A Rearrangement in the Structure of Phosvitin*

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The phosphoprotein phosvitin was found to release acid when incubated in aqueous solution at low alkaline *p*H values. This reaction was shown to lead to the disappearance of a titratable group having an apparent dissociation constant in the usual range of *pK* values for amino groups. Titrations in the presence of formaldehyde confirmed the indication that amino groups of the protein may be masked at alkaline *p*H. The disappearance of these groups was found to be at least partially reversible upon exposure to acid. At least two groups per molecule of protein appear to be involved in this reaction. The data are interpreted in terms of a hypothesis that phosphoryl groups may be shifted from hydroxyl to amino groups of the protein.

The migration of acyl groups between vicinal amino and hydroxyl functions of peptide-like substances has been known for some time (Bergmann *et al.*, 1923). In this reversible reaction, the amide linkage is formed in alkali and the ester linkage is favored in acid. A suitable orientation in space of the functional groups participating in such acyl transfer reactions was shown to be a prerequisite of a successful rearrangement (Fodor and Kiss, 1949). Other evidence indicated that similar rearrangements may occur in the covalent structure of proteins involving the alcohol function of hydroxyamino acid residues (Desnuelle and Casal, 1948; Elliott, 1952; Josefsson and Edman, 1956; Josefsson, 1958) although, with reference to some of this work (Josefsson and Edman, 1956; Josefsson, 1958), acylation of protein hydroxyl groups by the acid solvent rather than by acyl transfer from peptide to ester linkages was suggested as the more probable hypothesis (Smillie and Neurath, 1959). In phosphoproteins, the possibility of similar migrations of phosphoryl groups was envisaged (Linderstrøm-Lang, 1933). Shifts of dialkylphosphoryl groups from nitrogen to oxygen, at acid *p*H, were observed with derivatives of 1,2-hydroxyamino compounds (Plapinger and Wagner-Jauregg, 1953). As far as we are aware, there is no evidence either that the shift of dialkylphosphoryl groups may be reversible or that free phosphoryl groups may undergo similar migrations. Attempts to show such transfer reactions with *N*-phosphorylated derivatives of 1,2-hydroxyamino compounds were reported to lead instead to the hydrolytic cleavage of the phosphoramidate bond (Rathlev and Rosenberg, 1956). In this paper, we present evidence in support of the hypothesis that phosphoryl groups linked to the phosphoprotein phosvitin may be shifted from hydroxyl to amino groups of the protein.

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EXPERIMENTAL

Phosvitin was prepared by the method of Mecham and Olcott (1949) and was used without further treatment. The techniques used in elementary and amino analyses and some details of the use of the *p*H-stat were described in a previous publication (Connelly and Taborsky, 1961). Titrations were carried out with a *p*H-stat assembly either manually or automatically with a Radiometer Titrigraph. Experimental details for individual titrations are described under "Results." The experimental arrangement was subjected to control experiments which were repeated frequently to insure reliability and significance of the experimental data. Interference by atmospheric carbon dioxide was found to be insignificant by the measurement of the rate of alkali uptake by salt solutions at *p*H 10–11 for periods of several hours. Calibration of syringe burets (Agla Micrometer Syringes) and tests of the uniformity of the syringe bore were carried out by comparisons of standardized titrant solutions with various sections of a given syringe. Stability of *p*H readings within 0.02 *p*H units at *p*H 4, 7, and 10 was maintained for the duration of any one experiment. All titration curves were corrected for blank titrations obtained with appropriate salt solutions. The correction, compared with the total amounts of titrant required by the protein, was about 2% at *p*H 10 and about 15% at *p*H 11. The range of several blank values was ±0.1% and ±1%, respectively, at *p*H 10 and 11, again in terms of the amount of titrant consumed by the protein. The total blank variation was one order of magnitude smaller than the differences observed between protein titration curves described later in this paper. Titrations in the presence of

