is no pertinent evidence relating directly to changes in charge distribution. The alterations in dipole moment which take place could be entirely the result of a change in shape. On the other hand there is increasing evidence for unusual interaction of charges in proteins which may be sensitive to the physiological state of the protein. The classical picture of Debye in which a rigid sphere of fixed dipole moment rotates in solution no longer appears entirely satisfactory. Such a molecular picture is particularly inconsistent with Jacobsen's dielectric studies[22] on folded poly acid–base macromolecules. When these molecules in solution were fully oriented in an annulus across which there was a high shear gradient, the dielectric properties were the same whether measured across the long axis or across the short axis of the molecules. He also made comparisons between the dielectric relaxation time and rotational relaxation times obtained in other ways and found poor agreement. As a substitute for the rotational picture of Debye, he suggested that proteins have a large effect in organizing the structure of water and that it is this structure as modified by the protein which is seen in dielectric experiments. Recently Kirkwood and Shumaker[23] have proposed an entirely new molecular picture for proteins in which the response of the permanent dipole moment, if any, to an applied field is equalled or overwhelmed by proton fluctuations following the field. Since there are more basic sites in proteins than bound protons, the protons may migrate from site to site toward the end of the protein at which the negative field intensity is the greatest. This is somewhat similar to the Maxwell–Wagner theory in which the ionic atmosphere surrounding a polyelectrolyte migrates in an applied field. Another application of the Maxwell–Wagner theory to a polyelectrolyte recently has been made by Dintzis, *et al.*[23,24]

(22) B. Jacobsen, THIS JOURNAL, **77**, 2919 (1955).

(23) J. G. Kirkwood and J. B. Shumaker, *Proc. Nat. Acad. U. S.*, **38**, 855 (1952).

(24) H. M. Dintzis, J. L. Oncley and R. M. Fuoss, *ibid.*, **40**, 62 (1954).

Since the fluctuations of charge should also result in interprotein interactions, it has been possible to seek some verification of the Kirkwood–Shumaker theory from studies of these effects. Timasheff and co-workers[25] report some experimental confirmation of charge fluctuation as a source of protein interaction from light-scattering studies. It seems possible that a change in protein charge distribution on oxygenation might influence the charge fluctuation phenomenon and thus appear as a change in effective dipole moment. On the other hand the relaxation process in this theory seems to be attributable to the formation or dispersion of the hydration shells of the charges as they move about, and it is not at all clear how a change in charge distribution would alter the relaxation time. Unfortunately the testing of the various theories and the application of the newer ones to dielectric phenomena are not simple nor well advanced. It is thus not possible at present to interpret the behavior of hemoglobin in terms of a well-established picture of molecular events. In any event the experimental results reported here appear to be reproducible and free of artifacts. Furthermore at least two kinds of hemoglobin demonstrate the same behavior and thus serve to suggest a generality for the phenomenon under observation. It thus appears for at least one protein that physiological function is associated with drastic modifications in the properties of the solutions of that protein.

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(25) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, THIS JOURNAL, **79**, 782 (1957).

MINNEAPOLIS 14, MINN.

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA]

Dielectric Properties of Hemoglobin. III. Carbon Monoxide Addition to Horse Hemoglobin

BY SHIRO TAKASHIMA AND RUFUS LUMRY

RECEIVED AUGUST 28, 1957

The dielectric parameters have been determined for horse hemoglobin through the frequency region of anomalous dispersion as a function of the degree of saturation with carbon monoxide. The dipole moment of carboxyhemoglobin was 410 debye in excellent agreement with Oncley's value, but the mean relaxation time τ_0 was 10^{-7} sec., 15% larger than reported by Oncley. The real and imaginary parts of the dielectric constant plotted against each other formed good semi-circles in agreement with the equation of Cole and Cole. As carbon monoxide was added, the dielectric quantities showed the pattern of maxima and minima previously reported from oxygenation studies. The pattern was clearly marked in all parameters at 15° but only detectable in τ_0 at 25° and not in the dielectric increment or distribution parameter for relaxation time α. At 15° there are maxima at about 35 and 75% saturation and a minimum at about 55%. The maximum to minimum differences are considerably smaller than observed in oxygenation experiments. The distribution of relaxation times is broad at 15° and narrow at 25°. The difference in τ_0 at the two temperatures is not consistent with the difference in solvent viscosity.

