Valentine, R. C., Shapiro, B. M., and Stadtman, E. R. (1968), *Biochemistry 7*, 2143.
Woolfolk, C. A., Shapiro, B. M., and Stadtman, E. R. (1966), *Arch. Biochem. Biophys. 116*, 177.
Wulff, K., Mecke, D., and Holzer, H. (1967), *Biochem. Biophys. Res. Commun. 28*, 740.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.
Wyman, J. (1967), *J. Am. Chem. Soc. 89*, 2202.

Mechanism of the Oxidative Dephosphorylation of the Phosphoprotein Phosvitin*

Robert W. Rosenstein† and George Taborsky

ABSTRACT: The mechanism of the oxidative dephosphorylation of the egg yolk phosphoprotein phosvitin (Grant, C. T., and Taborsky, G. (1966), *Biochemistry 5*, 544) has been elucidated on the basis of isotopic tracer experiments. Essentially all of the more than 100 phosphoserine residues of phosvitin appear to be potentially oxidizable in a random and independent manner. The stable products of the oxidation are aminomalonic semialdehyde residues and inorganic orthophosphate. Although the overall reaction must pass through a transient state corresponding to the oxidation level of the enol phosphate derivative of phosphoserine, a direct search failed to yield evidence for the production of a stable phosphoenol (which was postulated in the earlier work cited above) and the demonstration of the stability of the C–H bond at the α-carbon of the oxidized residues ruled it out. The results, including the finding that phosphate release occurs by P–O bond cleavage, are wholly consistent with a mechanism by which an oxidatively generated carbonium ion derivative of phosphoserine is converted into a stable product by the direct formation of the free aldehyde and a monomeric metaphosphate ion, the latter reacting with water to yield inorganic orthophosphate. The possible biological significance of the oxidative activation of phosphoprotein ester groups is seen in the hypothetical action of phosvitin as an energy source during embryonic development in the hen egg, or in its service as a macromolecular model for the formation of a chemical intermediate in the energy conservation step of oxidative phosphorylation.

When various phosphate esters, including phosphoproteins, interact with Fe^{2+} and O_2, the metal and the phosphorylated ligand become oxidized: results obtained with phosvitin suggested that the oxidation of the protein occurs by α,β dehydrogenation of its phosphoser ineresidues, yielding the corresponding enolphosphate, followed by hydrolytic formation of P_i and the aldehyde derivative of serine, aminomalonic semialdehyde (Grant and Taborsky, 1966). Prompted by our interest in the possible biological significance of this reaction (see Discussion), we undertook an investigation of its chemical mechanism.

Experimental Procedure

Materials. Phosvitin was prepared according to Joubert and Cook (1958). It was analyzed for N, P, and Fe as before (Connelly and Taborsky, 1961), with results very similar to those of Mecham and Olcott (1949). The protein was rendered metal free and stored as described earlier (Taborsky, 1963).

[^{3}H]Water (New England Nuclear; 25 Ci/l.) was diluted for use up to twofold. [^{18}O]Water (Bio-Rad; 10 atom % excess) was diluted as given in Results. [^{32}P]ATP was prepared with carrier-free [^{32}P]P_i (Tracerlab) by the method of Glynn and Chappell (1964). At the time of its use, its specific activity was about 2×10^7 cpm/μmole. Several batches of [^{3}H]sodium borohydride (Nuclear-Chicago) had nominal specific activities of 80–500 Ci/mole. It has been the experience in our laboratory (T. S. Stashwick, 1967, personal communication) that [^{3}H]$NaBH_4$ may contain an appreciable fraction of acid-stable counts. The acid stability may be noted with the dry material as received, or it may develop upon cold storage in strongly alkaline solution. We store the reagent in the dry form and use only such preparations which have an acid-stable isotope content of no more than a few per cent. Its specific activity, in terms of its active H content, was measured on the basis of the ^{3}H content of lactic acid prepared from it and pyruvic acid. (The [^{3}H]lactic acid was purified according to Busch *et al.* (1952) and was assayed by the enzymic procedure of Lundholm *et al.* (1963).) The specific activity of [^{3}H]$NaBH_4$, when used, always exceeded 50 Ci/mole; any dilutions were made with $NaBH_4$ (Alfa Inorganics).

Gu·HCl (Ultra Pure; Mann), DPN (P-L Biochemicals), and lactic dehydrogenase (Sigma) were commercial products.

* From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520. *Received September 9, 1969.* Most of the data in this paper are taken from the dissertation presented by Robert W. Rosenstein to the faculty of the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The work was done in the former Department of Biochemistry of Yale University and was supported by a research grant from the U. S. Public Health Service (AM 03188).

† Predoctoral Trainee of the U. S. Public Health Service, 1964–1969. Present address: Department of Microbiology, College of Medicine, University of Illinois, Chicago, Ill. 60680.

Phosphoprotein kinase from beef liver was a preparation according to Dineen (1967).

Methods. Phosvitin was oxidized by stirring an aqueous solution of protein (usually 7 mg/ml, pH 7) and $FeSO_4$ (enough to saturate about 70% of the iron binding sites of the protein)[1] for 1 hr, in air. On adding $FeSO_4$ to the protein solution, the mixture becomes turbid, clarifies in about a minute, and then remains clear throughout the oxidation.

The oxidized protein was reduced with 5 equiv of $NaBH_4$ (based on the amount of Fe), added to the oxidation mixture which had been diluted with an equal volume of sodium borate buffer (0.3 M, pH 8); 0.5 hr later, the protein was precipitated and excess reagent destroyed with trichloroacetic acid (10%).

Occasionally, oxidation was carried out by the addition of the iron salt in increments spread over a period of many hours. On other occasions, a given oxidized and reduced protein was oxidized again. Then, the reduction mixture was first dialyzed against EDTA and water, and lyophilized.

