174°; $[\alpha]^{20}D$ −43.3° (*c*, 1.7, pyridine), $[\alpha]^{20}D$ −16.1° (*c*, 2.1, 20% ethanol).[13]

Anal. Calcd. for $C_{52}H_{65}O_{17}N_4P$: P, 2.96. Found: P, 2.75.

Discussion

In an effort to prove that the phosphate group was actually attached to carbon number four of the glucose, Raymond[3] compared the osazones and the rate of glucoside formation of his product with those of glucose-3- and glucose-6-phosphoric acids and found marked differences.

Steric considerations point to interaction between groups on carbons four and six as the most likely to be involved in rearrangement. The following data, when compared with values for analogous compounds to be found in the present paper, indicate that there has not been a shift of the phosphate group to carbon number six. Lardy and Fischer[7] report 1,2,3,4-tetraacetyl-6-diphenylphosphono-β-D-glucopyranose, m. p. 64–66°, $[\alpha]^{22}D$ 16.5° (*c*, 1.37, pyridine) and 1,2,3,4-tetraacetyl-β-D-glucose-6-phosphoric acid, m. p. 126–128° $[\alpha]^{25}D$ 18.7° (*c*, 1, pyridine). Robison and King[14] report the dibrucine salt of glucose-6-phosphoric acid, $[\alpha]^{20}D$ 20.6° (*c*, 0.84, water).

Both physical properties and presence of reducing power rule out a transfer of the phosphate group to carbon number one. Presumably the pyranose ring would remain intact in such a synthesis as the one presented and it would seem unlikely that carbon number five would be involved. However, until such time as glucose-5-phosphate can be studied this possibility cannot be ruled out and the nomenclature in this paper avoids specifying ring structure.

Acknowledgments.—We should like to express our sincere thanks to the Office of Naval Research for potentiating this investigation. The senior author was supported by Contract N6onr-218, Project NR-123-243.

Summary

Several derivatives of glucose-4-phosphoric acid have been obtained by an improved synthesis.

The properties of two new compounds have been reported.

(13) It should be noted that the rotation in pyridine is in good agreement with that reported by Raymond[3] but the rotation in alcohol is not. The value given here is that exhibited by several samples. For comparison, Raymond's values were −45.3 and −9.8°, respectively.

(14) R. Robison and E. J. King, *Biochem. J.*, **25**, 323 (1931).

EUGENE, ORE. RECEIVED APRIL 20, 1949

[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

Phosvitin, the Principal Phosphoprotein of Egg Yolk

BY DALE K. MECHAM AND HAROLD S. OLCOTT

A phosphoprotein preparation containing 10% phosphorus has been isolated from egg yolk in yield sufficient to account for at least 60% of the total protein phosphorus in yolk. The proposed name *"phosvitin"* indicates both its high phosphorus content and its source in the egg yolk. Details of the isolation procedures and the results of various chemical and physical studies will be described in this paper.

A number of investigators have separated polypeptides rich in phosphorus following enzyme digestion of both vitellin and casein.[2–13] The presence of such peptides was attributed to the occurrence of groupings in these proteins resistant to digestion. In the case of vitellin at least it now appears that such polypeptides were formed from a phosphoprotein of as high phosphorus content as any of the polypeptides isolated, and that most of the phosphorus was present in the form of the phosphoprotein to be described.

Isolation

Phosphoprotein fractions containing 7% or more phosphorus[14] were first obtained by the following procedure. Fresh liquid egg yolk was extracted with chloroform to remove lipids. The residual suspension was then washed with water to remove the "livetin" fractions, and finally extracted with 10% sodium chloride in the presence of chloroform to obtain the phosphoprotein. Salt was removed by dialysis. Apparently most of the other yolk protein components were insolubilized

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Miescher, *Medizinische-Chemische Untersuchungen*, **4**, 502 (1870), cited in Jukes and Kay.[40]

(3) Bunge, *Z. physiol. Chem.*, **9**, 49 (1885).

(4) Hugounenq and Morel, *Compt. rend. acad. sci.*, **140**, 1065 (1905).

(5) Posternak, *ibid.*, **184**, 306 (1927).

(6) Posternak and Posternak, *ibid.*, **184**, 909 (1927).

(7) Rimington, *Biochem. J.*, **21**, 1179 (1927).

(8) Damodaran and Ramachandran, *ibid.*, **35**, 122 (1941).

(9) Lowndes, Macara and Plimmer, *ibid.*, **35**, 315 (1941).

(10) Rimington, *ibid.*, **35**, 321 (1941).

(11) Posternak and Pollaczek, *Helv. Chim. Acta*, **24**, 1190 (1941).

(12) Nicolet and Shinn, Abstracts, 110th Meeting, American Chemical Society, September, 1946.

(13) Mellander, *Upsala Läkareforenings Förhandlingar*, **52**, 107 (1947).

(14) Unless otherwise stated, phosphorus analyses are in terms of non-lipid phosphorus not removable by dialysis. Since Plimmer (*J. Physiol.*, **38**, 247 (1909)) cited in Needham[39]) and Schmidt and Thannhauser (*J. Biol. Chem.*, **161**, 83 (1945)) found in yolk considerably less nucleic acid phosphorus than phosphoprotein phosphorus (the latter report 11 mg. and 116 mg., respectively, per 100 g. yolk), no attempt was made to distinguish between the two in most yolk fractions. As described in the text, there is no detectable nucleic acid in phosvitin.

by shaking with chloroform[15]; then in the presence of salt, phosphoprotein was dissociated from them. Yields by this method were low.

Phosvitin then was obtained by the method outlined in a preliminary report.[16] The fraction which separated from diluted yolk (1 part yolk to 2 parts water) in a Sharples centrifuge[17] was lyophilized, extracted with ether and dispersed in a solution containing 5 g. of sodium sulfate per 100 ml. of water. Addition of sodium sulfate to a concentration of 36 g. per 100 ml. of water precipitated the bulk of the proteins. After dilution of the soluble fraction to a concentration of 22 g. of sodium sulfate per 100 ml. of water, copper acetate was added to precipitate the phosvitin as the copper salt. The precipitate was dissolved in *p*H 5, 0.5 *M* citrate buffer, dialyzed against several changes of the same buffer to remove copper, and finally against distilled water to remove citrate. The material isolated had essentially the same composition and properties as that prepared by the method described below but the latter was more satisfactory both in yield and in economy of operation.

It was noted that addition of magnesium sulfate in small amounts to solutions of phosvitin caused formation of a precipitate which slowly dissolved at higher salt concentrations. This behavior was then used to obtain a phosvitin-rich fraction directly from yolk. An outline of the process and the distribution of protein solids and phosphorus during the various steps is shown in Fig. 1, and details are given below.

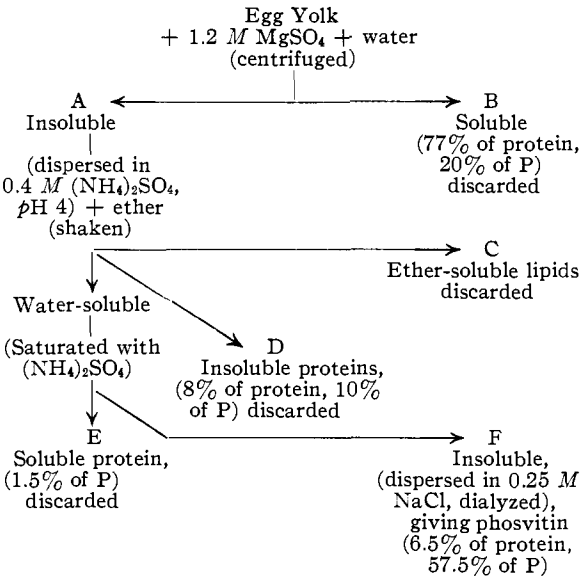


Fig. 1.—Preparation of phosvitin fraction from egg yolk. Analyses refer to dry, lipid-free protein and phosphorus of original yolk. Total recovery of protein was 92%, of phosphorus, 90%. Phosphorus contents of protein solids: original yolk, 1.09%; fraction B, 0.29%; fraction D, 1.36%; fraction F, 9.7%.

