The Generation of Labile, Protein-Bound Phosphate by Phosphoprotein Oxidation Linked to the Autoxidation of Ferrous Ion*

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ABSTRACT: The interaction of phosvitin, an egg yolk phosphoprotein, with Fe^{2+} and oxygen results in the conversion of Fe^{2+} to Fe^{3+} and the oxidation of the phosphoprotein. The two reactions appear to be linked. The major reaction involving the protein is the α,β-dehydrogenation of protein-bound phosphoserine to the corresponding enol phosphate derivative. This conclusion is based on the principal findings that (1) the Fe^{2+}–phosvitin complex consumes more oxygen than required by the autoxidation of iron, (2) there is no reversible oxygen binding by the Fe^{2+}–phosvitin complex, (3) there is no change in the state of oxidation of the protein-bound phosphorus, (4) the oxidative deamination of amino terminal residues or lysine side chains is unlikely, (5) the oxidation generates labile, protein-bound phosphate and nitrogen and leads to the formation of carbonyl groups, and (6) the oxidized protein may be reduced with sodium borohydride, labeled with tritium, resulting in the appearance of isotope in serine residues that were presumably regenerated by reduction of the enol product of the oxidation. The reaction occurs with other phosphoproteins (and low molecular weight phosphate compounds) but not with proteins free of phosphate. The potential biological significance of this oxidative reaction is discussed.

From published work concerned with phosvitin function, two specific hypotheses emerge. One, recently advanced by Greengard *et al.* (1964), ascribes an iron-carrier role to this protein. Indeed, a variety of iron-containing egg yolk phosphoprotein preparations, hematogen (Bunge, 1885), vitellinic acid (Levene and Alsberg, 1901), ovotyrine β_2 (Posternak and Posternak, 1927), the particulate fraction of the yolk (Schmidt *et al.*, 1956), have been described and are probably related to phosvitin. This protein is isolated containing firmly bound, nonheme iron (0.3–0.6%) (Mecham and Olcott, 1949; Connelly and Taborsky, 1961; Greengard *et al.*, 1964). It has the ability to bind very much larger quantities of iron: when it is saturated with the metal, it is one of the most iron-rich proteins known (8.9%) (Taborsky, 1963). Although there is little doubt about the identity of the iron carrier of the egg yolk, the nature of its involvement in the dynamic metabolic activity of the fertilized egg remains a matter of conjecture.

The work of Rabinowitz and Lipmann (1960) suggests another hypothesis: phosvitin may function as an energy-rich phosphate-carrier protein. They showed that phosvitin and adenosine 5′-triphosphate (ATP) or adenosine 5′-diphosphate (ADP) can interact reversibly in an enzymic reaction. The average free energy released upon hydrolysis of a phosvitin–phosphate linkage may not be far less than that released upon hydrolysis of a pyrophosphate bond of adenosine 5′-triphosphate. Other reports on kinases acting on phosvitin have been published (Sundararajan *et al.*, 1960; Rodnight and Lavin, 1964). Phosvitin is known to be phosphorylated also by respiring mitochondria (Pinna *et al.*, 1963; Moret *et al.*, 1964). These reports on phosvitin phosphorylation are of special interest for the following reason. Cellular phosphoprotein probably plays some role in the energy metabolism of tissues: it is well established that protein-bound phosphate undergoes rapid turnover which is dependent on substrate oxidation (Friedkin and Lehninger, 1949; Davidson and McIndoe, 1949; Johnson and Albert, 1953; Ahmed and Judah, 1963). This turnover, according to Rabinowitz and Lipmann (1960), is probably a manifestation of *reversible* phosphate transfer reactions which, in purified systems, could be demonstrated so far only with the "classical" phosphoproteins (casein and phosvitin) rather than of essentially *irreversible* phosphotransferase reactions such as those catalyzed by phosphorylase *b* kinase or hexokinase.

It occurred to us that these two hypotheses of phosvitin function might be related to each other: energy-rich phosphate could be generated oxidatively in a reaction in which iron would be involved in some way. This possibility suggested itself by the finding that upon the addition of a ferrous salt to a phosvitin solution,

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rapid oxygen consumption and iron oxidation results (Taborsky, 1963). In this paper, we present evidence that the autoxidation of iron is accompanied by an oxidation of the phosphoprotein itself. This oxidation probably consists of the conversion of protein-bound serine phosphate to the corresponding enol phosphate, an energy-rich structure (eq 1).

$$\begin{array}{l} CH_2OPO_3H_2 \\ | \\ RNHCHCOR' \end{array} \longrightarrow \begin{array}{l} CHOPO_3H_2 \\ \| \\ RNHCCOR' \end{array} + 2H^+ + 2e \quad (1)$$

Materials and Methods

Phosvitin was prepared by the method of Mecham and Olcott (1949). It was rendered metal free and stored as described earlier (Taborsky, 1963). Acetylated phosvitin was prepared with acetic anhydride. To a 1.0% solution of protein in 4 M sodium acetate about 50 μmoles of acetic anhydride/mg of protein was added in 15 portions over a period of about 4.5 hr. The reaction mixture was kept in an ice bath with constant stirring throughout the acetylation and its pH was maintained at 6.1 ± 0.8 by the occasional addition of sodium hydroxide. The reaction mixture was then dialyzed exhaustively against water and lyophilized. Deaminated phosvitin was the product of the nitrous acid reaction essentially under the conditions of the manometric amino acid analysis technique of Van Slyke (1929). This technique was used also to assess the success of both protein modifications quantitatively. It was found that 93% of the amino groups were substituted and 91% were removed in the acetylated and deaminated preparations, respectively. Other proteins were commercial preparations as follows: casein (Hammarsten, Mann), ovalbumin (five times crystallized, Pentex), ribonuclease (bovine pancreatic, five times crystallized, Mann), chymotrypsinogen A (three times crystallized, Worthington), catalase (crystalline suspension, Boehringer). [2-^{14}C]EDTA was a product of New England Nuclear Corp. (1 curie/mole). Tritium-labeled sodium borohydride (about 25 curies/g, Volk) was diluted, as needed, with the unlabeled metal hydride (Alfa Inorganics) in a chilled aqueous solution and used immediately. All other reagents were of the best available grade and were used without purification.