For the phosphorylation of phosvitin a typical mixture containing protein (2 mg/ml), [^{32}P]ATP (1 mM), $MgSO_4$ (5 mM), and phosphoprotein kinase in 1.0 M Tris-HCl buffer (pH 7.5) was incubated at 37°. The degree of phosphorylation was determined by the length of incubation time and enzyme concentration. Usually, the incubation lasted for 20 min and aliquots of an enzyme preparation of a specific activity of 250 units were used (a unit corresponds to the incorporation of 1 mμmole of P into 1 mg of phosphoprotein/mg of enzyme protein in 20 min). The phosphorylated protein was precipitated and washed four times with trichloroacetic acid (10%), redissolved in buffer, dialyzed against water, and lyophilized. Contamination of the phosphorylated protein with enzyme was negligible (less than about 4%).

Serine was isolated from complete protein hydrolysates (6 N HCl, 110°, *in vacuo*, 20 hr) with a Beckman, Model 120, amino acid analyzer (Moore *et al.*, 1958; Spackman *et al.*, 1958). Phosphoserine was isolated from partial protein hydrolysates (2 N HCl, 100°, *in vacuo*, 10 hr) based on the procedure of Lipmann and Levene (1932), by ion-exchange chromatography (Ahmed and Judah, 1967). The effluent solution containing phosphoserine was pooled, lyophilized, dissolved in water, and further purified by paper electrophoresis (Whatman No. 1 paper strip, 1 M acetic acid, 40 V/cm, 1.5 hr; Savant flat-plate system). The purity of the product was established by the detection of a single band with ninhydrin spray (0.2% in isopropyl alcohol), molybdate spray (Hanes and Isherwood, 1949), and—if radioactive—by scanning with a TMC-Vanguard, Model 880, chromatogram strip scanner. Phosphoserine was eluted from the paper with water.

^{3}H associated with the β-carbon of serine was measured in the dimedon derivative of formaldehyde (Frisell and Mackenzie, 1958) which had been produced by periodate oxidation of serine (Nicolet and Shinn, 1941). H atoms attached to the β-carbon of serine are not appreciably exchanged in this procedure (Mackenzie and Abeles, 1956).

P analyses were carried out with a scaled-down version of the Sumner (1944) procedure. Total P was determined after digestion according to Robertson and Boyer (1956). The P_i content of protein solutions was measured after removal of protein by precipitation with trichloroacetic acid (10%) or by ultrafiltration through Diaflo membranes (Amicon Corp.) UM-1, UM-2, or PM-10. In trichloroacetic acid filtrates, some protein may remain dissolved. If the aliquot used for P_i analysis is large, perceptible precipitation may occur on addition of molybdate. Recovery tests with added P_i indicated that no disproportionate losses of P are incurred if the precipitate is removed by centrifugation. However, we found recently that the precipitate has a small and apparently saturable capacity to entrap P_i. Thus significant losses of P will occur if the P concentration is low or the protein concentration is high. None of the experiments reported in this paper are subject to this limitation. Analysis for carbonyl groups in the oxidized protein was done by the method of Lappin and Clark (1951).

The ^{18}O content of P_i was determined after isolation of P_i and the conversion of its O atoms into CO_2 by mass spectrometric analysis according to Boyer and Bryan (1967) and Boyer *et al.* (1961) with a Consolidated Electrodynamics residual gas analyzer, Type 21–614. Radioactive samples containing ^{3}H or ^{32}P were analyzed with a Beckman, CPM-100, liquid scintillation system, using 0.5-ml samples in 10-ml portions of a modification of the scintillation fluid of Bray (1960), as described earlier (Grant and Taborsky, 1966).

Results

Nature of Reactive Sites and Oxidation Products. If aminomalonic semialdehyde is the oxidation product, as suggested earlier (Grant and Taborsky, 1966), then the serine, generated from the oxidized protein by reduction with [^{3}H]$NaBH_4$, should be labeled exclusively on the β-carbon. Periodate oxidation of such [^{3}H]serine and analysis of the serine fragments showed (in triplicate experiments) that this is the case for 97–99% of the serine-bound isotope.

The other known oxidation product, P_i, must be derived from phosphoserine residues, since the specific activities of phosphoserine in phosvitin (labeled with ^{32}P by reaction of the protein with [^{32}P]ATP in the presence of phosphoprotein kinase) and of the P_i released from the protein upon oxidation are very similar (Table I, expt 1).

Number of Reactive Sites. In five typical experiments, 0.89 ± 0.07 (standard deviation) residue of phosphoserine/mole of protein (based on a molecular weight of 35,000 (Taborsky and Mok, 1967)) was oxidized. The yield was measured in terms of the radioactivity of [^{3}H]serine (obtained from oxidized and then reduced protein) and the separately determined specific activity of the reducing agent (assuming the transfer of one hydride to the aldehyde). This is a very low yield in view of the high phosphoserine content of the protein (about 120 residues/mole; Allerton and Perlmann, 1965). However, the reactivity is known to extend over several residues of phosphoserine, as a minimum, if the experimental conditions are varied: the amount of O_2 consumed by the reaction can be increased (Grant, 1965), and so can the yield of various products. For example, when Fe^{2+} is added to the protein in increments over the whole time course of the reaction, the yields of carbonyl groups, aminomalonic semialdehyde residues, and P_i can be enhanced by factors of up to three. A cumulative increase in the oxidation yield can be observed when the protein is repeatedly oxidized (Table II).

[1] 100% saturation was taken to correspond to a P:Fe ratio of 2 (Taborsky, 1963).

TABLE I: Radioactivity of Phosphorus Derived from [^{32}P]Phosvitin.[a]

Expt	Pretreatment of Phosvitin[b]	Extent of Phosphorylation by [^{32}P]ATP[c] (mole/mole)	Sp Act. (cpm/μmole of P)		
			Protein Phosphate	Serine Phosphate	P_i Released by Oxidation
1	None	0.09	1,520	1,640	1,380
2	Oxidation, reduction	0.28	54,500	50,500	43,000
3	Oxidation, reduction	1.73	304,000	308,000	303,000

[a] All experiments were done with phosvitin pretreated as shown. The protein was incubated with [^{32}P]ATP and phosphoprotein kinase, isolated, completely hydrolyzed to yield protein phosphate, partially hydrolyzed to yield serine phosphate, or oxidized to yield P_i released by oxidation. [b] Oxidation and reduction, when indicated, were done as usual. [c] The extent of phosphorylation, varied by variation of the enzyme concentration and the time of incubation, was measured in terms of the radioactivity incorporated into the protein from an ATP preparation of known specific activity.