Preparative.—Yolks of fresh eggs[18] were washed in tapwater and rolled on cheesecloth to free them from adhering white. Chalazae were cut off. The yolk membranes were then punctured and the contents allowed to drain out through a single layer of cheesecloth. To 2400 g. of yolk contents (from 13 dozen eggs) were added 1200 ml. of 1.2 *M* magnesium sulfate solution (containing 120 mg. of Merthiolate[19]) and the mixture was stirred vigorously, but without producing foam, for one hour. Addition of 12 l. of water (containing 600 mg. of Merthiolate) then was started and completed in one and one-half hours, the mixture being stirred as before. After eighteen hours at room temperature (surface covered with toluene) the soft sticky precipitate was collected by centrifugation (A, Fig. 1), and the soluble fraction (B, Fig. 1) was discarded. The original yolk was at *p*H 6.0; the 0.09 *M* magnesium sulfate supernatant solution was at *p*H 5.9.

The precipitate was dispersed in 1600 ml. of 0.4 *M* ammonium sulfate with forty-five minutes of stirring. The dispersion was brought to *p*H 4.0 by the careful addition of 6 *N* sulfuric acid, and 80 ml. of *p*H 4, *M* acetate buffer was added. The dispersion then was shaken vigorously with 1500 ml. of ethyl ether and allowed to stand overnight at room temperature. The addition of ether was necessary in order to collect the non-phosvitin residue into a separable layer; filtration in the absence of ether did not remove all of the non-phosvitin protein. The slightly opalescent aqueous layer was drawn off and the remaining mixture was centrifuged to obtain some additional aqueous extract. The free ether layer (C, Fig. 1) was discarded. Three additional extractions of the residue (a gelatinous emulsion) were made with 0.4 *M* ammonium sulfate plus *p*H 4, *M* acetate in the ratio previously used, the successive volumes employed being 1,000, 800, and 800 ml.; 300 ml. of ether was added with the first of these, and no ether was then removed until after the final salt extraction. Only the last of these extracts was separated by centrifuging, the others being allowed to stand overnight before drawing off the aqueous layer. The gelatinous insoluble residue (D, Fig. 1) was discarded.

The *p*H 4, 0.4 *M* ammonium sulfate extracts were shaken in successive portions as obtained with one portion (approximately 200 ml.) of ethyl ether. After thirty to forty-five minutes, standing, the aqueous layers were drawn off and filtered through coarse paper. The filtrates were again shaken with 200 ml. of ethyl ether and the aqueous layers filtered through medium porosity sintered glass covered with one-half inch of Hy-flo filter aid (analytical grade).[19] The filtrate (4420 ml.; *p*H, 4.1) was perfectly clear and slightly yellow in color. It was next dialyzed overnight against two liters of saturated ammonium sulfate plus excess solid ammonium sulfate. A slow stirrer kept the solution agitated. The sacks then were transferred to two liters of saturated ammonium sulfate solution adjusted to *p*H 4 with glacial acetic acid, again with solid ammonium sulfate present.

After overnight dialysis, a white gelatinous solid had separated, most of which could be collected by centrifugation. The rest was collected by filtration through sintered glass. The saturated ammonium sulfate supernatant (E, Fig. 1) was discarded after it was found to contain only 2.5% of the phosphorus present in the 0.4 *M* ammonium sulfate extracts.

The precipitate was mixed with 50 ml. of 5 *M* sodium chloride, then diluted to 1000 ml. total volume. The viscous dispersion was dialyzed against several changes of 2 *M* sodium chloride, the first portion being made 0.01 *M* in sodium acetate to hasten solution of the precipitate, and then against water.

No systematic investigation of the effects of small variations in the separation procedures was made. In

(15) Sevag, Lackman and Smolens, *J. Biol. Chem.*, **124**, 425 (1938).

(16) Mecham and Olcott, *Fed. Proc.*, **7**, 173 (1948).

(17) Alderton and Fevold, *Arch. Biochem.*, **8**, 415 (1945).

(18) No difference in yield of phosvitin by the procedure given was found between eggs less than twenty-four hours old or grade A eggs purchased at retail markets.

(19) Mention of this material does not constitute endorsement.

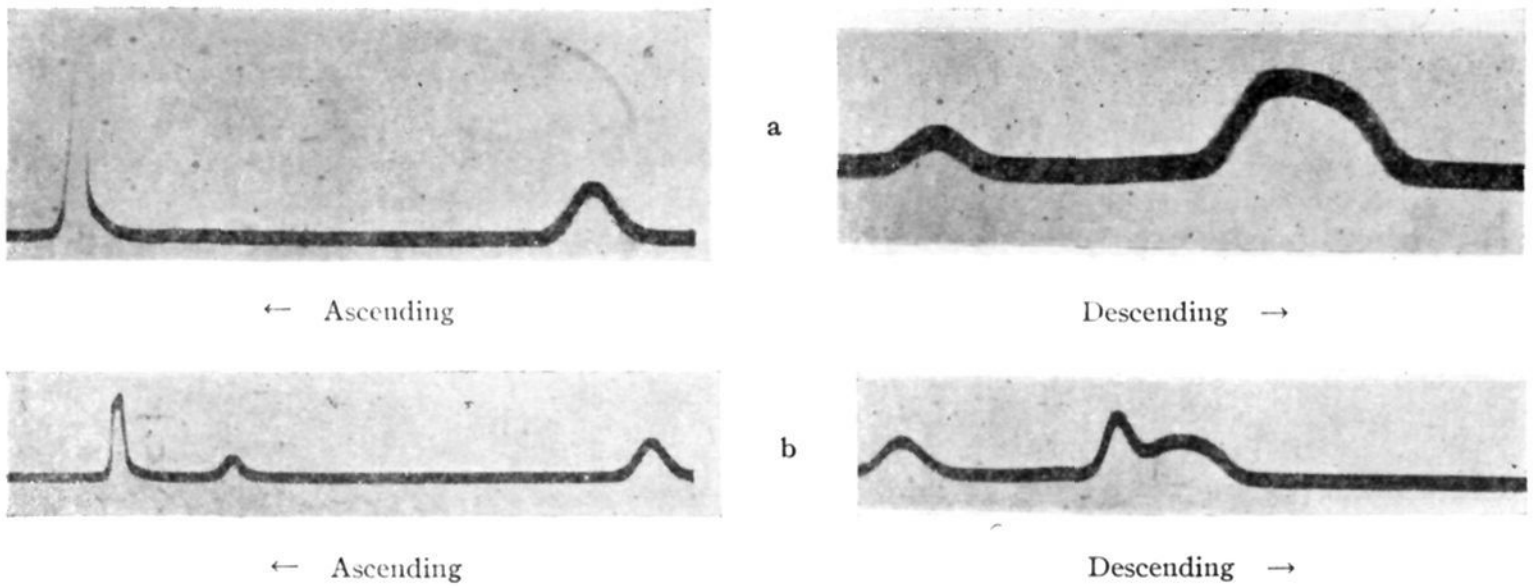


Fig. 2.—Svensson–Philpot electrophoresis records: phosvitin (citrate-dialyzed, magnesium sulfate method of preparation), 1.7% solution; "*a*" in sodium acetate buffer, *p*H 4.6, μ 0.1; mobility of rising boundary, $-12(\times 10^{-5})$ cm.^{2} volt^{-1} second^{-1} referred to 0°; delta boundary at right. Larger falling boundary represents unresolved components ranging in mobility from −8 to −10 units; epsilon boundary at left. "*b*" in sodium citrate buffer: *p*H 4.6, μ 0.1. The small slower moving rising boundary, about 25% of the whole, has a mobility of about −12 units. The faster 75%, which appears further resolvable, has a mobility of about −15 units; delta boundary at right. The slower moving falling component, comprising about 40% of the whole, has a mobility of −9 units; the faster 60%, about −12 units; epsilon boundary at left.

general, however, it was observed that in the magnesium sulfate precipitation step, dilution to 0.05 *M* concentration (instead of 0.09 *M*) caused precipitation of more non-phosvitin protein, while above about 0.3 *M* concentration, there was little precipitation of phosvitin. Also, after removal of the 0.09 *M* magnesium sulfate precipitate at a *p*H near 6, additional precipitate could be obtained by raising the *p*H to 8 or 9, but almost no phosvitin could be separated from this second precipitate. In the ammonium sulfate–ether extraction step, separation of the non-phosvitin fraction into the ether–gel layer became less complete as the *p*H was increased above 4 or the ammonium sulfate concentration was increased above 0.4 *M*. At lower salt concentrations (*ca.* 0.1 *M*) dissociation of phosvitin from the magnesium sulfate precipitate was quite incomplete.

