tion added. The ether solution was decanted from the precipitate and the ether removed under reduced pressure. The yellow viscous residue was distilled from a Hickman still at 0.07–0.08 mm. The fraction boiling at 106–107° was collected, yield 40 g. (69%). The color in the distillate, resulting from splashing of the residue, was removed by redistillation at 0.08 mm., $n^{25}D$ 1.5182.

Anal. Calcd. for $C_{12}H_{18}O_2$: C, 74.19; H, 9.34. Found: C, 74.43; H, 9.48.

Acid-catalyzed Cleavage of 2,3-Dimethyl-4-phenylbutane-2,4-diol (III).—Glacial acetic acid (8 ml.), 2 ml. of water, 0.6 ml. of concentrated sulfuric acid and 1.7 g. of III were mixed together and distilled through a 7-cm. glass helix packed column. The distillate coming over up to 98° was collected in a flask immersed in a salt–ice freezing mixture. The 2,4-dinitrophenylhydrazone of the distillate melted at 118–122° after two recrystallizations from 95% ethanol and gave a mixed m.p. with the 2,4-dinitrophenylhydrazone of acetone of 124–126°. When an acidified aqueous solution of the distillate and sodium nitroprusside was made alkaline with ammonia, a violet color appeared.

The undistilled portion was diluted with 35 ml. of water and extracted with 35 ml. of pentane. The pentane was washed with sodium bicarbonate solution and evaporated. The residue gave a semicarbazone melting at 220–221° identical with a benzaldehyde semicarbazone.

Cleavage of a Mixture of I and III.—A mixture of 1.1 g. of III and 2.0 g. of I was cleaved using the same procedure employed for III. The portion distilling up to 99° was collected. The 2,4-dinitrophenylhydrazone of the distillate melted at 121–144° after recrystallization from 96% ethanol.

1,2,2,3-Tetramethyl-1-phenyltrimethylene Oxide (IV).—A mixture of 43 g. (0.61 mole) of 2-methyl-2-butene and 56 g. (0.46 mole) of acetophenone was irradiated in a 120-cc. mercury vapor illuminator for 196 hours. The irradiation was discontinued every 24 hours and the tar on the coils removed. The excess 2-methyl-2-butene was removed and the unreacted acetophenone distilled off through a 23-cm. glass helix packed column at 70–72° (3.8 mm.). The residue was distilled through a semi-micro column at 0.05 mm. and the fraction boiling at 42° collected, $n^{25}D$ 1.5040, yield 3.8 g. (4.4%).

The infrared spectrum indicated the presence of small amounts of hydroxy and carbonyl compounds. These impurities were removed by passing a pentane solution of the product through a column of neutral alumina; principal infrared bands: 3.4, 6.0, 7.2, 7.3, 7.9, 9.38, 9.6, 11.15, 13.1, 14.3.

Anal. Calcd. for $C_{13}H_{18}O$: C, 82.06; H, 9.54. Found: C, 81.71; H, 9.41.

Acid-catalyzed Cleavage of IV.—When IV was added to 2,4-dinitrophenylhydrazine reagent a precipitate formed which melted at 246° after recrystallization from ethanol–ethyl acetate. No m.p. depression was observed when this precipitate was mixed with the 2,4-dinitrophenylhydrazone of acetophenone.

Compound IV (2.0 g.) was then hydrolyzed by the same method employed for III. The portion distilling up to 99° was collected. The distillate was acetaldehyde which gave a precipitate with 2,4-dinitrophenylhydrazine reagent, m.p. 149–154° after recrystallization from 95% ethanol.

***n*-Butyraldehyde-2-Methyl-2-butene Irradiation Product (V).**—A mixture of 196 g. (2.72 moles) of freshly distilled *n*-butyraldehyde and 174 g. (2.48 moles) of 2-methyl-2-butene was irradiated in a 600-cc. mercury vapor illuminator for 29 hours. The unreacted 2-methyl-2-butene was removed and most of the *n*-butyraldehyde distilled off through a 36-cm. glass helix packed column at atmospheric pressure. The residue was distilled through the same column at 22 mm. and the portion boiling at 59–61° collected, yield 23 g. (6.5%). This fraction was redistilled through a semi-micro column at 23 mm. (b.p. 62–64°), $n^{25}D$ 1.4178.

The infrared spectrum indicated the presence of small amounts of hydroxy and carbonyl compounds. These impurities were removed by passing a pentane solution of the product through a column of neutral alumina; principal infrared bands: 3.4, 6.8, 7.3, 9.5, 9.7, 10.4, 10.7, 11.6, 11.7.

Anal. Calcd. for $C_9H_{18}O$: C, 75.99; H, 12.76. Found: C, 75.79; H, 12.68.

Acid-catalyzed Cleavage of V.—Compound V was added to 2,4-dinitrophenylhydrazine reagent and the mixture allowed to stand for 10 minutes. The precipitate was filtered off and recrystallized twice from 95% ethanol, m.p. 120–121°, no m.p. depression when mixed with the 2,4-dinitrophenylhydrazone of *n*-butyraldehyde. The mother liquor was allowed to stand for 5 hours and the precipitate which formed recrystallized from 95% ethanol, m.p. 119–120°, no. m.p. depression when mixed with the 2,4-dinitrophenylhydrazone of *n*-butyraldehyde.

Compound V (2.0 g.) was hydrolyzed by the same method employed for III. No material distilled before *n*-butyraldehyde.

The completely precipitated 2,4-dinitrophenylhydrazone of V was dissolved in benzene and passed through a chromatography column filled with Florosil. All fractions melted at 120–121° after recrystallization from 95% ethanol and showed no m.p. depression when mixed with the 2,4-dinitrophenylhydrazone of *n*-butyraldehyde.