It is well known that the reactions of hemoglobin with carbon monoxide are qualitatively very similar to those with oxygen.[1] In previous papers we already have presented evidence that large changes of unknown molecular nature occur during the oxygenation of hemoglobin.[2,3] These changes pro-

(1) R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949.

(2) S. Takashima, THIS JOURNAL, **78**, 541 (1956).

(3) S. Takashima and R. Lumry, *ibid.*, **80**, 4238 (1958).

duce a distinct series of maxima and minima if one plots against the oxygen partial pressure the real part of the dielectric increment ϵ', which is related to the square of the dipole moment, the mean dielectric relaxation time τ_0, or the Cole–Cole parameter for the distribution of relaxation times α. Qualitatively the pattern is the same for horse and bovine hemoglobin. It is thus of some interest to determine whether or not similar variation in the dielectric parameters occurs during the addition of carbon monoxide. In this article we demonstrate that such is indeed the case for horse hemoglobin. The effects are considerably weaker than produced by oxygen but still reliably detectable even with the considerable errors of our particular bridge method for dielectric measurement.

Experimental

Preparation of the crystalline protein and the protein solutions has been described.[3] The final protein solutions contained from 10 to 15 grams of protein which was determined by weighing aliquots dried at 110°.

The hemoglobin solution was evacuated for 20–30 minutes and then exposed to a continuously flowing mixture of carbon monoxide and nitrogen. The solution was equilibrated with this mixture for 1–1.5 hours as required to secure constant bridge readings. After readings had been made at the several frequencies the optical absorption coefficients were determined rapidly at 640, 655 and 670 mμ in the presence of air. The degree of carbon monoxide saturation was established by comparison of these absorption coefficients with those for the carbon monoxide and oxygen forms of hemoglobin on the assumption that oxygen addition does not affect the carbon monoxide already bound to the heme groups. This assumption is justified if the optical measurements are made rapidly since the rate of desorption of carbon monoxide is very slow. Complete evacuation of the samples for short periods produced no change in the amount of carbon monoxide bound nor did the presence of oxygen accelerate the rate of desorption.

The impedance bridge and dielectric cell have been discussed, as has the method of their use.[3] Temperatures were held to ±0.2°. The solutions were unbuffered but contained KCl to about 10^{-5} *M*.

Results

In Fig. 1 are plotted the capacitance data for carboxyhemoglobin as determined in these experiments at 15 and 25° and as determined by Oncley[4] at 25°. In this figure the ordinate is $(\epsilon' - \epsilon'_\infty)/(\epsilon_0' - \epsilon_\infty')$, in which ϵ_0' and ϵ_∞' are the (real) dielectric constants at frequencies well below and well above the dispersion region. The agreement between the two experiments at 25° is good though our data yield a curve of slightly more abrupt sigma form. Oncley reported a dielectric increment, $\Delta\epsilon'/g = (\epsilon_0' - \epsilon_\infty')/g$, of 0.44, which is within the error of our mean value of 0.42. The critical frequency given by Oncley is 1.9 mc. which may be compared with 1.7 mc., the mid-point of curve b.

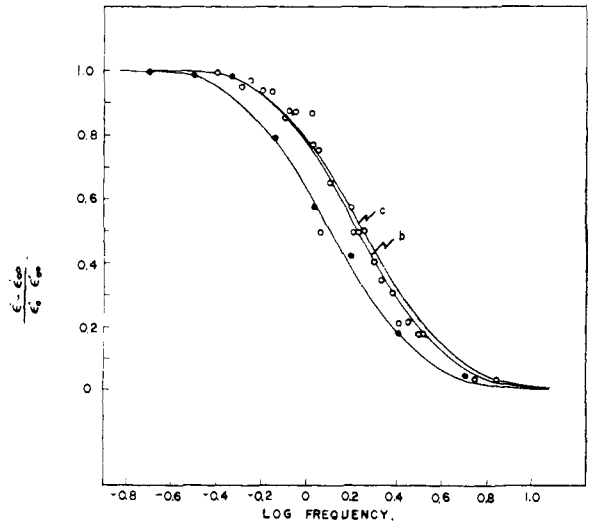

Fig. 1.—Dispersion of capacitance *versus* logarithm frequency for horse carboxyhemoglobin. Curve a is at 15°; curve b at 25°; curve c is that given by Oncley for 25° data.[7]