When it was found that phosvitin preparations were apparently unaffected by heat (solutions at levels of *p*H from 4 to 8 when heated for several hours at 100° did not precipitate nor show any other evidence of change), attempts were made to devise methods of preparation in which the non-phosvitin proteins of yolk were heat-denatured. Small amounts of phosvitin could be recovered but the procedures were definitely less satisfactory than those described above.

Analyses.—The dialyzed preparation (F, Fig. 1) was at *p*H 6.05, and contained 24.4 g. of solids. The solids contained 9.7% phosphorus and 11.9% nitrogen (molar ratio N/P, 2.72). Total lipids were 0.7%; total lipid phosphorus, less than 0.03% of the lipid; total inorganic phosphorus, about 0.01% of the total solids. Neither purine nitrogen (method of Hitchings[20]) nor pentoses (orcinol–ferric chloride method[21]) were detectable. Ninety-six per cent. of the phosvitin phosphorus was removed from a sample of the preparation by 0.25 *N* sodium hydroxide at 35° in twenty-four hours. This is the method used by Plimmer[22] to characterize phosphoproteins. Thus, practically all of the phosphorus of phosvitin is present as phosphoprotein phosphorus.

Attempts to obtain products containing still lower nitrogen-to-phosphorus ratios by further fractionation procedures applied to fraction F were unsuccessful. In each case the molar ratios were within the range of 2.5–2.9.

(20) Hitchings, *J. Biol. Chem.*, **139**, 843 (1941).

(21) Brown, *Arch. Biochem.*, **11**, 269 (1946).

(22) Plimmer and Scott, *J. Chem. Soc.*, **93**, 1699 (1908).

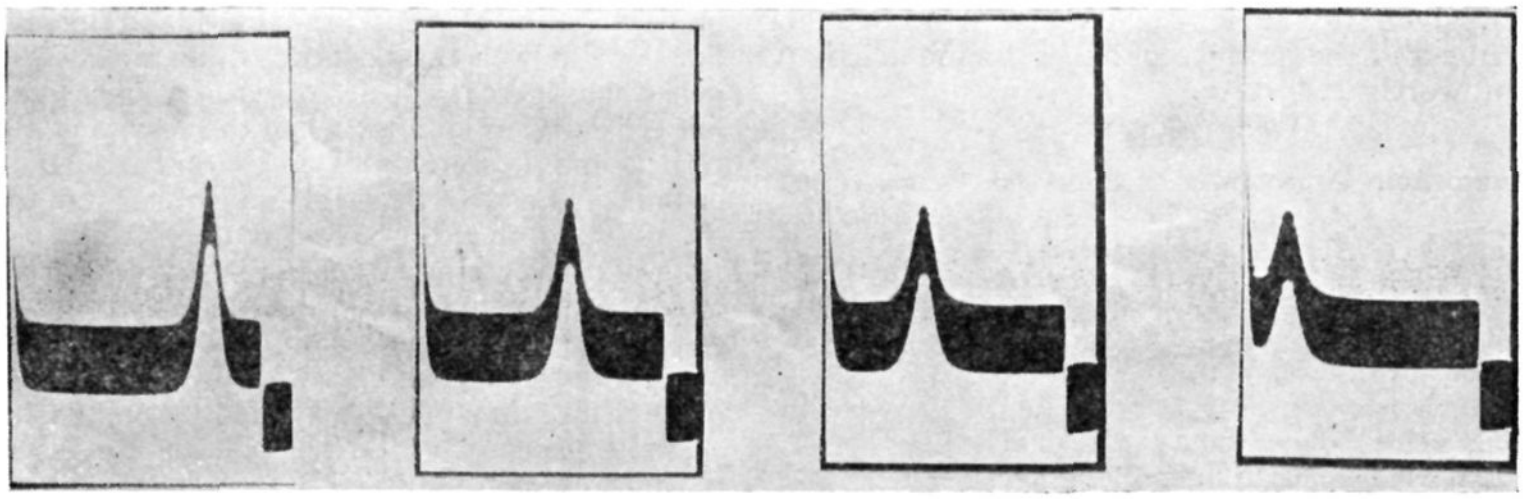
In preparations carried out on a smaller scale with more nearly complete recovery of the starting material, the amount of yolk protein phosphorus recovered as phosvitin phosphorus was consistently between 60 and 70%. In these preparations the 0.4 *M* ammonium sulfate extracts were separated by centrifuging; more complete extraction of phosvitin was indicated by a lower phosphorus content (about 1.1%) of the insoluble protein (D, Fig. 1). The phosphorus not precipitated by 0.09 *M* magnesium sulfate (B, Fig. 1) was consistently about 20% of the total.

Characterization

Physical Properties.—Typical electrophoretic patterns obtained by W. H. Ward with phosvitin are shown in Fig. 2. In sodium citrate buffer, two main components or groups of components were present. About 40% had a falling mobility of −9, and about 60%, −12 ($\times 10^{-5}$ cm.^{2} volt^{-1} second^{-1}) at *p*H 4.6, ionic strength, 0.1. In sodium acetate buffer the boundaries were not resolved and the mobilities were less by 2 or 3 units ($\times 10^{-5}$). Such data indicate that phosvitin preparations consist of at least two groups of substances, not easily resolved electrophoretically, with high mobilities centering about the values given above. Because of the high charge on this material, the use of higher ionic strength buffers would have been desirable.

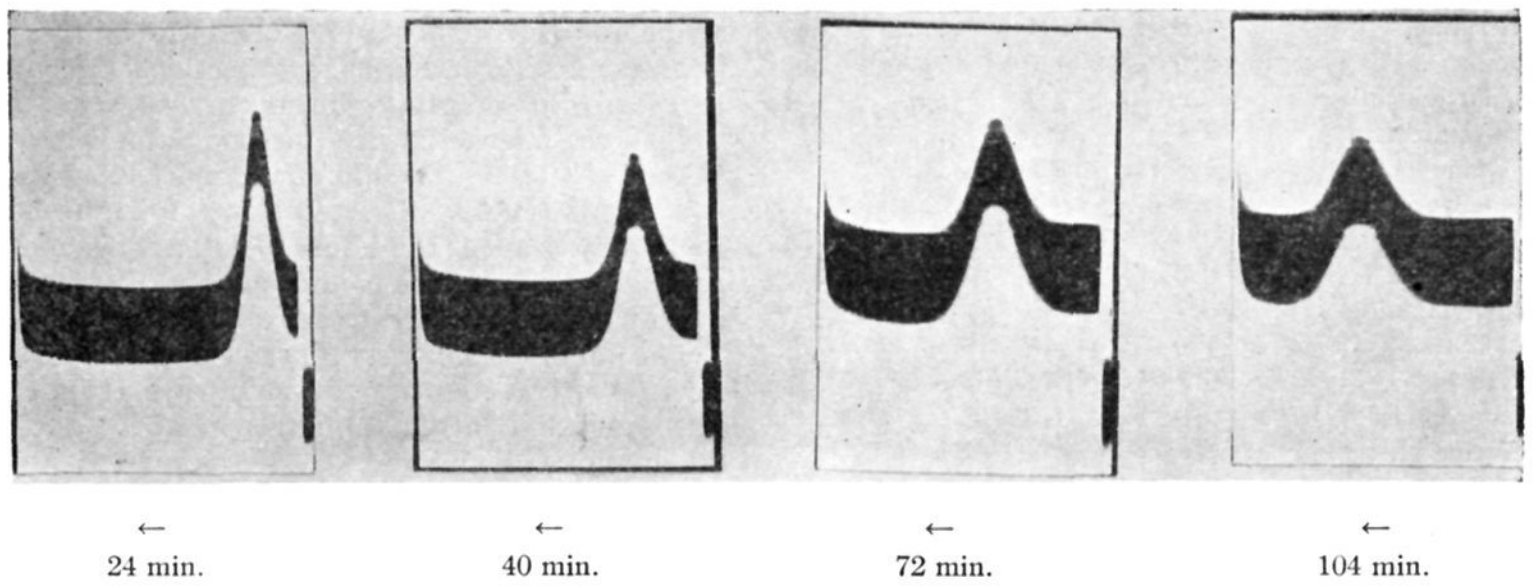
The osmotic pressure method of Bull[23] was used to estimate the molecular weight of phosvitin. Values ranging from 18,000 to 25,000 were obtained with samples prepared by the salt fractionation, copper precipitation method. The runs varied in sample concentration from 0.3 to 1.4%, in *p*H from 5.7 to 6.5, and sodium chloride concentration from 0.5 to 2.5 *M*. Observed molecular weights in the presence of 6.7 *M* urea and 0.5 *M* sodium chloride were also in the range given above.

