- Roberts, R. B. (1960). *Yearb. Carneg. Instn*, **59**, 229.
 Roberts, R. B. (1961). *Yearb. Carneg. Instn*, **60**, 263.
 Roberts, R. B. (1962). *Yearb. Carneg. Instn*, **61**, 244.
 Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Publ. Carneg. Instn: Studies of Biosynthesis in Escherichia coli*, no. 607, p. 5.
 Schachman, H. K., Pardee, A. B. & Stanier, R. Y. (1952). *Arch. Biochem. Biophys.* **38**, 245.
 Spahr, P. F. & Tissières, A. (1959). *J. molec. Biol.* **1**, 237.
 Tissières, A., Schlessinger, D. & Gros, F. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 1450.
 Tissières, A., Watson, J. D., Schlessinger, D. & Hollingworth, B. R. (1959). *J. molec. Biol.* **1**, 221.
 Ts'o, P. O. P., Bonner, J. & Vinograd, J. (1958). *Biochim. biophys. Acta*, **30**, 570.

Biochem. J. (1964) **90**, 555

Studies on a Herring-Egg Phosphoprotein

By T. E. BARMAN,* NGUYEN-KIM BAI AND NGUYEN-VAN THOAI
Laboratoire de Biochimie générale et comparée, Collège de France, Paris, France

(Received 26 August 1963)

Phosvitin is the major phosphoprotein of hen's-egg yolk. All its phosphorus (10 %) is monoesterified to serine (Mecham & Olcott, 1949) and blocks of phosphorylserine sequences make up an important part of its structure (Williams & Sanger, 1959). Schmidt & Laskowski (1961) suggest that in these blocks alkali-labilization of the phosphate groups occurs by enolization of the carbonyl groups. This may explain the susceptibility of the phosvitin phosphorus to alkali, a property common to other classical phosphoproteins (Plimmer & Scott, 1908), and its possible role as a source of energy (Rabinowitz & Lipmann, 1960).

Poorly characterized phosphoproteins have been obtained from the eggs of herring (Levene & Mandel, 1907), cod (Levene, 1901) and tunnyfish (Dezani, 1908) by methods relying on alkali extraction. Much dephosphorylation and degradation could have occurred under these conditions. Though there is evidence that rainbow-trout eggs contain a phosphoprotein (Ito, Fujii & Yoshioka, 1963), it is not known whether the phosphorus of this material (4 %) is alkali-labile and whether it is esterified to serine.

The present paper deals with the isolation and characterization of a phosphoprotein (herring-egg phosvitin) from the mature unfertilized eggs of the herring (*Clupea harengus*). This protein is of particular interest as its phosphorus content (12 %) is greater than that of any other phosphoprotein yet isolated.

MATERIALS AND METHODS

Preparation of herring-egg phosvitin. Herring was obtained at a local fish market during the winter months of 1963 and the eggs, which represented as much as one-sixth

of the weight of the fish, were cleaned of adhering material and blood and treated as shown in the following typical experiment.

Eggs (400 g.) were homogenized in a Waring Blendor, and the homogenate was diluted with 1.2M-MgSO₄ (1 vol.). The mixture was stirred for 30 min., separated by centrifugation and the residue re-extracted with 1.2M-MgSO₄ (0.5 vol.). The combined extracts were dialysed (at 4–5°) against several changes of distilled water in Visking tubing and the precipitate that formed was removed by centrifugation. The supernatant was made 80 % with respect to saturated (NH₄)₂SO₄ and the precipitate was collected by filtration on fluted filter paper overnight at 4–5°. The filtrate contained less than 5 % of the organic phosphorus of the egg. The precipitate was dissolved in 1.5 vol. of a 4:1 (v/v) mixture of 1.7M-NaCl and M-acetate buffer pH 4, and 0.75 vol. of butan-1-ol was added slowly, with stirring. The mixture was stirred at room temperature for a further 4 hr. After centrifugation, the organic top layer (containing herring-egg phospholipids) was poured off and the aqueous bottom layer was carefully collected from underneath the intermediate solid material by siphoning. The insoluble material was re-extracted with buffer and butan-1-ol but with half the volumes used above. The combined aqueous layers were extracted with ether (3 × 0.5 vol.) and clarified by filtration on fluted filter paper. Solid (NH₄)₂SO₄ was added until a permanent crystalline deposit was present; this entrained with it much anthrone-positive material, but little phosphorus, and was removed by centrifugation. The supernatant was dialysed against several changes of distilled water and the phosvitin was precipitated as its calcium salt by the addition of 2 ml. of 5M-CaCl₂. The material was washed several times with water and ethanol and dried under vacuum over CaCl₂ to provide a light white powder. The total yield was 210 mg., containing 12.0 % of phosphorus.