At least through three oxidation cycles, the increase is nearly linear, suggesting that the steps are equivalent. To gain a larger enhancement of the yield, an extension of recycling experiments to many cycles would be impractical; other efforts, by variation of temperature, pH, or O_2 pressure, failed. The question about the number of potentially oxidizable phosphoserine residues was approached, therefore, in an indirect way.

Oxidizability of Phosphoserine Residues: Question of Specificity. Our approach depended on the use of protein tagged with ^{32}P or ^{3}H or both. (The valid use of [^{32}P]phosvitin requires that certain conditions be met. That these experiments meet them will be shown in the following section.) In the experiments shown in Table I, the close agreement between the specific activities of P—protein bound, serine bound, and released by oxidation—implies that all phosphorylated serine sites may be equally susceptible to oxidation. Essentially the same agreement is found if labeling of the protein was accomplished by the phosphorylation of serine residues which had been either nonphosphorylated to begin with (expt 1), or additionally generated by oxidation (phosphoserine to aldehyde) and reduction (aldehyde to serine) (expt 2 and 3).

Evidence that enzymic phosphorylation of the serine residues generated by oxidation and reduction does indeed occur is given in Table III. It shows that, if [^{3}H]$NaBH_4$ is used in the reduction step, ^{3}H appears in phosphoserine upon phosphorylation. (The low specific activity of phosphoserine, compared with the level of ^{3}H in total protein-bound serine simply reflects incomplete phosphorylation of the free serine residues and will be of no consequence for our argument.) It may be noted also that the specific activities of protein-bound phosphoserine before and after oxidation are in good agreement (within ±5%), reenforcing the view that the residues oxidized represent a random sample of all residues. This view will be developed on the basis of the experiments of this section in the Discussion.

The experiment with the most highly phosphorylated and labeled preparation should be the best basis for setting limits on the size of the oxidizable pool of residues. In terms of that experiment (no. 3, Table I), the size of the oxidizable pool could differ from the total phosphoserine pool by no more than two to three residues. This is only about 2% of the total pool.

Phosphoprotein Kinase Action: Specificity for Serine Residues and Lack of P Exchange. A valid interpretation of the experiments with [^{32}P]phosvitin requires that the kinase-catalyzed phosphorylation be limited to serine residues and that phosphate, once introduced, should not be subject to random redistribution among all serine residues under the action of the enzyme.

The first requirement appears to have been met in view of the closely similar specific activities of total protein-bound P and of phosphoserine P, as shown by the three experiments in Table I.

The data in Table IV rule out appreciable randomization by some exchange reaction mediated, for example, by a phosphoenzyme. These experiments were done with [^{3}H]-phosvitin, the ^{3}H being in serine residues which had been formed by previous oxidation and reduction of the protein. With kinase alone, or with kinase and ADP, the apparent degree of phosphorylation of these [^{3}H]serine residues was only about one-third or less of the actual degree of phosphorylation attained with ATP present. The appearance of ^{3}H

TABLE II: Cumulative Increase in Oxidation Yield upon Repeated Reaction.[a]

No. of Reaction Cycles	Sp Act. [^{3}H]Serine (cpm/μmole)[b]		
	Expt 1	Expt 2	Expt 3
1	23,000	23,000	0
2	40,000	40,000	0
3	61,500	42,500	17,000

[a] The protein was oxidized and then reduced with $NaBH_4$, repeating this cycle three times. The oxidation yield was measured after each cycle in terms of [^{3}H]serine, derived from aminomalonic semialdehyde by reduction of the oxidized protein. [b] The three experiments differ only in the presence or absence of ^{3}H in the reducing agent used in given cycles. [^{3}H]$NaBH_4$ was used in all cycles of expt 1, int he first two cycles of expt 2, and only in the third cycle of expt 3. Experiments 1 and 2 are identical through the second cycle.

TABLE III: Radioactivity of [^{3}H]Serine and [^{3}H]Phosphoserine Derived from Oxidized, Reduced, and Phosphorylated Phosvitin.[a]

	Sp Act. (cpm/μmole)		
		Phosphoserine	
Expt	Total Serine	Before Oxidation	After Oxidation
1	22,900	1,780	1,950
2	22,900	5,800	6,250

[a] Oxidation, reduction with [^{3}H]$NaBH_4$, and phosphorylation with ATP in the presence of phosphoprotein kinase preceded the analyses. The oxidation referred to in the table is a second oxidation which was carried out *after* the phosphorylation step of the above-mentioned sequence of reactions. The two experiments shown here are based on the same oxidized and reduced preparation, hence the identical values for total serine. Aliquots of the reduced protein were subjected to kinase-catalyzed phosphorylation for different periods of time to attain different levels of esterification of the free serine residues: 0.28 and 1.73 moles of P per mole of protein, respectively, in the two experiments. (These experiments involve the same protein preparations that are described in expt 2 and 3 of Table I.) The explanation of the gross deviation of the specific activity ratio of the data given here for the two experiments (1780/5800 = 0.31) from the corresponding ratios of the degree of phosphorylation (0.28/1.73 = 0.16) or the [^{32}P]phosvitin and [^{32}P]phosphoserine specific activity data in Table I (54,500/304,000 = 0.18 and 50,500/308,000 = 0.16) lies in the fact that some phosphate appears to become randomly distributed among serine residues during partial acid hydrolysis which leads to phosphoserine liberation. For further details, see the next section on phosphoprotein kinase action.