formaldehyde were carried out as follows. Analytical reagent grade formaldehyde, containing 12% methanol for stabilization, was added to the protein solution to a given concentration. The protein remained completely soluble under these conditions. Usually 2.00 ml of a phosvitin solution (in 1 M KCl) was subjected to a given preliminary treatment, followed by adjustment of the *p*H to 8.50. Immediately, formaldehyde was added and the resulting lowered *p*H (to about *p*H 7) was raised again to 8.50 by titrant addition over a period of approximately 20 minutes. The end-points at *p*H 8.50 of both the initial and final *p*H adjustments were determined by identical settings of the autotitrator.

Results

Amino Acid Composition of Phosvitin.—To assess the quantitative significance of the results reported in this paper it was necessary to consider the amino acid composition of the protein. The data in the literature (Mecham and Olcott, 1949; Lewis *et al.*, 1950) had been obtained mostly by microbiological methods, and we considered it desirable to make use of the more recent chromatographic method of amino acid analysis (Moore *et al.*, 1958; Spackman *et al.*, 1958) in the expectation of greater accuracy. Table I shows the composition of phosvitin determined by this technique. The data of Lewis *et al.* (1950) are included for comparison. Phosvitin is clearly a highly unusual protein in which approximately 60% of all amino acid residues are accounted for by residues of serine, 15% by residues of basic amino acids, 10% by acidic amino acid residues, and only 15% by residues of the several amino acids found in other proteins. There is sufficient serine for all of the phosphate to be linked to the alcohol functions as phosphomonoester groups.

Table I
Composition of Phosvitin

Residues	Moles/10^3 g of Protein	
	This Work	Lewis *et al.*[a]
Serine[b]	3.72	3.15
Lysine	0.45	0.41
Arginine	0.31	0.28
Histidine	0.30	0.31
Aspartic acid	0.36	0.33
Glutamic acid	0.32	0.23
Glycine	0.16	0.21
Alanine	0.20	0.17
Valine	0.06	0.09
Leucine	0.07	0.08
Isoleucine	0.04	0.04
Proline	0.09	0.08
Threonine	0.12	0.12
Phenylalanine	0.07	0.03
Tyrosine	0.07	0.01
Tryptophan	0.03	0.03
Cystine	0.00	0.00
Methionine	0.00	0.02
Phosphate	3.23	3.13
Phosphate/serine	0.87	0.99
N recovered (%)[c]	95	89
Weight recovered (%)[d]	97	89

[a] The values given by Lewis *et al.* (1950) were recalculated in terms of moles/10^3 g of protein and the weight and amount of N accounted for were also calculated. Their values for tryptophan and cystine and the elementary analyses for P and N had been taken from Mecham and Olcott (1949). [b] Values for serine were corrected for 32% hydrolytic loss in this work (based on a control experiment with *O*-phosphoserine), and for 10% loss in the work of Lewis *et al.* (1950) (based on reported losses of serine). [c] The recovery of nitrogen was calculated without including an estimate of amide nitrogen. The destruction of serine or phosphoserine during hydrolysis leads to the formation of large amounts of ammonia, making the estimation of amide nitrogen quite uncertain. Mecham and Olcott (1949) reported 0.6 moles of ammonia per 10^3 g of protein, corrected for the estimated ammonia arising from hydroxyamino acid destruction. [d] The recovery of weight was calculated from the amino acid and phosphorus analysis, assuming all phosphate to be monoesterified and in form of the monosodium salt. The protein had been dialyzed against sodium chloride, followed by water, prior to lyophilization; the *p*H of an aqueous solution of this protein preparation was between 4 and 5, indicating that the primary acid function of the phosphate group had been neutralized.