CAMBRIDGE, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

Spectrophotometric Study of the Reaction of Protein Sulfhydryl Groups with Organic Mercurials[1]

BY P. D. BOYER

RECEIVED APRIL 12, 1954

A procedure is described for the sensitive and rapid spectrophotometric measurement of the extent and rate of reaction of various organic mercury compounds, particularly *p*-mercuribenzoate, with sulfhydryl groups. The procedure with *p*-mercuribenzoate is based on the increase in absorbancy in the 250 mμ region accompanying mercaptide formation. The rate of reaction of the sulfhydryl groups of egg albumin and β-lactoglobulin is more rapid at *p*H 4.6 than at *p*H 7; the rate with egg albumin is markedly affected by the nature of the ions present. The reaction of *p*-mercuribenzoate with β-lactoglobulin at *p*H 4.6 in acetate buffer and with egg albumin at *p*H 7 in phosphate buffer follows the course of a second-order reaction. By variation of the experimental conditions three different types of sulfhydryl groups may be recognized in egg albumin.

Organic mercurial compounds are the most specific and sensitive reagents now available for reaction with sulfhydryl groups, and have been widely used in studies of the biochemical function of sulfhydryl groups since the pioneer researches of Hellerman with *p*-mercuribenzoate.[2] Quantitative studies of the reaction of *p*-mercuribenzoate

(1) Supported in part by a grant from the National Science Foundation. Paper No. 3191, Scientific Journal Series, Minnesota Agricultural Experiment Station. A preliminary report was presented at the 123rd meeting of the Am. Chem. Soc., Los Angeles, March, 1953. The technical assistance of Mrs. Madelyn Ferrigan is gratefully acknowledged.

(2) (a) L. Hellerman, *Physiol. Revs.*, **17**, 454 (1937); *Cold Spring Harbor Symp. Quant. Biol.*, **7**, 165 (1939); (b) L. Hellerman, F. P. Chinard and V. R. Deitz, *J. Biol. Chem.*, **147**, 443 (1943). (c) The designation *p*-mercuribenzoate has been used herein for compounds in solution derived from added *p*-chloromercuribenzoate; the mercury compound in various solutions may be combined principally with hydroxyl or other anions instead of chloride ion.

with sulfhydryl groups have been made by titration techniques using the nitroprusside test as a measure of the end-point,[3] and mercaptide formation with silver ion has been followed by amperometric titration.[4] While giving useful results, the titrimetric procedures have limitations of convenience, sensitivity and adaptability to measurement of reaction rates as compared with spectrophotometric procedures.

Study of possible spectrophotometric procedures for measurement of the reaction of *p*-mercuribenzoate with sulfhydryl groups were initiated because of the need for such a method in investigations of sulfhydryl group function in enzyme catalysis.[5] It

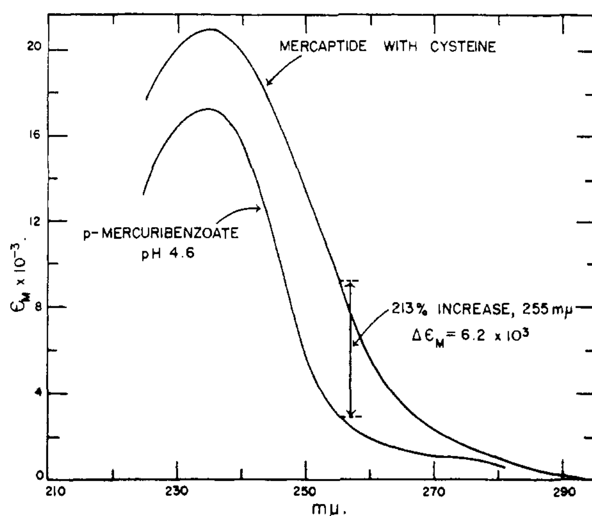

Fig. 1.—Absorbancy of *p*-mercuribenzoate and its mercaptide with cysteine in 0.33 *M* acetate, *p*H 4.6.

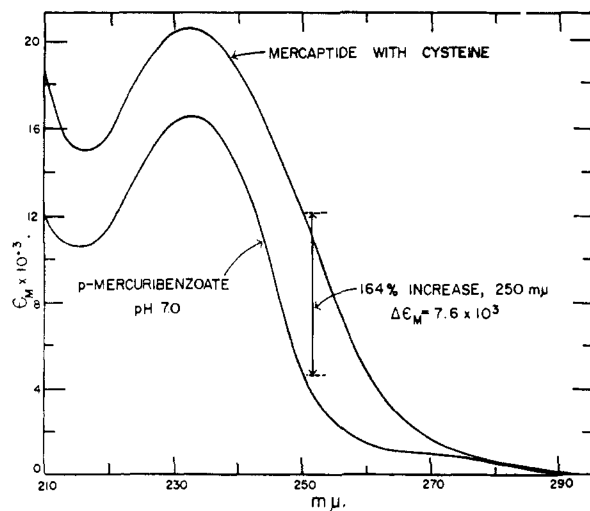

Fig. 2.—Absorbancy of *p*-mercuribenzoate and its mercaptide with cysteine in 0.05 *M* phosphate, *p*H 7.0.

was found that reaction of *p*-mercuribenzoate with sulfhydryl groups was accompanied by spectral shifts, and a useful procedure for measurement of the rate and extent of the reaction has been developed based on spectral shifts in the 250 mμ region. This paper describes the procedure developed and its applications to study of the properties of sulfhydryl groups of proteins.

Spectral Shifts.—Spectral shifts accompanying reaction of *p*-mercuribenzoate with an excess of cysteine at *p*H 4.6 and 7.0 are shown in Figs. 1 and 2. The absorption maximum of *p*-mercuribenzoate itself is increased and shifted slightly toward the visible by lowering the *p*H from 7.0 to 4.6. Further spectral shift occurs with additional *p*H decrease. Increase in the *p*H above 7.5 does not result in additional spectral change. Measurements in the region from *p*H 4.5 to 7.5 have been made in well-buffered media.

