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Addendum

Since this manuscript was submitted for publication, a monograph by P. F. HJORT (*Scand. J. Clin. & Lab. Invest.*, 9 (1957) suppl. 27) has come to our attention. HJORT presents data that strongly indicate that a phospholipid-containing particle is united through calcium ion to a plasma protein in the sequence of reactions leading to the clotting of blood plasma in the presence of brain thromboplastin.

VOLUME CHANGES IN OVALBUMIN AND BOVINE SERUM ALBUMIN ON ADDING ACID

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Polypeptide chains undoubtedly pack in certain more or less well-defined patterns in native protein molecules. If denaturation consists in the partial or complete replacement of these regular patterns by a more random arrangement of the chains, then one should expect that denaturation will be accompanied by characteristic volume changes that should parallel, at least in a general way, the changes in other properties, such as the optical rotation, which are also affected by the chain configuration. Several workers have, in fact, noticed that the destruction of the native configuration in proteins is accompanied by a decrease in the volume of the protein solution amounting to several hundred ml/100,000 g of protein[1-4]. The apparent volume occupied by a protein molecule in solution is also influenced by the charged groups present in the molecule because of electrostriction of the solvent surrounding the charged groups; if the configuration changes are accompanied by known changes in the charged groups of the protein, however, the effects of the latter can be taken into account by suitable model experiments[5,6].

The measurements reported in the present communication were performed with Carlsberg dilatometers of the type described by LINDERSTRØM-LANG[7], with the modifications suggested by JOHANSEN AND THYGESEN[8] and by JOHANSEN[9].

The dilatometers consist of an inverted "V", one arm containing the protein solution and the other containing the acid. The space above the solutions is filled with kerosene and a graduated capillary is attached by means of a suitably greased[8] ground-glass joint at the vertex of the "V". The inverted "V" is inserted into a well-controlled thermostat ($\pm$ 0.001°) and allowed to stand until thermal equilibrium has been reached. If the bath is warmer than room temperature, as will generally be the case, the kerosene meniscus will be at the top of the capillary tube and must be brought down to a suitable point in the graduated region by placing the inverted "V" momentarily in water slightly warmer than the thermostat, soaking up the excess kerosene that flows out of the top of the tube, and replacing in the thermostat. After thermal equilibrium has been re-established and the kerosene meniscus has reached a constant level, the two solutions are mixed.

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The resulting volume change is found from the change in the kerosene meniscus. With care, volume changes can be measured that are reproducible to within a few hundredths of a microliter, and a precision of about 20 ml/100,000 g can be attained using 100 mg samples of protein.

Figs. 1 and 2 show the volume changes that occur when hydrochloric acid is added to ovalbumin and to serum albumin. As is well known, when the pH is less than 4 the serum albumin molecule undergoes an immediate, reversible unfolding, that is accompanied by an increase in the viscosity and in the laevo-rotation and a decrease in the sedimentation constant[10,11]. The unfolding also has a pronounced effect on the shape of the titration curve[12]. All of these effects are especially marked when the salt concentration is low, and there is also some tendency for the effects to be greater when the protein concentration is low. On the other hand, the ovalbumin molecule undergoes no such drastic changes in configuration until the pH is less than about 2, where a gradual, irreversible unfolding takes place[13,14].

Fig. 1 shows that the volume changes observed for ovalbumin down to pH 2 correspond to an increase in volume of 11 ml for each proton bound by the protein. This corresponds to the well-known[1,5,6] volume change due to the neutralization of a carboxylate ion by a proton

$$-COO^- + H^+ \longrightarrow -COOH$$

which is undoubtedly the sole mechanism for the binding of protons in most proteins when the pH is less than about 5. Thus the ovalbumin molecule above pH 2 acts like a rigid object with ordinary carboxyl groups attached to it.

The volume changes observed for serum albumin, on the other hand, amount to 11 ml per bound proton only when the pH is not below 4. At lower values of the pH the expansion normally brought about by the neutralization of the carboxylate ions appears to be accompanied by a decrease in volume from some other process. (This difference between ovalbumin and serum albumin has recently been detected by CHARLWOOD[4], who compared the partial specific volumes of these proteins at pH values of *ca.* 5 and *ca.* 2.) The magnitude of this superimposed volume decrease is smaller in the absence of salt than when salt is present. It also seems to be somewhat larger when the protein is more dilute. In all cases the volume changes were complete within 2 or 3 min, which is the time required for thermal equilibrium to be re-established after the acid is added to the protein.

