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Effects of High pH and Sodium Dodecyl Sulfate on the Hidden Tyrosines of Human Serum Albumin†

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ABSTRACT: A stop-flow technique has been developed for distinguishing between the tyrosines of human serum albumin which are freely accessible to solvent (11 or 12 out of 18) and those which are hidden in the interior of the native molecule. When the latter are unmasked by raising the pH or by adding various quantities of sodium dodecyl sulfate, reversible changes in degree of ionization and in degree of unfolding both occur and may be distinguished from one another. Exposure to high pH enhances both. Exposure to detergent leads to more complex results; small amounts suppress both ionization and unfolding, and large amounts (up to 300 equiv) enhance unfolding. Unfolding produced at acid pH (N → F transition) has also been examined by this method. The pK of the accessible tyrosines, which may occur in two sets, is anomalously high. Preliminary explorations of the effects of low and high ionic strengths, temperature, and defatting procedures on the ionization and unfolding have also been made. The results with bovine serum albumin differ in detail from those with the human protein. Rate measurements of all the experiments show that a fast first-order process dominates the kinetics; the dependence of rate on pH increases rapidly at pH >11.4. The effects of detergent concentration are complex: *e.g.*, 20 equiv of dodecyl sulfate result in a slowed rate, only slightly dependent on pH, even when the reaction goes to completion at high pH, but the highest detergent concentrations enhance the rate at any pH.

It is now well known that some of the acidic and basic groups of many native proteins in solution are inaccessible to solvent or other solutes. Thus these groups do not contribute to protein titration curves if the latter are determined at very short time intervals after mixing. Some of the phenolic groups of tyrosine are almost always found in this "masked" category (Steinhardt and Reynolds, 1969, Table VI). Since dissociating a proton requires that the protein undergo a prior conformation change (which exposes the groups to solvent), their apparent dissociation constants contain factors contributed by the equilibria controlling these conformation changes. The discrepancy between the measured titration constants and those of normally accessible groups has often led to their being described as "abnormal" or "anomalous" tyrosines. However, since the conformation change is sometimes observably time dependent (for an example, see data on ribonuclease (Tanford *et al.*, 1955)), and since the ionization of phenolic groups is accompanied by large easily measured time-dependent changes in their ultraviolet (uv) spectra, the origin of the "abnormal" titration behavior of these tyrosines is fairly obvious. The use of stop-flow devices is well suited to reveal the time-dependent nature of the abnormal tyrosine dissociations when the reactions are more rapid than with ribonuclease. A suitable wavelength is 243 nm at which the extinction coefficient of tyrosine phenolic groups is close to zero, but at which the extinction coefficient of the corresponding phenoxy ion may be greater than 11,000 (Wetlaufer, 1962).

The time-dependent change in extinction coefficient (absorption) may be used to study the conformation change itself, the equilibria determining the initial and final states, and the kinetics of the reaction that transforms the native conformation to an unfolded state. The technique is not limited to studying denaturation by bases at pH values high enough to ionize the tyrosine. As long as the pH is high enough to cause partial ionization of the tyrosines which are already exposed in the native state (the "outside tyrosines"), the method may be applied to denaturation by sodium dodecyl sulfate or other detergents, and even to denaturation by acid. Application to denaturation by other means would probably also be feasible.

The technique depends on the ability to rapidly mix protein solutions with base, and on the fact that ionization changes may be regarded as instantaneous as compared with the time required for conformation changes. With sufficiently rapid mixing, the *initial* optical density can be used, after correction for instantaneous ionization changes brought about by the changes in pH, to characterize the initial conformation state at the *initial* pH, *i.e.*, the fraction of the total tyrosines which were free to ionize at the initial pH, *i.e.*, before the addition of base. The difference between the initial and infinite time optical densities at the same pH is then proportional to the increase in the number of ionizing groups brought about by the conformation change. The entire increase in optical density at a given high pH is due to the *increment* in the fraction of tyrosine groups which are exposed, and is given numerically by $\Delta OD/\alpha$, where Δ represents the increment from zero to infinite time and α is the degree of ionization of individual groups at the final pH of the experiment. When denaturation is brought about by, *e.g.*, the addition of detergent, there need be no change in pH. The actual sequence of

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events in experiments in which conformation changes are brought about by high pH or by dodecyl sulfate is given in the next section.

In all of the foregoing the assumption has been made that the transformation between native and denatured serum albumins relate to a two-state system with respect to effects on ionization and light absorption. It is also assumed in the case of combination with dodecyl sulfate that combination with the denaturing agent does not of itself sterically alter the accessibility of any group to hydrogen or hydroxyl ion.

Experimental Section

Materials. Crystallized human serum albumin (Pentex, lot 30) and bovine serum albumin (Nutritional Biochemicals, lot 3441) were deionized on a mixed-bed resin as in earlier work (Ray *et al.*, 1966; Reynolds *et al.*, 1967). In a few experiments, protein defatted by the method of Chen and Kernohan (1967) was used. Concentrations were determined spectrophotometrically, as in our earlier work.

Sodium dodecyl sulfate was a special custom synthesis from Mann Research. All other chemicals were reagent grade.

Methods. In most of the experiments deionized 0.2% solutions of serum albumin in 0.07 M phosphate buffer (pH 5.6) at 0.10 ionic strength (NaCl) were stored in one of the two storage syringes of the Gibson-Durrum stop-flow apparatus. The latter was fitted with a D-131 logarithmic amplifier so that readout in optical density units would be directly obtained. The other storage syringe contained phosphate or carbonate buffers at 0.07 ionic strength which would, on mixing equal volumes, bring the pH to values between 9.7 and 12.1, plus enough additional sodium chloride to bring the ionic strength after mixing to 0.24. In another series of experiments at lower ionic strength, the buffer ionic strength after mixing was 0.032 and no sodium chloride was added to either solution. Carbonate buffers were used only at pH below 10. The highest pH used, 12.1, required the addition of NaOH.

In many of the experiments various quantities of sodium dodecyl sulfate (10–300 equiv) were added to the syringe containing the alkaline buffer. These are not included in the ionic strengths given, partly because large fractions of this detergent are bound by the protein after mixing (up to 315 equiv can be bound (Reynolds and Tanford, 1970)),[1] and the remaining free detergent is not fully dissociated. Thus, only negligible contributions to ionic strengths result from the free detergent, except possibly in the experiments at 0.032 ionic strength.