The relative increase in the absorption maximum at 230–235 mμ accompanying mercaptide formation is small compared to the increase in the 250 mμ region as indicated in Figs. 1 and 2. The increase in absorption in the 250 mμ region at *p*H 4.6 and 7.0 accompanying mercaptide formation has been found to be a linear function of the amount of sulfhydryl compound added for cysteine, glutathione, mercaptosuccinate and various proteins, with the exception that cysteine at *p*H 7.0 gives anomalous results. The data in Fig. 3 illustrate the linear relationship found with mercaptosuccinate and the apparently anomalous results with cysteine at *p*H 7.0; curve I on Fig. 4 shows the linear relationship obtained with cysteine at *p*H 4.6. Both substances gave close to the same total spectral shift. The presence of 0.5 *M* Na_2SO_4, which accelerated the reaction of *p*-mercuribenzoate and egg albumin as mentioned later, did not eliminate the anomalous results obtained with cysteine at *p*H 7.0. This suggests that at *p*H 7.0 when *p*-mercuribenzoate is in excess more than one *p*-mercuribenzoate may react with each cysteine moiety. The linearity of the plot of spectral shift against molarity of –SH compound up to the equivalence point for all compounds tested at *p*H 4.6 and for all but cysteine at *p*H 7.0 is indicative of the small dissociation of the mercaptides formed.

The increase in absorption with mercaptide formation was also found to be linear with β-lactoglobulin at *p*H 4.6 and with egg albumin at *p*H 4.6 and 7.0. Results at *p*H 4.6 are illustrated in Fig. 4; the figures on the curves indicate the moles of *p*-mercuribenzoate which reacted with 1 mole of the –SH compound at the equivalence point under the conditions used. The total spectral shift accompanying mercaptide formation has been found to be slightly different with cysteine, egg albumin, β-lactoglobulin, serum albumin and glyceraldehyde-3-phosphate dehydrogenase. This makes necessary the use of the total possible spectral shift for a given protein as a basis for calculation of the total moles of *p*-mercuribenzoate which react with one mole of protein.

The specificity of the spectral shift as a measure of the reaction with –SH groups is shown by the small additional spectral changes when *p*-mercuri-

(3) (a) M. L. Anson, *J. Gen. Physiol.*, **24**, 399 (1941); (b) L. R. MacDonnell, R. B. Silva and R. E. Feeney, *Arch. Biochem. Biophys.*, **32**, 288 (1951); (c) J. Fraenkel-Conrat, B. B. Cook and A. F. Morgan, *ibid.*, **35**, 157 (1952).

(4) I. M. Kolthoff and W. E. Harris, *Ind. Eng. Chem., Anal. Ed.*, **18**, 161 (1946); R. Benesch and R. E. Benesch, *Arch. Biochem.*, **19**, 35 (1948).

(5) P. D. Boyer and H. L. Segal, in W. D. McElroy and B. Glass, "A Symposium on the Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, 1954, p. 520.

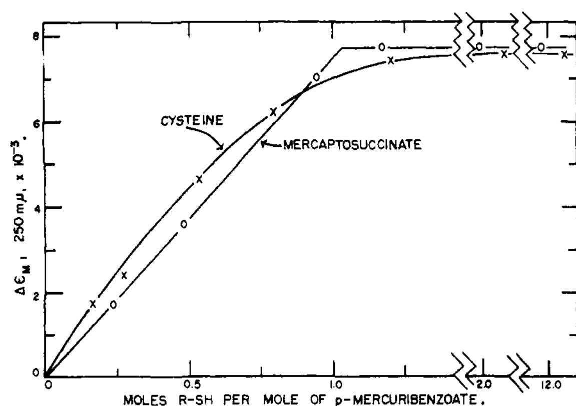

Fig. 3.—Increase in absorbancy at 250 mμ from reaction of 7 × 10^{-5} *M* mercuribenzoate with mercaptosuccinate or cysteine in 0.05 *M* phosphate, *p*H 7.0.

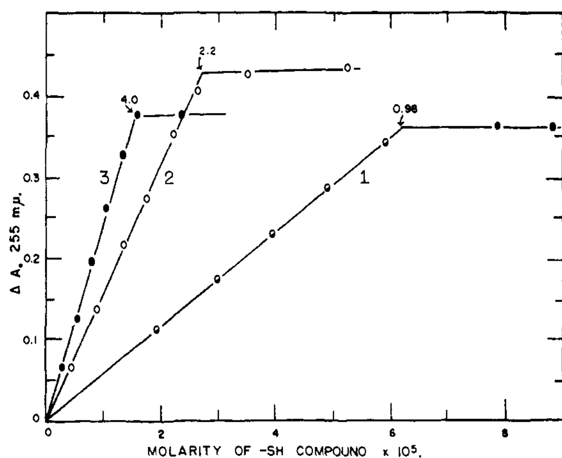

Fig. 4.—Increase in absorbancy at 255 mμ from reaction of 6 × 10^{-5} *M* *p*-mercuribenzoate with egg albumin (curve 3), β-lactoglobulin (curve 2) or cysteine (curve 1) in 0.33 *M* acetate, *p*H 4.6. Readings for cysteine and for egg albumin were taken approximately 15 minutes after mixing; those for β-lactoglobulin after 20 hours standing.

benzoate is added in excess of that required to react with the protein –SH groups. When a three-fold excess of *p*-mercuribenzoate was added to egg albumin at pH 4.6, there was no increase in the absorption at 250 mμ of the excess *p*-mercuribenzoate; at *p*H 7.0 with a four-fold excess, the excess *p*-mercuribenzoate showed a 6% increase in absorption. With a commercial bovine serum albumin preparation, the increase in absorption at 250 mμ of excess *p*-mercuribenzoate at *p*H 7 was 23%. Treatment of serum albumin with iodoacetamide and purification of the albumin nearly abolished the spectral shift of added *p*-mercuribenzoate. Thus although combination of *p*-mercuribenzoate with other groups or compounds in the protein preparations may occur, only the combination with the –SH groups results in the large increase in the molecular extinction coefficients at 250 mμ. Spectral measurements may thus serve to distinguish between combination of *p*-mercuribenzoate with –SH groups of other groups.