Hen's-egg phosvitin. This was prepared according to the method of Mecham & Olcott (1949).

Phosphorylserine and phosphorylthreonine. These were prepared according to the method of Neuhaus & Korkes (1958).

Nucleic acids. Yeast RNA (10.9 % phosphorus) and herring-sperm DNA (9.1 % phosphorus) were obtained from L. Light and Co. Ltd.

* Present address: National Institute for Research in Dairying, Shinfield, near Reading, Berks.

Human γ -globulins. Human γ -globulins (fraction II) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of partial acid hydrolysates. Herring-egg phosvitin was hydrolysed by 2N-HCl at 100° for 6 hr. The HCl was removed under vacuum over P_2O_5 and KOH and the residue was dissolved in a small volume of water, providing a colourless solution. This was ready for electrophoresis or paper chromatography.

Preparation of total acid hydrolysates. Herring-egg phosvitin was hydrolysed by 6N-HCl at 110° for 18 hr. The HCl was removed under vacuum over P_2O_5 and KOH and the residue dissolved in a small amount of water to provide a colourless solution. This was ready for chromatographic investigations.

Paper chromatography. Phosphoryl-amino acids and -peptides were separated on Whatman no. 1 or 3MM paper (which had been washed with 2N-acetic acid and then with water until the washings were neutral). The solvent used (solvent A) was aq. ammonia (sp.gr. 0.88)-pyridine-ethanol (95%, v/v)-water (3:3:3:1, by vol.). Amino acids were separated on Whatman no. 1 paper with phenol-water (4:1, w/v) (solvent B) or butan-1-ol-acetic acid-water (25:6:25, by vol.) (solvent C) as the solvent. Phosphorylamino acids were located by the molybdate reagent of Burrows, Grylls & Harrison (1952), and amino acids with ninhydrin [0.2% (w/v) in acetone containing 2% (v/v) of pyridine].

Paper electrophoresis. Samples were subjected to electrophoresis on acid-washed Whatman no. 1 or 3MM paper with formic acid-water (1:4, v/w), adjusted to pH 1.5 with NaOH, as the buffer system (Heald, 1961). A potential of 15–17 v/cm. was applied for 14–18 hr. at 0–1°. The papers were dried at room temperature; final traces of formic acid were removed in an oven at 100–105° for 5 min.

Purification of herring-egg phosphorylpeptides. Portions of partial hydrolysates of herring-egg phosvitin were subjected to chromatography on Whatman 3MM paper in solvent A and the positions of the phosphorylpeptides located by guide strips. The corresponding areas were cut out and the peptides eluted by capillary action with water. Further purification was carried out by paper electrophoresis at pH 1.5, after which the peptides were again eluted with water.

Isolation of phosphorylserine from a partial hydrolysate of herring-egg phosvitin. Portions of partial hydrolysates were subjected to paper chromatography and electrophoresis as described above; in each case the band corresponding to phosphorylserine was eluted. Ethanol (95%, v/v) was added to the final eluate to give a concentration of 70% (v/v). The mixture was left in the cold overnight and the

crystals of phosphorylserine were collected by filtration [m.p. 164.5° (decomp.), mixed m.p. 165° (decomp.), both uncorrected].