Warburg manometric measurements of oxygen consumption and carbon dioxide production were carried out with conventional equipment and techniques (Umbreit *et al.*, 1964). Phosvitin (typically about 20 mg or about 60 μmoles of protein-bound P) or other complexing agents were placed in the main compartment of the flask, and iron, as $FeCl_2 \cdot 4H_2O$, in the side arm. Iron concentrations were determined by direct analysis of iron salt solutions prepared freshly for each experiment. The solvent was 1 M KCl in order to suppress the charge effect exerted by the highly charged phosvitin molecule. The pH was controlled only by the buffering capacity of the complexing agent. (The iron interacted with a variety of buffer substances that had been tried.) Their solutions were adjusted initially to the desired pH value. This initial pH will be referred to as the pH of a given experiment. Final pH values were about 1 pH unit lower. All Warburg experiments were done at 37°. Whenever catalase was added to reaction mixtures to test for the possible presence of hydrogen peroxide, catalase activity under the particular conditions of the experiment was ascertained to be present by the demonstration of oxygen release from such mixtures on the addition of authentic hydrogen peroxide. Kjeldahl nitrogen analyses were done according to Miller and Houghton (1945). Phosphate was determined colorimetrically by the method of Sumner (1944). Iron was estimated by the *o*-phenanthroline method according to Sandell (1950). Ammonia analyses were carried out with the Conway (1958) microdiffusion technique. Carbonyl groups, in the form of their 2,4-dinitrophenylhydrazone derivatives, were estimated following the colorimetric procedure of Lappin and Clark (1951). We have been unable to reproduce their molar extinction values with a number of authentic compounds. Our results are based, therefore, on data obtained with pyruvic acid solutions as standards. Iodometric titrations were carried out according to Pierce and Haenish (1950). Ultraviolet spectra were measured with a Perkin-Elmer Model 350 recording spectrophotometer and infrared spectra with a Beckman Model IR-5 recording spectrophotometer. In the latter case, the potassium bromide disk technique was used as it is described in the manufacturer's manual. Radioactivity measurements were made either with a glass-flow detector (Nuclear Chicago, Model D 47) or with a liquid scintillation spectrometer (Packard, Tri-Carb System). Measurements with the Geiger counter were made with "infinitely" thick ^{3}H-containing samples (windowless), or with "infinitely" thin ^{14}C-containing samples (with a Micromil window). Scintillation counting was done with 0.5-ml aqueous samples, suspended in glass vials containing 10 ml of a scintillation mixture consisting of 200 g of naphthalene (recrystallized from alcohol, Eastman), 10 g of 2,5-diphenyloxazole (Packard), and 0.25 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (Packard) per l. of dioxane (certified, Fisher). To this mixture, silicon dioxide (Cab-O-Sil, Cabot Corp.) was added to a final concentration of 3%. Using the external standardization device of the instrument, it was found that there was no significant differential quenching of counts between samples, including blanks. Thus, all measured counts were directly comparable to each other. Protein was hydrolyzed with glass-distilled, constant-boiling HCl in sealed, evacuated tubes at 110° for 24 hr. Automatic ammonia and amino acid analyses were made with an amino acid analyzer (Spinco, Model 120 B) according to Moore *et al.* (1958) and Spackman *et al.* (1958). When the instrument was used in a preparative manner, the column effluent was collected with an external fraction collector. Thin layer chromatography of protein hydrolysates was carried out with commercial equipment (Desaga-Brinkmann) following the procedure of Fahmy *et al.* (1961) using their chloroform–

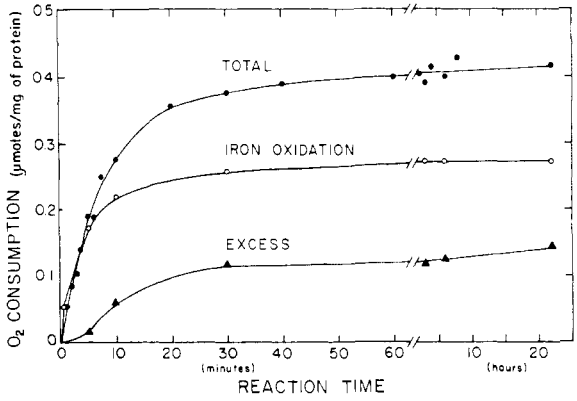


FIGURE 1: Rate of O_2 consumption by the Fe^{2+}–phosvitin complex. Reaction mixture (pH 5.6) containing metal-free phosvitin (18 mg, 54 μmoles of protein-bound P) and Fe^{2+} (20 μmoles). The total O_2 consumption rate (●) is the average obtained with six reaction mixtures which were used, one at a time, for the determination of the amount of Fe^{2+} oxidized at the times indicated. Those determinations made the calculation of the O_2 consumption rate due to Fe^{2+} oxidation (○) possible. The excess O_2 consumption rate (▲) is the difference between the other two rates.

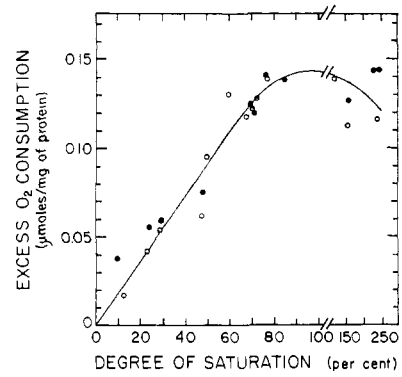


FIGURE 2: Dependence of the amount of O_2 consumed by the Fe^{2+}–phosvitin complex, in excess of the O_2 requirement of Fe^{2+} oxidation alone, on the degree of saturation of phosvitin with Fe. Reaction mixtures were similar to the mixture described in Figure 1 except for the amount of Fe which was varied. The excess O_2 consumption shown here represents the value measured after about 4 hr of incubation, that is, well after the reaction had been essentially completed. The different symbols represent different preparations of metal-free protein.

methanol–ammonia solvent system. The silica gel G plates were 0.25 mm thick and they were prepared, stored, developed, and sprayed with a ninhydrin–collidine–cupric nitrate solution according to Randerath (1963). The samples were applied to the plates as bands about 3 mm in height and 8 cm in width. After development and drying, one-half of the chromatogram was sprayed and the other half was scraped off the glass in bands of 1-cm height beginning from the bottom of the plate. These fractions were eluted with 0.5 ml of water each, over a period of 30 min, with occasional shaking, followed by centrifugation. To prepare samples for isotope analysis, the resulting supernatant fluids were dried on aluminum planchets under a heat lamp.

Results

Consumption of Oxygen by the Fe^{2+}–Phosvitin Complex. When a ferrous salt is added to a solution of metal-free phosvitin, in the presence of air, rapid oxygen consumption and the conversion of Fe^{2+} to Fe^{3+} ensues. Figure 1 shows that oxygen is consumed greatly in excess of the amount required for iron oxidation as calculated on the basis of the chemical equation

$$O_2 + 4Fe^{2+} + 4H^+ \longrightarrow 2H_2O + 4Fe^{3+} \qquad (2)$$

Excess uptake occurs only while iron is being oxidized. There is no oxygen uptake at all after the addition of a ferric salt to a phosvitin solution.

Phosvitin is assumed to be fully saturated with iron when 1 mole of iron is bound/2 moles of protein-bound phosphorus (Taborsky, 1963). Figure 2 shows that the excess oxygen consumption increases nearly linearly as the degree of iron saturation of the protein increases. Up to about 80% saturation, the solutions remain clear throughout the experiment. Beyond 80%, some of the protein–iron complex precipitates during the experiment and the oxygen uptake data become difficult to interpret. The linearity suggests that all of the phosvitin-bound metal ions may be equivalent at least insofar as the excess oxygen-consuming reaction is related to the oxidation of protein-bound iron.