The interference of impurities in commercial bovine serum albumin and of ethylenediaminetetraacetate are outlined briefly in the Experimental section. Mercuric chloride up to 10^{-3} *M* produced little or no spectral increase at 220 mμ or above when mixed with excess glutathione.

For application of the procedure it is fortunate that the maximum absorption changes associated with mercaptide formation by *p*-mercuribenzoate occur in a region where there is a minimum in the ultraviolet absorption of proteins. Measurements may also be made of mercaptide formation with *p*-mercuriphenylsulfonate and phenylmercuric nitrate as shown in Fig. 5. However, with these compounds the maximum spectral shifts occur in a region where the absorption of proteins is considerably higher than at 250 mμ. *p*-Mercuriphenylsulfonate was used by Velick,[6] and has the advantage of greater solubility than *p*-mercuribenzoate.

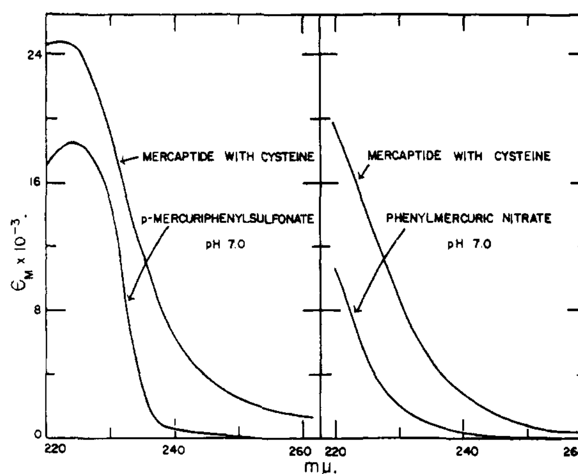

Fig. 5.—Absorbancy of phenylmercuric nitrate and *p*-mercuriphenylsulfonate and their mercaptides with cysteine in 0.05 *M* phosphate, *p*H 7.0.

Spectrophotometric measurements of mercaptide formation would be facilitated if compounds could be obtained which would show spectral shifts above 300 mμ associated with mercaptide formation. To this end, *o*-chloromercuriphenol and *o*-chloromercuri-*p*-nitrophenol were prepared. *o*-Chloromercuriphenol, which has an absorption maximum near 280 mμ showed a maximum increase in ϵ_M accompanying mercaptide formation with cysteine at *p*H 4.6 of only 0.75 × 10^3 in the region from 260 to 300 mμ. *o*-Chloromercuri-*p*-nitrophenol has an ultraviolet absorption maximum of 320 mμ in acid solution and 410 mμ in neutral solution. However, as shown in Fig. 6 the spectral shift accompanying mercaptide formation was small at *p*H 4.2; small spectral shifts were also obtained in neutral solution. These results distinctly limit the usefulness of *o*-chloromercuriphenol and *o*-chloromercuri-*p*-nitrophenol and probably of other related nitro derivatives for spectrophotometric measurements of mercaptide formation.

Ionic and *p*H Effects.—The rate of reaction of sulfhydryl groups of egg albumin with *p*-mercuribenzoate was markedly affected by the presence of various anions as shown by the results given in Figs. 7, 8 and 9. Increase of the *p*H above 7.6 decreased

(6) S. F. Velick, *J. Biol. Chem.*, **203**, 563 (1952).

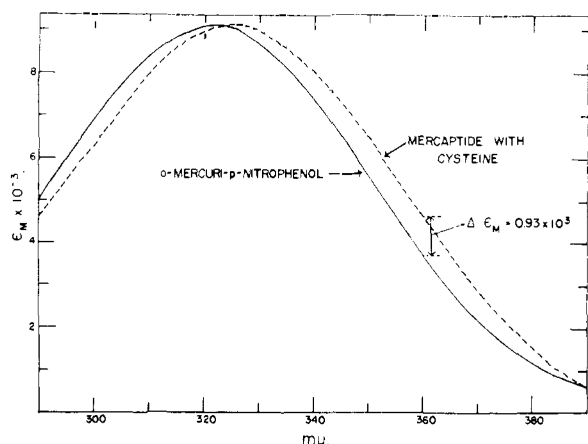

Fig. 6.—Absorbancy of *o*-mercuri-*p*-nitrophenol and its mercaptide with cysteine in 0.33 *M* acetate, *p*H 4.2.

the reaction rate somewhat as shown by comparison of the lower 3 curves and the curves for 0.5 *M* acetate at *p*H 7.6, 8.6 and 10.6 in Fig. 7. Figure 8 shows

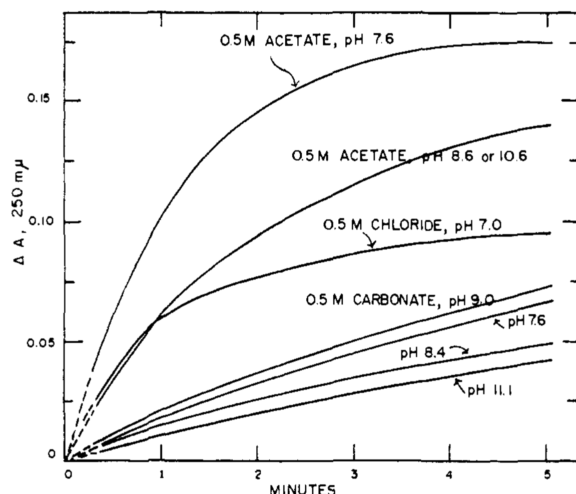

Fig. 7.—The effect of acetate, chloride, carbonate and hydroxide ions on the rate of reaction of *p*-mercuribenzoate with egg albumin. All solutions contained 0.67 × 10^{-5} *M* egg albumin, 10.4 × 10^{-5} *M* *p*-mercuribenzoate, 0.01 *M* phosphate and other sodium salts as indicated. The final *p*H was as indicated and the temp. 27°; the *p*H for the lower curves was adjusted with NaOH.

the effect of sulfate, phosphate, citrate and pyrophosphate ions and Fig. 9 shows the effect of increasing sodium sulfate concentration in the presence of 0.05 *M* phosphate on the reaction rate. The rate with 0.5 *M* sulfate in the presence of 0.05 *M* phosphate (Fig. 9) was greater than in the absence of the phosphate (Fig. 8).