Instability of Phosvitin at Alkaline pH.—Earlier, we reported that phosvitin liberates acid at alkaline *p*H values (Connelly and Taborsky, 1961). Incubation of a 1% phosvitin solution in 1 M KCl in the *p*H-stat showed that at *p*H values 4.9, 6.0, 7.0, and 8.1 the *p*H remained constant without the addition of titrant. When the *p*H was raised to 8.50 or higher, a steady consumption of alkali over a period of many hours was required to maintain constant *p*H. Figure 1 shows the results of such an experiment at *p*H 8.50. A relatively rapid reaction during the first hour was followed by a slower, steady acid formation. This slow reaction did not cease in as many as 18 hours.

Titration Curve of Phosvitin.—The acid-forming reaction which phosvitin can undergo at alkaline *p*H was expected to affect the *p*H-titration curve of the protein. Figure 2 shows the results of a titration experiment between *p*H 3 and 11. The protein exhibited appreciable buffering in the *p*H range 8 to 11 during the titration with alkali (curve A), but very little buffering was observed in this *p*H region during the following titration with acid (curve B). A group giving up protons in this *p*H range during the alkaline titration was no longer available for protonation during the acid titration. When such titrations were performed manually (titration rate approximately 10 minutes per *p*H unit), a downward drift of *p*H was noted between titrant additions in the *p*H range 9 to 10.5. This drift was observed only during the

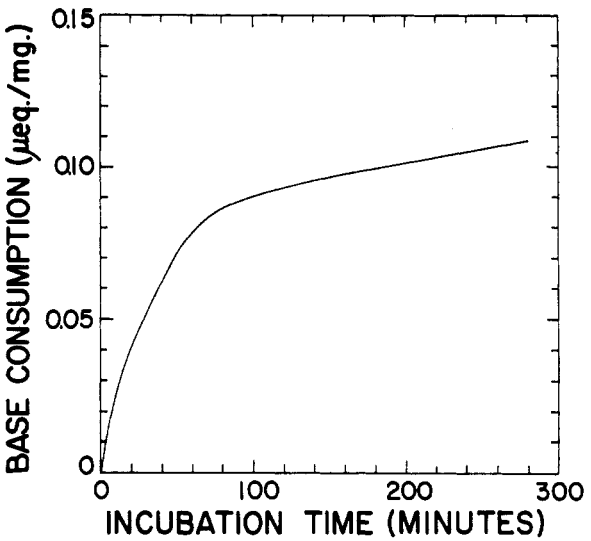


FIG. 1.—Rate of acid release by phosvitin at *p*H 8.50. Two ml of 1.00% phosvitin in 1.0 M KCl was adjusted to *p*H 8.50 and the *p*H was maintained with the *p*H stat (0.0791 N NaOH) at 37.0°.

