iron-free hemoglobin) is qualitatively much more stable than globin. Protoporphyrin as well as heme, therefore, appears to provide at least part of the stability of the natural conjugated protein, possibly because of an interior location similar to that of heme, such as has been postulated to account for the results of the experiments reported here. Should this be the case, the presence of an iron-protein bond, essential for combination with oxygen, may not be essential for a high degree of stability against acid denaturation of the protein. These experiments, as well as those reported here, render it unlikely that a "crevice" structure involving more than one bond between iron and protein is responsible for the stabilization. The "crevice" structure for heme proteins has also been criticized by others (see Nichols, 1962). Measurements of the rates of denaturation of globin (which must lack either the iron-imidazole bond or the physical presence of protoporphyrin as sources of stability) should therefore be highly relevant and will be undertaken.

ACKNOWLEDGMENT

The authors wish to thank Prof. G. Scatchard of the Department of Chemistry, Massachusetts Institute of Technology, for his generous provision of facilities and for numerous helpful discussions.

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Interaction Between Phosvitin and Iron and Its Effect on a Rearrangement of Phosvitin Structure*

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Received October 8, 1962

Earlier work showed that some amino groups of phosvitin become masked as a result of a rearrangement at alkaline *p*H. This paper reports that metal-free phosvitin does not rearrange and that the reaction recurs upon the addition of iron to the protein. Phosvitin promotes a rapid oxidation of ferrous ion, and it binds ferric ion strongly and extensively. The earlier hypothesis that phosphoryl groups may be shifted from hydroxyl groups to amino groups of the protein is amended by the suggestion that metals, such as ferric ion, may play a catalytic or stabilizing role in the transfer process. An alternative hypothesis that a direct substitution of some ligand of iron by amino groups of the protein is responsible for the observations made on alkaline solutions of the protein is rejected on the basis of a consideration of the probable nature and rate of such a ligand substitution reaction.

Phosvitin, one of the egg yolk phosphoproteins, liberates acid when incubated in alkaline solution. This acid formation is accompanied by a masking of amino groups and is not accompanied by the appearance of inorganic phosphate. This reaction was suggested to be related to structural changes, possibly of the nature of group migrations or changes in metal-protein interactions. Titrimetric evidence supported the hypothesis that, in alkaline solution, phosphoryl residues may migrate from hydroxyl oxygen to amino nitrogen

* This work was supported by a research grant (USPHS AM-03188) from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

(Connelly and Taborsky, 1961; Taborsky and Connelly-Allende, 1962). The question was raised next whether metals, which are known to be present in phosvitin, play a role in the structural rearrangement which occurs in alkali. The present paper describes the interaction of phosvitin with iron and the effect of the presence or absence of iron on the behavior of phosvitin in alkaline solution.

Experimental

Phosvitin was prepared by the method of Mecham and Olcott (1949). Its phosphorus content was 9.4%. Iron-free phosvitin was obtained by dialysis against several changes of 0.01 M ethylenediaminetetraacetic acid (disodium salt; Eastman) followed by several changes of glass-distilled water. Visking casing was used, and the dialysis was carried out with stirring at about 2° over a period of several days. This procedure reduced the iron content of phosvitin from 0.30% to less than 0.05%. The dialyzed protein was lyophilized and stored over $CaCl_2$ in the cold. Phosphate was determined by the method of Sumner (1944), and iron was estimated by the *o*-phenanthroline method according to Sandell (1950). Measurements of alkali uptake and titrations in the presence of formaldehyde were carried out as described previously (Taborsky and Connelly-Allende, 1962). In the formaldehyde titrations, final concentrations were 0.2–0.3% protein and 1 M formaldehyde. Iron concentrations were determined by direct analysis of the $FeCl_2 \cdot 4H_2O$ (Mallinckrodt, analytical reagent) solutions which were freshly prepared for a given experiment. Amino nitrogen analyses were made by the manometric nitrous acid method of Van Slyke (1929). The original method requires a reaction mixture containing 2.3 M $NaNO_2$ and 2.2 M acetic acid. In some cases, to be noted, this method was modified so that the final sodium nitrite concentration was 0.5 M and the addition of a 2 M sodium acetate buffer, *p*H 3.5, gave a final acetate concentration of 0.9 M. Spectrophotometric measurements were made with a Beckman Model DU spectrophotometer.