The reaction rate increases with increasing pH. At pH 3, the initial rate of oxygen consumption is of the order of 1 mμmole of oxygen/min per mg of protein. At about pH 6, the reaction rate becomes limited by the diffusion rate of oxygen into the solution: about 40 mμmoles of oxygen/min per mg of protein. Most of our experiments were conducted at about pH 5.5. The extent of the reaction does not appear to be affected by variations in pH. At pH values of 4.3, 5.5, 6.8, and 7.9, the oxygen uptake is completed within 3 hr and the variation in the amount of excess oxygen taken up in these experiments does not appear to be significant.

Chloride ion which was present in most reaction mixtures at a high concentration (1 M) may be omitted without consequence. The involvement of another possible component of the reaction mixtures in an oxygen-consuming reaction was also tested. Some EDTA which was used in the preparation of metal-free phosvitin may have remained associated with the protein and could serve as an oxidizable substance. Very rapid excess oxygen consumption does, indeed, occur when ferrous ion is added to an EDTA solution (C. T. Grant, unpublished data; see also Grau and Halliday,

1957). However, when phosvitin was dialyzed against [2-^{14}C]EDTA, followed by dialysis against water as usual, the metal-free protein contained isotope only to an extent that was equivalent to less than 0.0005 μmole of EDTA/mg of protein. This is a vanishingly small quantity of a potentially oxidizable substance compared with the observed excess oxygen uptake of the order of 0.1 μmole/mg of protein.

Test of Reversible Binding or Incomplete Reduction of Oxygen. Should the excess oxygen consumption be a manifestation of reversible oxygen binding, it would be expected that extremes of pH or the removal of iron from the protein with a competing chelating agent would cause any bound oxygen to be released. There is no significant release of oxygen, however, from reaction mixtures after completion of oxygen uptake, upon addition of HCl (0.03 N), NaOH (0.03 N), or EDTA (slight molar excess over the quantity of iron). The excess oxygen could be accounted for also by incomplete reduction of oxygen. The autoxidation of iron occurs presumably in steps (Weiss, 1953; George, 1954; Abel, 1955; Kaden and Fallab, 1961). The question may be raised whether the phosvitin–iron complex might not stabilize some partially reduced oxygen derivative. It appears unlikely that any intermediates corresponding to the oxidation levels of O_2H or OH could persist for long. Were hydrogen peroxide formed, however, it might be stable enough as part of a complex or even free in solution since the protein-bound iron might be prevented, on account of its being chelated, from catalyzing its decomposition (Baxendale, 1952). That this is not the case here can be shown by adding catalase to a protein–iron reaction mixture after oxygen uptake has been completed. There is no evolution of gas. If catalase is present in the reaction mixture from the beginning of the experiment, there is a slight decrease (about 10%) in the amount of excess oxygen uptake suggesting that hydrogen peroxide may be formed as an intermediate even if it is not a final product. Hydrogen peroxide and ferric ion do not react with phosvitin, however.

Test of the Conversion of Protein-Bound Phosphite to Phosphate, or Phosphate to Peroxyphosphate. The possibility that protein-bound phosphite groups may be oxidized to phosphate is ruled out by infrared spectroscopic data. Although an absorbance increase at about 10 μ and a decrease at about 11 μ observed upon oxidation, in the presence of iron, indicated at first the possibility of a phosphite–phosphate conversion (Scott, 1957), the reversibility of this change on removal of the metal (by dialysis against EDTA) suggests rather that the change in infrared spectrum is related to the binding of iron. If the excess oxygen consumption were a reflection of the elevation of phosphate groups to the oxidation level of peroxyphosphate, it might be expected that the addition of iodide to a phosvitin–iron reaction mixture, after oxygen uptake ceased, would result in the formation of iodine (Van Wazer, 1958). The only iodine found in such experiments, even in the presence of acid [added because of the reported increased reactivity of peroxyphosphates in acid (Simon and Richter, 1959; Crutchfield and Edwards, 1960)], was due to iodide oxidation by ferric ion.

Oxidation of the Phosphoprotein. The conclusion appears to be inescapable that the excess oxygen uptake must be solely a manifestation of the oxidation of the protein itself. A specific oxidative degradation of a particular kind of protein side chain, with one exception, is unlikely to account for the excess oxygen uptake, since the difference between the amino acid composition of oxidized phosvitin and that of the nonoxidized protein appears to be only of marginal significance (C. T. Grant, unpublished data). The exception is the possibility that oxidative deamination of N-terminal residues or lysine side chains might occur. Amino acid analyses might not be sensitive enough to detect small decreases in lysine content or greater decreases even in serine content (should serine be N terminal) since phosvitin contains more serine than all other amino acids combined and lysine is its second most abundant amino acid constituent (Taborsky and Connelly-Allende, 1962). However, nearly completely acetylated or deaminated phosvitin derivatives consume about as much excess oxygen, about as rapidly, as does the unmodified protein. Since the acetamido and the primary alcohol groups of the modified preparations are very unlikely to be able to replace the primary amino groups of the unmodified protein as substrates of the hypothetical oxidation, without affecting either the extent or the rate of the reaction, oxidative deamination is unlikely to be significant as the excess oxygen-consuming reaction.

Any oxidation involving the protein might be expected to lead to the formation of a variety of degradation products. The reaction is found, indeed, to be accompanied by the production of inorganic phosphate, ammonia, carbon dioxide, and carbonyl groups. The inorganic phosphate must arise from phosphate bound directly to the protein since phosvitin is known to be free of other phosphorus compounds such as nucleic acids or phospholipids (Mecham and Olcott, 1949). Ammonia, carbon dioxide, and carbonyl groups, but not inorganic phosphate, could arise from the 2% carbohydrate (half of which is glucosamine) which phosvitin has been reported to contain (Tunmann and Silberzahn, 1962). All of the observed products could be accounted for by a breakdown of phosphoserine residues. If the protein oxidation consisted of an α,β-dehydrogenation of phosphoserine residues (eq 1), then inorganic phosphate, free ammonia ion, and carbonyl groups could be products of simple hydrolytic reactions which the primary product, the enol phosphate residue, might be expected to undergo. Other products of such reactions might give rise to carbon dioxide as well (see Discussion). Indeed, such a degradation of intrachain phosphoserine residues could explain also the apparent fragmentation of the protein upon oxidation. Under conditions of dialysis when normally less than 10% of the total initial weight of nonoxidized protein is lost through the dialysis sac, about 60% of a repeatedly oxidized protein sample was found to diffuse through the dialysis tubing. The following experiments confirm the hypothesis that phospho-

TABLE I: Generation of Labile Protein-Bound Phosphorus by Autoxidation of the Ferrous Ion–Phosvitin Complex.