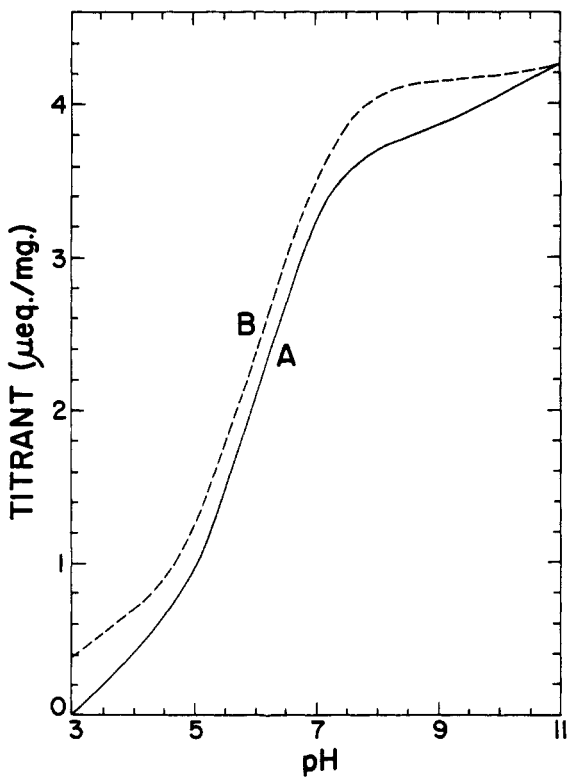


FIG. 2.—Titration curves of phosvitin. Two ml of 1.00% phosvitin in 1.0 M KCl was incubated at 25.0° and adjusted to *p*H 3.00. Titration with 1.96 N NaOH to *p*H 11.50 (curve A) was followed by titration with 2.33 N HCl to *p*H 3.00 (curve B). The curves were matched at *p*H 11.00, where the blank correction was reproducible and small compared with the total amount of titrant. The automatic titration rate was 20 minutes/*p*H unit. Final dilution by titrant was about 5%.

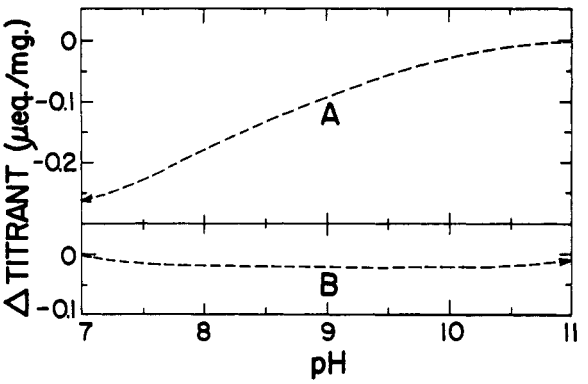


FIG. 3.—Difference titration curves of phosvitin (titrations between *p*H 7 and 11). Two ml of 1.02% phosvitin in 0.1 M KCl was incubated at 25.0° and adjusted to *p*H 2.97. Titration with 0.473 N NaOH to *p*H 11.48 was followed in turn by titration with 0.481 N HCl to *p*H 7.01 and again with base to *p*H 11. The difference between the acid titration and the first alkaline titration is shown by curve A, after matching at *p*H 11.00 (*cf.* legend to Fig. 2). The difference between the last alkaline titration and the acid titration is shown by curve B, after matching at *p*H 7.01. Thus, the sign of the values on the ordinate indicates whether titratable groups were gained or lost as revealed by the second of a given pair of titrations (the direction of the second titration is shown by the arrow). The manual titration rate was 20 minutes/*p*H unit, with 5 titrant increments per *p*H. Final dilution by titrant was about 20%.

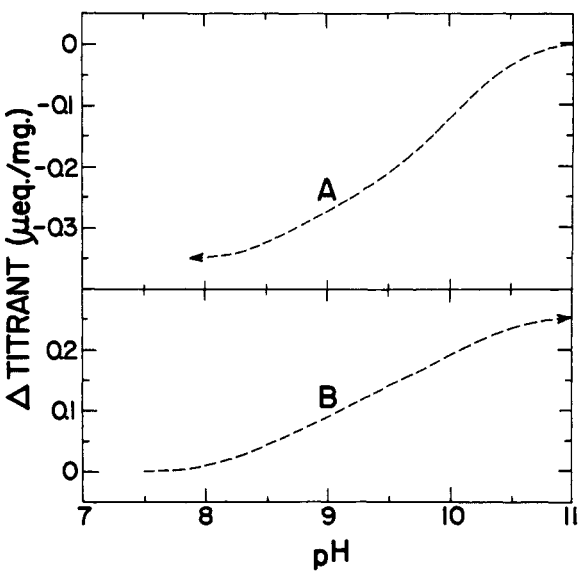


FIG. 4.—Difference titration curves of phosvitin (titrations between *p*H 3 and 11). Two ml of 1.00% phosvitin in 1.0 M KCl was incubated at 25.0° and adjusted to *p*H 3.00. Titration with 1.96 N NaOH to *p*H 11.50 was followed in turn by titration with 2.33 N HCl to *p*H 3.00 and again with base to *p*H 11. For the calculation of the curves see legend to Figure 3. (These curves were matched at *p*H 11.00 and 7.50 respectively.) The automatic titration rate was 20 minutes/*p*H unit. Final dilution by titrant was about 7%.

first titration with alkali and not during the following acid titration.