The changes in the chain configurations of proteins brought about by urea and other denaturing agents are invariably accompanied by an increase in the laevo-rotation and viscosity, and by a decrease in the sedimentation constant. These configuration changes in urea are also accompanied by a decrease in the volume of the protein solution. The fact that the swelling of serum albumin molecule in acid is not only accompanied by an increase in viscosity and levo-rotation, but also by a decrease in volume, lends further support to the belief that the changes in acid are essentially the same as those that occur in denaturation reactions in general.

It is significant, however, that the addition of salt is much more effective in quenching the changes in optical rotation and viscosity brought about by acid than it is in eliminating the volume decrease. This can be seen from Fig. 2, where the optical rotations corresponding to various conditions have been noted along the curves. (These measurements were made on solutions that were identical in composition to those used in the dilatometers. The rotations are slightly lower than those reported

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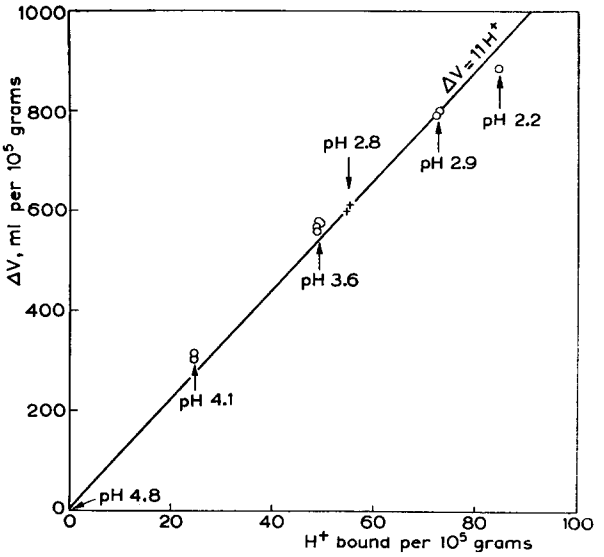

Fig. 1. Volume changes on adding hydrochloric acid to ovalbumin at 30.0°. Final protein concentration 4.02 %; initial protein concentration 8.04 %. + no salt present. o 0.15 *M* KCl present. The numbers written beside the points indicate the pH.

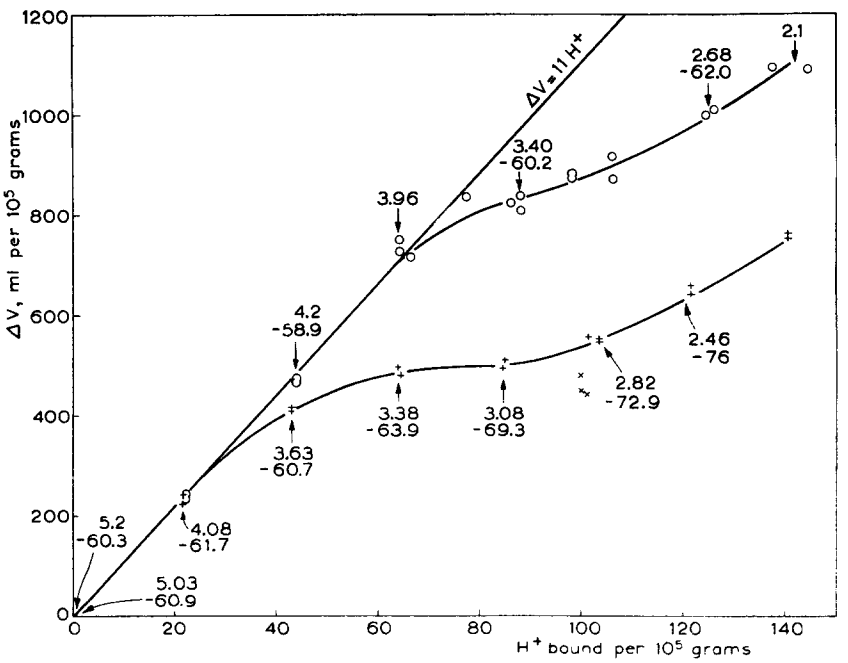

Fig. 2. Volume changes on adding hydrochloric acid to bovine serum albumin at 30.0°. + no salt present, final protein concentration 2.24 %. × no salt present, final protein concentration 1.50 %. O 0.15 *M* KCl present, final protein concentration 2.24 %. The numbers written beside the curves give the pH (upper number of each pair) and the specific rotation, $[\alpha]_D^{30}$ (lower number of each pair), when the protein concentration is 2.24 %.