On mixing, the course of the changes in optical density with time (up to four to eight half-periods) were recorded on Polaroid film, using at least two different time scales to assure adequate resolution at the rapid first stages of the reaction, as well as accurate determination of the equilibrium optical density, which may not be approximated closely until over five half-periods have elapsed. In each series of experiments the zero offset of the logarithmic amplifier read directly in optical density units, and deflections on the scope face represented known increments or decrements of the same units. Thus there was never any ambiguity as to the equilibrium optical densities at long times (usually a second or longer), which were not subject to errors other than slow drift in the calibration. Initial optical densities are less well known, because of a 2-msec dead time in mixing and in replacement of the previous solution in the flow cuvet by the new one. Were the reaction first order, extrapolation could be relied on to give reliable zero time values, but the kinetics are complex. The zero time values given in this paper are extrapolated, with little weight given to the first 4 msec; in some cases recourse has been had to an empirically useful observation that the rates are proportional to the inverse 2.2 power of the reactable residue (final optical density minus optical density at time t)$^{-2.2}$. The resulting uncertainties are small.

The optical densities given in text and figures of this paper are those given by the apparatus used. They must be multiplied by 1.18 to obtain the true values, as given by a Cary Model 14 spectrophotometer.

In a few experiments, a Biomation 802 transient recorder, reading out directly onto graph paper, was substituted for oscilloscope and camera. This change improved resolution and reduced analysis time importantly, as well as permitting the use of fewer changes of time base to survey accurately the complete course of a reaction.

In the experiments in which unfolding was brought about by acid rather than by base, protein at the same initial pH (5.6) was exposed for a few minutes to dilute HCl at pH values between 3.0 and 5.4 at 0.1 ionic strength, then placed in a storage syringe of the stop-flow apparatus and brought suddenly to the "viewing pH" (10.82) at which both initial and time-dependent tyrosine partial ionization could be observed.

In most experiments, buffer only or buffer plus detergent was mixed with protein. In a few experiments, the effect of detergent alone was determined in one of two different ways: (a) use was made of a pH value, (9.9–10.8) at which sufficient tyrosine ionization occurred to produce measurably phenoxy ion absorption at 243 nm, and the time-dependent increase in optical density was then monitored after mixing; (b) detergent was added to protein at pH 5.6 at various times before addition of alkaline buffers. The resulting *initial* optical density was always higher than when detergent was added at the same time as the base, but the *final* optical density was not affected. Information about the effect of various concentrations of detergent at pH 5.6 for various times could also be inferred from the zero time optical densities obtained after changing the pH to 10.8 or higher.

Results

Effects of pH. The two sets of data in Figure 1 show the initial and final optical densities of solutions of human serum albumin brought instantly from pH 5.6 to the pH shown at 0.24 ionic strength. If the unfolding reaction is not too fast, the initial values (dotted curve) should represent the tyrosine groups freely exposed to the solvent at pH 5.6 after ionization at higher pH. The increase in optical density with pH in this set should thus represent only the increasing ionization of these accessible groups at increasing pH.

The data for infinite time (continuous line) coincides with the data for zero time at pH values below 10.9, but are higher at pH >10.9. The difference between the two sets of data at any pH represents the extent to which the alkaline conformation change (unfolding) exposes the tyrosines which were inaccessible in the isoelectric native protein, multiplied by the degree of ionization at each pH. The maximum *change* in a 0.1% solution in a 2-mm cell which contains 18 ionized tyrosines per protein molecule is about 0.55 density unit when measured in a Cary Model 14 spectrophotometer

[1] No isotherms are available for the pH range of this study (9.3–12.1). Isotherms at pH between 3.8 and 7.5 show very little change at pH above 6 (Reynolds *et al.*, 1970, and unpublished work).

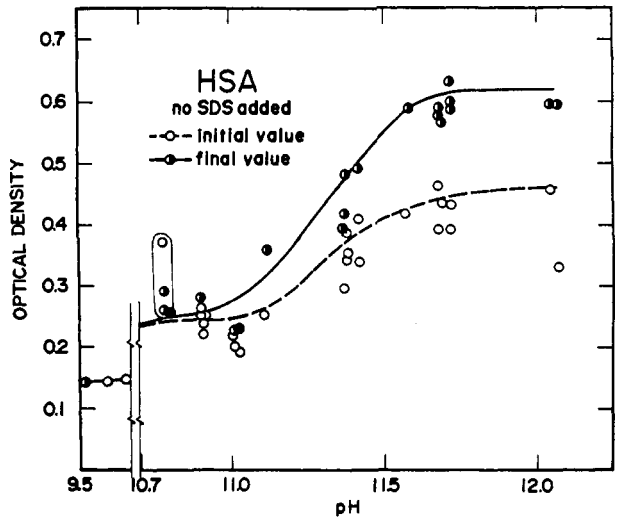

FIGURE 1: Effect of pH on initial and final uncorrected optica densities after rapid mixing of solutions of human serum albumin originally at pH 5.6 with various phosphate buffers at a final ionic strength of 0.24. The lower curve represents initial (zero time) values; the upper curve represents equilibrium values.

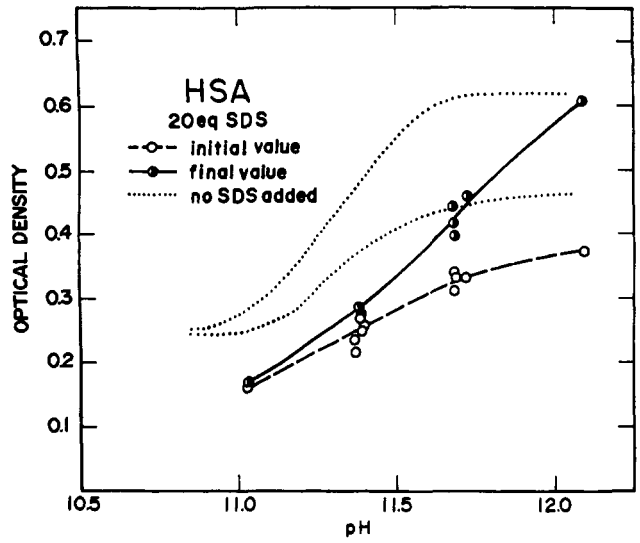

FIGURE 2: The same as Figure 1 with 40 equiv sodium dodecyl sulfate added with the alkaline buffer, so that the final concentration was 20 equiv. The data of Figure 1 (no detergent present) are represented as dotted and broken lines.