Alkali-lability experiments. Phosphoprotein, DNA, RNA or crude herring-egg phospholipid was incubated with N-NaOH at 37° for 18 hr. or M- Na_2CO_3 at 100° for 10 min. The inorganic phosphate liberated was determined by the method of Briggs (1932).

Acid-lability experiments. Herring-egg phosvitin was treated with N-HCl for 10 min. at 100° and the mixture assayed for orthophosphate.

Phosphorus and nitrogen determinations. Total phosphorus was obtained by digesting samples with the ammonium molybdate-perchloric acid reagent of Bolin & Stamberg (1944) and orthophosphate was determined in portions of the hydrolysate by the method of Briggs (1932). Inorganic phosphorus was determined after removal of protein by trichloroacetic acid (20%, w/v). Herring-egg-phospholipid fractions were assayed for orthophosphate after the removal of butan-1-ol under vacuum over $CaCl_2$.

Nitrogen was determined by the micro-Kjeldahl technique.

RESULTS

The calcium salt of the herring-egg phosvitin was soluble in 2N-hydrochloric acid, 2N-sodium hydroxide, 1.7M-sodium chloride or formate buffer, pH 1.5, indicating an isoelectric point of above 1.5. It was insoluble in water but dissolved readily on the addition of EDTA, a fact suggesting that, like hen's-egg phosvitin (Mecham & Olcott, 1949), an insoluble dimer is formed in the presence of Ca^{2+} ions.

The phosphorus content of the phosvitin was 12.0%, of which almost all was alkali-labile (Table 1). The alkali-stability of various herring-egg fractions was determined in the presence of M-sodium carbonate or N-sodium hydroxide. As shown in Table 1, the nucleic acids lost little phosphorus when treated with M-sodium carbonate for 2 hr. The phosvitins lost most of their phosphorus in only 12 min. in the presence of this reagent. Rather surprisingly, herring-egg phospholipids lost as much as one-third of their phosphorus as orthophosphate when treated with M-sodium carbonate for 12 min. and nearly 40% when incubated with N-sodium hydroxide for 24 hr. Phosphorylserine could not be detected by chromato-

Table 1. *Effect of alkali on phosphorus-containing macromolecules*

The results are expressed as the percentage loss (as orthophosphate) of organic phosphorus of the relevant macromolecule. Details are given in the Materials and Methods section.

Conditions of incubation			Yeast RNA	Herring- sperm DNA	Hen's-egg phosvitin	Herring-egg phosvitin	Herring-egg phospholipids
Alkali	Temp.	Time					
N-NaOH (pH ~ 14)	37°	18 hr.	—	—	97.0	98.3	36.1
M- Na_2CO_3 (pH ~ 11)	100°	6 min.	—	—	91.7	—	—
		12 min.	—	—	99.0	95.8	28.2
		60 min.	0.5	1.7	—	—	—
		120 min.	1.5	3.6	—	—	—

graphy in partial hydrolysates of lipid fractions. Herring-egg phosvitin lost little phosphorus (2%) when treated with hot *N*-hydrochloric acid.

The calcium salt of the phosvitin contained 7.6% of nitrogen; thus the N:P molar ratio of the material was 1.38.

The absence of nucleic acid in the phosvitin was shown by its negative reaction with the orcinol-ferric chloride reagent of Dische (1955), its ultraviolet-absorption spectrum (Fig. 1), the stability of its phosphorus in *N*-hydrochloric acid, and the lability of its phosphorus in alkali. No carbohydrate could be detected by the anthrone reagent of Morris (1948).

Fig. 1. Ultraviolet-absorption spectra of herring-egg phosvitin (---), hen's-egg phosvitin (-.-.-) and human γ -globulins (—). A solution (2.95 mg./ml.) of herring-egg phosvitin was obtained by adding EDTA to a suspension of its calcium salt in 0.5 M-tris-HCl buffer, pH 7.5. Excess of EDTA was removed by dialysis against the same buffer. Hen's-egg phosvitin (0.62 mg./ml.) and human γ -globulins (0.47 mg./ml.) were dissolved in 0.5 M-tris-HCl buffer, pH 7.7.