Reaction Mixture[a]	Moles of P_i Produced/Mole of Excess O_2[b]			
		After Additional Treatment with 1 N HCl[c,d]		
	After Oxidation[c]	30°, 3 hr	30°, 63 hr	90°, 3 hr
Iron-free phosvitin	0.00	0.03	0.47	1.78
Ferric–phosvitin complex	0.00	0.03	0.37	1.70
Ferrous–phosvitin complex	0.47	0.60	1.50	2.68
Net increase[e]	0.47	0.57	1.08	0.95

[a] The composition of the reaction mixtures was: phosvitin (19 mg, 60 μmoles of protein-bound P), iron (20 μmoles, where present), $pH_{initial}$ 7.0. [b] Oxygen was consumed only by the ferrous ion–phosvitin reaction mixture. The results of analyses of the two control solutions (containing ferric ion, or no iron) are expressed in terms of the excess oxygen uptake by the ferrous ion–phosvitin complex assuming thereby that the "blank" reaction is the same in all mixtures. All values were corrected for the small quantity of P_i found in phosvitin solutions prior to any incubation. [c] The data were collected in two separate experiments: one yielded the "after oxidation" values and another the "after additional treatment with 1 N HCl" ones. [d] These phosphate analyses were carried out with the Warburg incubation mixtures diluted with an equal volume of 2 N HCl and incubated for the indicated time periods at the given temperatures. [e] The average values obtained with the two control solutions were subtracted from the corresponding values pertaining to the ferrous complex.

serine residues are oxidized by α,β-dehydrogenation.

Generation of Labile, Protein-Bound Phosphorus and Nitrogen. If the oxidation gives rise to an enamine structure and the observed formation of inorganic phosphate and ammonia is a reflection of partial hydrolytic decomposition of the protein-bound enol phosphate, then oxidized phosvitin should release additional quantities of inorganic phosphate and ammonia when exposed to conditions that favor the hydrolysis of the acylated phosphoenol derivative yet are mild enough not to cause extensive destruction of unoxidized serine skeletons. The data in Tables I and II show this to be the case.

From Table I it is clear that protein-bound phosphate becomes acid labile as a result of the autoxidation reaction. As long as the temperature of the acidified reaction mixture is kept low (30°), the amount of inorganic phosphate formed in the phosvitin solution that contained Fe^{2+} initially is several times the quantity released in the two control mixtures. Under more drastic treatment (90°), the ratio of the extent of dephosphorylation of the reaction mixture to that of the controls becomes smaller. It is of interest to note that the absolute difference between control values and the value obtained with the oxidized mixture tends to remain unchanged at a level of about 1 mole of orthophosphate (1.08 and 0.95)/mole of excess oxygen.

Table II shows that a small but significant amount of ammonia is formed as a consequence of oxidation only. (Duplicate experiments, including the blank values, are shown separately in order to indicate clearly that the net changes are relatively small but, nevertheless, the net increase in free ammonia appears to be a reproducible phenomenon.) If the phosvitin-containing reaction mixture is subjected to complete acid hydrolysis, a large and variable quantity of free ammonia is formed with blank and experimental phosvitin samples alike, due mostly to partial destruction of serine. We believe, however, that the increments by which the experimental values are larger than the blank values are significant. They are small in relation to the total amounts of ammonia (12–13%), but large in absolute amount and, interestingly, the net increase as a consequence of oxidation is about 1 mole of ammonia (0.9 and 1.4)/mole of excess oxygen. This is the same amount of ammonia as the amount of labile phosphate. Interpreting this quantitative correspondence between labile phosphorus and nitrogen in terms of their postulated structural proximity within the protein, we can identify the labile phosphate and the labile nitrogen as belonging to the same phosphoserine residue originally.

Generation of Carbonyl Groups. The enol phosphate hypothesis, allowing for partial hydrolysis of the phosphoenol product of the oxidation, would predict the appearance of free aldehyde groups. Table III shows that reactive carbonyl groups are formed as a consequence of oxidation. No carbonyl derivative appears with the control samples containing ferric ion or no iron. Upon subsequent hydrolysis, under conditions similar to those which were used in the experiments designed to measure phosphate release, the number of carbonyl groups increases and, as in the case of phosphate, the specificity of the reaction (compared with the controls) is lost to a great extent. At 100°, in 1 N hydrochloric acid, the ferric complex and even the iron-free phosvitin solution show the formation of reactive carbonyl groups. The carbonyl content of the originally Fe^{2+}-containing reaction mixture, however, remains significantly higher than that of either of the two controls. To reassure ourselves that the color reaction

TABLE II: Generation of Labile Protein-Bound Nitrogen by Autoxidation of the Ferrous Ion–Phosvitin Complex.

Expt No.[a]	Ammonia Found after	Moles/Mole of Excess O_2[b] Blank	Exptl	Net Increase
1	Oxidation only[c]	0.27	0.48	0.21
2	Oxidation only[c]	0.28	0.46	0.18
3	Oxidation–hydrolysis[d]	7.1	8.0	0.9
4	Oxidation–hydrolysis[d]	11.7	13.1	1.4

[a] The reaction mixtures had a composition similar to those described in Table I, footnote *a*. [b] "Blank" solutions consumed no oxygen. The data pertaining to them are expressed in terms of the excess oxygen consumed by the corresponding "experimental" solutions (see also Table I, footnote *b*). [c] Incubation in the Warburg apparatus was followed immediately by analysis for free ammonia with the Conway technique. The "blank" values represent the ammonia content of phosvitin solutions before oxidation. [d] After incubation in the Warburg apparatus, an aliquot of each reaction mixture was subjected to complete protein hydrolysis (110°, 24 hr, 6 N HCl) and the ammonia in the hydrolysates determined with the amino acid analyzer. The "blank" values represent the ammonia content of hydrolysates of phosvitin–ferric ion solutions. Ferric ion was added to these "blank" solutions in order to allow for any effect the iron may have on the course of protein hydrolysis.

measures indeed carbonyl group appearance, we subjected the oxidized and acid-treated reaction mixtures to reduction with sodium borohydride [a reducing agent with a high degree of specificity for the reduction of carbonyl compounds (Gaylord, 1956)]. Measurement of the carbonyl content of these reduced reaction mixtures yielded negligible values, as expected. It is unlikely that the sodium borohydride or its decomposition products interferes with the carbonyl test in some unknown manner since a control test, carried out with sodium borohydride which had been decomposed with acid prior to its addition to the protein solution, gave carbonyl values which were nearly identical with those obtained with the same reaction mixtures before reduction.

