Biochem. J. (1971) **122**, 107–113
Printed in Great Britain

Chemical Composition of an Oestrogen-Induced Calcium-Binding Glycolipophosphoprotein in *Xenopus laevis*

By A. Q. ANSARI, P. J. DOLPHIN, C. B. LAZIER, K. A. MUNDAY and M. AKHTAR
Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 5NH, U.K.

(*Received* 30 *November* 1970)

1. Oestrogen treatment has previously been shown to induce the formation of large amounts of a serum protein, vitellogenin (xenoprotein), in *Xenopus laevis*. Vitellogenin was purified from serum by dimethylformamide precipitation and was shown to be homogeneous by a variety of electrophoretic techniques. 2. The molecular weight of vitellogenin was estimated by gel filtration to be about 6×10^5. The chemical constituents of vitellogenin were determined and lead to the characterization of this protein as a serum calcium-binding glycolipophosphoprotein. 3. The extractable lipid accounted for 12% of vitellogenin. Gas–liquid-chromatographic analysis of the saponified lipid moiety showed the presence of palmitic acid, palmitoleic acid, stearic acid, oleic acid and linoleic acid in the molecular proportions 6.8 : 1.5 : 1.0 : 3.6 : 1.4. 4. The carbohydrate moiety consisted of 0.4g of hexose, 0.77g of hexosamine and 0.18g of sialic acid/100g of vitellogenin. 5. The calcium and phosphorus contents were 0.85 and 1.65g/100g of vitellogenin respectively. 6. Serum from oestrogen-treated animals injected with $^{45}CaCl_2$ contained 9.7 times the radioactivity present in serum from untreated $^{45}CaCl_2$-injected animals. Of the radioactivity due to $^{45}CaCl_2$ in the serum of oestrogen-treated animals 72% was non-diffusible on dialysis. Of this activity 65.4% was associated with the vitellogenin band on cellulose acetate electrophoresis.

It has been shown that in response to oestrogen treatment the South African clawed toad *Xenopus laevis* synthesizes large quantities of a serum protein (Follett & Redshaw, 1967; Rudack & Wallace, 1968; Wallace & Dumont, 1968; Follett, Nicholls & Redshaw, 1968; Munday, Ansari, Oldroyd & Akhtar, 1968), which we referred to as xenoprotein (Munday *et al.* 1968). However, to avoid confusion in the literature we will adopt the generic term 'vitellogenin', as proposed by Pan, Bell & Telfer (1969) and adopted by Wallace (1970). *Xenopus* vitellogenin therefore refers to our xenoprotein, to the SLPP of Wallace & Jared (1968) and to the lipophosphoprotein of Follett *et al.* (1968). In our previous report (Munday *et al.* 1968) we showed that this protein could be isolated from the serum of oestrogen-treated animals by precipitation with dimethylformamide. In this paper we describe the chemical composition of vitellogenin and extend our previous work (Munday *et al.* 1968) on the calcium-binding property of this protein.

MATERIALS AND METHODS

Animals. Female *Xenopus laevis* were imported directly from South Africa and maintained at 22°C in large tanks supplied with constant running water. The animals were fed once every 7 days on diced ox liver.

Injections. Oestrogen-treated animals received 1mg of 17β-oestradiol (BDH Chemicals Ltd., Poole, Dorset, U.K.) in 0.2ml of olive oil injected intramuscularly or into the dorsal lymph sac. Control animals received the medium only.

Vitellogenin. Vitellogenin was obtained in preparative quantities from the serum of oestrogen-treated animals by the following method. Female *Xenopus laevis* (50) were injected intramuscularly with 1mg of oestradiol and killed 18 days later when vitellogenin production is known to be high (A. Q. Ansari, P. J. Dolphin, C. B. Lazier, K. A. Munday & M. Akhtar, unpublished work). The blood was obtained by cardiac puncture and centrifuged; the serum was pooled and cooled to 0°C. The serum was then treated with chilled 3.5M-dimethylformamide (3.7ml/10ml of serum) and the resulting suspension adjusted to pH 7–7.5 with 0.2M-acetic acid. The resulting thick precipitate was collected by centrifugation at 15000rev./min at 0°C for 45min and washed once with 2.5ml of 3.5M-dimethylformamide in 0.15M-NaCl. The gelatinous precipitate was collected as described above, dissolved in 0.3M-NaCl and dialysed for 12h at 5°C against four changes of water. The non-diffusible material was then freeze-dried (Munday *et al.* 1968). In some cases vitellogenin was stored at 5°C as the gelatinous precipitate obtained before dialysis. Vitellogenin prepared in the above fashion has a pronounced green colour and in addition to the typical protein absorption at 280nm has absorption maxima at 400 and 700nm. Most of the green pigment in vitellogenin is

extractable with the lipid moiety by using chloroform–methanol (2:1, v/v). Protein concentrations in NaCl solution were determined by the biuret method (Gornall, Bardawill & David, 1949).