(ϵ 10,630). This saturation value is almost attained at pH 11.7 and appears to be fully attained at pH 12.09. The corresponding change in absorbance found in the stop-flow experiments is only 0.47, and the maximum absorbance is 0.61; but application of the correction factor (see Methods) brings the value of the change to 0.555, essentially the same as in the Cary spectrophotometer. The corrected maximum total absorbance is 0.82.

The initial value at pH 11.7 is about 0.45 (corrected as above to 0.531). Almost all the tyrosine absorbance at this wavelength is due to phenoxy ions. If the accessible tyrosines are fully ionized at pH 11.7–12.1, which seems probable, then over half of the tyrosines (11–12), are accessible in the native state at pH 5.6, and 6–7 are buried in the interior of the molecule.[2] The solvent perturbation method of Herskovits and Laskowski (1962) indicated that over a pH interval from below 2 to above 8 (which includes the acid conformation change) only about 40% is accessible to either sucrose or ethylene glycol, but more could be accessible to the smaller hydroxyl or oxonium ions.

Attention is invited to the two points at the extreme left of Figure 1, which do not lie on the smooth curves. These data were obtained with protein initially exposed to pH 3.80, where the N → F unfolding reaction occurs (Foster, 1960). The zero time value lies *above* the equilibrium value; the latter is only slightly higher than the zero time values in other experiments in which the pH was raised from 5.6 to less than 9.6. Thus inaccessible tyrosines are made accessible in the N → F transition, and most of these unmasked tyrosines are reprotonated and folded back and made inaccessible in a time-dependent step again when the pH is raised. In a later paper this circumstances will be exploited to examine the N → F isomerization in greater detail.

It should be noted that the titration curve of the tyrosines covers a narrower pH range than would characterize "normal" tyrosine dissociations, *i.e.*, the curves are steeper. The deviations cannot, therefore, be explained as the result of electrostatic interactions (Tanford and Roberts, 1952), and implicate some kind of "cooperativity." The Discussion returns to the basis of this effect.

Effects of a Detergent Denaturant, Sodium Dodecyl Sulfate. When 20 equiv of sodium dodecyl sulfate, a potent protein unfolder, are added to human serum albumin solutions at the same time that the tyrosine is ionized by raising the pH, the values obtained at zero time (Figure 2 dotted curve) are lower than those obtained without detergent, except at the highest pH. The data obtained with 20 equiv of detergent can be described, if only to a first approximation, as a shift to higher pH in the titration curve of the tyrosines accessible to solvent at pH 5.6, as compared with their ionizations in the absence of the high-affinity anion, *i.e.*, an increase in the net negative charge of the protein results in a higher pK for the ionization of the accessible tyrosines. Such a shift might be expected solely on the basis of an increased negative charge from bound detergent anions in the vicinity of tyrosines.

The equilibrium (long-time) values can also be explained in this way, except possibly at the lowest (left-hand) extreme where more data, at lower pH, would be required to make the demonstration complete. Thus it may be said that the effect of the presence of 20 equiv of detergent is largely to raise the pH at which the accessible tyrosines ionize, whether they are initially accessible or not. At this concentration the detergent apparently does not increase the unfolding itself.

This conclusion must be modified where larger amounts of detergent are used. Figure 3A–D shows results with 50, 100, 200, and 300 equiv. With 50 equiv, for example, the suppression of ionization at zero time is not only larger, but appears to increase in size with increasing pH; thus it cannot be the result of a single simple electrostatic effect. On the other hand, with 100 and 200 equiv, the ionization of the accessible tyrosines does not increase at all up to about pH 11.5 and then increases only slightly, with between 200 and 300 equiv. The equilibrium absorbance value at 50 equiv is about the same as that with no detergent (the amounts exposed may be larger since the ionization has been partially suppressed)

[2] The fraction accessible is (0.45 − 0.14)/(0.61 − 0.14) = 0.66, equivalent to about 12 tyrosines. The figure 0.14 subtracted represents the non-tyrosine absorbance, which does not depend on pH. It is the absorbance given at any values between pH 8.8 and 9.6.