It is to be noted that the net production of carbonyl groups upon oxidation but without any subsequent acid treatment is greater than the net production of either inorganic phosphate or free ammonia noted earlier. This becomes understandable considering that we found an authentic enol phosphate, phosphoenolpyruvate, to be reactive in the carbonyl test without prior hydrolysis. Apparently, the conditions of the carbonyl test are such that the latent carbonyl function of the enol phosphate is also reactive. Under identical conditions, 65% of a sample of phosphoenolpyruvate reacted compared with the 100% reaction observed with pyruvic acid. If we assume, therefore, that a major proportion of any protein-bound enol phosphate would react as if it were a free carbonyl compound, in addition to any free aldehyde that may have formed by hydrolysis of enol phosphate, then we would predict that the results of carbonyl analysis after oxidation but *without* acid treatment should approach the values of inorganic phosphate analysis after oxidation *and* subsequent acid treatment. The results tend to agree with this prediction: 0.7 mole of carbonyl compound was measured compared with about 1.0 mole of phosphate/mole of excess oxygen. As a further consequence of this argument, we would not expect to find a larger difference between the carbonyl contents of the ferrous–

TABLE III: Generation of Carbonyl Groups by Autoxidation of the Ferrous Ion–Phosvitin Complex.

Reaction Mixture[a]	Moles of Carbonyl Group Produced/Mole of Excess O_2[b] Oxidation[c]	Hydrolysis[d]	After Reduction[e] Control	Experimental
Iron-free phosvitin	0.0	0.3	0.4	0.0
Ferric–phosvitin complex	0.0	0.8	0.7	0.0
Ferrous–phosvitin complex	0.7	1.3	1.2	0.2
Net increase[f]	0.7	0.5	0.5	0.2

[a] The composition of the reaction mixtures was similar to the composition described in Table I, footnote *a*. [b] See Table I, footnote *b*. [c] Immediately after oxygen consumption ceased. [d] The oxidized reaction mixture was subjected to 100° for 90 min in 1 N HCl before analysis. [e] An aliquot of the oxidized and acid-treated reaction mixture (1.4 mg of phosvitin/ml) was neutralized with NaOH, buffered with 0.1 M sodium acetate buffer, pH 4.6, and treated twice, 5 min apart, with $NaBH_4$ (0.03 M). Ten minutes after the second addition of reducing agent, the solution was acidified with HCl (1 N) and subjected to carbonyl analysis. The "control" data refer to aliquots of the reaction mixtures which had been treated identically with the "experimental" aliquots except that the reducing agent was destroyed by acidification before its addition to the buffered protein solution. [f] The values obtained with the ferric–phosvitin control solution were subtracted from the corresponding values pertaining to the ferrous complex.

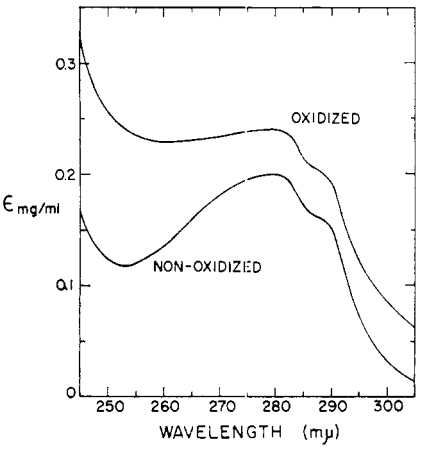


FIGURE 3: Ultraviolet absorption spectra of iron-free phosvitin before and after autoxidation of its ferrous complex. "Nonoxidized" phosvitin was an iron-free preparation (11.6% N, 9.2% P, <0.01% Fe). "Oxidized" phosvitin was the same preparation, subjected to a typical autoxidation experiment (similar to the one described in Figure 1), followed by extensive dialysis against EDTA and water, and lyophilization (12.0% N, 10.9% P, <0.01% Fe). The spectra were obtained with aqueous solutions of about 1.1 mg/ml (pH about 5). The data were corrected to a concentration of exactly 1.0 mg/ml.

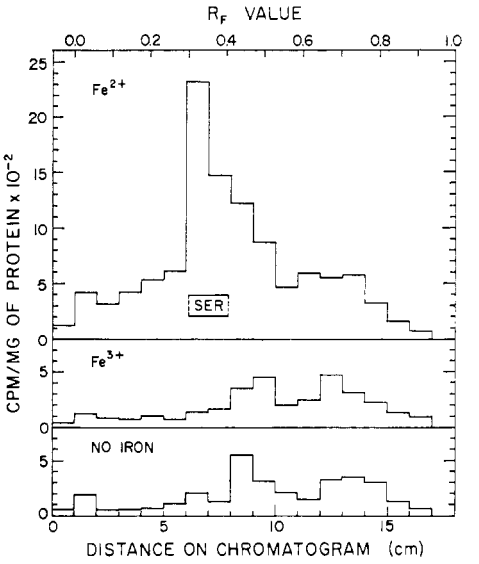


FIGURE 4: Thin layer chromatographic fractionation of the hydrolysate of tritium-labeled, reduced phosvitin. Phosvitin was oxidized as described in Figure 1. Control experiments without Fe or with Fe^{3+} were carried out in parallel with the phosvitin–Fe^{2+} experiment. Immediately after completed oxidation, the reaction mixtures were reduced with [^{3}H]$NaBH_4$, essentially as described in Table III, footnote *e*. The reduced reaction mixtures were subjected to hydrolysis and chromatography as described in the text. The actual quantity of material subjected to chromatography corresponded to about 0.8 mg of protein. The equivalent of half of this quantity was used for isotope analysis. The top portion of the figure depicts the results obtained with the hydrolysate of the reduced ferrous ion–phosvitin autoxidation mixture. The center and bottom parts show the results of the two control experiments as designated. The position of serine, as detected by spraying with ninhydrin, is indicated.

phosvitin and ferric–phosvitin reaction mixtures *after* hydrolysis than *before* hydrolysis. The differences we find are indeed nearly the same before and after hydrolysis (0.7 and 0.5 mole).

Finally, we should comment on the appearance of carbonyl groups in the control solutions upon hydrolysis. Destruction of intact serine residues may be expected to occur in hot acid solution. The product of this destruction would be expected to be a pyruvoyl peptide or free pyruvic acid, both expected to be reactive in the carbonyl test and also to be reducible by sodium borohydride.

Change in the Ultraviolet Absorption Spectrum of Phosvitin upon Autoxidation. Figure 3 shows the ultraviolet absorption spectra of oxidized and nonoxidized phosvitin solutions measured under similar conditions. The spectra differ from each other qualitatively in the same way in which the spectrum of phosvitin has been reported to change upon dephosphorylation in alkaline solution (Mecham and Olcott, 1949). The dephosphorylation has been suggested to occur by β-elimination of serine-bound phosphate, leaving behind peptide-linked α-aminoacrylic acid residues. The conversion of a phosphoserine residue to the corresponding enol phosphate produces a structure which is similar to the aminoacrylic acid residue and would be expected to have a similar spectrum.

The oxidized and nonoxidized phosvitin samples are different in more respects than the identity of their phosphorylated amino acid residues alone. This is reflected in their elemental analyses (Figure 3). The difference in elemental composition is a result of fragmentation upon oxidation and loss of peptide material on dialysis. The demonstrated absence of iron from either preparation precludes the possibility that the spectroscopic differences could be due to the presence of a protein–iron complex in one case and not in the other.