Determination of calcium and phosphorus. Ca^{2+} was determined by the method of Duthie & McDonald (1960). Protein phosphorus was determined by the method of Weil-Malherbe & Green (1951) with casein as a standard.

Carbohydrate determinations. Samples (50mg) of native and lipid-extracted vitellogenin were hydrolysed in 3ml of 5% (w/v) trichloroacetic acid at 100°C for 20min. Trichloroacetic acid has been employed for the release of sialic acids from glycoproteins (Swenson & Kern, 1968; Winzler, 1955). Time-course experiments showed that hydrolysis at 100°C for 20min secured the maximal release of sialic acid from vitellogenin. Recovery experiments showed that sialic acid was stable under the hydrolysis conditions used, the average recovery being 95%. After hydrolysis the protein suspension was centrifuged and the supernatant containing the sialic acids was extracted twice with ether to remove the trichloroacetic acid (Swenson & Kern, 1968). The aqueous phase was then assayed for sialic acids by the method of Svennerholm (1957). Purified *N*-acetylneuraminic acid (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was used as standard.

Samples (50mg) of native or lipid-extracted vitellogenin were hydrolysed in 2ml of 3M-HCl at 100°C for 7h. The hexosamine content of the hydrolysate was determined as glucosamine as described by Rondle & Morgan (1955), with the modification of Kraan & Muir (1957). The hydrolysis conditions employed did not cause significant destruction of the released hexosamines. Time-course experiments showed that 7h hydrolysis released maximal quantities of hexosamines.

Samples (30mg) of native or lipid-extracted vitellogenin were analysed for neutral sugars by the method outlined by Winzler (1955) with orcinol–sulphuric acid. An equimolar solution of galactose–mannose was used as a standard. Some turbidity was observed on the addition of the orcinol–sulphuric acid reagent to the native vitellogenin. This turbidity was partially removed by centrifugation in an MSE Minor bench centrifuge.

Extraction of lipid from vitellogenin. The delipidation of 0.5g of vitellogenin was carried out in a Soxhlet apparatus under N_2 reflux with 200ml of chloroform–methanol (2:1, v/v) for 12h. The chloroform–methanol solution containing the extracted lipids was then partitioned by the addition of water (50ml). The chloroform phase was dried over anhydrous sodium sulphate, evaporated to dryness and the ether-soluble residue was measured gravimetrically. The Soxhlet thimble containing the delipidated denatured protein was dried in a vacuum oven at 45°C for 6h and after air equilibration was re-weighed.

Saponification and methylation of vitellogenin lipid. Samples (60–120mg) of the lipid extracted from vitellogenin were saponified under N_2 reflux with 10ml of methanolic 5% (w/v) KOH for 3h; water (10ml) was then added. The non-saponifable lipid was extracted from the hydrolysate twice with ether–light petroleum (b.p. 40–60°C) (1:2, v/v). The aqueous phase was then acidified with HCl and the saponified free fatty acids extracted with 2×50ml of the ether–light petroleum mixture. The extract was evaporated to dryness, redissolved in 3ml of ether and treated with diazomethane. The ether solution containing the methylated fatty acids was dried over anhydrous sodium sulphate and evaporated to dryness under N_2. The residue was dissolved in a known volume of ethyl acetate and analysed by g.l.c.

Gas–liquid-chromatographic analysis. The samples were analysed on a Perkin–Elmer F 11 gas–liquid chromatograph fitted with a disc integrator. A 2.5m column containing polyethylene glycol–succinate ester on Chromosorb-P was used, with N_2 as carrier gas. The conditions were: oven temperature 180°C; N_2 pressure 16lb/in^2; H_2 pressure 15lb/in^2; air pressure 26lb/in^2. Identification and quantitation of the peaks was performed by using standard fatty acid methyl esters obtained from the Hormel Institute, University of Minnesota, Austin, Minn., U.S.A.