In contrast to the results with egg albumin, the rate of reaction of β-lactoglobulin with *p*-mercuribenzoate was less in 0.5 *M* sodium sulfate, *p*H 6.8, than in 0.05 *M* phosphate, *p*H 7.0. Reaction of the -SH groups of β-lactoglobulin is much slower than those of albumin and the rate limiting step with β-lactoglobulin may well be configurational changes which need to precede reaction with *p*-mercuribenzoate.

The effect of ions on the reaction rate probably

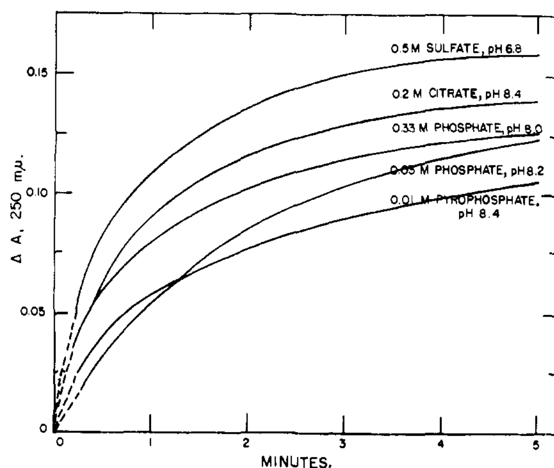

Fig. 8.—The effect of pyrophosphate, phosphate, citrate and sulfate ions on the rate of reaction of *p*-mercuribenzoate with egg albumin. All solutions contained 0.67 × 10^{-5} *M* egg albumin and 10.7 × 10^{-5} *M* *p*-mercuribenzoate, with sodium salts at the final *p*H as indicated, temp. 26°.

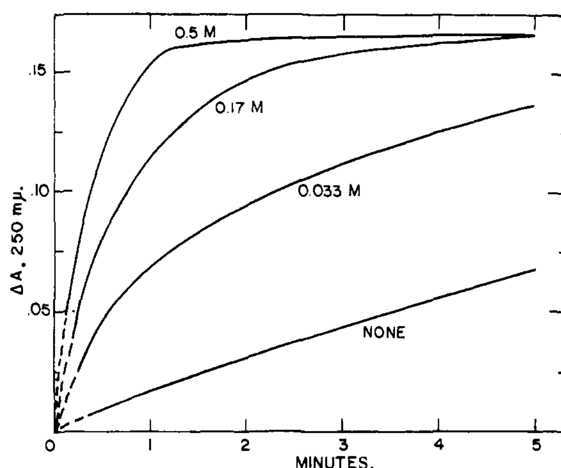

Fig. 9.—The effect of increasing sulfate concentration on the rate of reaction of *p*-mercuribenzoate with egg albumin. All solutions contained 0.67 × 10^{-5} *M* egg albumin, 9.5 × 10^{-5} *M* *p*-mercuribenzoate, 0.05 *M* phosphate (*p*H 7.0), and Na_2SO_4 as indicated; temp. 27°.

results in part from the displacement of an undissociated hydroxyl group from the mercury. That *p*-chloromercuribenzoate solubilized with dilute NaOH and brought to *p*H 8 exists chiefly as the undissociated hydroxide is shown by the observation that nearly two equivalents of acid per mole were required to titrate such a solution to *p*H 3.[7] Addition of pyrophosphate or sulfate to suspensions of *p*-mercuribenzoate at *p*H 7 to 8 will result in solubilization of the *p*-mercuribenzoate probably as a result of association of the anions with the mercury residue. The marked ability of *p*-mercuribenzoate and similar mercury derivatives to associate with various anions has been demonstrated by the polarographic studies of Benesch and Benesch.[8]

(7) Titration of the carboxyl group from *p*H 8 to 3 would be expected to consume only one equivalent of acid; the additional acid consumed is therefore attributable to titration of a hydroxyl group combined with the mercury.

(8) R. Benesch and R. E. Benesch, private communication.

The pH had a marked effect on both the rate and extent of reaction of egg albumin with p-mercuribenzoate. At pH 4.6 in 0.33 M acetate 4.0 moles of p-mercuribenzoate reacted rapidly with one mole of egg albumin (46,000 g.); at pH 7.0 in 0.05 M phosphate only 3.2 moles of p-mercuribenzoate reacted in 24 hours. Increase of the pH above 7.0 decreased slightly the rate but not the extent of reaction of the egg albumin. Decrease in pH from 7.0 to 4.6 markedly increased the rate of reaction of β-lactoglobulin with p-mercuribenzoate.

The reaction of the –SH groups of egg albumin at pH 7.0 in phosphate buffer or of β-lactoglobulin at pH 4.6 in acetate buffer with p-mercuribenzoate follows a second-order course, as shown by the data in Figs. 10 and 11. The second-order velocity constants were as indicated on the figures.