Results

Effect of Metal Removal on the Instability of Phosvitin at Alkaline pH.—One of the manifestations of the reaction which leads to a masking of some of the free amino groups of phosvitin is the instability of the protein in alkaline solution. A continuous titration with alkali is required to keep the *p*H of a phosvitin solution constant over a period of several hours at *p*H values of 8.5 or higher. It was of interest to determine whether removal of metal from phosvitin affected this acid formation in any way.

Figure 1 shows the rates of acid release at *p*H 8.5 by phosvitin samples before (curve *A*) and after (curve *B*) dialysis against ethylenediaminetetraacetic acid. Clearly, dialysis against the chelating agent resulted in a considerably decreased rate and extent of the acid-forming reaction.

Titration of Phosvitin in the Presence of Formaldehyde and Iron.—The finding of increased stability in alkaline solution upon dialysis against the chelating agent led to the question whether the iron-free protein would also show a reduced loss of amino groups on exposure to alkali. Formaldehyde titrations were carried out, therefore, with solutions of phosvitin which had been dialyzed against ethylenediaminetetraacetate. Iron, as $FeCl_2$, was added to such protein solutions in graded amounts. One portion of each solution was adjusted to *p*H 11 with sodium hydroxide, followed by readjustment to *p*H 8.5 with hydrochloric acid. Another portion of each solution was adjusted directly to *p*H 8.5. Formaldehyde titration was carried out with both solutions of a given pair, containing identical amounts

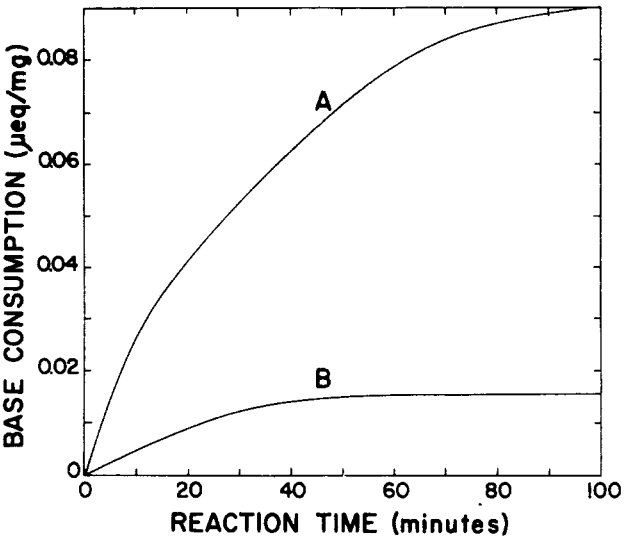

FIG. 1.—Rate of acid release by phosvitin at *p*H 8.50 before (*A*) and after (*B*) dialysis against ethylenediaminetetraacetate. Two ml of 1.00% phosvitin in 1.0 M KCl was adjusted to *p*H 8.50 and the *p*H was maintained with the *p*H-stat (0.08 N NaOH) at 37.0°. The ordinate denotes μeq base consumption per mg of protein.