Identification of the Serine Residue as the Major Site of Autoxidation. The apparently successful reduction of the carbonyl groups generated by the autoxidation of the iron–phosvitin complex suggested a way to identify the oxidized sites in the protein. Reduction of the α-aminomalonic semialdehyde residue should regenerate the serine residue. Reduction of the reaction mixture, after complete oxidation, was undertaken with sodium borohydride labeled with tritium. The reaction mixture containing the reduced protein was subjected to complete acid hydrolysis and the hydrolysate was dried in a rotary evaporator (at about 50°). The residue was

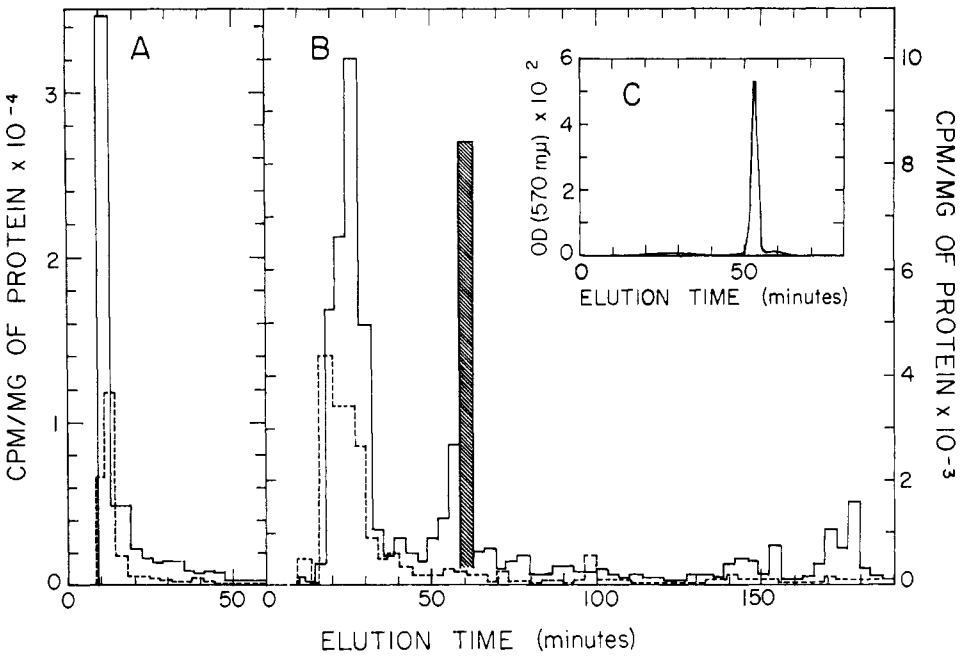


FIGURE 5: Ion-exchange chromatographic fractionation of the hydrolysate of tritium-labeled, reduced phosvitin. The hydrolysates used were those described under Figure 4. The equivalent of about 0.5 mg of protein was applied to each column. The solid line depicts the results obtained with the hydrolysate of the reduced ferrous ion–phosvitin autoxidation mixture. The broken line shows the results of the control experiment with ferric ion. Part A represents the "short" column, fractionating the basic amino acids of which the first one, lysine, appeared at about 25 min. Part B represents the "long" column, fractionating the neutral and acidic amino acids of which the first one, aspartic acid, appeared at about 45 min; no significant ninhydrin-positive material was eluted prior to this time. An aliquot of the fraction, which is shown shaded in part B, was rechromatographed on the same column. Its elution pattern, in terms of ninhydrin-positive material, is shown in part C (insert).

redissolved in water and dried repeatedly in order to remove any hydrogen isotope that would be exchangeable with water. The hydrolysate was then fractionated chromatographically by two different methods. Finally, the chromatographic fractions were analyzed for ninhydrin-positive material and for the presence of isotope. Similar experiments were carried out with control solutions of phosvitin, containing ferric ion from the beginning, or no iron at all.

Figure 4 shows the results obtained by thin layer chromatography. The isotope is heavily concentrated in the R_F region of the chromatogram where serine is expected to be (Fahmy *et al.*, 1961). This was confirmed under our experimental conditions with an authentic sample of serine. The position of the serine band is indicated in Figure 4. Of other amino acid components of the hydrolysate, aspartic acid and histidine could overlap slightly with the radioactive band. On the chromatogram which is the basis of Figure 4, aspartic acid moved with an R_F of 0.28–0.31 and histidine with an R_F of 0.41–0.46. The serine band is spread out, as shown, with an R_F of 0.30–0.40. Since the counts given in Figure 4 (recalculated to a milligram of protein basis) were obtained with samples at "infinite" thickness, the data are proportional to the specific activity of the samples. Upon numerical integration of the areas under the three curves, we found 3100 ± 100 cpm/mg of protein on the iron-free control chromatogram, 3200 ± 200 cpm/mg of protein on the Fe^{3+} control chromatogram, and 11,000 ± 200 cpm/mg of protein on the chromatogram of the hydrolysate of the Fe^{2+}–phosvitin reaction mixture. Nearly four times as much isotope is present in the product of the Fe^{2+}–phosvitin reaction than in either of the controls, and this large excess of radioactive material is clearly concentrated in the serine band of the chromatogram. By determining the isotope content of separate samples of the total hydrolysate of the Fe^{2+}–phosvitin reaction product (corrected for the counts in the total hydrolysate of the Fe^{3+}–phosvitin control mixture), an estimate was made of the true specific activity of the hydrolysate, based on its self-absorption (determined with the hydrolysate) and an estimated detector efficiency of 15% (assuming 2π geometry). From this estimate and the specific activity of the reducing agent (accepting the manufacturer's value, corrected for experimental dilution), it followed that the incorporated isotope is equivalent to about 0.5 mole of carbonyl compound/mole of excess oxygen consumed in the oxidation. Considering the crudeness of this estimate, its agreement with the analytical value (0.7 mole of carbonyl group/mole of excess oxygen; Table III) is very satisfactory.