The close conformity of the reaction with p-mercuribenzoate at pH 7 of three –SH groups per egg albumin molecule to second-order kinetics indicates that under the conditions used the three –SH groups are homogeneous with respect to their reactivity with p-mercuribenzoate. That the three –SH groups do not react at the same rate under all conditions appears probable from the results obtained in the presence of 0.5 M NaCl (Fig. 7) and 0.1 M pyrophosphate (Fig. 8); a distinct decrease in reaction rate is evident after reaction of approximately $^1/_3$ of the –SH groups. Thus of the 4 –SH groups per egg albumin molecule which react with p-mercuribenzoate in the preparation used, three different types may be recognized on the basis of their rate of reaction. Some of the –SH groups present react much more rapidly at pH 4.6 than at pH 7.0, and of the groups reacting at pH 7 some react more rapidly than the remainder in presence of certain ions. The factors which determine the reactivity of –SH groups in proteins have not been well established although steric effects have frequently been considered as explanations for the decreased reactivity of –SH groups often observed in native proteins. Reactivity of protein –SH groups might also be affected by hydrogen bonding of the –SH[9] or by H bonding in connection with the proximity of an aliphatic side chain.[10]

The number of moles of p-mercuribenzoate found to react with egg albumin agrees well with the data of MacDonnell, *et al.*[3b] These workers using a titrimetric procedure of pH 5.3 found reaction of 2.9 and 4.1 moles of p-mercuribenzoate per mole native and guanidine denatured egg albumin, respectively. From the results in Fig. 4, curve 3, it is evident that 4 –SH groups of egg albumin react readily with p-mercuribenzoate at pH 4.6 in acetate buffer without treatment of the albumin with a denaturing agent such as guanidine. The decreased reactivity of the –SH groups of the native egg albumin at pH 7.0 as compared to pH 4.6 probably accounts for the observations of MacDonnell, *et al.*,[3b] that their titration values were erratic and low at pH values above 5.3. Discussion of various values which have been obtained for the number of –SH groups in egg albumin has been given by MacDonnell, *et al.*,[3b] and need not be repeated here.

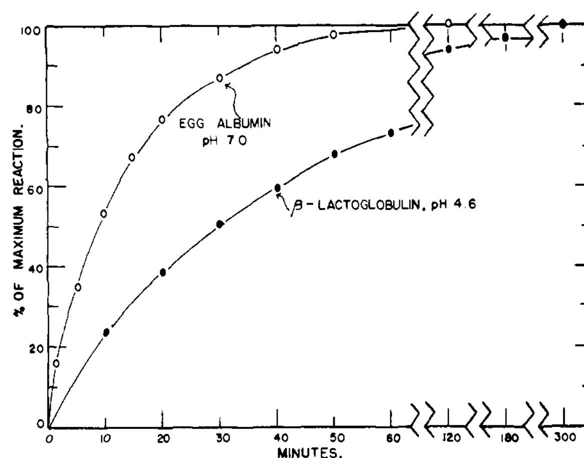

Fig. 10.—The rate of reaction of p-mercuribenzoate with egg albumin and β-lactoglobulin. Egg albumin 2.3 × 10^{-5} M, p-mercuribenzoate 6.8 × 10^{-5} M, 0.05 M phosphate, pH 7.0, 28°. β-Lactoglobulin 3.3 × 10^{-5} M, p-mercuribenzoate 6.8 × 10^{-5} M, 0.33 M acetate, pH 4.6, 28°.

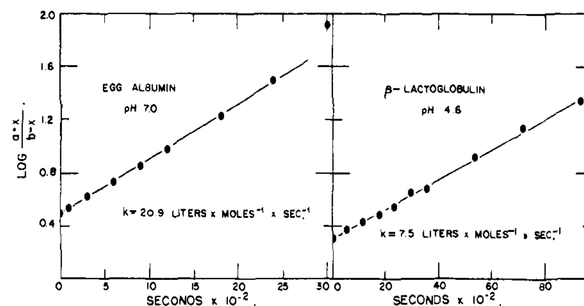

Fig. 11.—Second order plots for the reaction of p-mercuribenzoate with egg albumin and β-lactoglobulin. Data used are shown in Fig. 10.

The moles of p-mercuribenzoate which reacted with β-lactoglobulin at pH 4.6 in 20 hours were equivalent to 2.2 –SH per 35,500 g.[11] of β-lactoglobulin. Using a titrimetric procedure, Fraenkel-Conrat, *et al.*,[3c] found that 1.8 moles of p-mercuribenzoate reacted with 35,500 g. of β-lactoglobulin. Other values recently reported for moles of –SH per 35,500 g. of β-lactoglobulin have ranged from 1.6 to 3.9[3c,12]; there thus appears to be considerable variation in the reactivity of the –SH groups of β-lactoglobulin. Higher values for total –SH found by oxidation procedures might result from overoxidation or side reactions; alternatively the p-mercuribenzoate might not react with all the –SH groups of β-lactoglobulin under the conditions used.

Experimental

p-Chloromercuribenzoate.—A preparation of p-chloromercuribenzoate several years old made by the procedure of Whitmore and Woodward[13] was suspended in water and

(9) R. O. Cecil, *Biochem. J.*, **47**, 572 (1950).

(10) R. E. Benesch and R. Benesch, THIS JOURNAL, **75**, 4366 (1953).

(11) The molecular weights for β- and β_1-lactoglobulin are 35,000 and 36,100, respectively, as reported by B. D. Polis, H. W. Schmulker, J. H. Custer and T. L. McMeekin, *ibid.*, **72**, 4965 (1950).

(12) J. T. Hutton and S. Patton, *J. Dairy Sci.*, **35**, 699 (1952); M. L. Groves, N. J. Hipp and T. L. McMeekin, THIS JOURNAL, **73**, 2790 (1951); L. K. Christensen, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **28**, 37 (1952); B. L. Larson and R. Jeanness, *J. Dairy Sci.*, **33**, 890 (1950).

(13) F. C. Whitmore and G. E. Woodward, "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1941, p. 159.

treated with sufficient 1 M NaOH to dissolve the bulk of the material; a small amount of insoluble matter was removed by centrifugation. The solution was treated with sufficient 1 M HCl to precipitate the p-chloromercuribenzoate, centrifuged, and the precipitate was again dissolved in dilute NaOH. The product was reprecipitated twice more, washed 3 times with distilled water by centrifugation, and dried in a thin layer in an evacuated desiccator over P_2O_5. A commercial preparation (Sigma Chemical Co.), similarly purified, was used for some studies.