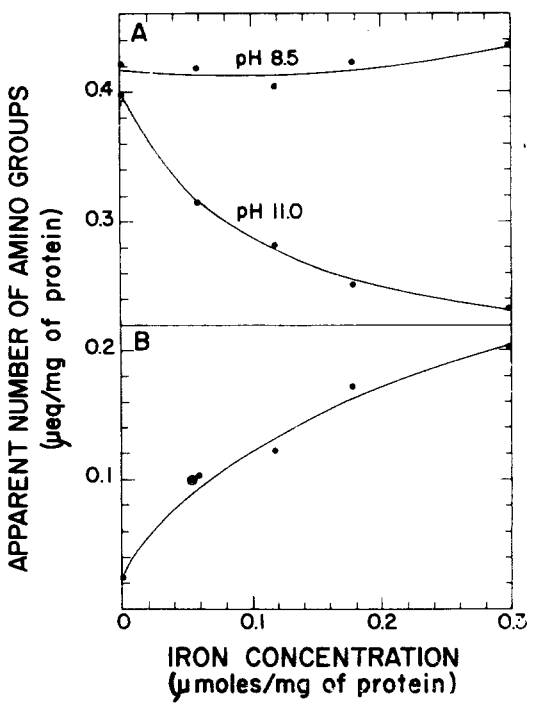

FIG. 2.—Titration of metal-free phosvitin in the presence of formaldehyde and iron. The apparent number of amino groups (μeq/mg of protein) is based on the amount of base consumed during readjustment of the *p*H of a given solution to 8.50 after the addition of formaldehyde. *A*, the apparent number of amino groups in the presence of increasing amounts of iron after preliminary exposure to *p*H 8.5 or 11.0. *B*, the curve representing the net loss of amino groups upon exposure to *p*H 11.0 as the difference between the two curves of *A*. The single value ⊙ represents the result obtained with phosvitin which had not been dialyzed, containing iron corresponding to the indicated concentration.

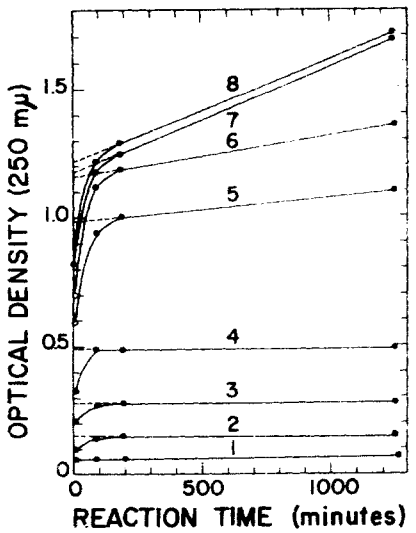

FIG. 3.—Rate of optical density change after mixing of phosvitin and ferrous chloride. To a phosvitin solution in 1 M KCl at *p*H 6 a solution of $FeCl_2 \cdot 4H_2O$ in 1 M KCl was added at zero time to result in a clear solution containing 0.20 mg of protein per ml and the following concentrations of iron (μmoles/mg of protein): 1, none; 2, 0.090; 3, 0.226; 4, 0.451; 5, 1.130; 6, 2.260; 7, 3.390; 8, 4.510. Optical density measurements were made at about 24° against 1 M KCl as the blank.

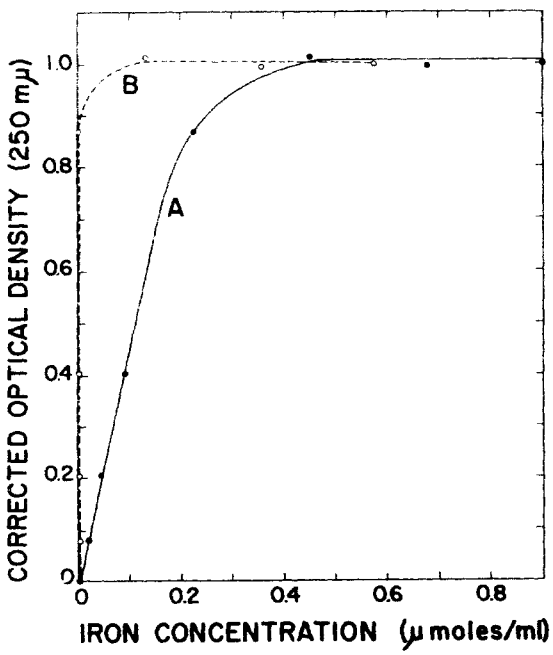

FIG. 4.—Optical density of phosvitin solutions containing iron as a function of iron concentration. The optical density values shown are those of Fig. 3 after suitable corrections were carried out as described in the text. *A*, *total* iron concentration; *B*, *free* iron concentration.

of iron. The results of these experiments are summarized in Figure 2. Part *A* shows the apparent number of amino groups as a function of iron concentration in the two protein samples, one exposed to *p*H 11.0 and the other only to *p*H 8.5. The apparent number of amino groups in the sample pretreated at *p*H 8.5 was essentially invariant with iron concentration.[1] Pretreatment at *p*H 11.0, however, resulted in a progressive decrease in the number of amino groups as the iron concentration increased. The difference between these two curves is shown in part *B*.