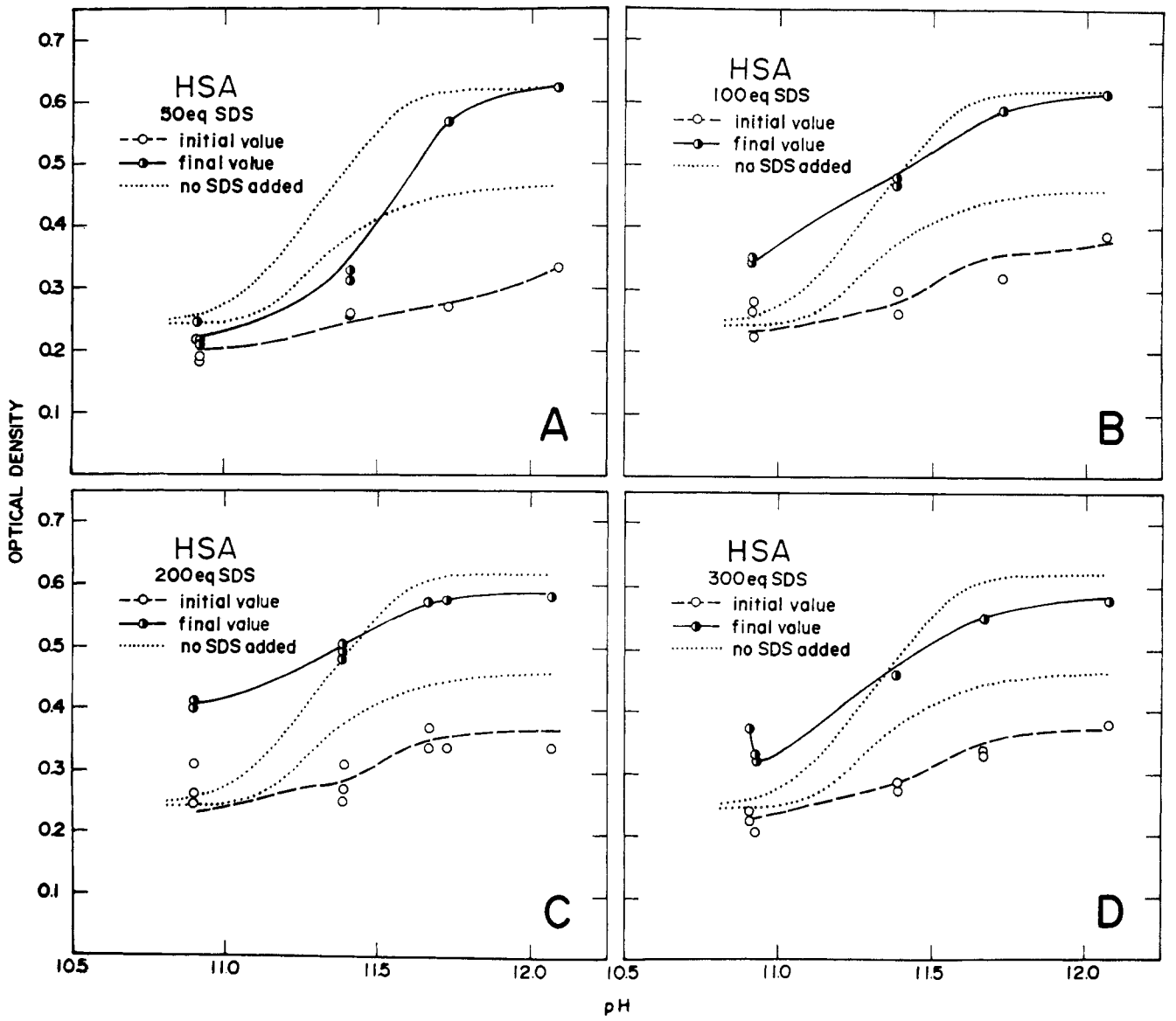

FIGURE 3: The effect of pH on initial and final uncorrected optical densities when (A) 50, (B) 100, (C) 200, and (D) 300 equiv of detergent are added with the alkaline buffers. The data obtained without detergent (Figure 1) are represented as dotted and broken lines.

and rises to the maximum value more rapidly than at either 0 or 20 equiv. This is more dramatically evident with 100, 200, and 300 equiv. There is the *same* amount of unfolding or ionization or combinations of both at all pH values from 11.4 on, and *greater* ionization or unfolding at pH 10.9–11.0 than in the absence of detergent.[3]

Some of the data in Figure 3, identified in the figure legend, were obtained by adding the dodecyl sulfate a few minutes before the pH was raised, instead of concurrently. In each case the zero time absorbance was raised, although there was no change in the equilibrium absorbance; it is clear that addition of large amounts of detergent by itself can cause unfolding, and that this unfolding becomes visible when the tyrosines are permitted to ionize by raising the pH.

Thus it appears that binding small amounts of the detergent anion suppresses both ionization and unfolding; however, binding larger amounts enhances unfolding, without any substantial effect on degree of ionization. This reversal in effects between small and large quantities of detergent has manifested itself in other work, and is considered further in the Discussion.

The interplay between the effects of pH (in deprotonation and/or unfolding) and concentration of dodecyl sulfate (suppression of deprotonation and either suppression or enhancement of unfolding) is further shown in the four panels of Figure 4, each of which represents a different constant pH. At pH 10.9 (panel A, upper left), at which very little unfolding occurs without detergent, there is a slight minimum in ionization (formation of charged phenoxy groups) at 20 equiv where there is also a sharp minimum in unfolding. The amount of unfolding *by detergent* increases only between 75 and 200

[3] Since the ionization cannot increase beyond that of 18 tyrosines it is clear that with 100–300 equiv shifts of ionization to a higher pH cannot be invoked to explain the data; the suppression evident with 20 equiv must be reversed instead of being increased when larger amounts of detergent are present. Thus all the effects just described for 50–300 equiv must be due to an effect of detergent in enhancing unfolding. On the other hand, the effect of 20 equiv cannot be due importantly to suppression of unfolding (since at pH 10.9 there is no unfolding) and must be attributed to suppression of ionization.