Iodometric titration[2b] was used as a check on the purity of a p-chloromercuribenzoate sample prepared as described above. The p-chloromercuribenzoate was dissolved with a minimum of dilute NaOH and a 5-ml. aliquot containing 0.206 millimole by weight was mixed with 2 ml. of acetate buffer (0.5 M NaAc, 0.5 M HAc), 8 ml. of 95% ethanol and 3 ml. of a standard iodine solution containing 0.290 millimole. After standing for 1 minute or 10 minutes the excess iodine was titrated with standard thiosulfate; the calculated iodine consumption was 1.02 and 1.03 moles per mole of p-mercuribenzoate, respectively. At pH 7.0 the consumption of iodine increased rapidly with time of standing with the result that more than 1 mole of iodine was consumed per mole of p-chloromercuribenzoate. This extra consumption of iodine was minimized at pH 4.6 but may be reflected in the slightly higher than theoretical iodine consumption.

For spectrophotometric standardization weighed amounts of a well-dried preparation were completely dissolved in a slight excess of dilute sodium hydroxide and diluted with an appropriate buffer. p-Chloromercuribenzoate solutions deteriorate slowly on standing; solutions used were prepared within several days of their use and were mixed with an appropriate buffer after preparation to avoid standing at high pH. Because of the difficulty in insuring that the p-chloromercuribenzoate is completely dissolved, solutions prepared routinely were centrifuged and standardized by measurement of the absorption at 232 mμ, pH 7 (ϵ_M 1.69 $\times 10^4$), or 234 mμ, pH 4.6 (ϵ_M 1.74 $\times 10^4$), following dilution of an appropriate aliquot. If solutions are filtered, error may result by introduction from the filter paper of materials absorbing in the 230 mμ region.

Solubilization of p-mercuribenzoate may be aided by inclusion of pyrophosphate or sulfate ions or to a lesser extent of phosphate or citrate ions. For example, 1 ml. of a suspension containing 8.5 micromoles of p-chloromercuribenzoate at pH 7.0 was solubilized by addition of 0.3 ml. of 0.15 M sodium pyrophosphate, final pH 8.1, or by addition of 0.1 ml. of 4 M $(NH_4)_2SO_4$, final pH 7.0.

o-Chloromercuriphenol.—This was prepared as described by Whitmore and Hanson.[14]

p-Mercuriphenylsulfonate.—This was prepared by use of the diazonium borofluoride of sulfanilic acid essentially as described by Dunker, *et al.*[15] A suspension of 12.7 g. of $SnCl_2 \cdot 2H_2O$, 7 g. of $HgCl_2$, 25 ml. of acetone and 13 ml. of water was mixed slowly with 7.1 g. of the diazonium borofluoride. After effervescence had ceased, the mixture was heated in near-boiling water for 10 minutes, cooled, and 1:1 NH_4OH together with about 200 ml. of water was added until the mixture was alkaline to litmus. The copious precipitate of metallic hydroxides was filtered off. The yellow-brown filtrate after addition of 2.5 volumes of ethanol and standing overnight yielded 3.2 g. of light brown crystals. Recrystallization from hot water gave a colorless product which was dried under vacuum over P_2O_5. The product as isolated did not give a test for chloride or for ammonia, and was sparingly soluble in water but readily soluble in acetate or phosphate buffer. The product thus probably corresponded to the inner salt $+HgC_6H_4SO_3-$; Whitmore and Ehrenfeld[16] isolated the inner salt of o-mercuritoluene-p-sulfonate by crystallization from hot water.

p-Nitro-p-chloromercuriphenol.—This was prepared by direct mercuration of p-nitrophenol with mercury acetate.[17] The product was isolated as the chloride by recrystallization from water acidified with HCl.

(14) F. C. Whitmore and E. R. Hanson, ref. 13, p. 161.

(15) M. F. W. Dunker, E. B. Starkey and G. L. Jenkins, This Journal, **58**, 2308 (1936).

(16) F. C. Whitmore and L. Ehrenfeld, *ibid.*, **48**, 789 (1926).

(17) E. Fourneau and A. Vila, *J. Pharm. Chim.*, **6**, 433 (1912); *C. A.*, **7**, 2285 (1913).

Phenylmercuric Nitrate.—The Eastman product was used as received.

Protein Preparations.—The egg albumin was prepared as described by Sorensen and Hoyrup[18] and recrystallized 2 times, dialyzed until free of sulfate and dried from the frozen state. Concentrations were calculated from the absorption at 280 mμ, based on the absorption at 280 mμ of a solution of known dry weight. The β-lactoglobulin was a five-times recrystallized preparation[19] kindly made available by Prof. R. Jenness. The β-lactoglobulin concentration was calculated from the absorbancy at 280 mμ, using a value of $E^{1\%}_{1\text{ cm.}}$ of 9.5, intermediate between the values of 9.3 and 9.7 reported for β- and β_1-lactoglobulin, respectively, by Polis, *et al.*[11] The crystalline bovine serum albumin was a commercial product (Armour); its concentration was calculated for the absorbancy at 280 mμ using a value of $E^{1\%}_{1\text{ cm.}}$ of 6.6.[20]

Cysteine.—The hydrochloride was recrystallized from 20% HCl,[21] dried under vacuum over P_2O_5 for 1 to 2 days and stored in a well-stoppered bottle. As noted in Fig. 3, the product gave close to a stoichiometric reaction with p-mercuribenzoate. Hellerman[2b] has noted that cysteine hydrochloride preparations may give slightly high values for sulfhydryl sulfur, possibly due to loss of some HCl to form free cysteine. Titration of the preparation used with brom cresol green indicator showed that 1 mole of cysteine hydrochloride was equivalent to 0.985 mole of NaOH.

Spectrophotometric Measurements.—Measurements were made in the conventional manner with a model DU Beckman spectrophotometer or with a spectrophotometer equipped with a special amplifier and recording potentiometer for automatic recording of absorbancy changes.[22] For measurements at pH 4.6 the p-mercuribenzoate was added as a dilute neutral or slightly alkaline solution because of its limited solubility in acid solutions; the excess of acetate buffer present brought the pH of the resulting mixture to the desired value. For initial estimations of the –SH content of a given protein solution, determinations were made with amounts of protein less than and greater than that which would react with all the p-mercuribenzoate present. This was necessary to calculate the total increase in absorbancy accompanying mercaptide formation with a given protein. Subsequent determinations with a protein required only one absorbancy determination with p-mercuribenzoate in excess.