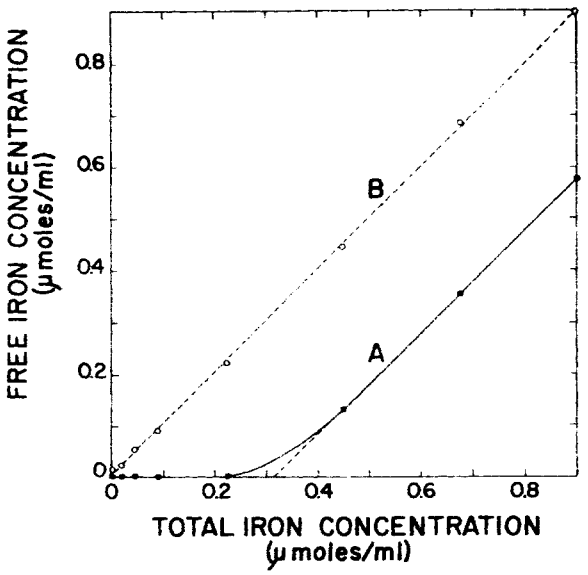

FIG. 5.—Plot of free iron concentration *vs.* total iron concentration in solutions containing phosvitin. For experimental conditions see the legend under Fig. 3 and the text. *A*, hydroquinone absent; *B*, hydroquinone present. Data for both curves were obtained with aliquots of the same protein-iron mixtures.

Estimation of the Extent and Strength of Iron Binding by Phosvitin.—In view of the effect of iron on the formaldehyde titration of phosvitin it was of interest to obtain an estimate as to what extent and how strongly phosvitin may bind this metal. An initial finding of interest was that the ferrous form of iron was oxidized very rapidly after its addition to the protein solution and that ferric ion was bound by the protein. Ferrous ion, below a certain concentration, could not be detected in phosvitin solutions by the *o*-phenanthroline method. A ferrous–*o*-phenanthroline complex formed slowly when a suitable reducing agent (hydroquinone) was present in excess in the iron-phosvitin solution. Ferrous ion was oxidized by molecular oxygen. A solution of ferrous chloride and phosvitin consumed oxygen (in a Warburg manometric apparatus) rapidly in a quantity sufficient to account completely for the amount of ferrous ion oxidized. Neither ferrous chloride nor phosvitin alone took up any oxygen under the same conditions. The oxidation of ferrous ion was accompanied by a very large increase in the optical density of the iron-phosvitin solution in the region 240–340 mμ. These findings formed the basis of the following experiments.

Figure 3 shows the rate of change of the optical density of phosvitin solutions at 250 mμ after the addition of varying amounts of ferrous chloride. At low iron concentrations, the optical density became stable within a short time after mixing, but at higher concentrations a very rapid rise in the optical density was

[1] The apparent number of amino groups in metal-free phosvitin (0.42 μeq/mg) is lower than the value reported earlier for phosvitin (0.51–0.54 μeq/mg; Taborsky and Connelly-Allende, 1962). In order to conserve material, protein concentrations used in these experiments were lower than before. In turn, the formaldehyde concentration had to be lowered to keep blank corrections relatively small. The equilibrium of the formaldehyde-amino group reaction might be expected to be affected unfavorably, but the effect on the significant quantity, the difference between pairs of samples, would not be expected to be appreciable (*cf.* Table II in earlier paper). The Van Slyke nitrogen analysis, yielding a value of 0.57 μeq/mg, is the more reliable one for the absolute determination of amino groups.

followed by a slow rise. The rate of this rise increased as the iron concentration increased. It should be noted that the first point shown with each curve was obtained at 2 minutes. By this time, evidently most of the fast reaction had already taken place. It appeared reasonable to suppose that the fast reaction reflects the primary interaction between iron and phosvitin, and for this reason all curves were extrapolated to zero time, as shown. These optical densities, after correction for the absorption of protein and ferrous chloride, are equivalent to the net change resulting from mixing iron and protein and are a measure of the extent of binding. A plot of these values as a function of total iron concentration is shown in Figure 4 (curve *A*). Beyond a total iron concentration of about 0.4 μmoles/ml, there is no further net optical density change, signifying no further iron binding.