The hydrolysates of the reaction mixture and the two control mixtures were fractionated also by ion-exchange chromatography. Samples of each hydrolysate were chromatographed twice, alternating the operation of the column between analytical and preparative modes, in order to test the performance of the apparatus, especially in terms of the reproducibility of peak positions. Aliquots of the fractions collected in the preparative experiments were subjected to scintillation counting. The distribution of isotope by this chromatographic method is shown in Figure 5. Only the results obtained with the two iron-containing reaction mixtures are shown, in order to simplify the presentation. The difference between the Fe^{3+} and the iron-free control experiments is negligible (as it is in the case of the thin layer chromatographic fractionation, shown in Figure 4). With the "short" column (Figure 5A), nearly all of the radioactivity appears at the solvent front and there is no indication of a specific incorporation of isotope into any of the basic amino acids. With the "long" column (Figure 5B), the major portion of radioactivity is again at the solvent front but a large peak of radioactivity appears at a time which corresponds to the serine position in the analytical experiments. To affirm the identity of this radioactive fraction, an aliquot of it (shaded fraction in Figure 5B) was rechromatographed analytically. The effluent pattern is shown in Figure 5C. The fraction with the high radioactivity contains ninhydrin-positive material which is essentially pure in terms of this chromatographic analysis. Without any doubt, this material is serine. No other amino acid present in phosvitin could account for the size of the peak shown in Figure 5C. The only possible alternative apparent to us is that the radioactivity is associated with some ninhydrin-negative material that is eluted from the column in the same position as serine. In view of the coincidence of the radioactivity with the serine position in two different chromatographic systems, we prefer to dismiss this alternative as a most improbable one. Figure 5B shows also that small amounts of isotope may have become incorporated into the aromatic amino acids that are eluted at about 170–180 min. Some of the large amount of isotope eluted without retardation by the column is likely to be born by a lactic acid derivative. It would be expected to migrate with the solvent front and it would also be expected to be formed reductively from any pyruvic acid derivatives that may be produced as a consequence of the oxidation reaction. We have no explanation for the significant isotope incorporation into material moving with the solvent front in the control experiments. The extent of incorporation into this material is not much less (about 75%) than that into the corresponding fast-moving fraction obtained with the hydrolysate of the Fe^{2+}–phosvitin reaction mixture. Thus, most of this incorporation does not appear to be a consequence of the oxidation reaction.

Specificity of the Reaction. In order to assess the degree of specificity which may characterize the oxidation reaction involving phosvitin, the rate and extent of oxygen consumption by mixtures of ferrous ion and two additional phosphoproteins (casein and ovalbumin) and two nonphosphoproteins (ribonuclease and chymotrypsinogen) were measured. All three phosphoproteins react similarly. Indeed, casein and ovalbumin appear to undergo more extensive oxidation than phosvitin, although somewhat more slowly. In contrast, the mixtures of ferrous ion and proteins free of phosphate are completely unreactive. Additional experiments, under similar conditions, were carried out with low molecular weight substances that are representative of the presumed phosphorylated side chain of the phosphoproteins. Phosphoserine and phosphoethanolamine interact with ferrous ion and oxygen quite analogously with phosvitin, although higher phosphate concentrations (by 1–2 orders of magnitude) are required with these model substances to attain the rapid reaction rate that is observed with a given concentration of phosvitin-bound phosphate. These reactions are also accompanied by the production of inorganic phosphate and ammonia, indicating that the course of the reaction taken by the model substances is probably similar to the course of the phosphoprotein reaction. The extent of the degradation of these simple phosphate esters is greater (3–6 times), in terms of the quantities of these products relative to the amount of excess oxygen consumed, than the extent of phosvitin degradation measured by the same criterion.

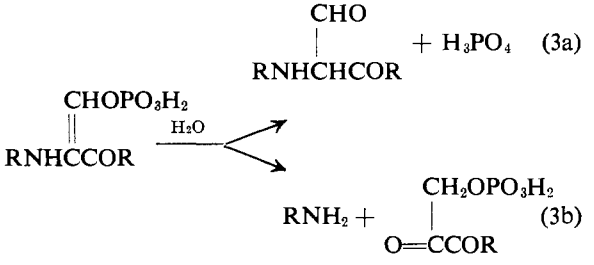
Discussion

The autoxidation of the ferrous complex of phosvitin (and of certain other phosphate esters) involves not only a conversion of Fe^{2+} to Fe^{3+} but also the oxidation of the complexing agent. The autoxidizability of phosphoproteins appears to be a hitherto unknown reaction. Indeed, the vast literature dealing with the autoxidation of ferrous complexes (for partial reviews, see Warburg, 1914; Smythe, 1931; Wieland, 1932; Szent-Györgyi, 1938; Oppenheimer and Stern, 1939; Kaden and Fallab, 1961) rarely makes mention of reactions of any type of phosphorus compounds. Pertinent to our findings may be the report of the oxidation of phosphite to phosphate (Manchot and Schmid, 1932). In addition to the possibility of the generation of protein-bound phosphate from a lower oxidation state of phosphorus, the formation of a peroxy derivative of phosvitin–phosphate could not be excluded *a priori*. Our infrared spectroscopic observations and results of iodine titrations, however, make it very unlikely that such reactions would account for the oxidation of the Fe^{2+}–phosvitin complex. Literature reports concerning the autoxidation of *organic* phosphorus compounds in the presence of iron provide no clues as to the nature of the Fe^{2+}–phosvitin oxidation. The autoxidation of lecithin (Warburg and Meyerhof, 1913) is probably of no consequence in the context of our work since the phosphatide autoxidation does not appear to depend on its phosphorus content (Page and Bülow, 1934). Thymus nucleic acid, egg albumin (Warburg and Meyerhof, 1913), hexose diphosphate, and glycerophosphate (Banga, 1938) have been reported to be unreactive themselves although some of them promote the autoxidation of ferrous iron. Egg

yolk has been found to promote iron autoxidation, ascribed to the presence of phosphatides in the yolk (Tompsett, 1940). A slow, light-catalyzed oxidation of a variety of phosphorus-containing (and other) substances has been noted (Neuberg, 1910). The phosvitin reaction occurs, however, in the dark as well as in daylight. As a special case, the oxidation of carbohydrates in the presence of ferrous pyrophosphate may be mentioned (Spoehr, 1924).

The evidence for phosphoserine residues being the reactive protein components and the indicated close relationship between iron and protein oxidation suggests that the phosphorus-bearing sites, the iron-binding sites, and the sites of oxidation overlap. The promotion of the Fe^{2+} to Fe^{3+} conversion by complex formation with inorganic phosphate is a well-established phenomenon (King and Davidson, 1958). Phosphoserine is oxidizable when mixed with Fe^{2+}, as we have noted earlier, and it is known to form a strong complex with ferric ion, having the composition Fe^{3+}–(phosphoserine)$_2$; for iron binding, it is reported to utilize probably only the phosphate and not the amino or carboxylate groups (Österberg, 1959). The iron complex of phosvitin is likely to be constituted similarly, utilizing two phosphorus-bearing side chains for the binding of each iron atom (Taborsky, 1963). Furthermore, good iron chelators, such as polybasic or hydroxy acids, are well known to be prone to undergo autoxidation (Smythe, 1931; Banga, 1938; Grau and Halliday, 1957; Kaden and Fallab, 1961). When compared with the autoxidizability of carboxylic acids, however, the reactivity of the phosphoprotein shows an interesting selectivity. Oxygen cannot be replaced by hydrogen peroxide, without or with added iron (in the ferrous or ferric form), as the oxidant of the phosphoprotein, whereas citrate, for example, will react with hydrogen peroxide as well, in the presence of ferric ion (C. T. Grant, unpublished data).