TABLE IV: Test of Serine-to-Serine Migration Catalyzed by Phosphoprotein Kinase.[a]

	Sp Act. of [^{3}H]Phosphoserine (cpm/μmole)	
Additions to Reaction Mixture	Found	Theory for Random Equilibration
None	760	
Kinase	800	22,900
Kinase + ADP	510	22,900
Kinase + ATP	1,780	22,900

[a] The tests were carried out by incubating [^{3}H]phosvitin (obtained by oxidation followed by reduction with [^{3}H]$NaBH_4$) with Mg^{2+} and added components as indicated. Except for these, the conditions of each incubation were identical. The degree of serine-to-serine shift of phosphate is measured by the extent to which nonphosphorylated [^{3}H]serine appears to have been transferred to the phosphoserine pool. The theoretical maximum of phosphoserine specific activity corresponds to completely random distribution of [^{3}H]serine residues among all serine residues whether they are phosphorylated or not.

in phosphoserine in the experiments *without* ATP cannot represent kinase-catalyzed shifts of P from phosphoserine to [^{3}H]serine because approximately the same low level of radioactivity was moved from the serine pool into the phosphoserine pool in the absence of kinase. This small degree of phosphate shift, to an extent of about 3% of the theoretical maximum, is probably associated with the exposure of the protein to acid (see, for example, Khorana, 1961) during its partial hydrolysis to yield phosphoserine.

Test of the Formation of a Stable Enol Phosphate Intermediate. In the original hypothesis of the oxidation mechanism, the formation of the enol phosphate derivative of phosphoserine (by α,β dehydrogenation) was postulated. Our interest being centered on the reaction mechanism, we made a search for more direct evidence for this hypothetical intermediate.

An authentic enol phosphate, phosphoenolpyruvate, condenses with 2,4-dinitrophenylhydrazine as if a fraction of its latent carbonyl function would be reactive, presumably as a consequence of its partial hydrolysis under the hot, acidic conditions prevailing in the carbonyl assay: 0.1–0.2 equiv of carbonyl group and P_i were produced after 5 min, and about 0.6 carbonyl group equiv after about 20 min. In contrast, with an acidic reaction mixture of 2,4-dinitrophenylhydrazine and oxidized protein, the yield of P_i rose not at all after 10 min, and only barely significantly after 30 min at 100°, relative to the P_i yield of the oxidation reaction itself (and corrected for the small amount of hydrolysis measured with a control sample of nonoxidized phosvitin).

Mercuric salts are known to bring about a rapid cleavage of the enol phosphate linkage (Lohmann and Meyerhof, 1934). But, when oxidized phosvitin (from which over 90% of its Fe^{3+} content had been removed by repeated ultrafiltration in the presence of EDTA) was treated with $HgCl_2$, no P_i was liberated. Under similar conditions, phosphoenolpyruvate released essentially all of its phosphate.

Were a protein-bound phosphoenol residue to have drastically altered reactive properties, compared with a "typical" enol phosphate, ^{3}H incorporation into serine might conceivably occur *via* the reduction of such an "atypical" phosphoenol residue. Some enol esters are reducible with $NaBH_4$ (Dauben and Eastham, 1951), but phosphoenolpyruvate is not. When reaction mixtures of [^{3}H]$NaBH_4$ and either pyruvic acid or phosphoenolpyruvate were subjected to paper electrophoresis, the former mixture yielded exclusively [^{3}H]lactic acid, while the latter contained no radioactive product only an acidic one with the same mobility as that of the starting material. Its identity with the starting material was confirmed by the demonstration that it released 0.97 equiv of P_i upon treatment with $HgCl_2$. It might be argued that protein-bound phosphoenol could exist as two tautomers, A and B, of which B, being a Schiff's base, could be reducible. But reduction of B with [^{3}H]$NaBH_4$ would place ^{3}H on the α-carbon, contrary to our finding.

TABLE V: Test of Sodium Borohydride Effect on Phosvitin Oxidation.[a]

Expt	Iron Added at Start of Oxidation	Nature of Reducing Agent Added		Specific Activity			
		Before Oxidation	After Oxidation	[^{3}H]Phosvitin[b] (cpm/optical density unit)			[^{3}H]Serine (cpm/μmole)
				15 min	30 min	60 min	60 min
1	Fe^{2+}	None	[^{3}H]$NaBH_4$	29,000	32,900	32,800	48,000
2	Fe^{2+}	[^{3}H]$NaBH_4$	$NaBH_4$	24,800	30,800	30,200	47,500
3	Fe^{2+}	$NaBH_4$	[^{3}H]$NaBH_4$	8,480	8,170	7,020	8,800
4	Fe^{3+}	[^{3}H]$NaBH_4$	$NaBH_4$	6,910	7,700	7,740	9,850

[a] The oxidation was carried out as usual with Fe^{2+} or Fe^{3+} (control) and the inclusion of $NaBH_4$ or [^{3}H]$NaBH_4$ in the oxidation mixture as indicated. At given times, aliquots were removed, subjected to the action of a second portion of reducing agent, acidified to stop the oxidation and destroy excess reagent, dialyzed, lyophilized, and subjected to complete hydrolysis. The hydrolysates were evaporated to dryness, taken up in water, and aliquots were subjected to ninhydrin analysis or to the serine isolation and assay procedure. [b] The specific activity of the protein is expressed in terms of the radioactivity (counts per minute) associated with a quantity of protein hydrolysate giving an optical density of 1.00 in the ninhydrin assay.

These experiments provide no support for the hypothesis that the phosphoenol derivative of phosphoserine is a detectable intermediate product of the oxidation. We reinvestigated, therefore, the earlier finding on which, in part, the hypothesis had been based. Phosvitin was oxidized as usual, yielding about 1 mole of P_i/mole of protein, as expected. It was then subjected to mild acid treatment and assayed for the formation of additional P_i and carbonyl groups. The conditions of these experiments were: exposure to 1 N HCl, at 30°, in the absence and in the presence of 0.5 M KCl (Grant and Taborsky (1966) reported additional P_i release under the latter conditions), or incubation in sodium citrate buffer (pH 3.8) at 50° (conditions under which phosphoenol hydrolysis might proceed more rapidly (Weil-Malherbe and Green, 1951)). Any increase in P_i noted over a period of 2–3 days was matched by an increase in a control mixture containing nonoxidized phosvitin and Fe^{3+}. Also, the specific activity of [^{3}H]serine, isolated from oxidized protein that had been in contact with 1 N HCl for 3 and 9 hr and was then reduced with [^{3}H]$NaBH_4$, remained constant (101 and 92%, respectively, of the value obtained before acid treatment).