A typical Cole–Cole plot for carboxyhemoglobin at 25° is shown in Fig. 2 (see also Fig. 5). It is

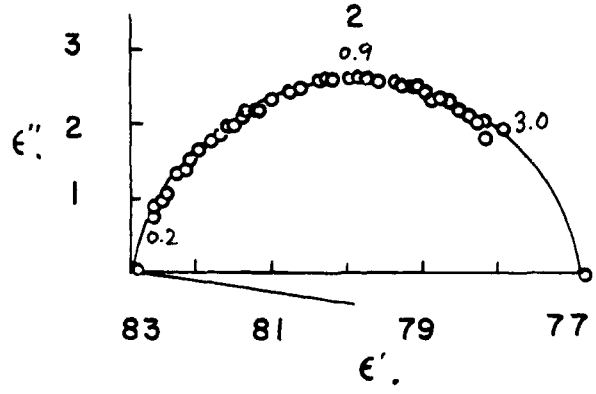

Fig. 2.—A typical Cole–Cole plot for horse carboxyhemoglobin at 25°. The small numbers are the frequencies in megacycles.

apparent that these plots adequately describe the data. The fit was particularly good with carboxyhemoglobin at 25° and was found equally satisfactory with other carbon monoxide pressures at this temperature. Points at high frequencies are omitted since they were not within the range of reasonable precision of the bridge. The very high frequency points were calculated in the manner previously described.[3] For carboxyhemoglobin α is 0.20 and 0.12 at 15 and 25°, respectively. These values suggest that the distribution of relaxation times is relatively narrow though considerably larger than a single such time in which case α would be zero. Oncley did not estimate α, but a rough comparison may be made on the basis of capacitance data from the shapes of the curves in Fig. 1. The shapes of these curves are dependent on α and since all three curves have about the same shape, α must have been of about the same size.

The principal concern in these experiments has been with the variation of the dielectric parameters as the degree of saturation with carbon monoxide is varied. The variation of dielectric increment and τ_0 observed at 25° is given in Fig. 3; that at 15° in Fig. 4. The pattern of maxima and minima is considerably less pronounced at 25° than at 15°. The dielectric increment is constant within experimental error at the higher temperature but the relaxation times at that temperature do show two maxima at about 25 and 75% saturation. The pattern is reliably presented in both parameters by the 15° data. The exact positions

(4) J. L. Oncley, *ibid.*, **60**, 1115 (1938).

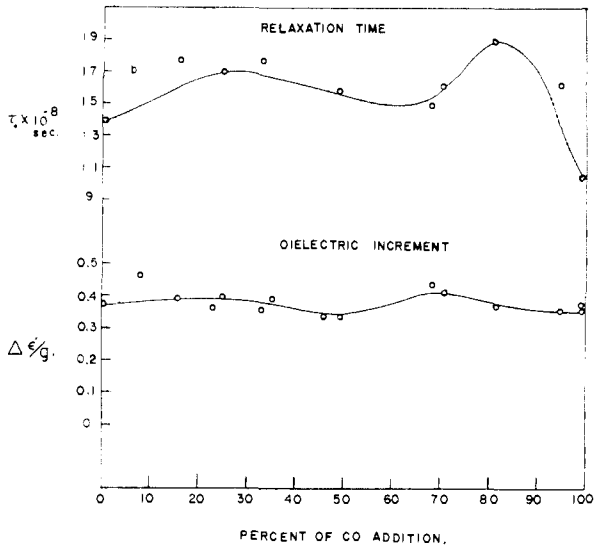

Fig. 3.—The variation of dielectric increment, $\Delta\epsilon'/g$, and mean relaxation time, τ_0, as a function of the degree of carbon monoxide saturation of horse hemoglobin at 25°.