(23) Bull and Currie, THIS JOURNAL, **68**, 742 (1946).



80 min. 144 min. 208 min. 272 min.

Phosvitin in 0.1 *N* sodium chloride, *p*H 5.



24 min. 40 min. 72 min. 104 min.

Phosvitin in 0.1 *N* sodium chloride–0.05 *M* magnesium chloride, *p*H 5.

Fig. 3.—Ultracentrifuge (Spinco) analysis of 0.8% solution of phosvitin (upper series of pictures) in 0.1 *M* sodium chloride at *p*H 5. Computed sedimentation constant, 1.84 *S* (10^{-13} second) referred to distilled water at 20°. This confirms an earlier measurement made for us by W. B. Dandliker, University of California. The lower series of records represents an analysis made in 0.1 *M* sodium chloride with 0.05 *M* magnesium chloride at *p*H 5. Computed sedimentation constant, 3.79 *S*, referred to distilled water at 20°. The discontinuity at the right of each record shows the position of the liquid surface.

The behavior of phosvitin in the ultracentrifuge (Spinco) was determined and interpreted by W. H. Ward. When magnesium ions were present, an apparent aggregation of phosvitin was observed both in the ultracentrifuge and with osmotic pressure measurements. A preparation obtained by the magnesium sulfate method and citrate-dialyzed was centrifuged at 0.8% concentration in 0.1 *M* sodium chloride, *p*H 5.0, solutions in the absence and presence of 0.05 *M* magnesium chloride. Patterns are shown in Fig. 3. Corrected sedimentation constants (S_{20}) of 1.84 and 3.79 were obtained. These correspond to minimum molecular weights of approximately 12,000 and 39,000, in the absence and presence, respectively, of magnesium. By osmotic pressure measurements the corresponding values were 21,000 and 38,000. The data suggest that, under these conditions, the protein acts as a dimer in the presence of magnesium ions. Possibly magnesium forms a bridge between phosphate groups on adjacent molecules.

The optical rotation of a 2% phosvitin solution in water was measured at *p*H 7.5 and 4.5; values for $[\alpha]^{20}D$ of −71 and −51° were found. In 2 *M* sodium chloride, corresponding values were −68 and −46°. Hewitt[24] found that the optical rotation of a serum albumin preparation (−67°) was the same at *p*H 4.9 and 7.2; Almquist and Greenberg[25] reported that the optical rotation of egg albumin (−31°), livetin (−39°) and serum albumin (−49°) did not vary in the *p*H range 4.5 to 10. The positive effect of *p*H on the rotation of phosvitin probably reflects the change of ionization state of the phosphate group.

Amino Acid Composition.—Results of amino acid determinations on a phosvitin preparation

(24) Hewitt, *Biochem. J.*, **21**, 216 (1927).

(25) Almquist and Greenberg, *J. Biol. Chem.*, **105**, 519 (1934).

are given in Table I. The large amount of serine and the absence of sulfur amino acids are noteworthy.

TABLE I

AMINO ACID COMPOSITION OF PHOSVITIN PREPARATIONS[a]

Component	Untreated %	Untreated Residues per 10^4 g.	Dephosphorylated[b] %	Dephosphorylated[b] Residues per 10^4 g.
Total nitrogen	11.9	85	..	..
Amino nitrogen	0.7	5	..	..
Total phosphorus	9.7	31	..	..
Total sulfur	<0.1	...	..	..
β-Hydroxy-α-amino acids[c,d]		29	..	36
Ammonia nitrogen[c,d]		13	..	6
Serine[c,d]	....	...	32.3	31
Threonine	....	...	Trace	..
Arginine	4.8	2.8	...	..
Arginine[e]	5.8[e]	...	...	..
Histidine	4.8	3.1	...	..
Lysine	5.9	4.0	...	..
Aspartic acid	4.4	3.2	...	..
Glutamic acid	3.4	2.3	...	..
Glycine	1.6	2.1	...	..
Leucine	1.0	0.8	...	..
Methionine	0.4	.3	...	..
Methionine[e]	0.3[e]	.2	...	..
Phenylalanine	0.7	.4	...	..
Proline	1.0	.9	...	..
Tyrosine	0.1	< .1	...	..
Tryptophan[c]	0.6	.3	...	..
Cystine plus cysteine	0.0	.0	...	..

[a] With exceptions noted, all analyses are on a single preparation of the partial sodium salt (*p*H 6), moisture-free basis. Samples were hydrolyzed by refluxing sixteen hours with 6 *N* hydrochloric acid (except for tryptophan). Amino acid analyses are by microbiological methods except as noted. [b] Eighty per cent. dephosphorylated with grapefruit phosphatase. Values are expressed in terms of the original untreated phosvitin. The dephosphorylated product was not isolated; aliquots of the dephosphorylation digest were frozen and dried in vacuum in the vessels subsequently used for hydrolysis. [c] Determined by chemical methods (see methods section). [d] Observed values corrected for estimated 10% destruction of hydroxy amino acids during hydrolysis, and a corresponding increase in ammonia nitrogen. [e] Values obtained on different phosvitin preparation.

It was of particular interest to determine whether a sufficient number of serine residues were present to permit all of the phosphorus to be present as the serine phosphate ester, since the isolation by Levene and co-workers,[26,27] of phosphoserine from yolk indicated that at least part of the phosphoric acid was present in this form.

It is usually assumed that about 10% of the total serine in proteins is destroyed during acid hydrolysis.[28] Lowndes, *et al.*,[9] showed that destruction of serine in hydrochloric acid was somewhat accelerated in the presence of added phosphoric acid. However, the comparative stability in acid of peptide-linked serine and phosphoserine residues is not well established; Lipmann and Levene[26] considered residues of both kinds approximately equal in lability, while the results of Posternak and Posternak,[6] Damodaran and Ramachandran[8] and Nicolet and Shinn[29] suggest that the phosphorylated residues are more labile. Results of the determination of the total hydroxyamino acid and ammonia contents of hydrolysates of phosvitin and dephosphorylated phosvitin (see Table I) were in agreement with the latter authors in showing that phosphorylated hydroxyamino acid residues of phosvitin are somewhat less stable to acid than non-phosphorylated. Therefore, serine was determined only in enzyme dephosphorylated samples. Correction of the serine values for 10% destruction during hydrolysis brings the serine and phosphorus contents (of phosvitin) to equivalence. Microbiological determinations of serine and threonine gave values of 28–35 equivalents per 10^4 g., and traces, respectively (not corrected for destruction during hydrolysis). Although the microbiological methods were not considered completely satisfactory in the case of these two amino acids,[30] the results are in agreement with the chemical methods, in indicating both a preponderance of serine, and a serine content approximately equivalent to the phosphorus present.