The amount of free iron in the protein-iron mixtures was determined by taking advantage of the fact that in the absence of a reducing agent apparently only unbound ferrous ion was detectable. The results of these analyses are also shown in Figure 4 (curve *B*). No free ferrous ion could be detected up to about 0.2 μmoles Fe ml. The question might be raised whether this is in fact a reflection of binding and not simply a result of the oxidation of ferrous to ferric ion. The latter possibility was rejected on grounds of the observation that the addition of hydroquinone, which reduces free ferric ion almost instantaneously, resulted only in a very slow appearance of the ferrous-*o*-phenanthroline complex. In 1 hour, less than two thirds of the apparently bound iron was freed to react with the chelating agent. All iron was recovered, however, after 17 hours. Therefore, the iron could not have been free in solution in the presence of the protein. A plot of free iron *vs.* total iron concentration is given in Figure 5. Curve *A* shows the data obtained in the absence of hydroquinone, while curve *B* illustrates that on protracted reduction all iron appears as "free" iron. Curve A enables one to estimate the binding capacity of phosvitin by an extrapolation of the curve to the zero ordinate, as indicated. The extrapolated value, 0.315 μmoles Fe/ml, corresponds to 1.58 μmoles of iron per mg of protein, or an iron content of 8.85%. It is instructive to compare this iron content with the phosphorus content, which amounts to 3.03 μmoles/mg (9.4%). The molar phosphorus-iron ratio equals 1.92.

Curve *B* in Figure 4 allows an estimate to be made of the minimal value of the apparent over-all binding constant. The maximal optical density change was about 1.0. This change may be assumed to correspond to 100% saturation of the protein with iron. Assuming further a single set of equivalent, noninteracting binding sites and ignoring other equilibria (such as acid-base dissociation), the minimal over-all binding constant may be estimated from the fact that at about 90% saturation (corresponding to an optical density change of 0.9) the free iron concentration was still lower than the sensitivity of the assay, which would have detected iron at a concentration of 0.01 μmoles/ml, or 10^{-5} M. From the relationship

$$K' = \frac{[\text{iron-phosvitin}]}{[\text{iron}][\text{phosvitin}]}$$

it follows that the value of K' must be at least of the order of 10^6 M^{-1}.

Estimation of the Number of Primary Amino Groups of Phosvitin.—Since data reported here, and also earlier, suggested the involvement of some of the amino groups of phosvitin in a rearrangement reaction,

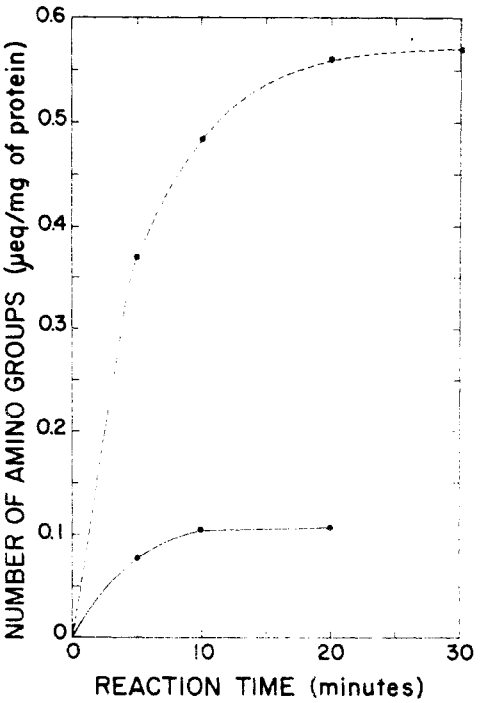

FIG. 6.—Amino group analysis of phosvitin. ···· original Van Slyke method; — modified Van Slyke method. All reactions carried out at room temperature. For experimental details see text.