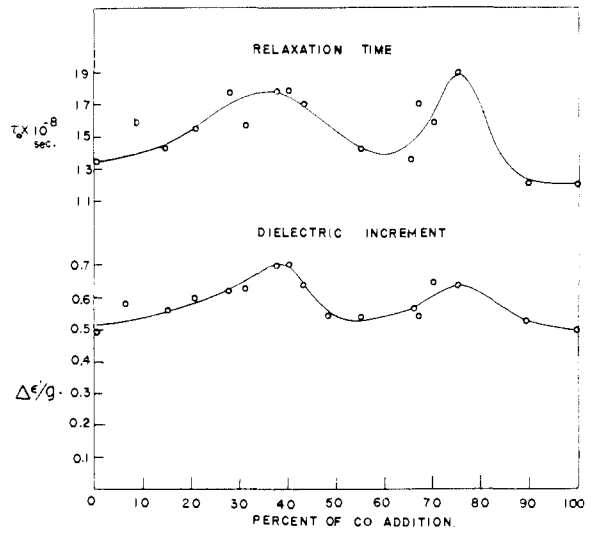

Fig. 4.—The variation of dielectric increment, $\Delta\epsilon'/g$, and mean relaxation time, τ_0, as a function of the degree of carbon monoxide saturation for horse hemoglobin at 15°.

of the extrema are not established readily for carbon monoxide compounds because the total variation is small. The extrema in dielectric increment and relaxation time curves occur at sufficiently similar degrees of carbon monoxide saturation to make their patterns of change identical within present errors. The pattern is the same as observed on oxygenation though the minimum to maximum variation is considerably smaller. The maximum and minimum values of the dipole moment and relaxation time from the oxygen[3] and carbon monoxide studies are given in Table I. The two maxima appear at about 35 and 75 to 80% saturation and the minimum at 55 to 60%. These values may be compared with 25, 75 and 50% obtained for the several extrema in oxygenation experiments.

Values of the dielectric quantities for points near the maxima are quantitatively less reliable than other points as a consequence of the long extrapolations to low and high frequencies which are necessitated by the broad distribution of relaxation times at these points.

TABLE I

HORSE HEMOGLOBIN. THE MAXIMUM AND MINIMUM VALUES OF THE DIPOLE MOMENT AND RELAXATION TIME AT 15°

	Dipole moment, debye		Relaxation time 10^{-8}, sec.	
	Max.	Min.	Max.	Min.
Oxyhemoglobin	750	380	61	10
Carboxyhemoglobin	510	430	18	10

Cole–Cole plots of data secured at several degrees of saturation are given in Fig. 5. The variation in α with degree of carbon monoxide saturation is shown for the two temperatures in Fig. 6. Again the effects are much larger at the lower temperature. The minima and maxima occur at somewhat different degrees of saturation than those of dielectric increment and relaxation time. This is particularly true at the second maximum.

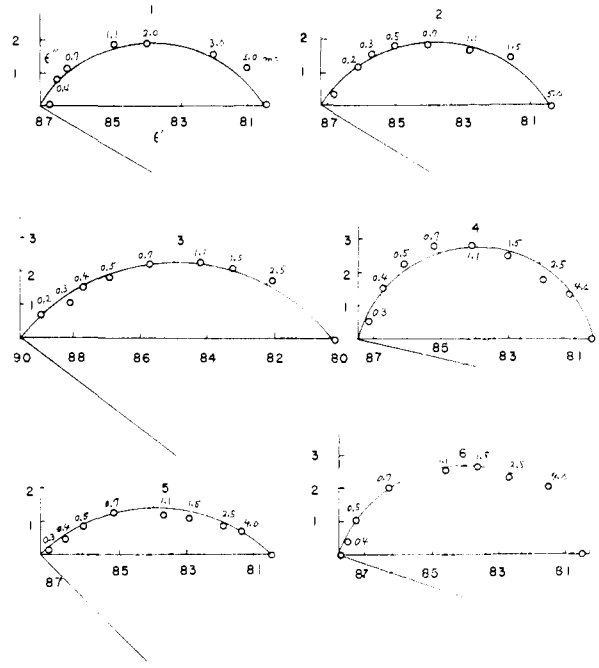

Fig. 5.—Cole–Cole plots of carboxyhemoglobin data at several levels of carbon monoxide saturation: (1) 0%; (2) 14%; (3) 37%; (4) 50%; (5) 70%; (6) 100%. The small numbers on the graph are the frequencies in megacycles. The temperature was 15°.