Determination of molecular weight. Bio-Gel A1.5m (100–200 mesh) was obtained from Calbiochem Ltd., London W.1, U.K. A glass column (25mm×760mm) was coated twice with dichlorodiethylsilane (1% solution in benzene) to minimize wall effects. The settled bed height was 710mm. The exclusion limit of the gel was 1.5×10^6 daltons. The column was equilibrated with 1.0 M-NaCl at a constant flow rate of 30ml/h maintained by a constant-pressure-head device. The void volume was determined with Blue Dextran 2000 [Pharmacia (G.B.) Ltd., London W.13, U.K.] and the final volume with L-tyrosine (BDH Chemicals Ltd.). Protein samples (10–20mg of each protein) were placed on the column in 1.5ml of 1.0M-NaCl containing 10% (w/v) sucrose. The absorption of the column effluent was read and recorded automatically at 260nm with an LKB Unicord type 4701A monitor. A column of identical dimensions containing Sepharose 4B (Pharmacia Ltd.) was prepared in a similar fashion to the above. The exclusion limit of the Sepharose was 20×10^6 daltons.

Bovine thyroglobulin type 1, pepsin (twice recrystallized), catalase (bovine), bovine serum albumin type F and haemoglobin (bovine) type 1 (twice recrystallized) obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., were used as standards. The elution volume for pepsin was determined in the absence of all other proteins.

Cellulose acetate electrophoresis. This was performed as described by Smith (1968*a*), with 0.037M-sodium barbitone buffer, pH8.6. After electrophoresis the cellulose acetate strips were stained with Ponceau S. Stained protein bands were cut into strips, dissolved in 1ml of methylene chloride–ethanol (9:1, v/v) and their radioactivities counted in 10ml of scintillator containing 0.8g of 5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole (Koch–Light Ltd.) in 100ml of sulphur-free toluene. Radioactivity was determined in a Beckman CPM 200 liquid-scintillation spectrometer and the results were corrected to constant counting efficiency.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel columns (0.6cm×6.0cm) were prepared in Perspex tubes by following the procedures outlined by Shandon Scientific Co. Ltd. (London N.W.10, U.K.) in the instruction book accompanying their analytical polyacrylamide-gel electrophoresis apparatus. The running pH of all gels was 8.5, with 0.05M-tris–glycine as the reservoir buffer. Concentrations of 4.0, 6.0 or 7.5% acrylamide were used in the small-pore gel. All small-pore gels were prepared with

ammonium persulphate as initiator, and large-pore spacer gels were prepared with riboflavin as the initiator of polymerization. In a typical electrophoretic run 300–500 μg of protein was applied to the gel columns in 0.05 M-tris–glycine buffer containing 10% (w/v) sucrose. Electrophoresis was carried out for 40 min at a constant current of 5 mA/tube. After the run, gels were fixed in 7% (v/v) acetic acid, stained with 1% (w/v) Amido Black in acetic acid, electrolytically destained and scanned in a Joyce Loebl U.V. Polyfrac at 265 nm.

RESULTS

Preparation of vitellogenin and assessment of its purity. Vitellogenin was prepared from the serum of oestrogen-treated animals essentially as described by Munday *et al.* (1968) and evaluated for its homogeneity by a variety of electrophoretic techniques. Fig. 1(*a*) shows a scan of serum from oestrogen-treated animals, 3 days after injection, run on 4% polyacrylamide gel. The magnitude of the response to oestrogen is readily seen by comparison with the pattern obtained from the serum of untreated animals (Fig. 1*b*). In a typical preparative procedure serum from animals injected with oestradiol 2–3 weeks previously was cooled to 0°C, treated with dimethylformamide and the pH adjusted to 7–7.5, when a thick gelatinous precipitate was formed. About 100 mg of vitellogenin can be obtained in this way from 1 ml of serum. The protein prepared by the above method runs as a single band when analysed by electrophoresis on starch gels (Plate 1), cellulose acetate strips or polyacrylamide gels (containing 4, 6 or 7.5% acrylamide). Fig. 2(*a*) shows a scan of vitellogenin precipitated from oestrogen-treated animals, 18 days after injection, and run on 4% acrylamide gels (also see Plate 2). The small albumin impurity (2–4%) present in the dimethylformamide precipitate can be readily removed by gel filtration. The purest samples of vitellogenin are obtained when the precipitation is performed with large volumes of serum (20 ml). The concentration of dimethylformamide and the adjustment of the pH to 7–7.5 appear to be critical factors in the purification and are sometimes inadequately achieved with smaller volumes of serum (1–2 ml).