Experiments on the Reversibility of the Effect of Alkali on Phosvitin.—The titratable group which had disappeared on exposure to alkali did not reappear on subsequent exposure to *p*H 7. Curve A in Figure 3 shows that about 0.25 μeq/mg more titrant was needed to cover the *p*H range 7 to 11 in the first titration than in the following titration from *p*H 11 to 7. When a third titration, again from *p*H 7 to 11, was carried out, the titration curve was essentially identical with the preceding *p*H 11 to 7 titration curve (curve B). An additional pair of titration curves (not shown in the figure) confirmed this reversibility *after* the first titration. Figure 4 shows another example of the irreversibility of the initial change. Curve A depicts the loss of about 0.35 μeq/mg in the course of an initial titration sequence *p*H 7 to 11 to 7. In this experiment, however, the second titration (from *p*H 11 down) was continued beyond *p*H 7 to *p*H 3 and the third titration went from *p*H 3 to 11. Curve B shows that in this case, after the initial titration to *p*H 11, the lowering of the *p*H to 3 led to the recovery of part of the buffering capacity in the region *p*H 8 to 11. The third titration in this sequence (*p*H 3 to 11) required a larger amount of titrant in the alkaline *p*H region than did the preceding second titration (*p*H 11 to 3). Thus, Figures 3 and 4 show that the group which became masked by the first exposure of phosvitin to alkali remained masked when the *p*H was lowered to only 7, but that it could be unmasked, at least partially, when the *p*H was lowered further to 3. It may also be noted that quantitatively the experiments described in Figures 3 and 4 differed somewhat, since the total amounts of groups masked and the apparent *pK* value of such groups was not quite the same. A known difference between the two experiments was the difference in the ionic strengths of the two test solutions (0.1 and 1.0 M KCl, respectively).

Titration of Phosvitin in the Presence of Formaldehyde.—Since phosvitin appeared to have a dissociable group which became masked during titration with alkali in the *p*H region 8 to 11, it was of interest to establish whether changes occurred as well in the reactivity of amino groups which are expected to have *pK* values in this *p*H region. Formaldehyde is known to react with amino groups, effecting a lowering of their *pK* values (French and Edsall, 1945). Phosvitin was therefore titrated in the presence of formaldehyde before and after exposure to alkali. The results of these experiments are compared in Table II. These data show that in all experiments more groups were made available for titration at *p*H 8.50 in the presence of formaldehyde before treatment with alkali than after such treatment. Furthermore, it was found to be immaterial whether alkaline treatment involved a short exposure to *p*H 11 (experiments 1–4) or a longer exposure to *p*H 8.50 (experiment 5). It may be noted that when the formaldehyde concentration was high enough (experiments 2–5) the control samples consumed 0.51–0.54 μeq of base per mg of protein, in reasonably close agreement with the value of 0.50 μeq/mg. This is the amount expected on the basis of the estimated number of free amino groups per mg of phosvitin The lysine content is 0.45 μmoles per mg (*cf.* Table I) and 0.05 μeq of an α-amino group per mg of protein may be assumed for a single polypeptide chain having a molecular weight of 21,000 (Mecham and Olcott, 1949). The values for the "masked groups," 0.08–0.10 μeq/mg, are equivalent to about 2 groups per protein molecule on the same basis. It should also be noted that the amount of masked groups in experiment 5, 0.08 μeq/mg, is similar to the amount of base, 0.11 μeq/mg, consumed

TABLE II

PHOSVITIN TITRATION IN THE PRESENCE OF FORMALDEHYDE[a]