The dielectric parameters and dipole moments are listed in Table II for representative points near the extrema. Especially noteworthy are the small values of α at 25° for all degrees of saturation.

Direct comparison of the relative effects of oxygen and carbon monoxide on the dielectric increment are provided by Fig. 7 for which instead of adding carbon monoxide to unoxygenated hemoglobin, that gas was added to oxyhemoglobin. If the two gases produced effects of the same size, the

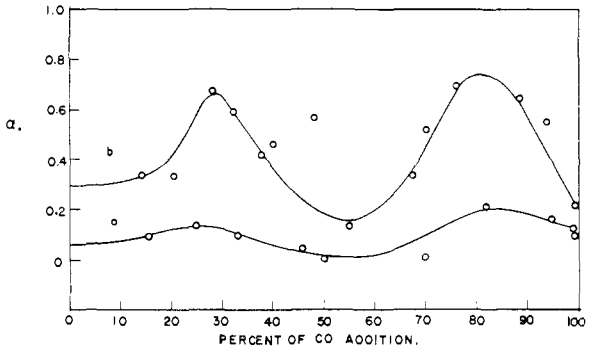

Fig. 6.—The variation in the distribution parameter for relaxation times, α, as a function of the degree of saturation with carbon monoxide: curve a, 25°; curve b, 15°.

curve would be approximately flat. The height of the curve at each point gives a direct measure of the difference in effectiveness of the two gases in producing the dielectric changes. As may be seen, oxygen is most effective relative to carbon monoxide at the two peaks and least at the minima.

TABLE II

SUMMARY OF THE DIELECTRIC PARAMETERS AND DIPOLE MOMENTS OF HORSE HEMOGLOBIN ON CARBON MONOXIDE ADDITION AT 15 AND 25°

CO, %	$\Delta\epsilon/g$	μ, debye	$\tau_0 \times 10^{-8}$, sec.	α
		15°		
0	0.50	430	13.4	..
28	.62	480	17.7	0.68
38	.70	510	17.8	.41
48	.53	450	..	.59
70	.65	490	15.9	.52
100	.50	430	12.1	.21
		25°		
0	0.41	400	14.2	..
25	.40	390	17.0	0.13
50	.36	370	15.9	.0
71	.41	400	16.2	.04
82	.35	370	19.2	.21
100	.41	400	10.0	.12

Discussion

Studies of the dielectric properties of hemoglobin previously have been confined to the form fully saturated with carbon monoxide. Studies by Errera[5] and by Arrhenius[6] predated the more complete investigation of Oncley[4] and of Shack.[7] Oncley's dipole moment was 410 debye as compared with our mean value of 400 debye. The relaxation times are 8.5×10^{-8} sec. according to Oncley and 10^{-7} sec. from Table II. The disparity in relaxation times is not surprising since our precision is not high and in neither investigation was it possible to cover the full frequency range in which anomalous dispersion takes place. Differences in age and method of preparation of the hemoglobin could also be reflected in the different values.

(5) J. Errera, *J. chim. phys.*, **29**, 577 (1932).
(6) S. Arrhenius, *Physik. Z.*, **39**, 559 (1938).
(7) J. Shack, Ph.D. dissertation, Harvard University, 1939.