In retrospect, it seems clear that the original observations dealt with an artifact. In view of the problem associated with the use of trichloroacetic acid filtrates (see Experimental Procedure) which had been used in the earlier work, the control values for nonoxidized protein must have been "too small" because the size of aliquots used was larger for blank analysis than for the analysis of oxidation mixtures (in order to keep absolute amounts of P_i more nearly equal). Hence, an apparent net gain in P_i in the oxidation mixture could have arisen as a consequence of a false correction for the blank value. In this context, it is of interest that ultrafiltrates (prepared with P-10 Diaflo membranes) of oxidation mixtures show a nearly negligible P_i content unless the mixtures are acidified before filtration: some acid-labile interaction between P_i and phosvitin may be implied.

Test of Proton Incorporation into Oxidized Protein from Water. Among possible mechanisms of the oxidation, some would require that protons derived from the aqueous medium appear in the stable oxidation product. In an attempt to determine if such incorporation occurs, we had to reckon with the possibility that protons may exchange between the aldehyde product and water because of enolization. The oxidation was carried out, therefore, in the presence of $NaBH_4$ in the expectation that the reducing agent would react with the aldehyde as rapidly as it was formed, trapping it as a derivative (serine) with only nonexchangeable carbon-bound H atoms. This deviation from the usual composition of the oxidation mixture required that we ascertain whether there is any interference with the oxidation proper by the added borohydride. Table V summarizes the results of this test. In terms of the gross incorporation of ^{3}H into protein from [^{3}H]$NaBH_4$, it appears to be of little consequence whether the reducing agent is added only after completed oxidation, as usual (expt 1), or is present from the beginning (expt 2). Neither the rate and extent of the reaction, in terms of protein activity data, nor the specific activity of the final product, [^{3}H]serine, are appreciably affected.

(Parenthetically, we may note that this result is not without some intrinsic interest from the point of view of that aspect of the oxidation which falls outside the scope of this paper, namely the linkage of protein oxidation to iron autoxidation. Since borohydride reduces Fe^{3+} to Fe^{2+} (Gaylord, 1956), *extensive* additional protein oxidation might be expected as long as reducing agent was available to regenerate Fe^{2+} *in situ*. This does not seem to occur, in view of the similar yields of the labeled oxidation products in expt 1 and 2 (Table V). That *some* additional oxidation might occur in this fashion is suggested, nevertheless, by the formation of labeled product (to an extent of about 20% of the normal yield) in the two control experiments (3 and 4). However, the similarity of the yields in these experiments does not permit a straightforward interpretation because of the difference in the timing of the exposure to labeled reagent.)

For the actual proton incorporation test, the incubation was shortened to 15 min in order to ensure that an effective

TABLE VI: Incorporation of Protons from Solvent into the Oxidation Product.[a]

Expt	Variable Components of the Reaction Mixture	Number of Hydrogen Atoms Incorporated (atoms/mole)			
		Phosvitin		Serine[b]	
		Actual Value	Cor for Complete Oxidation[c]	Cor for Complete Oxidation[c]	Cor for Blank Incorp
1	Phosvitin + $NaBH_4$	0.18	0.34	0.38	0.08
2	Oxidized phosvitin + $NaBH_4$	0.27	0.27	0.30	
3	Oxidized phosvitin	0.27	0.27	0.30	

[a] The indicated form of protein was incubated in $[^3H]H_2O$ with or without $NaBH_4$ as shown. In expt 1, Fe^{2+} was added at the start of incubation as the last component of the reaction mixture. In the other experiments, the protein had been oxidized previously and it contained bound Fe^{3+} as a result, comparable in quantity with the amount of Fe^{2+} used in expt 1. The period of incubation was 15 min. The period of prior oxidation in expt 2 and 3 was 45 min. [b] The results in terms of moles of oxidized serine are based on the value of 0.89 oxidized residue/mole of protein, determined in replicate experiments and referred to earlier in the text. [c] For the significance of the correction for complete oxidation, see text.

concentration of the reducing agent survived to the termination of the reaction. The experiments are described in Table VI. Experiment 1 is the principal experiment in which oxidation was initiated by the addition of Fe^{2+} to a solution of phosvitin and $NaBH_4$ in $[^3H]H_2O$. It was stopped by the addition of trichloroacetic acid and the precipitated protein was washed free of tritiated water, yielding material in which only stably bound isotope remained. "Blank" incorporation, not associated with oxidation, was measured in expt 2. Experiment 3 shows that the presence of $NaBH_4$ in expt 2 does not affect the blank incorporation. The first column of data in Table VI shows the actual number of H atoms incorporated. These numbers may be corrected for differences in the extent of protein oxidation: any isotope incorporation which is a direct reflection of an obligatory step of the oxidation is likely to be in a stoichiometric relationship with the extent of oxidation

TABLE VII: Incorporation of ^{18}O from Water into P_i Released by Oxidation.[a]

Expt	^{18}O Content (atom % excess)			No. of Solvent Oxygen Atoms Incorp into P_i
	P_i (per 4 O atoms)	P_i[b] (per 1 O atom)	Solvent Water	
1	2.26	9.04	9.50	0.95
2	2.04	8.16	8.11	1.01
3	0.234	0.936	1.01	0.93

[a] The given values of ^{18}O atom % excess represent averages of four or more analyses in every case. The values for P_i had been corrected for dilution by carrier: the dilution factors were about 9, 4, and 2 in the order of the experiments. [b] These values are fourfold multiples of those in the column to the left, taking account of the fact that the release of P_i must be accompanied by the net incorporation of only 1 atom of O/mole of P_i.