On presentation of our results on the formation of a variety of fragmentation products as a consequence of phosvitin oxidation, we suggested that their production is readily explicable in terms of likely hydrolytic reactions following the primary event, the conversion of the alcohol phosphate to the enol phosphate. It appears to be a most reasonable assumption on general chemical grounds that the primary oxidation (as shown in eq 1) could be followed by spontaneous hydrolysis resulting in either the free aldehyde residue and inorganic phosphate, or a peptide amide and a phosphohydroxypyruvoyl peptide, or both.



This scheme, augmented by further, obvious possibilities of hydrolytic and oxidative reactions involving the products of reactions 3a and 3b, is consistent with all of our data pertaining to phosphoprotein oxidation. For example, inorganic phosphate would be produced by (3a) and also by further hydrolysis of the product of (3b) or derivatives of it. Ammonia would be formed by hydrolysis of the amide formed in (3b). Either reaction 3a or 3b would yield a carbonyl compound and various additional carbonyl derivatives could be formed by further reactions involving these products. Carbon dioxide could result from the further oxidation of either of the two primary carbonyl products. Reaction 3b would lead to fragmentation of the peptide chain. Analogous postulates might explain the degradation of phosphoethanolamine and phosphoserine. The phosvitin reaction appears to produce, in general, much less of the same products than do the model reactions. The reason for this appears to be that the presence of the enol phosphate residue within a peptide chain is expected to confer on it a degree of stability so that relatively little further degradation, *via* (3a) and (3b), occurs. This stability would be expected of the acyl enamine structure within the protein, but the free enamine structure in the product of a reaction analogous to (1) would be expected to be very labile.

A particular alternative to reaction 1 might seem to be preferable, the oxidative formation of an imide. This reaction, however, would fail to account for the greater amounts of inorganic phosphate than of ammonia yielded by phosphoethanolamine. It would also necessitate the postulation of an additional reaction, the tautomerization of the imide to the enol derivative. Otherwise, the formation of a carbonyl derivative, from which serine may be regenerated reductively, could not be explained. For these reasons, the enol phosphate pathway appears to be the simplest, reasonable hypothesis that is consistent with all data. The proposed dehydrogenation of a carbon–carbon bond is certainly not without precedent. Peroxide treatment of acetate, for example, is known to yield succinic acid, a reaction analogous to dehydrogenation (Waters, 1964).

Assuming that the formation of each mole of enol phosphate (2 electron equiv) requires 0.5 mole of oxygen, the molar ratio of enol phosphate:excess oxygen should be 2, if enol phosphate formation were the sole oxygen-consuming reaction involving the protein. In fact, this ratio is near 1, as shown in terms of the molar ratios of either labile phosphate, or labile nitrogen, to excess oxygen. Accordingly, about half of the excess oxygen may be used for the formation of enol phosphate and the other half must be consumed in other reactions. Such reactions are likely to include the oxidative degradation of derivatives of the enol phosphate, produced by spontaneous hydrolysis (*e.g.*, α-aminomalonic semialdehyde, hydroxypyruvic acid, and their derivatives). Far-reaching degradation of such products is chemically possible and could be of such a nature that multiple oxidations would occur affecting the derivatives of a single enol phosphate residue. Thus, further degradation of only a relatively small fraction of the

first-produced enol phosphate would suffice to account for the amount of oxygen taken up beyond the quantity required by the primary dehydrogenation. As an additional possibility, a limited occurrence of those reactions which we have ruled out as being of major importance cannot be dismissed.

In typical experiments with phosvitin, about 0.1 mole of excess oxygen was consumed/mole of bound iron. This amount of oxygen corresponds to about an equimolar amount of enol phosphate, as we just discussed. Expressed in terms of a protein molecule that is fully saturated with iron (1.6 μmoles/mg of protein), this amounts to nearly six residues of enol phosphate/mole of protein [based on a molecular weight of 36,000 (Mok *et al.*, 1961)]. The protein molecule contains about 110 phosphoserine residues (based on a P content of about 10% and assuming all of the phosphorus to be serine bound). Assuming that each iron-binding site contains two phosphate groups (Taborsky, 1963), about 10% of all iron-binding sites reacted in these experiments to produce 1 mole of enol phosphate each. The potential extent of the reaction is probably higher since we know that the excess oxygen consumption for a given amount of bound iron can be enhanced, for example, by the gradual addition of ferrous ion to the protein instead of in the form of a single batch.

It is of interest to consider that if this oxidative reaction were to occur under enzymic direction, as one step in a chain of biochemical events, then any spontaneous reactions degrading the enol phosphate further might be eliminated on account of alternative, specific reactions occurring with enzyme catalysis. We are considering especially the possibility that such a chain of events might be of a cyclic nature such as the involvement of phosphoprotein in a sequence of oxidation, transphosphorylation, reduction, and phosphorylation steps (not necessarily in this order). Such a cycle would regenerate the "normal" phosphoprotein periodically. Were components of the electron-transport chain to serve as the oxidant and reductant in these reactions, and were adenosine 5′-diphosphate and inorganic phosphate the eventual phosphate acceptor and donor, respectively, then the phosphoserine–enol phosphate conversion could represent a key step in the mechanism of oxidative phosphorylation, the phosphoprotein itself being the substrate of the coupling process.

Acknowledgments

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Studies on the Mechanisms Underlying Adaptive Changes in Rat Liver Phosphoenolpyruvate Carboxykinase*

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ABSTRACT: The regulation of hepatic phosphoenolpyruvate carboxykinase activity *in vivo* was studied by measuring the activity of the enzyme in the soluble fraction of livers obtained from rats subjected to various alterations in their hormonal or metabolic status. Puromycin and actinomycin D were employed in some experiments to inhibit, respectively, protein and ribonucleic acid synthesis. The data indicate that the tissue activity of the enzyme is regulated not only by changes in the rate of biosynthesis of the enzyme, but also in one or more other ways which may involve activation processes or changes in the rate of degradation of the enzyme. Treatment of rats with hydrocortisone or D-mannoheptulose apparently results in an enhanced synthesis of carboxykinase. The administration of glucose to fasted rats depressed carboxykinase activity by 28% in 4 hr, whereas administration of puromycin caused a 9% decrease in activity in the same period. When both puromycin and glucose were given, activity fell by only 10%. These data were interpreted as signifying that protein synthesis is required for expression of the depressive action of glucose on the enzyme. Neither adrenal cortical hormone nor insulin appear to have a direct function in the regulation of carboxykinase.

The adaptation of many of the enzymic activities of mammalian tissues in response to alterations in the endocrine, nutritional, or environmental status of animals has been intensively studied in recent years (Freedland and Harper, 1957; Hechter and Halkerston, 1965; Potter and Ono, 1961; Smith and Haijer, 1962; Weber, 1963). In no case, however, has the precise mechanism of control been established. The problem is very complex, for changes in the tissue activity of an enzyme may occur in a number of ways. Segal *et al.* (1965) have listed several possibilities. They are: "changes in the rate of biosynthesis of the enzyme, changes in the degradation rate of the enzyme, changes in the level of activators or inhibitors, direct interaction of the hormone and the enzyme, interconversion of active and

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