Expt. No.	Phosvitin (%)	Formaldehyde (M)	Preliminary Treatment[b]			Masked Groups[c] (μeq/mg)
			None	*p*H 8.50	*p*H 11.00	
			Titrant Consumption (μeq/mg)			
1	1.0	0.2	0.40	—	0.30	0.10
2	1.7	1.6	0.51	—	0.43	0.08
3	1.7	2.1	0.53	—	0.44	0.09
4	1.6	2.5	0.54	—	0.45	0.09
5[d]	6.1	1.9	0.53	0.45	—	0.08

[a] This table shows the amounts of alkali needed to readjust the *p*H of a given phosvitin solution to 8.50 after the *p*H had dropped upon addition of formaldehyde to the protein solution at *p*H 8.50. All data were corrected for alkali uptake due to formaldehyde alone (about 5% of the total uptake). In each experiment the control and test samples consisted of aliquots of the same protein solution. [b] "Preliminary treatment" refers to treatment of the protein solution prior to the addition of formaldehyde. "*p*H 8.50": exposed to this *p*H for 17 hours in the *p*H-stat; "*p*H 11.00": exposed to this *p*H for 2 hours before readjustment to *p*H 8.50. [c] The amount of "masked groups" was calculated as the difference between the titrations of the test and control samples. [d] In this experiment, the increased protein concentration was required for increasing the significance of the measurement of the rate and extent of alkali consumption during the preincubation at *p*H 8.50 for 17 hours. The amount of base consumed was 0.11 μeq/mg.

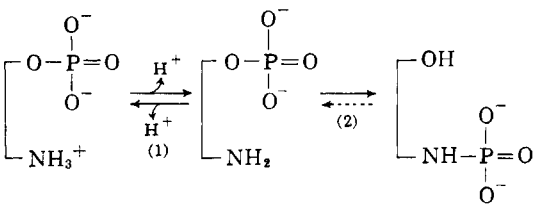
during the preincubation of the protein solution at *p*H 8.50 in the same experiment.

Test of Inorganic Phosphate Release During Phosvitin Titration.—Phosvitin released no significant amount of orthophosphate during incubation at *p*H 2.9 and 25° for 20 hours. Exposure to *p*H 11 for 21 hours led to the release of 0.01–0.02 μmoles of phosphate per mg of protein. Exposure to *p*H 11 for 1 hour, followed by exposure to *p*H 3.0 for 20 hours, resulted in the formation of 0.01 μmoles of phosphate per mg. Thus the extent of orthophosphate formation during the titration experiments (in which exposure to these extremes of *p*H was limited to only a few minutes) was smaller at least by one order of magnitude than the amount of titratable group which disappeared: the titrimetrically detectable change in the protein did not appear to be accompanied by a stoichiometric release of inorganic orthophosphate.

Discussion

Sixty years ago, it was suggested (Levene and Alsberg, 1901) that a protein fraction of egg yolk, corresponding in all probability to phosvitin in current terminology, had phosphate esterified to it. Later experimental observations led first to the inference (Posternak and Posternak, 1928), then to the demonstration (Lipmann and Levene, 1932) that the hydroxyl group of serine residues may be involved in this phosphate linkage. More recently, other evidence (Mecham and Olcott, 1949; Williams and Sanger, 1959) has supported this conclusion. It should be noted that this conclusion rests on experiments in which the protein preparation or the experimental procedure itself depended on the use of acid conditions. It is known, however, that some substituted phosphoryl groups, in acid solution, can shift from amide to ester linkages (*cf.* "Introduction") and that phosphoramidates are noted for their acid lability (*e.g.* Chanley and Feageson, 1958). A degree of uncertainty must be attached therefore to the conclusion that the native protein contains all of its phosphorus in the form of *O*-phosphoseryl residues. The study of the reversibility of the *p*H-titration of phosvitin suggested itself to us as a suitable approach to this problem.