The inhomogeneity of phosvitin indicated by electrophoresis (but not by the ultracentrifuge) is also suggested by the amino acid analyses. Of the amino acids determined, methionine, tyrosine and tryptophan were definitely present but in amounts too small to permit the presence of one residue in a molecule of molecular weight 21,000. Small amounts (less than 1%) of isoleucine and valine were present but were not determined accurately. Alanine was not determined, but might be expected (by elimination of other common amino acids) to be present in considerable amounts.

Subtraction of amide nitrogen (corrected for destruction of hydroxyamino acids) and of the non-α-amino nitrogen of arginine, histidine and lysine from the total nitrogen gives a value for α-amino nitrogen which on a molar basis is about double that of the phosphorus present. Consequently, there is about one phosphate group for each two amino acid residues.

Rietz, *et al.*,[31] found that when proteins are treated with cold concentrated sulfuric acid the aliphatic hydroxyl groups of serine and threonine residues form acid sulfate esters. A sample of phosvitin was treated by this technique; 86% of the original nitrogen was recovered after dialysis. In the recovered material, only one sulfur atom per thirty phosphorus atoms was present. This

(26) Lipmann and Levene, *J. Biol. Chem.*, **98**, 109 (1932).

(27) Levene and Schormüller, *ibid.*, **103**, 537 (1933).

(28) Rees, *Biochem. J.*, **40**, 632 (1946).

(29) Nicolet and Shinn, Abstracts, 104th Meeting, American Chemical Society, Sept., 1942.

(30) Meinke and Holland (*J. Biol. Chem.*, **173**, 535 (1948)) have shown that excess serine interferes with the microbiological determination of threonine.

(31) Rietz, Ferrel, Fraenkel-Conrat and Olcott, THIS JOURNAL, **68**, 1024 (1946).

result indicates that practically all of the hydroxy-amino acid residues in phosvitin are phosphorylated, and that the sulfuric acid treatment does not replace phosphate with sulfate groups.

Ultraviolet Absorption.—Nicolet and Shinn[32] have presented evidence that serine in peptide linkage forms dehydroalanyl residues upon treatment with dilute alkali; phosphorylated serine residues might be expected to form dehydroalanyl residues even more readily.[29] Since Carter and Greenstein[33] reported that dehydropeptides absorb light very strongly in the ultraviolet range, the ultraviolet absorption spectra of phosvitin, enzyme-dephosphorylated phosvitin, and alkali-treated phosvitin were determined. Results are presented in Fig. 4. There was nearly a tenfold increase in density at 250 mμ with 36% dephosphorylation in four hours of treatment with 0.25 *M* potassium hydroxide at 35°. An additional twenty hours of treatment, with 95% dephosphorylation, gave a relatively small additional increase in density, possibly because of degradation beyond the dehydroalanyl stage.

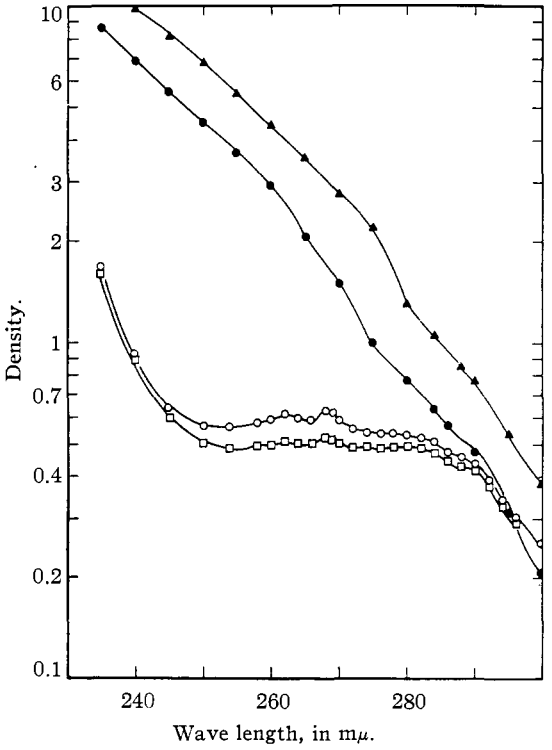


Fig. 4.—Ultraviolet absorption of phosvitin samples (density calculated to sample concentration equivalent to 165 micrograms of phosphorus per ml.; solvent, 0.1 *M* potassium chloride; samples at *p*H 6.5–6.7): □, untreated (citrate-dialyzed) phosvitin; O, enzyme dephosphorylated (80% dephosphorylated); ●, four hours, 0.25 *M* potassium hydroxide, 35° (36% dephosphorylated); ▲, twenty-four hours, 0.25 *M* potassium hydroxide, 35° (95% dephosphorylated). Enzyme and potassium hydroxide digestions made with citrate-dialyzed phosvitin at a concentration of 825 micrograms of phosphorus per ml.

(32) Nicolet and Shinn, *J. Biol. Chem.*, **142**, 609 (1942).
(33) Carter and Greenstein, *ibid.*, **165**, 725 (1946).

Titration.—Phosvitin samples were titrated in an attempt to determine whether all of the phosphorus was present as mono-esterified orthophosphate. Phosvitin prepared by the magnesium sulfate method was citrate-dialyzed, then electrodialyzed. Electrodialysis was very slow; ten days were required before the current through the cell failed to increase markedly from the time water was added until it was replaced (eighteen hours).

Titration curves for aliquots of the resulting solution containing 6.24 mg. of phosphorus are shown in Fig. 5. Although the inflection points (points of maximum slope) occurred at different *p*H values in the different solutions, the equivalents of alkali required to reach these points were in reasonable agreement.

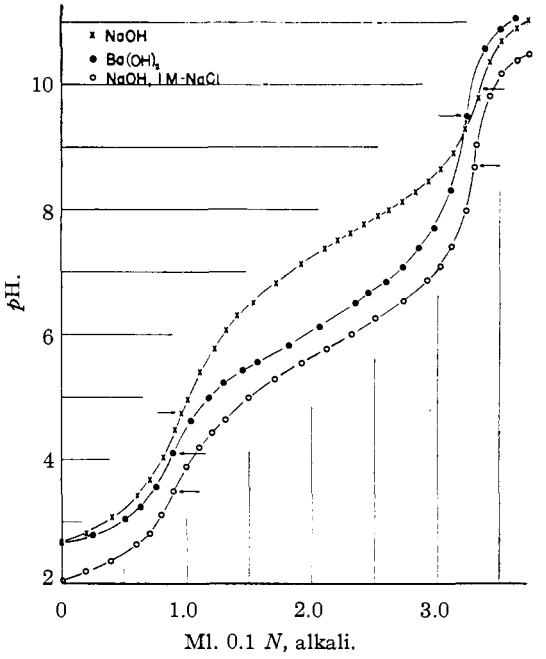


Fig. 5.—Electrodialyzed phosvitin titrations: 10-ml. solution (6.24 mg. phosphorus) titrated with 0.101 *N* sodium hydroxide (X); 0.101 *N* sodium hydroxide in the presence of 1 *M*, final concentration, sodium chloride (O); and 0.131 *N* barium hydroxide (●). Calculated to 0.100 *N* base before plotting. Arrows indicate inflection points.