a direct estimate of the number of free amino groups in this protein appeared to be desirable. The Van Slyke nitrous acid method in its original form leads to the quantitative production of nitrogen gas from all α-amino groups of amino acids and proteins in a few minutes. The ε-amino group of lysine will also react quantitatively but appreciably more slowly (Van Slyke, 1911). The large difference in the reactivity of α- and ε-amino groups led to a modification of the method allowing the determination of the faster-reacting α-amino groups without a significant conversion of ε-amino groups. Such specificity had been observed earlier at low temperatures (Sure and Hart, 1917). In the present case, advantage was taken of the effect of reactant concentrations on reaction rates. Figure 6 shows the results of Van Slyke analyses obtained with phosvitin under original and modified conditions. Of the total of 0.57 μeq of amino groups per mg of protein, 0.45 μeq may be accounted for by the ε-amino groups of lysine (Taborsky and Connelly-Allende, 1962). By difference, the data suggest 0.12 μeq of α-amino groups per mg of phosvitin. This value is closely approached by the result obtained under the modified conditions (0.11 μeq). The experiments described in Figure 7 support the assignment of these analytical data to α- and ε-amino groups, respectively. Under the modified conditions, glycylglycine (Sigma) (*pK* of amino group 8.3; Edsall and Wyman, 1958) reacted almost completely in less than 10 minutes, while leucine (Calbiochem) (*pK* of amino group 9.7; Cohn and Edsall, 1943) was much more sluggish. Leucine reacted quantitatively in 3 minutes under the original conditions. Ribonuclease, which is similar to phosvitin in its large lysine content (10 ε groups/1 α group; Hirs *et al.*, 1954), released nitrogen equivalent to almost 11 moles per mole of protein under the original conditions, while only 1 mole per mole was formed under the modified conditions. All the data are consistent with the suggestion that amino groups with relatively low amino *pK* values (such as α-amino

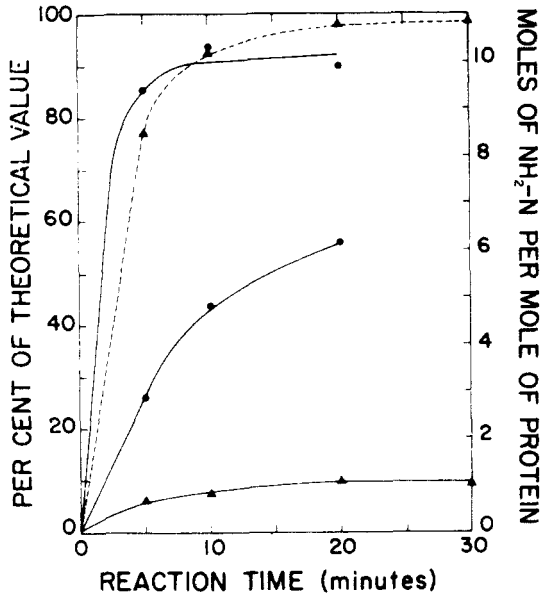

FIG. 7. Amino group analysis of various substances. Solid lines, modified Van Slyke method; broken line, original Van Slyke method. ●, glycylglycine (top), leucine (bottom); ▲, ribonuclease. All reactions were carried out at room temperature. For experimental details see text. The ordinate on the right applies only to ribonuclease.

groups of proteins and peptides) would react under the modified conditions, while amino groups with higher *pK* values (such as α-amino groups of free amino acids or ε-amino groups) would react sluggishly or not at all.