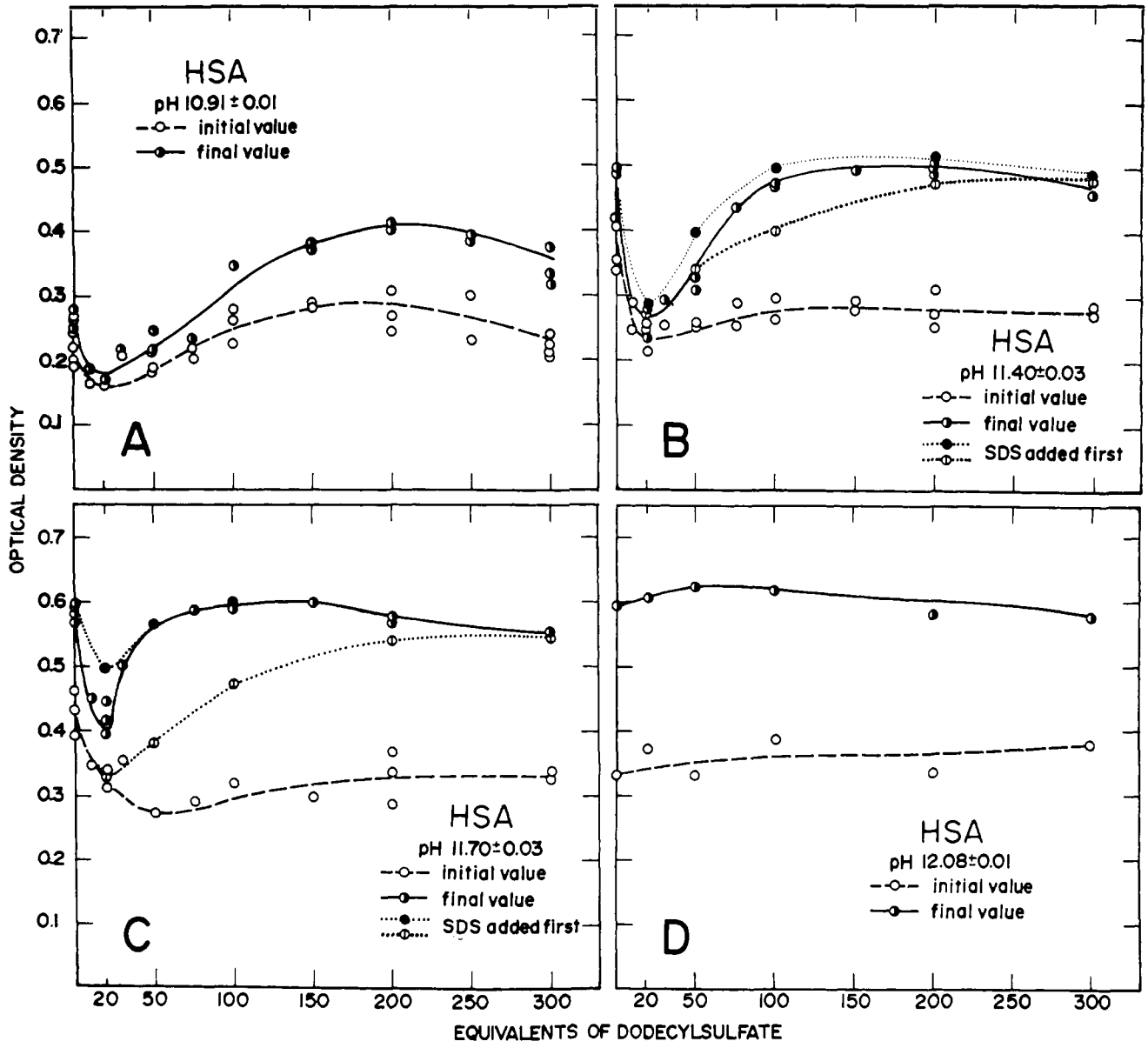

FIGURE 4: The effect of number of equivalents of dodecyl sulfate on initial and final uncorrected optical densities at (A) pH 10.9, (B) pH 11.38–11.42, (C) pH 11.67–11.70, and (D) pH 12.09.

equiv and remains constant thereafter. The amount of unfolding at high detergent concentrations (47% time-dependent increase in absorbance) is not much different than the amount (43%) produced by the rise in pH alone.

It will be made clearer in the discussion of Figures 4B–D that the changes in Figure 4A must be viewed as being brought about by partial suppression of phenolic ionization, combined with *protection* against unfolding, at low amounts of detergent; with a loss of both at larger amounts bound (the loss is complete at about 100 equiv).

At pH 11.38–11.40 (Figure 4B) two sets of results are shown, one for exposure to various amounts of dodecyl sulfate before addition of base, and the other, corresponding to the conditions in Figure 4A, simultaneous addition of base and detergent. Although the absorbance at zero time in the latter, due to ionization without unfolding, is increased at the higher pH the increase does not persist when 100 or more equiv of detergent are present—in fact the absorbance at over 100 equiv is almost exactly the same as at pH 10.9. Certain of the accessible tyrosines must be completely ionized at pH 10.9 and therefore cannot increase the light absorption at pH 11.38. The other *accessible* tyrosines are either not ionized at the latter pH, or their ionization is suppressed by the negative charge due to binding many dodecyl sulfate ions.

At pH 11.38 the *unfolding* also is substantially larger than at pH 10.9, about 77% rather than 47%, when measured at 100 equiv or more of detergent where the protective effect of smaller quantities has been outrun. This protective effect is maximal at 10–30 equiv; it seems to involve both suppression of ionization and of unfolding as in the data for pH 10.9.

The other data in Figure 4B, obtained by adding base a few minutes after adding dodecyl sulfate, show once more that the detergent unfolds the protein even at pH 5.6. The initial (zero time) optical densities are higher than when detergent and base are added together; therefore the unfolding due to the subsequent addition of base is much smaller, in fact al-

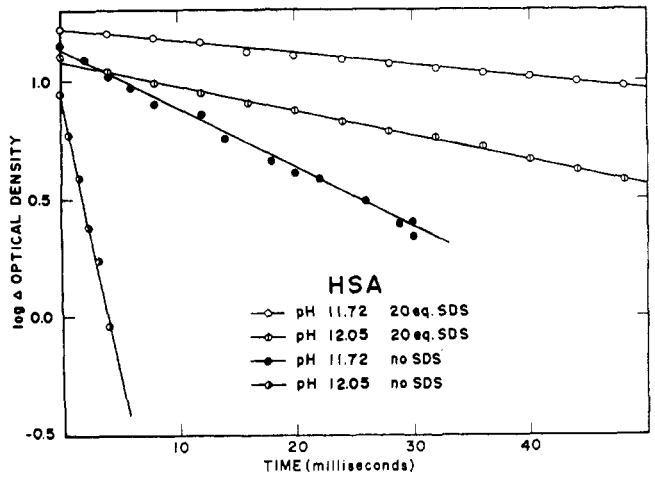

FIGURE 5. Kinetics of unfolding when the pH is suddenly raised from 5.6 to 11.7 or 12.05 plotted as a first-order process, in the absence of detergent and in the presence of 20 equiv of dodecyl sulfate.

most nonexistent at over 75 equiv of detergent. Sharp protective effects at 20 equiv of detergent are nevertheless very conspicuous in the data for both zero and infinite time.

Two sets of data are also given for pH 11.67 (panel 4C). Here the equilibrium values are almost identical at all values of detergent present. The zero time values are higher than those found at 11.38, at both the largest and smallest amounts of detergent, but the higher pH has no effect at quantities between 75 and 150 equiv. Thus, there appears to be a gradual suppression of ionization of phenolic groups as the result of accumulating negative charges; the latter do not affect the ionization brought about at pH 11.67 in the absence of detergent, and the suppressive effect appears to be lost when the protein is in the unfolded form. However, it is well to note again that the largest optical density at zero time, and hence the largest ionization of the accessible tyrosines, occurs in the total absence of detergent. This is not true at infinite time. There is a sharp inhibition of unfolding when the amount of detergent present is approximately 20 equiv. With no detergent, or with over 75 equiv, the optical density found corresponds to ionization of all the groups.[4]

At the highest pH investigated (12.09, see Figure 4D) the results are easily summarized. The zero time optical density, hence the dissociation of the accessible tyrosines, is only weakly dependent on the amount of detergent present. Suppression of ionization by the bound detergent anions reduces the optical density from about 0.41 with detergent absent to about 0.37.