The milliequivalents of the basic amino acids present with 6.24 mg. (0.201 millimole) of phosphorus, calculated from their ratio obtained from Table I were arginine 0.018, lysine 0.026 and histidine 0.020. It was assumed that all the basic amino acid residues neutralized their equivalent of acidity at *p*H values below the first inflection point, but only the arginine and about one-half the lysine ε-amino groups (of *pK* values around 12.5 and 10, respectively) did so at the *p*H of the second inflection point. The titration results expected, with all the phosphorus present as mono-esterified orthophosphate, then can be given as follows (in milliequivalents): to the first inflection point, acidity from phosphorus, 0.201, neutralized by basic residues, 0.064, net expected 0.137; total to the second inflection point from phosphorus, 0.402, neutralized by basic groups, 0.031; net expected, 0.371. Actually, only 0.092 and 0.328 milliequivalent of alkali were required. Because

the discrepancy between the acidity expected and found occurred in titration to the first inflection, rather than between the first and second inflections, the results indicated failure to remove all metal ions from the sample rather than the existence of diesterified orthophosphate.[34,35]

Accordingly a 500-mg. lyophilized portion of phosvitin prepared by the magnesium sulfate method, not citrate-dialyzed, was suspended in 10 ml. of 1 *N* hydrochloric acid in absolute ethanol, let stand thirty min., centrifuged, and the extraction with acidic ethanol repeated four times more. (The first and second extracts were definitely yellow, presumably because of extraction of iron and copper (see below)). Residual solvent was removed from the solid *in vacuum* over potassium hydroxide at room temperature; 490 mg. of solid was recovered. Portions were weighed out, titrated (results given in Fig. 6), and phosphorus and nitrogen contents of the titrated solutions determined. Nitrogen–phosphorus ratios were not significantly changed by the acid–alcohol extractions (2.72 in the original preparations; 2.69, 2.64 in the washed solids).

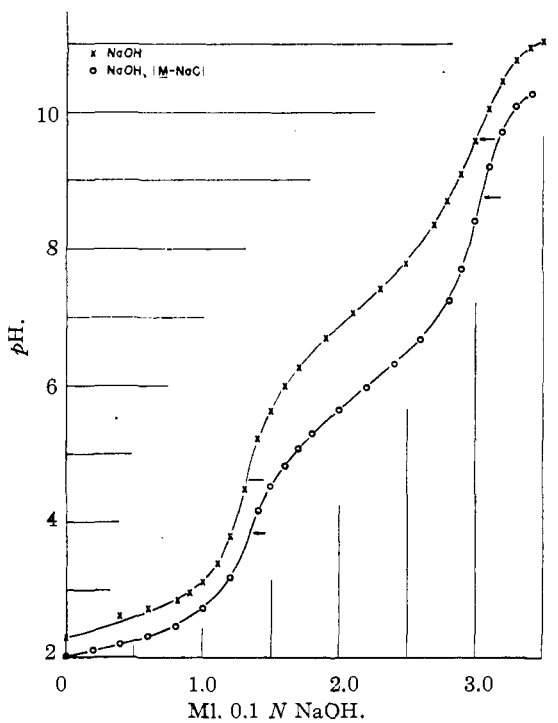


Fig. 6.—Acidic ethanol-washed phosvitin titrations: ×, 0.100 *N* sodium hydroxide, 4.2 mg. of phosphorus in sample; ○, 0.100 *N* sodium hydroxide in presence of 1 *M*, final concentration, sodium chloride, 4.4 mg. of phosphorus in sample. The solid samples, suspended in 10 ml. of water at the start of the titration, did not dissolve completely until about 1.0 ml. of alkali had been added. Arrows indicate inflection points.

In the case of these samples, it was assumed that all of the basic amino acid residues would be present as hydrochlorides at the start of the titration, but that the histidine imidazole and approximately one-half the lysine ϵ-amino groups would, at higher *p*H values, release hydrochloric acid and cause an increase in alkali requirement over that expected; the increased requirement would occur between the first and second inflection points. Calculated to the same quantity of phosphorus as the preceding titrations, the average results then were as follows: To the first inflection, expected acidity from phosphorus, 0.201 milliequivalent; found, 0.194; total titration expected from phosphorus, 0.402, acid released from basic amino acid hydrochlorides during titration, 0.033; found, 0.436. Thus, in the case of the acid–ethanol washed samples the results are interpretable as indicating that all of the phosphorus is present as mono-esterified orthophosphate.

Heavy Metals.—Miescher,[2] Bunge[3] and Hugounenq and Morel[4] found about one-twentieth as much iron as phosphorus in the products of high phosphorus content which they separated from pepsin digests of vitellin. Phosvitin prepared by the magnesium sulfate procedure was found to contain 0.4% iron. By spectrographic examination of the ash of phosvitin, copper and calcium were found to be present in amounts approximately equal to that of iron. No other metallic elements except sodium were present in more than trace amounts. No magnesium was found, despite its use in the preparation. Samples of phosvitin then were dialyzed against *p*H 5 citrate buffer followed by water, the procedure used previously[16] for removal of copper from the copper–phosvitin precipitate. Only traces of iron, copper and calcium remained in the phosvitin. The nitrogen–phosphorus ratio was not changed by the citrate dialysis. The affinity of phosphate esters for heavy metals is well-recognized. Hence it is possible that phosvitin preparations picked up these ions not only from the yolk but also from impurities in the large amounts of salts used in the fractionation sequence.

Susceptibility to Enzyme Action.—Phosphatases in general have not been found capable of dephosphorylating intact phosphoproteins. However, Axelrod[36] showed that a purified orange phosphatase would dephosphorylate phosvitin. An unfractionated preparation of grapefruit flavedo press-juice solids subsequently was found to be a satisfactory phosphatase source for this purpose. It is of interest that casein also was dephosphorylated by the grapefruit preparation. The grapefruit and orange phosphatases therefore fall into the class of phosphoprotein phosphatases. Harris[37] demonstrated phosphoprotein phosphatase activity in frogs' eggs.

Typical results obtained using the grapefruit preparation and a bone phosphatase preparation are given in Table II. Below phosvitin concentrations of 1%, dephosphorylation occurred rapidly with citrus phosphatase preparations. A sweet potato extract fraction capable of hydrolyzing *p*-nitrophenyl phosphate rapidly was nearly inactive on phosvitin.

In some cases, amino nitrogen determinations were obtained before and after dephosphorylation of phosvitin samples with the grapefruit preparations. A slight increase occurred during dephosphorylation; typical values were 5.3% amino nitrogen of the total nitrogen originally, 7.3% after 89% dephosphorylation. Comparable values for casein digests were 5.8% of the total nitrogen originally, 10.1% after 82% dephosphorylation. Thus the grapefruit preparation appears to have caused some proteolysis, but so little that, at least with phosvitin, the dephosphorylation of a protein rather than of a peptone is indicated.

The dephosphorylated phosvitin was insoluble, separating slowly during the later stages of the re-

(34) Reid, Mazzeno and Buras, in preparation for press.
(35) Kumler and Eiler, THIS JOURNAL, **65**, 2355 (1943).
(36) Axelrod, *J. Biol. Chem.*, **167**, 57 (1947).
(37) Harris, *ibid.*, **165**, 541 (1946).

TABLE II

PHOSPHOPROTEIN DEPHOSPHORYLATION BY PHOSPHATASE PREPARATIONS[a]

Phosphatase Source	Concentration mg. per ml.	Substrate	Protein phosphorus, mg. per ml.	Digestion conditions Hr.	°C.	pH	Dephosphorylation, %
Bone	6.0	Phosvitin	1.86	72	25	9.1	2
Grapefruit	0.6	Phosvitin	1.84	72	25	6.7	36
	.6	Casein	0.17	48	25	6.6	71
	.26	Phosvitin	0.87	64	35	6.6	90

[a] Bone phosphatase preparation according to Martland and Robison, *Biochem. J.*, **23**, 237 (1929), from veal shank bone. Grapefruit preparation, flavedo press-juice solids. Relative activity of preparations against *p*-nitrophenyl phosphate : bone (*p*H 9.1), 12 (not changed by addition of 0.003 *M* magnesium sulfate); grapefruit (*p*H 5), 70. The bone phosphatase preparation was not completely soluble in the digestion mixtures; such digests therefore were shaken gently throughout the digestion period. Phosvitin dephosphorylation was not changed by the presence of 0.003 *M* magnesium sulfate in either grapefruit or bone preparation digests.

action as a gel. In this property it is similar to sericin, the hot-water soluble protein of silk, which it also resembles in amino acid content (high serine, absence of sulfur amino acids).

The numerous reports of isolation of phosphopeptones[2-13] from enzyme digests of both egg yolk and milk proteins indicate that peptides containing phosphorylated amino acid residues are resistant to protease activity, consequently it was thought that phosvitin might not be as rapidly digested by trypsin and pepsin as are proteins containing little or no phosphorus. A single experiment was performed (Table III) along this line.