DISCUSSION

Some amino groups of phosvitin become masked upon exposure to alkaline *p*H, and this reaction is accompanied by the release of acid. It has been shown in this paper that neither of these phenomena occurs with metal-free phosvitin but the addition of iron results in the recurrence of both. The migration of phosphoryl residues from hydroxyl groups to amino groups had been suggested as a likely reaction accounting for the masking of amino groups, the release of acid, and the irreversibility of the *p*H-titration of phosvitin in the alkaline range (Taborsky and Connelly-Allende, 1962). It appears now that if such a migration occurs it must involve the participation of ferric ion. A metal may be expected to promote such a transphosphorylation in view of the fact that metals are known to be involved in enzyme-catalyzed phosphate transfer reactions (Calvin, 1954). Chelation may represent a structural rearrangement such that the likelihood of a successful migration is improved kinetically, or the product of such a transphosphorylation reaction may be stabilized by chelation, thus shifting the equilibrium of the reaction. We have discussed the stabilization of the product of such a transphosphorylation reaction in connection with our earlier data (Taborsky and Connelly-Allende, 1962).

Since the chelation of a metal ion by basic ligands itself may result in proton release, this alternative must be considered. The reaction occurs at *p*H values above 8.5. In this *p*H region, phenolic hydroxyl groups, water, and ammonium ion could be involved. The tyrosine content of phosvitin is insufficient to account for the amount of acid released (Taborsky and Connelly-Allende, 1962). The formation of metal hydroxide would be inconsistent with the disappearance of a number of amino groups approximately equivalent to the amount of acid released. It may also be noted that no precipitation occurs in phosvitin-iron solutions except when iron is present in excess of the amount bound maximally by the protein. The interaction of iron with amino groups could explain both acid release and masking of amino groups, but this alternative to the transphosphorylation hypothesis is considered less likely for the following reasons. Ferric ion is known to be chelated preferentially by oxygen-containing ligands (Martell and Calvin, 1952). Phenolate ion is implicated in the ferric conalbumin complex (Warner and Weber, 1953), and amino acids are known not to have an affinity for ferric ion (Albert, 1950) with the exception of *O*-phosphoserine (Österberg, 1959). The slow rate of acid release by phosvitin in alkali makes the chelation of the iron by amino groups even more unlikely. It may be assumed that, of the six coordination positions of the ion, four are filled by phosphate oxygen atoms. Indeed, this is suggested by the P Fe ratio of about 2 in phosvitin, which is fully saturated with iron, and is also consistent with Österberg's (1959) finding of the same ratio in the phosphoserine complex. The remaining two coordination positions are probably occupied by water or some anion present in the solution (*e.g.*, chloride). If amino groups were to displace water or other anions, the over-all reaction would be expected to be rapid in view of the known lability of the aquated ferric ion or of chloride and ammine complexes in ligand-exchange reactions (Taube, 1952).

The presence of 0.11–0.12 μeq of α-amino groups per mg suggests, on the basis of reported molecular weights of 21,000 (Mecham and Olcott, 1949) or 27,000 (Joubert and Cook, 1958), that the protein may consist of two or three polypeptide chains per mole. It is of interest in this connection that this number of α-amino groups is very similar to the number of amino groups masked in alkali in phosvitin as it is obtained from the egg yolk, containing reproducibly about 0.3% iron (*cf.* Fig. 2, and Connelly and Taborsky, 1961; Taborsky and Connelly-Allende, 1962).

The rapid oxidation of ferrous ion in the presence of phosvitin might be expected in view of the readily occurring autoxidation of ferrous complexes involving oxygen-containing ligands (Kaden *et al.*, 1960).

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Studies on the Structure of α_1-Acid Glycoprotein. IV. Optical Properties*

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Received August 8, 1962

An extensive study of the optical properties of α_1-acid glycoprotein has been carried out. The specific optical rotation was measured over the *p*H range from 1.5 to 13 and its reversibility determined. This property was studied in the presence of several polar organic reagents and in the presence of a wide range of concentrations of NaCl, LiBr, urea, and guanidine hydrochloride. The change in the rotation took place within a limited change in concentration of the latter three reagents. The optical dispersion (3028 to 5893 A) of α_1-acid glycoprotein measured in water, 4 M LiBr, 6 M urea, and 5 M guanidine hydrochloride was plotted according to the single-term Drude and the Moffitt equations. It was concluded that the net helical content of α_1-acid glycoprotein is negligible. The optical rotation of the enzymatically modified glycoprotein (removal of sialic acid and galactose) was determined in water and in the presence of 8 M urea. The oligosaccharide units of this protein do not seem to contribute to the conformation or to modify greatly certain solubility properties of the α_1-acid glycoprotein. It is proposed that the special conformation of the single polypeptide chain of α_1-acid glycoprotein is nonhelical and of an as yet unknown type.