The infinite time (unfolded) values at this pH are also nearly independent of the concentrations of dodecyl sulfate. There is no trace of a protective effect at 20 equiv; thus, pH 12.1 is able to unfold the protein to make all the tyrosines accessible, even in the presence of a protecting mechanism (see Discussion). In this case, no difference would be expected between the results obtained when the protein is exposed to dodecyl sulfate before raising the pH, and those obtained when base and detergent are added together.

Effect of Changing Detergent Concentration at Constant pH. Experiments in which the pH is not changed when detergent is added are necessarily performed at pH values high enough to permit some tyrosine ionization to occur, and low enough to avoid unfolding in the absence of dodecyl sulfate. Such experiments have been conducted at pH 9.7–10.9. At pH 9.7 where less than 5% of the accessible tyrosines are ionized, even the largest amounts of added detergent have caused no detectable unfolding. At 10.9 only minimal amounts of unfolding (time-dependent change in optical density) are observed.[5] Nevertheless it has already been shown that exposure of the protein to dodecyl sulfate solutions at pH 5.6 produces large amounts of unfolding, which can be demonstrated after the pH is raised (in our experiments to the range 11.38–11.71). One need not postulate that the changes produced by dodecyl sulfate ion at pH 5.6 make the protein more susceptible to unfolding at pH values well over 10, and would not occur if the pH were raised only to 9.7–9.9; it is much more plausible that the unfolding occurs, but cannot be observed with certainty when only a small fraction of the tyrosines ionize at pH values below 10. Thus, one sees a change in absorbance of only 0.02 when the pH is raised from 5.6 to 9.9. Even complete unfolding would add only about 0.016 absorbance unit. The results at pH 11.38–11.71 are far clearer because much more tyrosine ionization takes place.

Kinetics of Unfolding. Figure 5 shows typical experiments in which unfolding occurred when sodium dodecyl sulfate and alkaline buffer were added simultaneously. The time course of the reactions is complex, possibly because of the well-known microheterogeneity of the protein; an initial fast phase (50–80% completion) is followed by a slower phase. In Figure 5 only the initial phase is shown. The data can be resolved into the sum of two parallel first-order reactions, but the significance of this success is rendered doubtful by the fact that a third still slower phase must be invoked when the data are carried nearer to the equilibrium point. Much of the data are acceptably fit with a straight line empirically by plotting the minus 2.2 power of $(x_\infty - x)$, the reactable residue, against time, where x_∞ is the equilibrium value of the optical density (infinite time) and x is the optical density at time t. The slope of such a plot may be taken as a rate without attempting to assign any particular mechanism to the reaction, simply in order to describe how the unfolding rates depend on pH, dodecyl sulfate concentration, or other parameters.

A rate comparison based on the initial first-order rate is given in Figure 6 but use of the -2.2 power isotherm where applicable, would not change the comparison in the figure materially. Two features stand out: (a) there is only a small effect of pH below 11.4, and a very large effect above this value; and (b) stabilizing concentrations of dodecyl sulfate, particularly values near 20 equiv/mol of protein, greatly reduce the pH effect on rate of unfolding. Since at pH 12.1 the extent of unfolding at equilibrium depends on pH, and pH 12.1 produces a maximum unfolding effect on ionization even when 20 equiv of dodecyl sulfate is present, it is clear that the rates and equilibria do not run parallel, *i.e.*, there is an effect of detergent on the *refolding* as well as on the *unfolding* reaction. Stabilizing concentrations of detergent that reduce the rate of the unfolding process at a given pH, must also reduce the rate of the *refolding* reaction, although to a smaller extent. Finally, and not shown in the figure, stabilizing concentrations of detergent, although they may retard the rate of the initial fast phase, have a larger effect on the slower phase that follows. They may thus reduce the extent to which the data may be fit by a first-order process. Stabilizing concentrations reduce the velocity by a larger factor the higher the pH.

[4] There is a small reduction at 300 equiv.

[5] At pH 10.8, 100 equiv of detergent produce only a 7% time-dependent increase in absorbance.

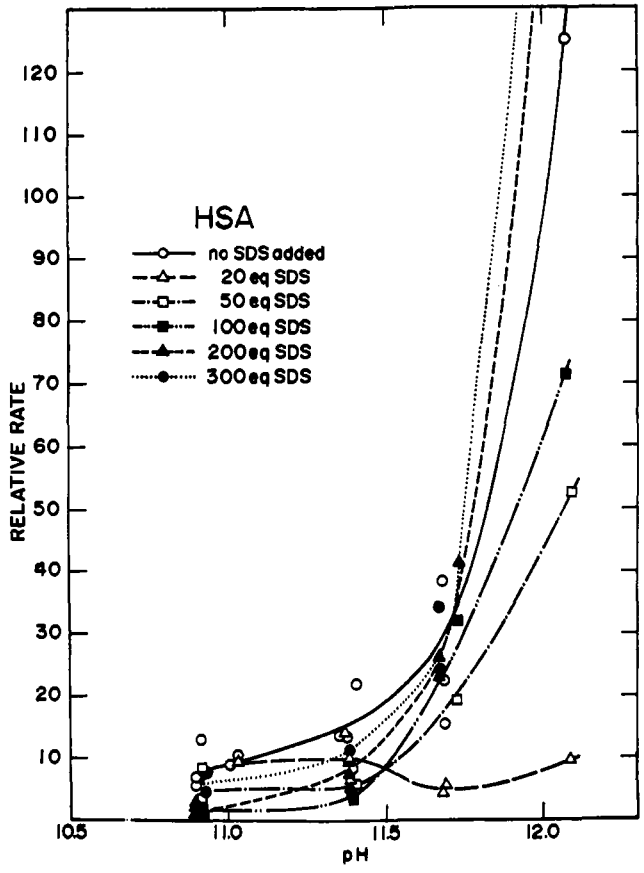

FIGURE 6: Dependence of the rate of unfolding on pH at a number of different concentrations of sodium dodecyl sulfate added at the same time that the pH is raised.