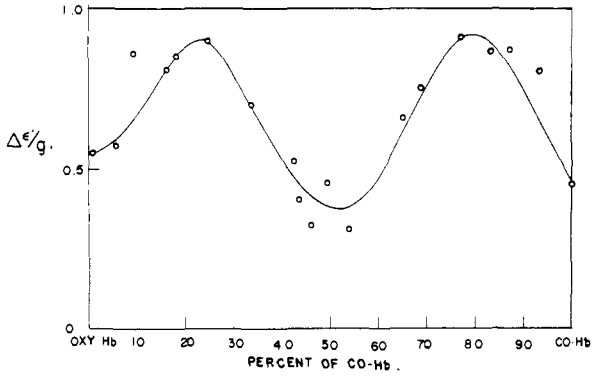

Fig. 7.—The dielectric increment change on replacing oxygen by carbon monoxide; horse hemoglobin at 25°. All hemoglobin is present either as oxyhemoglobin or carboxyhemoglobin. 100% CO addition means that bound O_2 has been replaced completely by CO.

Although Shaw, *et al.*,[8] found their data on β-lactoglobulin were well fitted by Cole–Cole plots, Oncley's hemoglobin data cannot be fitted even approximately by such plots. His capacitance data (ϵ') have been shown to be very similar to ours in Fig. 1; his conductance data (ϵ'') are very different.

The pattern of change in the parameters on addition of carbon monoxide is sufficiently sharply defined at 15° (Figs. 4 and 6) to be compared with that of oxygenation experiments.[3] It is quite clear that the qualitative patterns are identical. Though the oxygen-produced effects are considerably greater than those produced by carbon monoxide (Fig. 7), it may be concluded safely that the similarity which exists between the oxygen and the carbon monoxide compounds of hemoglobin is carried over into the dielectric behavior.

The general fit of Cole–Cole plots to the data at both temperatures is quite satisfactory and lends confidence to the measured values of α obtained from these plots. The minimum value of α at both temperatures appears at the central minimum but is significantly smaller at the higher temperature. Increased temperature not only diminishes the maxima and minima relative to each other but also sharpens the distribution of relaxation times. Thus, at the higher temperature the protein appears to be nearly homogeneous dielectrically throughout the range of carbon monoxide pressures despite the fact that there must be a number of hemoglobin species with different numbers of bound carbon monoxide molecules present at intermediate pressures.

Comparisons of the relaxation times at the two temperatures is illuminating. On the basis of the Debye picture of dielectric relaxation as a molecular rotation process, the ratio of the mean relaxation times at the two temperatures should be the same as the ratio of the solvent viscosities if the solutions are dilute. The latter ratio for water at 15 and 25° is 1.275.[9] The ratio of the relaxation

(8) T. M. Shaw, E. F. Jansen and H. Lineweaver, *J. Chem. Phys.*, **12**, 439 (1944).
(9) O. D. Hodgman, editor "Handbook of Chemistry and Physics," 35th Ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1954, p. 1993.

times for carboxyhemoglobin at the two temperatures is 1.21 (Table II). However even the approximate agreement here is misleading since the ratio of relaxation times at other degrees of carbon monoxide saturation is never this large and frequently less than 1. At zero saturation, for example, the ratio is 0.95. Thus the temperature dependencies of the other molecular processes involved hide the effect of temperature on viscosity if indeed the phenomena under consideration are viscosity dependent.

As in the case of oxygen control, the dielectric parameters undergo changes only in the range of pressures at which hemoglobin adsorbs the added gas. At higher pressures, above saturation, the parameters remain constant. The changes with both gases are thus linked directly to the compound-forming reactions. As in the case of oxygen, it would be attractive to relate the minima and maxima to the various intermediate compounds of carbon monoxide with hemoglobin. In the first paper of this series[2] Takashima was able to predict the oxygenation isotherm with some accuracy from dielectric increment changes. However, reasons have been given to doubt the validity of this treatment[3] and they apply with equal strength to carbon monoxide uptake.

The mechanism whereby carbon monoxide controls the dielectric properties of hemoglobin is no better understood than that through which oxygen exerts control. The several possibilities have been discussed for oxygen and may be extended to the case of carbon monoxide.[3]

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MINNEAPOLIS 14, MINN.