Table 2. R_f values and mobilities on paper of some phosphoryl-amino acids and -peptides

Details are given in the Materials and Methods section.

	R_f (in solvent A)	Mobilities towards anode (pH 1.5) (cm.)
Phosphorylserine	0.25	6.8
Phosphorylthreonine	0.27	6.2
Band A	0.20	9.0
Band B	0.25	6.8
Band C	0.32	5.5

When a total hydrolysate of the phosvitin was subjected to chromatography in solvent B or solvent C only five amino acids could be detected, namely aspartic acid, valine, glutamic acid, threonine and serine. Phosphorylserine was present in large amounts. No aromatic or sulphur-containing amino acids were detected by this method. The absence of aromatic amino acids in herring-egg phosvitin was supported by its low absorption in the ultraviolet region (Fig. 1).

When a partial hydrolysate was subjected to chromatography in solvent A or electrophoresis at pH 1.5, three bands were detected which reacted with both the ninhydrin and molybdate reagents. The R_f values and mobilities of these bands (A, B and C) are shown in Table 2. Serine was the only amino acid detected by chromatography in total hydrolysates of eluates of the bands.

Bands A and C. From their mobilities at pH 1.5 it is suggested that band A is due to a phosphoryl-peptide of the type (Ser-P)_n and that band C is due to a partially dephosphorylated phosphorylpeptide such as Ser,Ser-P.

Band B. From its chromatographic behaviour in solvent A and its mobility at pH 1.5, band B appears to be due to phosphorylserine. The presence of phosphorylserine in partial hydrolysates of the phosvitin was established by its isolation.

DISCUSSION

The results presented above establish the existence in the herring egg of a phosphoprotein closely resembling hen's-egg phosvitin. Both proteins contain much phosphorus, all of which appears to be monoesterified to serine and is alkali-labile. From their methods of preparation, however, it is clear that the phosvitins are bound to different types of complexes in their respective biological sources. Thus the method of Mecham & Olcott (1949), which relies on the precipitation of a phosvitin-lipoprotein complex with the aid of Mg^{2+} ions, was unsuccessful when applied to the herring egg; the phosphoprotein could only be precipitated when it had been freed from its lipoprotein complex. Further, whereas hen's-egg phosvitin could readily be liberated with butan-1-ol at alkaline pH (Sundararajan, Sampath-Kumar & Sarma, 1960), herring-egg phosvitin could only be freed from its complex at an acid pH. At pH 7 or 8 no alkali-labile phosphorus was liberated. Heald & McLachlan (1963) showed that, although hen's-egg and plasma phosvitins are identical, the complexes in which they exist are not.

Hen's-egg phosvitin contains 17 amino acids (Mecham & Olcott, 1949); in total hydrolysates of herring-egg phosvitin only five could be detected. In both serine predominates, and glutamic acid

and aspartic acid are important. This is in keeping with the suggestion of Perlmann (1955) that phosphoproteins and peptides are rich in glutamic acid and aspartic acid in addition to serine. About 20 % of the amino acids of hen's-egg phosvitin are basic; none could be detected in herring-egg phosvitin. This would explain the low N:P molar ratio of the herring-egg phosvitin (1.38; that of hen's-egg phosvitin is 2.72). Another fish-egg protein, obtained by Ito *et al.* (1963), contains only six amino acids; of these four are common to herring-egg phosvitin, namely serine, aspartic acid, glutamic acid and valine.

The lack of aromatic amino acids in the phosvitins results in their showing unusual ultraviolet-absorption spectra when compared to that of a mixture of 'typical' proteins, namely human γ -globulins (Fig. 1). The low plateaux in the 250–290 $m\mu$ region are probably due to light-scattering and the increase in extinction at lower wavelengths is probably due to the peptide bond (Beaven & Holiday, 1952).