by YANG AND FOSTER[10], but the changes are closely parallel to theirs.) It will be seen, for instance, that when 120 protons have been bound per 100,000 g of protein, the specific rotation in the absence of salt has been changed by about 15° (normal value about —61°, changed to —76° when 120 protons are bound). At the same degree of binding in 0.15 *M* KCl the laevo-rotation is increased by only 2° over the normal value. The observed expansion in the absence of salt is 640 ml/100,000 g when 120 protons have been bound. If the protein were a rigid obejct, the expansion at this degree of binding would have amounted to $11 \cdot 120 = 1320$ ml/100,000 g, so there must have been a contraction of $1320 - 640 = 680$ ml/100,000 g in the absence of salt. When 0.15 *M* KCl is present this contraction amounts to $1320 - 970 = 350$ ml/100,000 g. Thus 0.15 *M* KCl eliminates all but 13% of the increased laevo-rotation induced by the binding of 120 protons/100,000 g, whereas 51% of the contraction persists in 0.15 *M* KCl with the same amount of proton binding. Apparently the changes that take place in the two properties are not directly related and we are forced to conclude that the two properties are sensitive to somewhat different aspects of protein structure. This is encouraging because it means that volume changes may be able to tell us something about proteins that optical rotations do not reveal. Just what this new feature is cannot be ascertained without further investigation.

This result has some bearing on a hypothesis recently proposed by YANG AND DOTY[15], who suggest that the polypeptide chain in proteins exists in two basic types of structure. The one type of structure is a regular coil, probably the α-helix, whereas the other structure is irregular, resembling on the average a random coil. YANG AND DOTY interpret the changes in the optical rotatory parameters of proteins and synthetic polypeptides in different solvents by assuming that these two structures make independent contributions to the parameters. In this way they claim to be able to estimate the relative amounts of helical structures present in many native proteins. It is, however, clear that such a hypothesis cannot be tested adequately without making use of a number of properties that depend in different ways on the molecular configuration. The apparent volume of the protein is ideal for this purpose, because it should be just as intimately related to the chain configuration in proteins as is the optical rotation. The fact that the volume changes that occur on unfolding do not quantitatively parallel the optical rotation changes shows that if there are only two basic types of structure, these cannot make independent contributions to the volume of the protein in the manner postulated by YANG AND DOTY in their interpretation of the optical rotatory parameters. It is difficult to understand why independent contributions should be made in one case and not in the other, and it therefore seems likely that the situation is more complex than thas been assumed by YANG AND DOTY.

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SUMMARY

When hydrochloric acid is added to an isoelectric solution of ovalbumin in a dilatometer an expansion of about 11 ml per bound proton is observed. The expansion is caused by the neutralization of carboxylate ions by protons (WEBER AND NACHMANSOHN). A similar experiment with bovine serum albumin yields much smaller expansions when the pH is below 4, where the optical rotation and the viscosity also undergo anomalous changes. Evidently the molecular changes that occur below pH 4, bring about a decrease in the partial specific volume, $\bar{v}$, similar to that observed in protein denaturation, and this decrease in $\bar{v}$ is superimposed on the increase in $\bar{v}$ that normally occurs when carboxylate ions are discharged by protons. It is significant, however, that whereas 0.15 *M* potassium chloride almost completely eliminates the optical rotation change below pH 4, it is much less effective in preventing the anomalous contribution to $\bar{v}$. This fact raises questions about the hypothesis of protein structure recently made by YANG AND DOTY.

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A CELLULAR THYROXINE-BINDING PROTEIN FRACTION

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The observation of GORDON, GROSS, O'CONNOR AND PITT-RIVERS[1] that thyroxine is bound to a specific serum protein fraction in man, has been followed by extensive work on diverse aspects of this binding[2,3]. The specific protein fraction has also been detected in the blood of various mammals and reptiles and in other human extracellular fluids[2,4]. It is commonly referred to as thyroxine-binding protein or TBP. This protein fraction has the same electrophoretic mobility as serum α-globulin and is also capable of binding 3:5:3′-triiodo-L-thyronine and structurally related substances to a lesser degree. When the thyroid hormones are added in larger amounts they are then "spilled over" and bound to serum albumin. But, on the other hand, except for the precipitation of tissue iodine with proteins, only very little is known as yet about the interaction between thyroid hormones and tissue proteins[4,5]. This

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