The latter was measured in separate experiments in terms of the specific activity of [^{3}H]serine, as usual. These experiments were identical with those in Table VI except that ^3H was present in the reducing agent and not in the water. In expt 1 (after 15-min reaction), the oxidation extent was estimated to be 54% of the extent of oxidation of the protein used in expt 2 and 3. The second column of data in Table VI shows the incorporation data after correction for complete oxidation. The third column gives the results per mole of oxidized phosphoserine. The fourth column shows the *net* incorporation after correction for the blank value. Unfortunately, it seems that to devise a completely unobjectionable "blank" experiment is actually beyond reach in this instance. It is uncertain, for example, whether the blank value obtained in expt 2 might not have been more fairly applied *before* the correction for complete oxidation had been made: the blank incorporation may not be proportional to the extent of oxidation and may even be independent of it. In that case, the net value would be actually small but negative. In any case, the level of incorporation—even without the refinement of a blank correction—is so much lower (0.38) than what the minimal stoichiometry would require (1.00) that it seems most reasonable to conclude that there is no obligatory proton incorporation associated with the oxidation directly.

Bond Cleavage Associated with P_i Release. Since the overall process from phosphoserine to the aldehyde and P_i involves the cleavage of a phosphate ester, it is of material importance for the establishment of the mechanism to discover on which side of the bridge O atom the cleavage occurs. The nature of the products implies that the elements of water are consumed in the reaction. The results given in Table VII show that O derived from water appears to an extent of nearly exactly 1 atom/mole of P_i, indicating essentially exclusive P–O bond cleavage.

Discussion

Four aspects of the reaction between phosphoprotein, Fe^{2+}, and O_2 invite discussion: (1) the nature of the sites and products of the reaction, (2) the oxidation yield, (3) the

mechanism of the oxidation, and (4) its possible biological significance.

Nature of Reactive Sites and Oxidation Products. The original hypothesis (Grant and Taborsky, 1966) that the products, P_i and a carbonyl derivative of serine, arise from a common source, phosphoserine, did not rule out the possibility of alternative, separate sources. For example, phosphothreonine and carbohydrates (which may be phosphorylated) are known components of phosvitin (Allerton and Perlmann, 1965; Tunmann and Silberzahn, 1962), as are nonphosphorylated residues of serine (Rosenstein and Taborsky, 1970). However, the hypothesis appears now to rest conclusively on (1) the implications of the highly probable overlap between oxidation sites, Fe^{2+} binding sites, and phosphorylated sites;[2] (2) the finding that isotope (^3H) on the β-carbon of serine residues, introduced by reduction of oxidized protein, appears to be unaffected upon repeated oxidation; and (3) the demonstration that P_i is derived mostly, if not solely, from phosphoserine residues.

Oxidation Yield. The quantitative aspects of the oxidation are puzzling on first sight. The finding that, in any actual experiment, no more than a few of a vast number of phosphoserine residues become oxidized at best, raises the question of specificity. The existence of phosphoserine residues with distinguishable reactivities is possible, but such distinctions would have to reflect features of the primary structure of the protein since it is normally, including these experiments, devoid of secondary structure.[3] An obvious possibility for a distinction between phosphoserine residues is implicit in their primitive arrangement in linear sequences of up to six residues uninterrupted by other amino acids (Williams and Sanger, 1959; Belitz, 1965): the reactivity of terminal and internal residues might be different (Rabinowitz and Lipmann, 1960; Mano and Lipmann, 1966). However, our observations seem to be consistent, in a straightforward fashion, only with the view that essentially all of the phosphoserine residues are potentially oxidizable in an independent and random fashion.

The premise of our argument is that any nonphosphorylated serine residues in a given phosvitin preparation represent a small, distinct class of all serines (phosphorylated or not), and that their kinase-catalyzed phosphorylation converts them into a small, distinct class of phosphoserines. This class can be labeled if the phosphorylation is carried out with ^{32}P. Were the oxidation to show preference for any distinct class of phosphoserines, then the specific activity of this oxidizable class of residues (and P_i released by them) should, in general, differ from the average specific activity of all phosphoserine residues. Equal specific activities of the oxidizable set of residues and all residues could occur only (1) in the highly unlikely event that the labeled and the oxidizable classes overlap fortuitously to a particular degree (determined by the size of the fraction of all residues falling into the oxidizable class), or (2) in the much more likely event that the oxidizable set overlaps completely with the set of all residues. The data in Table I show that the specific activities of the oxidizable class (assessed as P_i) and of all phosphoserines are so similar that we must conclude—dismissing the possibility given under 1) above as totally improbable—that most, if not all, phosphoserines must be oxidizable.

It may be argued that our basic premise is arbitrary and may be invalid for expt 1 (Table I) since we do not know how any of the nonphosphorylated serine residues in the untreated protein may have arisen: they may or may not represent a special class.[4] But expt 2 and 3 are not objectionable on this ground. Indeed, the additional free serine residues generated by oxidation and reduction are specifically known to be derived from readily oxidizable phosphoserine residues, and upon rephosphorylation the latter should be regenerated.[5] Then the existence of a distinct, preferentially oxidizable class of residues would require that upon reoxidation the specific activity of P_i should be greater than that of all residues. This, clearly, is not the case.

Thus, we are still faced with the contrast implied by the actual oxidizability of only a few among many, apparently equivalent residues. Its explanation has been suggested by Grant (1965) to be related to the presumed linkage between iron autoxidation and protein oxidation. If the dehydrogenation of a phosphoserine residue occurs by reaction with a derivative of O_2, "activated" by its interaction with the complex between Fe^{2+} and the iron binding sites of the protein,[6] then it may be envisaged that the dehydrogenation competes for that oxidant with the oxidation of other ferrous ions. The partitioning of the common oxidant between the two reactions may be expected to be governed by spatial aspects of the protein–iron–oxygen complex. In this complex, the competing reductants are most likely confined to short segments of the protein chain composed of phosphoserine residues. Displacement of the competition in favor of one or another reductant should be possible by changing their relative abundance within such segments. Although the phosphoserine concentration is fixed in any segment, the iron concentration is variable. The finding that a slow, gradual addition of Fe^{2+}—ensuring a low Fe^{2+}: phosphoserine ratio—is effective in displacing the competition in favor of phospho-

[2] The autoxidation of Fe^{2+} is an obligatory corollary of the protein oxidation (Grant and Taborsky, 1966), presumably because it is in the course of the former that the activation of O_2 occurs (Stewart, 1964), yielding the actual oxidant consumed in the latter. Phosphorylated sites appear to be the only functional groups in phosvitin present in sufficient number to interact with the high number of bound Fe^{2+} (Taborsky, 1963).