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

Reactivity of Sulfhydryl and Disulfide in Proteins. III. Oxidation with Ferricyanide of Sulfhydryl in Native and Denatured Bovine Serum Albumin

BY I. M. KOLTHOFF AND ADA ANASTASI[1]

RECEIVED MARCH 8, 1958

Sulfhydryl in native bovine serum albumin (BSA) is not oxidized by ferricyanide (Feic), but it is after denaturation with guanidine hydrochloride (GHCl) or urea. Since the oxidation of BSA with Feic is unspecific for sulfhydryl, other groups being also oxidized, the oxidation of sulfhydryl was followed by allowing the denatured BSA to react with an excess of Feic and then determining the remaining sulfhydryl by amperometric mercurimetric titration. The rate of oxidation is faster at *p*H 9 than 7 and at the same *p*H faster in 4 *M* GHCl than in 8 *M* urea. In the presence of these denaturing agents the reaction is greatly accelerated by copper(II) in a concentration equimolar to that of protein. Additional evidence of disulfide(dimer) formation is derived from viscosity measurements. Upon fivefold dilution of a freshly prepared denaturation mixture of *p*H 7 or 9, the sulfhydryl is no longer oxidizable with ferricyanide; in this respect the denaturation is reversible.

Several authors[2-5] have described changes in reactivity of sulfhydryl and disulfide[6] groups upon denaturation of proteins. In a previous study[7] with bovine serum albumin (BSA) it was pointed out that the reactivity of sulfhydryl groups may vary with the kind of reagent used. Upon denaturation of BSA in 4 *M* guanidine hydrochloride (GHCl) solution the reactivity of sulfhydryl with mercaptide binding agents (silver nitrate, mercuric chloride) remains unchanged. These experiments have now been repeated in 8 *M* urea solution and the same results were obtained. For example, after 5 hr. of denaturation at *p*H 7 and 9 at 25°, 0.68 mole of sulfhydryl per mole of albumin was titrated. On the other hand, sulfhydryl in solutions of native BSA was found stable toward oxygen, while in 4 *M* GHCl it was oxidized to disulfide, the rate of reaction being greater at *p*H 9 than at *p*H 7. In the present paper are reported results on the oxidizability with ferricyanide (Feic) of sulfhydryl in BSA in the native state and in the denaturing media which were 4 *M* in GHCl or 8 *M* in urea.

In preliminary work it was shown that sulfhydryl in low molecular weight compounds, like cysteine and β-mercaptoethanol, can be titrated amperometrically at the rotated platinum electrode (RPM) with ferricyanide. At *p*H 7 to 9 the reaction is rapid and the end-point is detected with satisfactory accuracy and precision. Upon application of this method to denatured BSA solutions, results were obtained which depended upon the speed of addition of Feic. The reason for the variation of the results, which has been recognized by previous workers,[8-10] is that Feic is not specific for sulfhydryl and can oxidize other groups in the protein molecule during its reaction with sulfhydryl. Efforts to improve the situation by accelerating the reaction by addition of one mole of copper(II) per mole of albumin were unsuccessful because the copper also acted as an accelerator of the side reactions.

Instead of determining the amount of Feic consumed, the amount of sulfhydryl which had not reacted with Feic was titrated successfully by the amperometric mercurimetric technique.[7] The final procedure which gives accurate and reproducible

(1) On leave from S. A. Farmitalia, Milano, Italy.

(2) M. L. Anson, *Advances in Protein Chem.*, **2**, 361 (1945).

(3) E. S. G. Barron, *Advances in Enzymol.*, **11**, 201 (1951).

(4) W. A. Belitser, Communications et Rapports au III^e Congres International de Biochimie, Bruxelles, 1–6 August, 1955.

(5) F. W. Putnam, "The Proteins," Vol. IB, Academic Press, Inc., New York, N. Y., 1953, p. 807.

(6) I. M. Kolthoff, Ada Anastasi and B. H. Tan, THIS JOURNAL, **80**, 3235 (1958).

(7) I. M. Kolthoff, *et al.*, *ibid.*, **79**, 5162 (1957).

(8) A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **19**, 451 (1936).

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(10) A. E. Mirsky, *ibid.*, **24**, 709 (1941).