Our data show that at alkaline *p*H values free amino groups of phosvitin became masked. On brief exposure to high *p*H (for example *p*H 11) the buffering capacity of the protein decreased appreciably in the *p*H-range where amino groups are expected to act as buffers. The involvement of amino groups was shown more directly by titration of alkali-treated phosvitin in the presence of formaldehyde. Exposure of the protein for a longer period to lower *p*H values (for example *p*H 8.5), falling just within the usual range of amino group dissociation, also led to a decrease of the amount of the formaldehyde-affected groups. The disappearance of amino groups must be a result of a reaction which blocks them, rather than a reaction which abolishes them, in a case when the disappearance may be reversed simply by a suitable *p*H change. A substitution reaction which leads to a readily reversible loss of the basic character of the group appears to be best suited as a hypothesis. Acylation is most likely to serve as an amine-masking reaction in a protein. Under the relatively mild conditions of our experiments, acylation by carboxyl groups may be ruled out. Phosphate groups, on the other hand, suggest themselves as likely alternatives. The accompanying scheme would accommodate our experimental observations.



Reaction (1), representing the ammonium ion–amine equilibrium, would provide reaction (2), an O-to-N phosphoryl migration, with the nucleophilic acyl group acceptor. This is in analogy to the migrations discussed at the beginning of this paper. Reaction (2) would proceed readily if the equilibrium of reaction (1) is displaced far in favor of the free amino group (for example at *p*H 11). It might also proceed at lower *p*H values (for example at *p*H 8.5) so long as the equilibrium position of reaction (1) is such that a significant concentration of the free amino group is maintained. The release of acid observed at low alkaline *p*H (or the downward *p*H-drift seen during manual titrations) would be the result of the continuous release of protons in reaction (1) as the free amine is drawn off by reaction (2) at a finite rate. To assume a simple equilibrium reaction for (2) would lead one to expect a reversal of the over-all change equally at *p*H 7 and 3, both *p*H values being well below the *pK* characteristic of a dissociation reaction such as (1). This is contrary to the observations. However, the product of reaction (2) might be stabilized in a manner dependent on the presence of the negative charges of the phosphoramidate. The re-formation of the ester bond might then be contingent upon the neutralization of one of the negative charges which should require a *p*H lower than 7. (N-Substituted phosphoramidates are, in fact, more reactive in hydrolytic reactions in the singly charged than in the doubly charged state [Chanley and Feageson, 1958].) The fact that migrations of phosphoryl groups from O to N could not be shown to occur in model substances, we ascribe to the more exacting nature of the three-dimensional aspects of protein structure, more likely to provide the most favored spatial arrangement for an intramolecular shift in competition, for example, with the intermolecular alternative of hydrolysis in 55 M water.

Finally, it may be suggested that such an intramolecular rearrangement could be related to the manner in which phosvitin is metabolized. The results presented in this communication may be relevant to the findings of Rabinowitz and Lipmann (1960), who observed that when partially dephosphorylated phosvitin was phosphorylated by adenosine triphosphate (labeled with P^{32}) in the presence of protein phosphokinase, followed by a measurement of the P^{32}-transfer from the labeled protein to adenosine diphosphate (catalyzed by the same enzyme), there was a preferential transfer of the labeled phosphate from the protein to the nucleotide. In other words, the phosphate introduced into phosvitin in the presence of the kinase did not fully become part of a uniform phosphate "pool." Possibly, this is a reflection of the heterogeneity of phosvitin as shown by its chromatographic fractionation (Connelly and Taborsky, 1961). It is also possible, however, that the labeled phosphate groups in individual phosphoprotein molecules are not transferred by the enzyme at random, but rather by a specific mechanism in which amino groups may accept phosphate from favorably located serine hydroxyl groups and transfer the phosphate from the newly formed phosphoramidate group to the nucleotide diphosphate. Such amino groups may represent specific sites on the phosvitin molecule where phosphate groups might interact with either serine hydroxyl groups of the protein or the nucleotide diphosphate, depending on the direction of the reversible reaction catalyzed by protein phosphokinase. The report of Williams and Sanger (1959) that phosvitin contains sequences of adjacent phosphoserine residues suggests the speculation that phosphate groups attached to serine residues far removed from the reactive amino groups may be transferred to such amino groups reversibly as a result of a series of transphorylation reactions in which a given esterified phosphate may displace the phosphate in a neighboring ester linkage, and so on, until the final esterified phosphate would be transferred to the reactive amino group. Thus, such a "lateral" displacement of phosphate groups along the polypeptide chain would explain why phosphate which has been accepted last will be donated first by the phosvitin molecule.

