carboxyl groups of pepsin. However, the dissociation of hydrogen ion from the phenolic groups of this protein has recently been studied in this laboratory (17). Calculations based on these data are shown in Figure 2. The molecular weight has been taken as 35,000. The charge at any pH has been estimated from the amino-acid composition, in conjunction with the data on the dissociation of phenolic groups. Both this estimate, and the data themselves, are relatively unprecise. However, a reasonable straight line can be drawn through the data of Figure 2, giving a constant value of $w$ (0.038 at ionic strength 0.15) and a $\Delta F^{\circ}_{int}$ value of 12,400 calories.

*Serum Albumin*: More thermodynamic studies have been made for reactions of serum albumin than for those of other proteins. The dissociation of hydrogen ion from the carboxylic acid groups of human serum albumin and from the phenolic groups of bovine albumin has been studied by the present author (14) (16). Plots of $\Delta F^{\circ}$ *versus* $Z_p$ based on these studies are shown in Figure 3. A molecular weight of 69,000 has been used, with 108 carboxyl groups in human albumin, and 21 phenolic groups in the bovine product. The ionization of imidazole and amino groups and the binding of chloride ions has had to be taken into consideration in computing $Z_p$. The first two were estimated from the complete titration curve previously reported (14), and the binding of chloride ions was calculated from the equation given by Scatchard, Scheinberg and Armstrong (18). For our purposes, this equation, which depends on a calculated value of $w$, was considered an empirical equation relating the number of chloride ions bound to the charge, $Z_p$. From all these data the values of $Z_p$ at any pH could be obtained by successive approximation. The values for bovine and human albumin have been assumed interchangeable.

Figure 3 shows that the value of $w$ is not constant for either of the reactions studied, but decreases with increasing charge (positive in one case, negative in the other). This is especially interesting

because it has been found that the free energy/charge relationships of three entirely different reactions of serum albumin in the region of $Z_p$ between 0 and –50 require a single value of $w$ (0.031 at ionic strength 0.15). The reactions are the dissociation of $H^+$ from the imidazole and amino groups (14), and the combination of the protein with chloride ion (18) and with zinc (6). According to the theoretical treatment given above, $w$ should have the same value for all reactions at a given $Z_p$, and it is indeed found that the curves of Figure 3 can be reasonably extrapolated into the region of $Z_p$ between 0 and –50 to give straight lines the slope of which corresponds to a constant value of 0.031 for $w$.

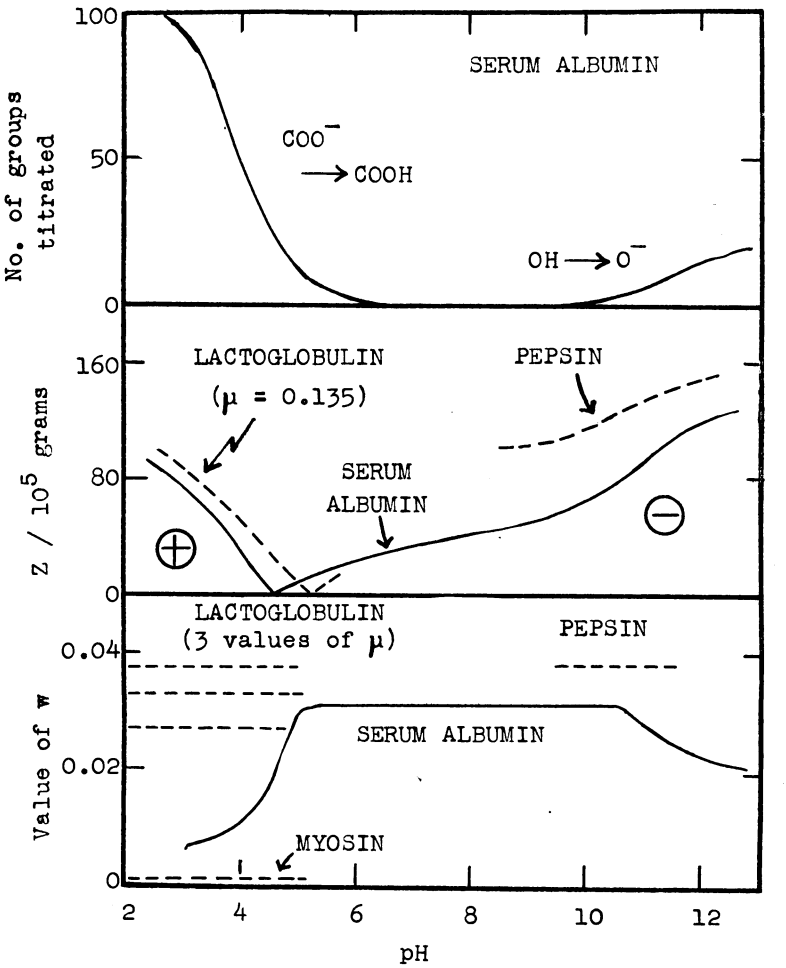


Figure 4. Variation of $w$ and related quantities as a function of pH.