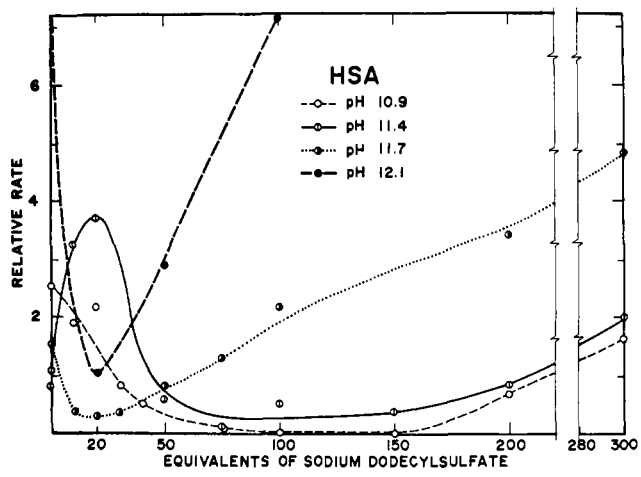

FIGURE 7: Dependence of velocity of denaturation on the number of equivalents of detergent present at four different pH values. The ordinate represents the slope of a linear plot of $(x_\infty - x)^{-2.2}$ against time in milliseconds.

Figure 6 shows directly the dependence of the rates at constant pH on the concentration of dodecyl sulfate. The highest concentrations of detergent always increase the rates, but the levels to which they do so are usually roughly those which are found when no detergent at all is present, except at pH 11.7 where they add to the pH effect. The protective effect (expressed in lowering this rate) is at a maximum at concentrations of 100–150 equiv at the lower pH values, and sharply at 20 equiv at the two higher pH values. With the two lower pH values 20 equiv produce a sharp *local minimum* in rates.

Effect of Ionic Strength. Repetition of most of the experiments at a reduced ionic strength (0.032 instead of 0.24) gave smaller amounts of ionization and smaller amounts of unfolding. The results are such as would be produced by an upward shift to a higher pH of about 0.1 unit in the titration curve of the tyrosines; and by a larger reduction in the susceptibility to unfolding. At pH 11.67 in the absence of detergent the absorbance at equilibrium at low ionic strengths reaches only 0.41 whereas it is almost 0.60 at 0.24 ionic strength. The addition of less than 50 equiv of detergent reduces the absorbance still further, and only a slight increase, to approximately the value obtained with no detergent at all, is found with 200–300 equiv. The effects at low ionic strength are so small at pH values below 12.1 that no further effort has been made to analyze them here.

Effect of Temperature. A reduction in temperature to 14.6° resulted in approximately halving the rate of the unfolding reaction at a given pH. The pH of alkaline solutions is strongly temperature dependent so such a comparison cannot be made without changing the buffer. The initial absorbance values and the total time-dependent change in absorbance were both much reduced. Thus temperature affects both ionization and unfolding. Because of the temperature effect on solubility of sodium dodecyl sulfate results were obtained with concentrations of the latter up to 50 equiv only.

Comparison of Results with Human, Defatted Human, and Bovine Serum Albumins. Experiments similar to those with human albumin have been made with bovine albumin and with charcoal-defatted human albumin (Chen and Kernohan, 1967). When no detergent is present, or less than 10 equiv, defatted human albumin is unfolded about half as rapidly as our deionized human albumin preparations, but in the presence of higher concentrations of detergent, it is unfolded almost twice as rapidly. Bovine albumin is unfolded much more slowly than human albumin at 20–100 equiv of dodecyl sulfate, but at other concentrations the difference is not large.

Discussion

The effects of high pH on serum albumin are of at least two kinds: (a) accessible tyrosines are ionized, possibly in two sets; and (b) a reversible time-dependent change in conformation (which makes the other tyrosines accessible) is induced which procedes to an equilibrium endpoint which in turn depends on pH. The rate of unfolding always increases with increasing pH, but does so much more markedly at pH values over about 11.3–11.4 (at 25°). On the other hand, the effects of detergent are more complex: (a) binding detergent partially suppresses ionization of accessible tyrosines by shifting p*K;* (b) it inhibits or protects protein from unfolding when present in amounts below about 50 equiv; and (c) binding larger amounts causes the protective effect to disappear and may even enhance the unfolding effect of pH alone. Finally the effect of detergent on *rate* is quite complex (Figure 7).

Unmasking of hidden tyrosines at high pH is a now well-known phenomenon (see Steinhardt and Reynolds, 1969, Chapter 5; Tanford, 1959). Other hidden groups are also unmasked: disulfide and thiol (Anson, 1941; Mirsky, 1941) imidazoles (Beychok and Steinhardt, 1957; Breslow and Gurd, 1962), and occasionally carboxyl (Vijai and Foster, 1967).

The lower curve in Figure 1 (properly corrected) should represent part of the titration curve of the accessible tyrosines; the upper curve does *not* give the titration curve of all the

tyrosines since at the lower pH values unfolding is not complete. The lower curve does not extend to zero absorbance because some of the absorption at 243 nm is due to 1 tryptophan, 32 phenylalanines, and 16 cystines and does not change importantly with pH or detergent concentration (Polet and Steinhardt, 1968). At pH 8.2–9.3 this absorption is only about 0.14. If the difference (0.31) between 0.14 and 0.45 is accounted for by the accessible tyrosines, the pH at which one-half this difference (0.155 + 0.14 = 0.295) is realized, namely, 11.22, should be the apparent pK of the *accessible* groups if they occurred as a single set, *i.e.*, with a single intrinsic pK.[6] This is very high for a side-chain tyrosine—values of 9.9–10.3 are common (Steinhardt and Reynolds, 1969, Chapter 5). Avruch *et al.* (1969) have shown that the spectrophotometric titration curve of the tyrosines of bovine albumin is fully consistent with such a high pK, but that the pK drops to the more normal value 10.3 after the lysines are acetamidated. The other prototropic groups are essentially unaffected by acetamidation. Thus, if the acetamidation process does not itself alter the tertiary structure deeply—and it does not appear to do so in other respects—lysines and tyrosines must interact in the native protein in such a way as to prevent tyrosine from ionizing until lysine deprotonation is largely completed. If some of the lysine ϵ-ammonium groups and some of the carboxyl groups of the native protein are internally associated to stabilize the native structure, as envisioned by Foster (1960), this interaction may result in few or *none* of the tyrosines of bovine and human albumin being accessible when the protein is at pH values between 5 and 9 (about 50% of normal accessible tyrosines should ionize at pH 10). Since only about 10% do so it is possible that only about one-fifth of the tyrosines are free from a stabilizing interaction with the lysines at or near neutral pH. After acetamidation deprotonation of the modified lysine side chains does not occur at pH values below 12, but the longer side chain of the acetimidated lysine may be sterically incapable of interacting strongly with the charged carboxyl groups to stabilize as described above and postulated by Foster for normal lysine.