Two specific points require further comment. The electrostatic effect with a protein carrying such a high negative charge is expected to be exceptionally large. Indeed, in 0.1 M KCl it is not minimized as well as in 1.0 M KCl (compare broad curve A of Figure 3 with steeper curve A of Figure 4). The relative displacement of these curves on the *p*H-axis is less readily explained at this time. The electrostatic effect would be expected to raise the apparent pK, contrary to what was found. A local electron deficiency might explain this, but it seems more likely that the reason will be found in different kinetics of reaction (2) in different media, since the apparent pK values suggested by Figures 3 and 4 must be considered to refer to the over-all reaction, including reaction (2). The same reasoning may apply to the difference in the extent of the change (0.25 and 0.35 μeq/mg, respectively). The second point to note is the quantitative difference between the number of masked groups as determined by the *p*H-titration curves (about 0.3 μeq/mg) and as derived from the formaldehyde experiments (about 0.1 μeq/mg). We suggest at this time that this discrepancy is the result of the competition of formaldehyde for the same free amino group which acts as an acceptor in reaction (2). It could thus effect a partial reversal of this reaction, lowering thereby the apparent amount of masked groups.

References

Bergmann, M., Brand, E., and Weinmann, F. (1923), *Z. physiol. Chem. 131*, 1.

Chanley, J. D., and Feageson, E. (1958), *J. Am. Chem. Soc. 80*, 2686.

Connelly, C., and Taborsky, G. (1961), *J. Biol. Chem. 236*, 1364.

Desnuelle, P., and Casal, A. (1948), *Biochim. et Biophys. Acta 2*, 64.

Elliott, D. F. (1952), *Biochem. J. 50*, 542.

Fodor, G., and Kiss, J. (1949), *Nature 164*, 917.

French, D., and Edsall, J. T. (1945), *Advances in Protein Chem. 2*, 277.

Josefsson, L. (1958), *Arkiv Kemi 12*, 183.

Josefsson, L., and Edman, P. (1956), *Acta Chem. Scand. 10*, 148.

Levene, P. A., and Alsberg, C. (1901), *Z. physiol. Chem. 31*, 543.

Lewis, J. C., Snell, N. S., Hirschmann, D. J., and Fraenkel-Conrat, H. (1950), *J. Biol. Chem. 186*, 23.

Linderstrøm-Lang, K. (1933), *Ergeb. Physiol. u. exp. Pharmakol. 35*, 415.

Lipmann, F., and Levene, P. A. (1932), *J. Biol. Chem. 98*, 109.

Mecham, D. K., and Olcott, H. S. (1949), *J. Am. Chem. Soc. 71*, 3670.

Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.

Plapinger, R. E., and Wagner-Jauregg, T. (1953), *J. Am. Chem. Soc. 75*, 5757.

Posternak, S., and Posternak, T. (1928), *Compt. rend. 187*, 313.

Rabinowitz, M., and Lipmann, F. (1960), *J. Biol. Chem. 235*, 1043.

Rathlev, T., and Rosenberg, T. (1956), *Arch. Biochem. Biophys. 65*, 319.

Smillie, L. B., and Neurath, H. (1959), *J. Biol. Chem. 234*, 355.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Williams, J., and Sanger, F. (1959), *Biochim. et Biophys. Acta 33*, 294.