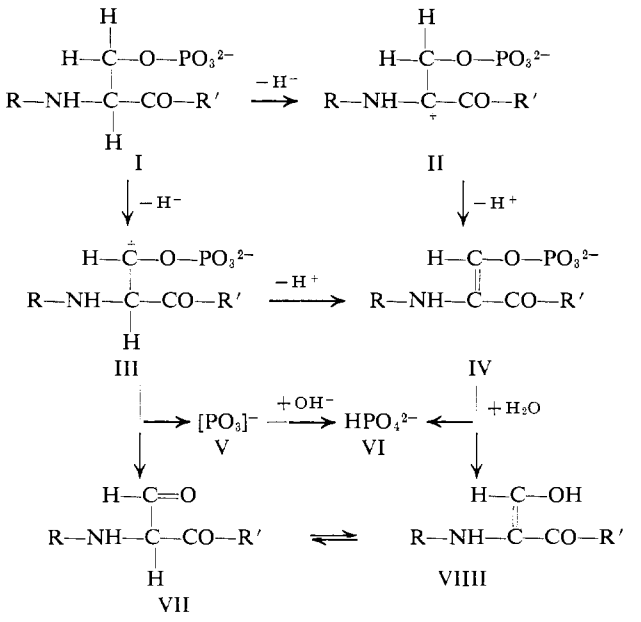
[3] The conformation of phosvitin is unordered over a wide range of conditions (Jirgensons, 1966; Perlmann and Allerton, 1966; Taborsky, 1968) and remains so upon addition of Fe^{2+}: solutions comparable with the reaction mixtures described in this paper show circular dichroic spectra which are very similar to those shown by iron-free phosvitin solutions in the region between 195 and 250 mμ (G. Taborsky, 1968, unpublished data).

[4] For example, if phosphate-free serine were to arise, plausibly, by random hydrolysis of fully phosphorylated "native" protein during its isolation or storage, then the assumption would be ostensibly invalid that these residues represented a special class. This would be so even if the kinase possessed relative specificity: to re-form randomly oxidizable phosphoserine residues, it would be necessary only that kinase-catalyzed phosphorylation proceed at all and not that it occur at a rapid rate.

[5] We assume that, in view of the absence of any ordered structure in phosvitin in these experiments,[1] any aspect of the chemical reactivity of the protein is rooted in the primary structure alone and that limited reaction has no other effects than the production of specific, localized, covalent changes.

[6] For detailed discussions of the role which iron chelates may play in oxidations see, for example, Kaden and Fallab (1961), Stewart (1964), and Green *et al.* (1956); for additional references, see Grant and Taborsky (1966).

SCHEME I



serine, is clearly consistent with this view of the linked oxidation system. Examples of similar competitive reactions are known. The yield of H_3PO_3, in the case of the autoxidation of Fe^{2+} and H_3PO_2, varies with the P:Fe ratio (Wieland, 1932); Fe^{2-} and glycine appear to compete for the same oxidant in the reaction between Fe^{2+}, glycine, O_2, and H_2O_2 (Maxwell and Peterson, 1957). Such competition may even be resolved exclusively in favor of ligand oxidation, not because of the concentration factor but as a consequence of the great stability of the complex between unreacted metal and oxidized ligand, as in the autoxidative conversion of the Fe^{2+}–dipiperidyl complex into its dehydrogenated derivative (Krumholz, 1953). If the phosphoprotein oxidation were to have a biological counterpart, it could readily occur with such an extremely uneven partition ratio either as a result of a controlled supply of Fe^{2+} or because of suitable enzymic direction.

Mechanism of the Oxidation. Scheme I depicts our view of the reaction mechanism. Assuming that the first event is the removal of a hydride ion or its electronic equivalent from phosphoserine (I), the oxidation could involve either the α- or the β-carbon, producing one or another carbonium ion derivative of the protein (II or III). Keeping in mind that the eventual product is the free aldehyde, further reaction may be supposed to occur in one of two ways. (Other possibilities are not considered since they would invariably lead to other than the aldehydic product.) A proton loss from either II or III would complete an α,β dehydrogenation and produce the enolphosphate (IV) which, by hydrolysis, would yield the final products P_i (VI) and aldehyde (VII) (through the enolic form, VIII, of the latter). A metaphosphate (V) loss from III would produce the aldehyde directly and P_i upon solvation of the hypothetical metaphosphate monomer. Even though both pathways would lead to the known products, only one accommodates all of the experimental observations. There is no evidence for enolphosphate formation, and there is evidence against the incorporation of solvent protons into the final product. Thus, any pathway *via* IV and VIII must be ruled out, leaving only the direct path which involves no stable phosphorylated intermediate. Whether the reaction from III to VI and VII occurs stepwise or in a concerted fashion cannot be decided, but the evidence for P–O cleavage requires that a nucleophilic attack by water (or OH^-) occur on phosphorus and not on carbon. It may be noted that the proposed mechanism would leave the configuration around the α-carbon intact.

The mechanism is analogous to the oxidative labilization of the phosphate groups of various hydroquinone esters (Clark *et al.*, 1961; for additional references, see Bruice and Benkovic, 1966). Evidence that the hypothetical, monomeric metaphosphate produced by phosphoprotein oxidation can phosphorylate substances other than water has not yet been obtained. In this context, however, it is of some interest to recall that unless the solution of oxidized protein is acidified before removal of protein and analysis for P_i, the apparent yield of P_i may be vanishingly small. The composition of phosvitin and its partially known sequence are suggestive of the possibility of the formation of acid-labile phosphoramidate bonds: basic amino acids are the second most dominant components of the protein, after phosphoserine (Allerton and Perlmann, 1965), and they tend to occur at the terminal residues of polyphosphoserine blocks (Belitz, 1965).