TABLE III

DIGESTION OF PHOSVITIN BY TRYPSIN AND PEPSIN[a]

Substrate	Concn., mg. N per ml.	Original *p*H	Enzyme	Concn., mg. per ml.	Amino N as % of total N 0 hr.	4 hr.	24 hr.
Phosvitin	1.0	8.4	Trypsin	0.06	6.3	8.8	9.6
Heated phosvitin	1.0	8.4	Trypsin	.06	...	..	9.4
Dephosphorylated phosvitin	0.8	8.4	Trypsin	.06	8.4	13.6	14.8
Casein	1.1	8.5	Trypsin	.06	6.5	11.8	13.0
Phosvitin	1.0	1.9	Pepsin	.11	6.3	7.1	7.6
Dephosphorylated phosvitin	0.8	1.8	Pepsin	.11	8.6	10.1	11.0
Casein	1.1	1.9	Pepsin	.11	6.5	11.4	14.0

[a] To 8 ml. of substrate solution at the *p*H indicated, 1 ml. of enzyme solution (trypsin in 0.001 *N* hydrochloric acid, pepsin in water) was added. After the indicated period of digestion at 35°, trypsin samples were acidified with glacial acetic acid, pepsin samples were neutralized with sodium hydroxide, made to 10 ml., and analyzed. The trypsin and pepsin were crystallized preparations.

With trypsin, phosvitin was less rapidly attacked than was either casein or dephosphorylated phosvitin. Heated phosvitin was digested only to the same extent as the unheated sample, hence it appears that phosvitin resists the action of trypsin rather than inhibits it in the manner of ovomucoid, the trypsin-inhibitor of egg white.[38] The results with pepsin do not permit strict comparison between phosvitin and casein, because the former gelled shortly after being adjusted to *p*H 1.9 and after twenty-four hours had formed a fairly dense gel occupying approximately one-half the volume of the digest.

Discussion

Extensive reviews of the literature on yolk proteins are available.[39,40] It was generally considered that there were two fractions, vitellin, the water-insoluble part, accounting for about 80% of the total, and livetin, the water-soluble fraction, accounting for the remaining 20%. Vitellin was a lipoprotein and contained 0.8–1.2% protein phosphorus,[41,42,43,44] livetin contained no lipid and no phosphorus.[45] Evidence that lipovitellin is not homogeneous was furnished recently by Alderton and Fevold[17] and Fevold and Lausten,[46] who separated the lipoprotein fraction of yolk into two fractions, one containing 2% and the other, 0.4%, protein phosphorus.[47]

It has now been shown that 60 to 70% of the protein phosphorus of yolk can be obtained by mild fractionating procedures in the form of phosvitin.[48] Hence all past vitellin preparations probably contained 5–10% phosvitin, an amount sufficient to influence markedly many of the reported properties. Phosvitin shows a high affinity for other proteins,[49] presumably through electrostatic bonds. Consequently a reasonable explanation for the differing phosphorus contents reported for vitellin is that more or less phosvitin was carried down in the lipovitellin precipitation as salt was removed to differing extents and at differing rates. In fact, Alderton and Fevold[17] reduced the pro-

(38) Lineweaver and Murray, *J. Biol. Chem.*, **171**, 565 (1947).

(39) Needham, "Chem. Embryology," Cambridge Press, 1931, Vol. I, pp. 287–294.

(40) Jukes and Kay, *J. Nutrition*, **5**, 81 (1932).

(41) McFarlane, *Biochem. J.*, **26**, 1061 (1932).

(42) Blackwood and Wishart, *ibid.*, **28**, 550 (1934).

(43) Chargaff, *J. Biol. Chem.*, **142**, 491 (1942).

(44) Calvery and White, *ibid.*, **94**, 635 (1931).

(45) Kay and Marshall, *Biochem. J.*, **22**, 1264 (1928).

(46) Fevold and Lausten, *Arch. Biochem.*, 11, 1 (1946).

(47) Shepard and Hottle (*J. Biol. Chem.*, **179**, 349 (1949)) have recently shown electrophoretically that livetin contains three main components, an observation in agreement with unpublished work of this Laboratory.

(48) The results with yolk proteins suggested that the phosphorus of casein also might occur as a separable high phosphorus phosphoprotein. However, attempts to prepare a phosphorus-enriched fraction from a casein solution or from skim milk by the magnesium sulfate precipitation procedure were unsuccessful.

(49) Equal weights (100 mg.) of phosvitin and protamine sulfate in solution formed a precipitate containing 95% of the phosphorus when mixed (*p*H 6.7; total volume, 140 ml.). The precipitate could be dissolved in 2 *M* sodium chloride. When dialyzed against 2 *M* sodium chloride the protamine nitrogen was eventually all removed with no loss of phosphorus. Since sodium chloride solutions of less than 2 *M* concentration would not dissolve the precipitate completely, the electrostatic bonds must be relatively strong. Phosvitin (2 mg. per ml.) and bovine serum albumin (18 mg. per ml.) in the absence of salt formed a precipitate at *p*H 6 but not at *p*H 4. In the presence of sodium sulfate (0.25 *M*), no precipitate formed at either *p*H.

tein phosphorus content of their lipoprotein precipitate from 2 to 1% by repeated solution in 5% sodium chloride and precipitation by dialysis, a procedure which probably left some phosvitin in solution with each repetition.

Similarly, Francis and Wormall[50] reported that only 25% of the total phosphorus of a precipitate of lipovitellin–antilipovitellin was protein phosphorus, although protein phosphorus accounted for 53% of the total in the original antigen preparation. It appears likely that this difference may represent a separation of the originally antigenic lipoprotein from phosvitin.

The separation of phosphopolypeptides from yolk proteins and casein during enzyme hydrolysis has been mentioned.[2-13] Presumably the vitellinic acid preparations of Levene and Alsberg[51] were somewhat less degraded fractions, very similar in composition to phosvitin. Vitellinic acid was prepared by exposure of vitellin to 12% ammonia for two hours. The yields were low; Jukes and Kay,[40] quoting unpublished results of Kay and Marshall, state that the maximum yield was 35% of the phosphorus of vitellin (of 1% original content). The low yields, together with the fact that no evidence was ever presented indicating that the material might be of higher molecular weight than the phosphopeptones separated from enzymic digests, probably were responsible for the failure to appreciate the possible significance of such material in the yolk proteins.

Whether phosvitin functions in the egg as a storehouse for phosphate, or for metals, or whether it plays an active role during metabolism remains to be determined.[52] Chargaff[53] found that radioactive phosphorus injected into a hen as disodium phosphate was more rapidly incorporated into the vitellin fraction than into the phospholipids of yolk. He therefore suggested that phosphorylated serine residues might be involved in phospholipid synthesis.

Analytical Methods.—Total and inorganic phosphorus was determined usually by the method of Allen[54]; isobutanol extraction was used if samples became turbid in the inorganic phosphorus determination. The methods of Fontaine, Pons and Irving[55] and of Lowry and Lopez[56] also were used occasionally for the determination of inorganic phosphorus. Total nitrogen was determined by the micro-Kjeldahl method recently shown not to require lengthy digestion times,[57] or by macro-Kjeldahl. Amino nitrogen was determined by the manometric Van Slyke method[58] with a fifteen minute reaction time. In order to determine moisture content, samples were dried to constant weight at 78° *in vacuo* over phosphorus pentoxide (Abderhalden pistol heated with 95% ethanol). Total lipid was determined as follows: The material was extracted with absolute ethanol (Soxhlet) for eighteen hours followed by ethyl ether for two hours. After careful evaporation of these solvents, the residue was taken up in chloroform, filtered from insoluble material, dried and weighed. Total sulfur was determined by a sodium peroxide fusion method.[59] Iron was determined by a thiocyanate method.[60]

Dialyses were performed with Visking tubing. Protein preparations were dried by lyophilization. The glass electrode was used for *p*H determinations.