$\Delta F^{\circ}_{int}$ for $H^+$ dissociation from the carboxylic acid groups is found to be 5460 calories, while for the phenolic groups it is 13,700 calories. Using these values, $w$ has been computed at all values of the charge, and the results are plotted in Figure 4.

## Discussion

As Figure 4 shows, $w$ varies for serum albumin in pH regions where it is constant for the other proteins studied: it decreases outside the region of $Z_p$ between 0 and –50. Such a decrease implies an expansion of unfolding of the albumin molecule (*cf.* the very small value for the very large molecule, myosin). The molecular weight of serum albumin is known to remain unchanged in these pH regions, so that the process occurring (which is instantaneous and reversible) must correspond to a rupture of internal bonds, with a consequent uncoiling of the normally compact structure. The process cannot be due to the mutual repulsion of like charges, for on the acid side it begins at zero charge, while on the basic side the compact structure is maintained to a charge of –50. In addition the charge per unit mass (the partial specific volumes of these proteins are virtually identical (4), so that this is also the charge per unit volume) is actually lower in the pertinent regions for serum albumin than for pepsin and lactoglobulin (Figure 4), yet no expansion occurs in these proteins.

The expansion must therefore be attributed to the direct rupture of internal bonds by hydrogen or hydroxyl ion. On the acid side the hydrogen ions added are taken up exclusively by carboxyl groups; on the basic side ($-Z_p > 50$) the hydroxyl ions added remove $H^+$ from amino and phenolic groups. Thus either $-COO^- {}^+H_3N-$ electrostatic bonds, or $-COO^- \cdots H\text{-}O-$ hydrogen bonds may be considered essential to the compact structure for serum albumin. The subsequent argument will show that the data are incompatible with the former, and strongly confirm the latter.

The reason for the expansion is then that the addition $H^+$ to $COO^-$ groups, or the removal of $H^+$ from OH groups, will rupture these essential hydrogen bonds. Figure 4 shows in support of this a good correlation between the number of groups so titrated and the change in $w$. It should be noted particularly that on the acid side all of these bonds must be broken, since all of the carboxyl groups become protonated. On the basic side, however, only the 21 phenolic groups ionize, and the bonds between carboxyl and aliphatic hydroxyl groups are unaffected. This accounts for the fact that the overall change in $w$ on the basic side is less than that on the acid side (Figure 4).

The existence of carboxyl-hydroxyl hydrogen bonds in serum albumin is also indicated by the $\Delta F^{\circ}_{int}$ values computed in this paper. For the dissociation of $H^+$ from acetic acid or any of its homologues $\Delta F^{\circ}$ is close to 6600 calories. Substitution of a polar group, such as an ester group or a peptide link, in the $\alpha$-position greatly reduces this figure (ROOC-$CH_2$-COOH 4560 cal., glycylglycinehydantoic acid 4830 cal.). Substitution of such a group in the $\beta$ or a more distant position, however, has a much smaller effect: an ester group in the $\beta$ position gives 6170 cal., in the $\delta$ position 6270 calories. (These and similar figures are contained in a compilation by J. T. Edsall (3)). Virtually all of the carboxylic acid groups of the proteins discussed in this paper derive from aspartic and glutamic acid side chains. They are therefore COOH groups with a polar group, the peptide bond, in the $\beta$ and $\gamma$-position, respectively. The standard free energy change for $H^+$ dissociation in either case is thus expected to be virtually identical, and equal to 6200 to 6300 calories. This standard free energy change should be identical with the $\Delta F^{\circ}_{int}$ computed from the dissociation of these groups in proteins, since all factors except charge have been taken into account. In confirmation, Table 1 shows that $\Delta F^{\circ}_{int}$ for the dissociation of $H^+$ from the COOH groups of both $\beta$-lactoglobulin and myosin does indeed have this value.

If, now, the product of ionization is *not* a free carboxyl group, but instead a hydrogen-bonded one, the free energy of which might be about 1000 calories lower (9), then $\Delta F^{\circ}_{int}$ for the ionization process would be expected to be lower by about 1000 calories. The value of 5460 calories obtained for serum albumin (Table 1) is therefore striking confirmation for the existence of hydrogen-bonded carboxyl groups in that Protein.