In the description of Figure 4B, it was suggested that a few of the accessible tyrosines were fully ionized at pH 10.9, *i.e.*, were completely normal. A division of accessible tyrosines into two classes similar to those discussed here is suggested by Latovitzki *et al.* (1971) on the basis of work with lysozyme. One noteworthy peculiarity of the ionization data presented in the present paper is the anomalously narrow range of pH in which the largest changes occur. Such "cooperativity" usually indicates conformation changes;[7] if it does so here, not all of the tyrosines we have labelled "accessible" (those responsible for the zero time curves) are actually accessible at pH <10.8.

The reversibility of the unfolding process is indicated by the existence of equilibria between intact and unfolded forms which depend on pH, dodecyl sulfate concentration, temperature, and ionic strength; and by direct regeneration experiments which will be reported elsewhere. The end points in either direction do not depend on the order in which the pH and detergent concentration are changed, although the initial state (zero time optical density) is greatly affected.

Numerous reports of protection of serum albumin against unfolding by binding small numbers of equivalents of hydrocarbons or detergents have been summarized by Steinhardt and Reynolds (1969, pp 117–121, 287–294); only part are cited here. Boyer *et al.* (1946) found that low concentrations of dodecyl sulfate protects human albumin against attack by urea and guanidine hydrochloride although the viscosity is raised by larger amounts, which also unmask disulfide groups, inaccessible in the native state. These observations were confirmed and enlarged by Markus *et al.* (1964) who concluded that protection was afforded only when the lysine side chains were protonated although the binding of dodecyl sulfate was little affected by this protonation. Aoki (1958) found that the acid isomerization (N → F) was prevented by binding small amounts of detergent. Numerous investigators found that fatty acids gave protection against urea or high temperature (Duggan and Luck, 1948; Markus and Karush, 1957), or acid (Aoki, 1958; Klotz and Heiney, 1957). Butane and pentane also afforded protection against acid (Wishnia and Pinder, 1964; Wetlaufer and Lovrien, 1964).

Lovrien (1963) determined the ionization of tyrosines spectrophotometrically, while following unfolding by means of changes in viscosity. Ten equivalents of sodium dodecyl sulfate was shown to shift the ionization of the tyrosines to higher pH, and also inhibit unfolding. The anomalous abrupt rise in the titration curve in the absence of detergent could be accounted for by reducing the w term in the Linderstrom–Lang titration equation from 0.025 to 0.014. *i.e.*, by a conformation change to a less compact form. The effect of the presence of detergent was not confirmed to a "pK shift" (a change in the wZ term due to the binding of anions), but also prevented the drop in w from 0.025 to 0.014, *i.e.*, changed the direction of the effect of the wZ term in this range. Thus, both a change in the position of the titration curve and an inhibition of unfolding were involved. The shift in titration curve when large amounts of detergent are bound was observed and analyzed by Vijai and Foster (1967); an analogous effect of fatty acids on the titration of the tyrosines has been measured by Zakrzanski and Goch (1968).

A mathematical model of a binding mechanism has been devised that predicts that binding small numbers of these ions will decrease the free energy of the native conformation, although the binding of larger amounts will lead, on the contrary, to total unfolding (Decker and Foster, 1966; Reynolds *et al.*, 1967).[8] Briefly, the model depends on the possibility on binding at many more sites on the unfolded than on the native protein. Small quantities stabilize because the binding on a limited number of sites on the native protein has a somewhat larger association constant than that of the binding on the more numerous sites on the unfolded or partially unfolded forms.

In accordance with this model, the binding of large amounts of detergent (possibly at $\bar{\nu}$ over 12, certainly at $\bar{\nu}$ over 40) should cause extensive unfolding at any pH at which such binding can occur (Reynolds *et al.*, 1970). The present investigation confirms this conclusion by making visible at, *e.g.*, pH 11.7, the exposure of tyrosines caused by unfolding at pH 5.6 when detergent is added before base. However, the amounts of additional unfolding produced when even 200–300 equiv of detergent are added without a rise in pH is paradoxically small, *i.e.*, the optical density rises by about 10–15%

[6] They do not occur as one set. The apparent pK of the second and larger set is 11.33.

[7] The appearance of cooperativity results when the first dissociation induces a conformation change which increases the number of dissociating groups (Steinhardt and Reynolds, 1969).

[8] This model has been extended to the case of two-stage unfolding such as is found in the albumins (Steinhardt *et al.*, 1972; Steinhardt *et al.*, unpublished data).

where up to 50% should be expected from analysis of the isotherm. Further work is in progress to determine whether this apparent paradox is an indication that the model is incomplete.

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Spectroscopic and Magnetic Studies of Iron(III) Phosvitins†

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ABSTRACT: Studies of the binding of iron(III) by phosvitin, the phosphoglycoprotein of avian egg yolk, have led to the isolation of two derivatives, green and brown iron(III) phosvitins, that contain 6.9 (±0.2)% by weight of iron. The coordination structure of the bound iron(III) in each form has been investigated by infrared and visible absorption spectroscopy, electron paramagnetic resonance (epr), and the temperature dependence of the magnetic susceptibility. In the green form of iron(III) phosvitin the coordination structure of the great majority of the bound iron(III) ions is tetrahedral, $[Fe(III)O_4]_{tet}$. The available oxygen-donor ligands are most probably contributed by the numerous serine phosphate residues that are deduced to be arrayed in the β structure of pleated sheets. The magnetic and epr data indicate that antiferromagnetic coupling extends among several iron(III) ions in polynuclear clusters. The brown form of iron(III) phosvitin also binds iron(III) ions in large polynuclear clusters but, unlike the green form, the coordination structure of most of the iron binding sites is octahedral.

Phosvitin is a phosphoglycoprotein of mol wt 35,000 (Taborsky and Mok, 1967) containing 6.5% carbohydrate and a large number of phosphoserine residues (side chain: $-CH_2-OPO_3H_2$) arranged, at least in part, in linear sequences of up to eight residues uninterrupted by other amino acids (Joubert and Cook, 1958; Shainkin and Perlmann, 1971). The interaction of iron with phosvitin previously has been investigated with respect to a postulated rearrangement of the structure

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