Amino acids were determined chemically as follows: serine, threonine and ammonia by periodate procedures,[61,62,63] tryptophan by the method of Horn and Jones,[64] methionine by the method of Horn, Jones and Blum,[65] cystine and cysteine by the method of Mecham,[66] arginine by the method of Brand and Kassel.[67] The microbiological determinations were carried out by methods to be described elsewhere by J. C. Lewis and N. Snell.

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Summary

Sixty to seventy % of the phosphoprotein phosphorus of egg yolk can be isolated in a protein fraction (the phosvitin fraction) representing 6.5 to 7.0% of the yolk protein and containing about 10% phosphorus. The separation is made by dispersing whole fresh yolk in 0.4 *M* magnesium sulfate solution and diluting to approximately

(50) Francis and Wormall, *Biochem. J.*, **42**, 469 (1948).

(51) Levene and Alsberg, *Z. physiol. Chem.*, **31**, 543 (1900).

(52) A series of sulfated proteins[30] was found by Kazal, Spicer and Brahinsky (Abstracts, 114th Meeting, American Chemical Society Sept., 1948) to possess blood anti-clotting activity, a possibility suggested by the fact that heparin contains acid sulfate groups. It was thought that phosvitin might also possess such activity, particularly since Doyon and Sarvonat (*Compt. rend. soc. biol.*, **65**, 368 (1913)) reported that an extract of "haemotogen" (a phosphopeptone obtained from a pepsin digest of vitellin), prepared according to Hugounenq and Morel,[4] prevented coagulation of blood *in vitro*. However, a phosvitin preparation was not active in a preliminary trial. Injection of 10 mg. of phosvitin caused no apparent ill effects in mice.

(53) Chargaff, *J. Biol. Chem.*, **142**, 505 (1942).

(54) Allen, *Biochem. J.*, **34**, 858 (1940).

(55) Fontaine, Pons and Irving, *J. Biol. Chem.*, **164**, 487 (1946).

(56) Lowry and Lopez, *ibid.*, **162**, 421 (1946).

(57) White, Secor and Long, *J. Assoc. Official Agr. Chem.*, **31**, 657 (1948).

(58) Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).

(59) Direction Booklet 116, Parr Inst. Co., Moline, Ill., p. 138.

(60) Snell, "Colorimetric Methods of Analysis," Vol. I, 3rd printing, p. 294.

(61) Nicolet and Shinn, *J. Biol. Chem.*, **142**, 139 (1942).

(62) Van Slyke, Hiller and MacFadyen, *ibid.*, **141**, 681 (1941).

(63) Boyd and Logan, *ibid.*, **146**, 279 (1942).

(64) Horn and Jones, *ibid.*, **157**, 153 (1944).

(65) Horn, Jones and Blum, *ibid.*, **166**, 313 (1946).

(66) Mecham, *ibid.*, **151**, 643 (1943).

(67) Brand and Kassel, *ibid.*, **145**, 359 (1942).

0.09 *M* magnesium sulfate concentration. The 23% of the total yolk protein that precipitates contains 80% of the phosphoprotein phosphorus. Phosvitin is then extracted from the precipitate with 0.4 *M* ammonium sulfate at *p*H 4; ethyl ether is added to coagulate non-phosvitin components. A typical preparation contained 11.9% nitrogen, 9.7% phosphorus (molar ratio N/P = 2.72). The phosphorus is present as mono-esterified orthophosphate.

Amino acid analyses indicated (in equivalents per 10^4 g.): total β-hydroxy-α-amino acids, 33, and serine, 28 (not corrected for destruction during hydrolysis), compared to 31 moles of phosphorus. There is approximately one phosphoserine residue for each two amino acid residues. Basic amino acids account for nearly one-third of the remaining amino acids.

Osmotic pressure measurements indicated a molecular weight of about 21,000. The best preparations were homogeneous in the ultracentrifuge but showed appreciable boundary spreading on electrophoresis. Inhomogeneity was also indicated by the presence of small amounts (less than 1 g. equivalent per 21,000 g.) of tyrosine, methionine and tryptophan.

A pronounced aggregating effect of magnesium ions was shown by both osmotic pressure and ultracentrifuge measurements; in 0.05 *M* magnesium chloride, the molecular weight found was 38,000 and 39,000, respectively, by the two methods.

Phosvitin was dephosphorylated by citrus phosphatases but not by a bone phosphatase preparation. Alkaline dephosphorylation caused an increase in ultraviolet absorption indicative of the formation of dehydroalanyl residues. Trypsin and pepsin acted more slowly on phosvitin than on casein.

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1,5-Anhydrolactitol and 1,5-Anhydromaltitol

BY HEWITT G. FLETCHER, JR., LEONORE H. KOEHLER[1] AND C. S. HUDSON

A previous publication[2] from this Laboratory described the synthesis of 1,5-anhydro-4-(β-D-glucopyranosyl)-D-glucitol (synonym, 1,5-anhydrocellobiitol) and 1,5-anhydro-6-(β-D-glucopyranosyl)-D-glucitol (synonym, 1,5-anhydrogentiobiitol) through the reductive desulfurization of the appropriate aryl 1-thioglycosides with Raney nickel. The present communication describes the extension of this synthetic method to the lactose and maltose series which was undertaken in order to obtain data for certain generalizations regarding the relationships between rotatory power and configuration among the various 1,5-anhydroglycitols and certain related compounds.[3]

Several well-characterized 1-thioglycosides suitable for the present purpose have been reported in the literature for the lactose and maltose series. Purves described phenyl 1-thio-β-lactopyranoside and its heptaacetate[4] as well as phenyl 1-thio-β-maltopyranoside heptaacetate (III).[5] Haskins, Hann and Hudson[6] prepared by the procedure of Purves, among other 1-thioglycosides, 2′-naphthyl 1-thio-β-lactopyranoside (I) and its heptaacetate as well as 2′-naphthyl 1-thio-β-maltopyranoside heptaacetate.

The reductive desulfurization by Raney nickel of 2′-naphthyl 1-thio-β-lactopyranoside (I) has now been carried out to give crystalline 1,5-anhydro-4-(β-D-galactopyranosyl)-D-glucitol (II), which may be assigned the alternative name of 1,5-anhydrolactitol. The specific rotation of this substance in water, $[\alpha]^{20}D$, proved to be +49.4°. Comparison of the molecular rotations of β-cellobiopyranose, β-lactopyranose and 1,5-anhydrocellobiitol listed in Table I shows that 1,5-anhydrolactitol would be expected to be dextrorotatory. Calculation based upon the isorotation hypothesis affords a numerical value of (7040 + 9550) ÷ 326 = +50.9°.

TABLE I

COMPARISON OF SOME MOLECULAR ROTATIONS IN THE CELLOBIOSE, LACTOSE AND MALTOSE SERIES

	Mol. wt.	$[\alpha]_D$ (H_2O)	$[M]_D$	Difference
β-Lactopyranose	342	+34.9°	+11,900	
				+ 7,040
β-Cellobiopyranose	342	+14.2°	+ 4,860	
1,5-Anhydrolactitol	326	+49.4°	+16,100	
				+ 6,550
1,5-Anhydrocellobiitol	326	+29.3°	+ 9,550	
		($CHCl_3$)		
Methyl β-maltopyranoside heptaacetate	651	+53.5°	+34,800	
				+51,500
Methyl β-cellobiopyranoside heptaacetate	651	−25.7°	−16,700	
1,5-Anhydromaltitol heptaacetate	621	+82.0°	+50,900	
				+48,400
1,5-Anhydrocellobiitol heptaacetate	621	+ 4.0°	+ 2,500	

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(2) H. G. Fletcher, Jr., and C. S. Hudson, THIS JOURNAL, **70**, 310 (1948).

(3) H. G. Fletcher, Jr., and C. S. Hudson, *ibid.*, **71**, 3682 (1949).

(4) C. B. Purves, *ibid.*, **51**, 3619 (1929).

(5) C. B. Purves, *ibid.*, **51**, 3631 (1929).

(6) W. T. Haskins, R. M. Hann and C. S. Hudson, *ibid.*, **69**, 1668 (1947).