When measurements of rates of reaction were made with protein solutions, the reaction was started by addition of 0.1 ml. of either a protein or of a p-mercuribenzoate solution with a convenient stirrer-adder[5] to 3.0 ml. of solution containing buffer and p-mercuribenzoate or protein, respectively, using a solution containing buffer alone for the blank setting. The Δ at 250 or 255 mμ for mercaptide formation was calculated by subtracting from the resulting absorbancy that due to the added substance and the initial absorption of the solution, corrected for the small dilution accompanying the addition.

The interference in spectrophotometric measurements by ethylenediaminetetraacetate limits use of this agent to decrease oxidation of –SH groups in proteins. The presence of 5 $\times 10^{-5}$ M ethylenediaminetetraacetate gave with 7 $\times 10^{-5}$ M p-mercuribenzoate 5 and 48% as much absorbancy change as na excess of cysteine at pH 4.6 and 7.0, respectively. Addition of 10^{-2} M $MgCl_2$ eliminated the interference of ethylenediaminetetraacetate but decreased by $^1/_3$ the Δ 250 mμ for mercaptide formation obtainable at pH 7.

A solution of bovine serum albumin (Armour lot N-66706) when freshly prepared gave upon addition of p-mercuribenzoate a Δ 225 mμ, pH 4.6, equivalent to 1.2 –SH per mole of serum albumin (69,000 g.). Reprecipitation of the albumin with ammonium sulfate reduced the measured amount of –SH to 0.85 group per mole. Treatment of an

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approximately 10^{-3} *M* solution of the reprecipitated albumin at *p*H 9.5 with 5×10^{-3} *M* iodoacetamide overnight at room temperature, followed by acidification and reprecipitation twice with ammonium sulfate, gave a product with a measured Δ 255 mμ following *p*-mercuribenzoate addition equivalent to only 0.07 –SH per mole. A control preparation similarly treated but without iodoacetamide additions gave a value of 0.89 –SH per mole. Reprecipitation of the iodoacetamide treated albumin was necessary because of formation in the reaction of substances which interfered in the spectrophotometric test. The nature of the impurity in the bovine albumin is not known.

ST. PAUL, MINN.

[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY, UNIVERSITY OF CALIFORNIA]

Adsorption Chromatography of Hypophyseal Growth Hormone and Other Proteins on Hyflo Super-cel Columns

BY HUBERT CLAUSER[1] AND CHOH HAO LI

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The adsorption behavior of hypophyseal growth hormone (somatotropin), bovine serum albumin (BSA), methylated BSA, ovalbumin, ribonuclease and adrenocorticotropic hormone (ACTH) on a Hyflo Super-Cel column, has been investigated. It was found that BSA, methylated BSA and somatotropin can be adsorbed onto the column with buffers of a *p*H below the isoelectric point of these proteins, whereas ovalbumin, ribonuclease and ACTH could not be adsorbed at any *p*H. The adsorbed proteins could be eluted into separate peaks by increasing, either continuously or discontinuously, the *p*H of the developing solvent. No loss of biological activity was observed after somatotropin had been repeatedly subjected to this adsorption and elution procedure. It was further demonstrated that the hormone protein exhibits homogeneity according to this chomatographic method. The mechanism for the adsorption of proteins onto Hyflo Super-cel is discussed.

The adsorption and elution of proteins, and particularly of enzymes, have been subjects for study for many years.[2,3] While proteins of relatively low molecular weight and high isoelectric point have recently been chromatographed successfully,[4–8] proteins of high molecular weight have been submitted to chromatographic investigation with less striking success. Shepard and Tiselius[9] introduced the technique of "salting-out adsorption," whereby serum proteins were adsorbed on silica gel. Frontal analysis of hemoglobin, bovine plasma albumin and egg proteins on cation-exchange resin Dowex-50 has been reported by Sober, Kegeles and Gutter.[10] Although in the past Hyflo Super-Cel usually has been considered as a rather poor adsorbent,[11] celite products have been used in several instances to fractionate protein mixtures.[12–18]

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In the present work, adsorption and elution analyses of hypophyseal growth hormone and several other proteins on Hyflo Super-Cel were performed. The separation of these proteins was carried out with both continuous and discontinuous *p*H gradient techniques.

Experimental

Protein Preparations.—The growth hormone preparations were isolated from ox anterior pituitary glands by the methods previously described[19–21]; they had been shown to behave as homogeneous substances according to electrophoretic, sedimentation and diffusion studies[19,21] and have recently been found to be essentially homogeneous by zone electrophoresis on starch[22] and end group analysis.[23] Growth-promoting activity of the hormone was estimated by the tibia test in hypophysectomized rats.[24]

The ACTH fraction employed was a peptic digest of a concentrate prepared from sheep glands, obtained by procedures previously described.[25] It possessed an activity of approximately 50 I.U. per mg. The crystalline proteins, bovine serum albumin, ribonuclease and ovalbumin were commercial products; the two former preparations were obtained from Armour and Co., the latter from the Worthington Biochemical Laboratories. The methyl ester of bovine serum albumin was kindly prepared for us by Dr. I. I. Geschwind by the acid methanol technique.[26]

Adsorbents.—Among the various celite products[27] investigated, Hyflo Super-Cel was found the most suitable for the present investigation. Before use, the adsorbent (200 g.) was pre-treated by washing twice in 6 hours with approximately 1 liter of 2 *N* HCl, filtered on a büchner funnel, washed with 1 liter of distilled water, suspended once more in 1 liter of distilled water and re-filtered twice. This procedure of washing with distilled water was repeated until the acidic reaction of the eluate disappeared, usually after 3 or 4 washings. Then the Hyflo Super-Cel was oven dried

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