It is probable that herring-egg phosvitin is an important reserve of both phosphorus and energy for the growing embryo, a reserve which would be particularly important in the herring egg with its long period of incubation (10 months) and its impermeability to orthophosphate (Needham, 1931).

SUMMARY

1. A phosphoprotein (herring-egg phosvitin) has been isolated from the eggs of the herring by a procedure involving extraction with butanol-1-ol of a water-soluble lipoprotein complex.

2. The calcium salt of the phosvitin contained 7.6 % of nitrogen and 12.0 % of phosphorus.

3. The protein contained only five amino acids, namely serine, aspartic acid, glutamic acid, valine

and threonine. Only serine was phosphorylated in the intact material.

T.E.B. was a recipient of a C.N.R.S. and N.A.T.O. fellowship during this research.

REFERENCES

- Beaven, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 319.
 Bolin, D. W. & Stamberg, O. E. (1944). *Industr. Engng Chem. (Anal.)*, **16**, 345.
 Briggs, A. P. (1932). *J. biol. Chem.* **53**, 13.
 Burrows, S., Grylls, F. S. M. & Harrison, J. S. (1952). *Nature, Lond.*, **170**, 800.
 Dezani, S. (1908). *G. Accad. Med. Torino*, **71**, 114. Cited in *Chem. Abstr.* (1908), **3**, 2011.
 Dische, Z. (1955). In *The Nucleic Acids*, vol. 1, p. 301. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
 Heald, P. J. (1961). *Biochem. J.* **80**, 510.
 Heald, P. J. & McLachlan, P. M. (1963). *Biochem. J.* **87**, 571.
 Ito, Y., Fujii, T. & Yoshioka, R. (1963). *J. Biochem., Tokyo*, **53**, 242.
 Levene, P. A. (1901). *Hoppe-Seyl. Z.* **31**, 543.
 Levene, P. A. & Mandel, J. A. (1907). *Hoppe-Seyl. Z.* **49**, 262.
 Mecham, D. K. & Olcott, H. S. (1949). *J. Amer. chem. Soc.* **71**, 3670.
 Morris, D. L. (1948). *Science*, **107**, 254.
 Needham, J. (1931). *Chemical Embryology*, vol. 2, p. 1247. Cambridge University Press.
 Neuhaus, F. C. & Korkes, S. (1958). *Biochem. Prep.* **6**, 75.
 Perlmann, G. (1955). *Advanc. Protein Chem.* **10**, 1.
 Plimmer, R. H. A. & Scott, F. H. (1908). *J. chem. Soc.* **93**, 1699.
 Rabinowitz, M. & Lipmann, F. (1960). *J. biol. Chem.* **235**, 1043.
 Schmidt, G. & Laskowski, M. (1961). In *The Enzymes*, vol. 5, p. 14. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
 Sundararajan, T. A., Sampath-Kumar, K. S. V. & Sarma, P. S. (1960). *Biochim. biophys. Acta*, **38**, 360.
 Williams, J. & Sanger, F. (1959). *Biochim. biophys. Acta*, **33**, 294.

Biochem. J. (1964) **90**, 558

Lipid Metabolism in Liver Injury Caused by Carbon Tetrachloride in the Rat

By A. S. AIYAR, PREMA FATTERPAKER AND A. SREENIVASAN
Central Food Technological Research Institute, Mysore 2, India

(Received 17 June 1963)

The fundamental metabolic deviation that results in fatty degeneration of liver in rats given carbon tetrachloride is not yet fully understood, though the biochemical and histological alterations in liver caused by this hepatotoxin have been investigated intensively in recent years.

Extensive studies have indicated that the administration of carbon tetrachloride causes damage to the integrity and function of mitochondria, resulting in an impaired ability to retain cofactors (Christie & Judah, 1954; Judah & Rees, 1959) and a marked uncoupling of oxidative phosphorylation