Similar calculations can be made for the ionization of phenolic groups, though with less certainty, since no detailed studies have been made on substituent effects in the ionization of phenols. In addition, the $\Delta F^{\circ}_{int}$ values reported here for this reaction are less

**Table 1**

Summary of Thermodynamic data

| | COOH Groups | Phenolic Groups | |
|---|---|---|---|
| | $\Delta F^{\circ}_{int}$ cal. | $\Delta F^{\circ}_{int}$ cal. | $\Delta H$ cal. |
| Expected Values | 6250 | 13,000 | 6,000 |
| $\beta$-Lactoglobulin | 6260 | ---------- | -------- |
| Myosin | 6320 | ---------- | -------- |
| Pepsin | -------- | 12,400 | 6,500 |
| Serum Albumin | 5460 | 13,700 | 11,500 |

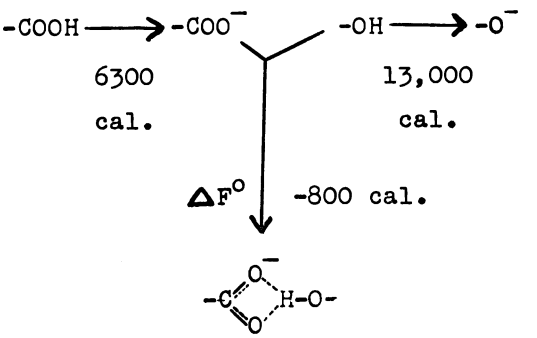


Figure 5. Thermodynamics of Carboxyl-Hydroxyl Bond Formation.

precise than those for COOH groups, because phenolic ionization occurs so far from the iso-electric point that the extrapolation to $Z_p = 0$ is of greatly reduced accuracy. Nevertheless, the figures of Table 1 shows that $\Delta F^\circ_{int}$ for the phenolic groups of serum albumin is about 1000 calories greater than the value found in pepsin, or the "expected" value. The change is in the direction to be expected, as seen from Figure 5. Table 1 also shows the heats of ionization of the phenolic groups, which show an even more striking difference. A fuller discussion of the $\Delta H$ values has been given elsewhere (16).

As was mentioned earlier, it is possible that electrostatic carboxyl-amino bonds, rather than carboxyl-hydroxyl hydrogen bonds, could produce the variation in $w$ found in serum albumin. If this were so, $\Delta F^\circ_{int}$ for ionization of the amino groups of serum albumin should be higher than normal by about 1000 calories. Actually the reverse is true: $\Delta F^\circ_{int}$ for these groups in serum albumin is a few hundred calories *lower* than would be expected (14). The possibility that such electrostatic bonds are important is therefore excluded. To account for the low value of the intrinsic free energy change, it is not unreasonable to suppose that in a negatively-charged protein an $H_2N\cdots H\text{-}O$- hyrogen bond will be somewhat more stable than a carboxyl-hydroxyl bond, since a free carboxyl group could better attract gegenions for charge stabilization. Simultaneously with the rupture of carboxyl-phenolic bonds, therefore, amino groups may be replacing carboxyl groups in the intact bonds. This would have no effect on the calculations made earlier in this paper, but would produce a low value of $\Delta F^\circ_{int}$ for the amino groups.

## Conclusions

The calculations presented here indicate that serum albumin is folded into a compact molecule by means of carboxyl-hydroxyl hydrogen bonds. Rupture of these bonds by titration of either of the participating groups leads to instantaneous, but reversible unfolding. In alkaline solutions amino groups may replace carboxyl groups in these bonds.

Pepsin (on the alkaline side) and $\beta$-lactoglobulin and myosin (on the acid side), on the other hand, depend on their structure on as yet unidentified bonds which are not broken by acid or base titration. Titration data for pepsin on the acid side are currently being initiated in this laboratory.

It is interesting to note that work on urea denaturation by Kauzmann and coworkers (12) has led to similar conclusions as regards serum albumin and pepsin. The former is easily and reversibly unfolded by urea, an agent which would be expected to break hydrogen bonds, while pepsin is completely unaffected.

Finally, however, it should be mentioned that the work here described can be no more than a beginning in the elucidation of internal bonding in proteins. Egg albumin, for example, shows a constant value of $w$ on both the acid and basic sides (1), but its phenolic groups cannot be titrated at all, except at very high pH, and the ionization then leads to irreversible denaturation (5). The titration of carboxyl groups in hemoglobin is exceedingly complex, resulting in irreversible changes (13). Even $\beta$-lactoglobulin, whose carboxyl groups appear to react in a completely normal fashion, probably shows more complex behavior in other respects (2) (12). All of these cases still await adequate explanation.

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