Recent studies of the optical properties of nonconjugated proteins have shown that the helical content and the screw sense of the helix of some of these macromolecules can be calculated on the basis of the Moffitt equation (Symposium, 1960; Proceedings, 1960; Blout, 1960; Urnes and Doty, 1962; Moffitt and Yang, 1956; Urnes *et al.*, 1961; Kendrew *et al.*, 1961). Glycoproteins have been investigated only to a small extent with respect to their optical characteristics. They raise, in addition to the problem of determining the conformation of their polypeptide chains, the question as to what extent their oligosaccharide moieties contribute to the three-dimensional structure and stability of such protein molecules.

α_1-Acid glycoprotein (Schmid, 1953; Weimer *et al.*, 1950), because of the ease with which it can be prepared in a state of extremely high purity and because of its special chemical composition and unique solubility properties, represents an almost ideal protein for the study of these aspects. Prior to this study, Jirgensons (1957, 1958) reported on some optical rotation and dispersion measurements of α_1-acid glycoprotein which we confirmed here. In the present paper further optical properties of this protein are described and answers to some of the above-mentioned problems are presented.

* Publication No. 323 of the Robert W. Lovett Memorial for the Study of Crippling Disease, Harvard Medical School at the Massachusetts General Hospital, Boston. This work has been supported by grants from the Lilly Research Laboratories, Eli Lilly and Company, National Science Foundation (G-16253) and from the National Institute of Arthritis and Metabolic Diseases (A-3564 [C2]), National Institutes of Health, United States Public Health Service. This study was carried out during the tenure of an Established Investigatorship (by K.S.) from The Helen Hay Whitney Foundation, New York. Part of this work was presented at the 137th Meeting of the American Chemical Society held in Cleveland, Ohio, April 5 to 14, 1960 (Abstract, p. 25C).

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Material and Methods

α_1-Acid glycoprotein was isolated from pooled normal human plasma by a procedure described earlier (Schmid, 1953). On free moving boundary electrophoresis at *p*H 8.6 it revealed 98 to 100% α_1-globulin.

The isoionic form of α_1-acid glycoprotein was prepared by deionization with the aid of ion-exchange resins (Dintzis, 1952). The sialic acid-free derivative of this glycoprotein was obtained by removal of the sialic acid with neuraminidase. From this protein derivative approximately 75% of the galactose was cleaved off with a bacterial β-galactosidase. The concentration of the enzymatically modified glycoproteins was obtained by dividing the optical density at 278 mμ of the solutions by the E_{278} value of native α_1-acid glycoprotein of 8.93. Thus, the optical rotation refers to the same polypeptide content.

Partial but irreversible denaturation of α_1-acid glycoprotein was effected at *p*H 6 by incubation at 37° in an ethanol-ether-water mixture (1:1:1 v/v) for 3 months. Ultracentrifugation of the resulting preparation showed the following analysis: Three components with sedimentation coefficients of 3.3, 11, and 20 *S* and with relative concentrations of 36, 60, and 4%, respectively. These data indicated that, as judged by the sedimentation behavior, approximately one-third of the protein had not been altered.

For the measurements of the optical rotation a polarimeter (F. Schmidt and Haensch, Berlin) equipped with a Rudolf photoelectric polarimeter attachment, Model 200, and a sodium lamp were employed. All measurements were carried out in 10-cm tubes at 22° to 24°. Eleven readings were taken of each solution and the specific optical rotation was calculated from their average. The obtained $[\alpha]_D$ values were not corrected for refractive index of the solvent. A Rudolf photoelectric spectropolarimeter (high precision) served to measure the optical rotation at wave lengths from 3028 to 5893 A. The reduced mean residue