Possible Biological Significance. It is one of our working hypotheses that phosvitin may provide the developing chick embryo with a potential phosphorylating agent which becomes activated by oxidation. Alternatively, phosphoprotein oxidation may be looked upon as a specific macromolecular model of the energy conservation step of the earliest hypothesis of the mechanism of oxidative phosphorylation, proposed by Lipmann (1941) (see also Slater, 1966). Modifying the earlier speculation (Grant and Taborsky, 1966) on the basis of the mechanism proposed above, it seems to us worth noting that such a model implies a phosphorylated, energy-rich intermediate which would be expected to be undetectable because of its transient character as a carbonium ion. Even its energy-poor form (phosphoserine) might not exist in appreciable concentration. Depending on the respiratory state of the oxidatively phosphorylating system, the intermediate might be oxidized and already dephosphorylated, or reduced but not yet rephosphorylated. Esterification of P_i to an alcohol group is energetically unfavorable and would need to be driven by the net free-energy yield of the rest of the cycle.

Acknowledgments

We are grateful to Dr. Denis M. Dineen for his generous gifts of [^{32}P]ATP and phosphoprotein kinase. We also wish to thank our colleagues for the use of certain instruments: Dr. Michael Caplow (mass spectrometer and liquid scintillation counter), Dr. Thomas F. Emery (liquid scintillation counter), and Dr. William Konigsberg (amino acid analyzer).

References

Ahmed, K., and Judah, J. D. (1967), *Methods Enzymol. 10*, 777.

Allerton, S. E., and Perlmann, G. E. (1965), *J. Biol. Chem. 240*, 3892.

Belitz, H.-D. (1965), *Z. Lebensm. Untersuch. Forsch. 127*, 341.

Boyer, P. D., and Bryan, D. M. (1967), *Methods Enzymol. 10*, 60.

Boyer, P. D., Graves, D. J., Suelter, C. H., and Dempsey, M. E. (1961), *Anal. Chem. 33*, 1906.
Bray, G. (1960), *Anal. Biochem. 1*, 279.
Bruice, T. C., and Benkovic, S. (1966), Bioorganic Mechanisms, Vol. 2, New York, N. Y., Benjamin, p 91.
Busch, H., Hurlbert, R. B., and Potter, V. R. (1952), *J. Biol. Chem. 196*, 716.
Clark, V. M., Hutchinson, D. W., Kirby, G. W., and Todd, A. R. (1961), *J. Chem. Soc.*, 715.
Connelly, C., and Taborsky, G. (1961), *J. Biol. Chem. 236*, 1364.
Dauben, W. G., and Eastham, J. F. (1951), *J. Amer. Chem. Soc. 74*, 4463.
Dineen, D. M. (1967), Ph.D. Thesis, University of East Anglia, Norwich, England.
Frisell, W. R., and Mackenzie, C. G. (1958), *Methods Biochem. Anal. 6*, 63.
Gaylord, N. G. (1956), Reduction with Complex Metal Hydrides, New York, N. Y., Interscience, p 60.
Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90*, 147.
Grant, C. T. (1965), Ph.D. Dissertation, Yale University, New Haven, Conn.
Grant, C. T., and Taborsky, G. (1966), *Biochemistry 5*, 544.
Green, S., Mazur, A., and Shorr, E. (1956), *J. Biol. Chem. 220*, 237.
Hanes, C. S., and Isherwood, F. A. (1949), *Nature 164*, 1107.
Jirgensons, B. (1966), *J. Biol. Chem. 241*, 147.
Joubert, F. J., and Cook, W. H. (1958), *Can. J. Biochem. Physiol. 36*, 399.
Kaden, T., and Fallab, S. (1961), *Helv. Chim. Acta 44*, 714.
Khorana, H. G. (1961), Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest, New York, N. Y., Wiley, p 63.
Krumholz, P. (1953), *J. Amer. Chem. Soc. 75*, 2163.
Lappin, G. R., and Clark, L. L. (1951), *Anal. Chem. 23*, 541.
Lipmann, F. (1941), *Advan. Enzymol. 1*, 99.
Lipmann, F. A., and Levene, P. A. (1932), *J. Biol. Chem. 98*, 109.
Lohmann, K., and Meyerhof, O. (1934), *Biochem. Z. 273*, 60.
Lundholm, L., Mohme-Lundholm, E., and Vamos, N. (1963), *Acta Physiol. Scand. 58*, 243.
Mackenzie, C. G., and Abeles, R. H. (1956), *J. Biol. Chem. 222*, 145.
Mano, Y., and Lipmann, F. (1966), *J. Biol. Chem. 241*, 3834.
Maxwell, C. R., and Peterson, D. C. (1957), *J. Amer. Chem. Soc. 79*, 5110.
Mecham, D. K., and Olcott, G. S. (1949), *J. Amer. Chem. Soc. 71*, 3670.
Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.
Nicolet, B. H., and Shinn, L. A. (1941), *J. Biol. Chem. 139*, 687.
Perlmann, G. E., and Allerton, S. E. (1966), *Nature 211*, 1089.
Rabinowitz, M., and Lipmann, F. (1960), *J. Biol. Chem. 235*, 1043.
Robertson, H. E., and Boyer, P. D. (1956), *Arch. Biochem. Biophys. 62*, 380.
Rosenstein, R. W., and Taborsky, G. (1970), *Biochemistry 9*, 658.
Slater, E. C. (1966), *in* Comprehensive Biochemistry, Vol. 14, Florkin, M., and Stotz, E. H., Ed., Amsterdam, Elsevier, p 355.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stewart, R. (1964), Oxidation Mechanisms, New York, N. Y., Benjamin, p 147.
Sumner, J. B. (1944), *Science 100*, 413.
Taborsky, G. (1963), *Biochemistry 2*, 266.
Taborsky, G. (1968), *J. Biol. Chem. 243*, 6014.
Taborsky, G., and Mok, C.-C. (1967), *J. Biol. Chem. 242*, 1495.
Tunmann, P., and Silberzahn, H. (1962), *Z. Lebensmitt. Untersuch. Forsch. 116*, 340.
Weil-Malherbe, H., and Green, R. H. (1951), *Biochem. J. 49*, 286.
Wieland, H. (1932), On the Mechanism of Oxidation, New Haven, Conn., Yale University, p 86.
Williams, J., and Sanger, F. (1959), *Biochim. Biophys. Acta 33*, 294.