Results in Table 1 suggest that the dimethylformamide precipitation method is primarily dependent on the chemical characteristics of vitellogenin. No precipitate is obtained when this method is applied to serum from untreated animals. When serum from untreated animals is mixed with different proportions of serum from oestrogen-treated animals, addition of dimethylformamide and adjustment of the pH to 7–7.5 results in the precipitation of theoretically expected quantities of vitellogenin.

Stability of vitellogenin. Samples of vitellogenin can be stored at 4°C in the form of a gelatinous precipitate for 4 weeks without any change in the electrophoresis pattern. However, several treatments, including exposure to low pH, storage in solution or gel filtration, resulted in the formation of two types of rearranged proteins with characteristic electrophoretic behaviour. Fig. 2(*c*) shows that storage for 14 days at 4°C of a 30 mg/ml saline solution of vitellogenin (originally containing band II only) resulted in the appearance of a protein of lower electrophoretic mobility (band I) than the main vitellogenin peak (band II). Band I is also present in low concentrations (up to 10% of band II) in unfractionated serum of animals that had been injected with oestradiol 3 weeks previously and were producing vitellogenin at maximal rates (A. Q. Ansari, P. J. Dolphin, C. B. Lazier, K. A. Munday & M. Akhtar, unpublished work). Preliminary evidence from ultracentrifugal analysis suggests that band I may represent an oligomeric form of vitellogenin.

Fig. 2(*b*) shows another peak (band III), which is absent from the original dimethylformamide precipitate but which can be derived from it by exposure to low pH. Addition of 0.08 ml of 0.2 M-acetic acid to a 1 ml sample of vitellogenin (15 mg/ml) that was originally homogeneous resulted in a precipitate, which on electrophoresis showed a 32%

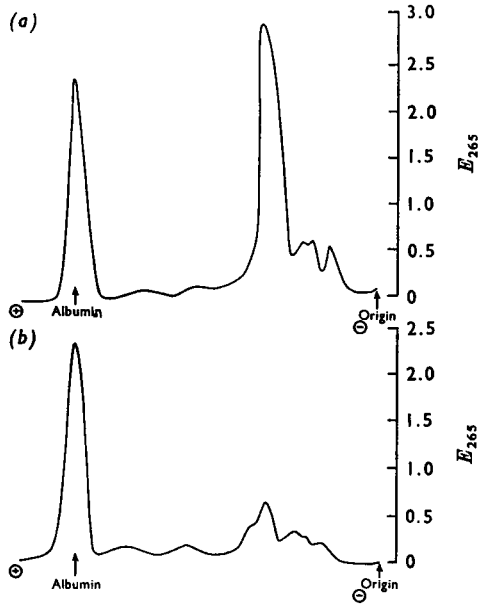


Fig. 1. Electrophoretograms of 4% polyacrylamide gels, stained with Amido Black. (*a*) Serum (10 μl) from oestrogen-treated female *Xenopus laevis*, 3 days after injection; (*b*) serum (10 μl) from untreated female *Xenopus laevis*.

Table 1. *Precipitation of vitellogenin from mixtures of serum from oestrogen-treated and non-oestrogen-treated animals by the dimethylformamide method*

Samples of serum from oestrogen-treated animals obtained 4 weeks after injection were mixed in various quantities with serum from non-oestrogen-treated animals to give a final volume of 1.0ml. Protein was then precipitated by the dimethylformamide method (see the Materials and Methods section).

Serum from non-oestrogen-treated animals (ml)	Serum from oestrogen-treated animals (ml)	Total protein concentration (mg/ml)	Protein precipitated (mg)	Theoretically expected precipitate (mg)
1.0	0.0	38.0	0.0	—
0.0	1.0	97.1	89.1	—
0.9	0.1	44.0	8.7	8.9
0.8	0.2	49.9	19.0	17.8
0.7	0.3	55.7	27.0	26.7

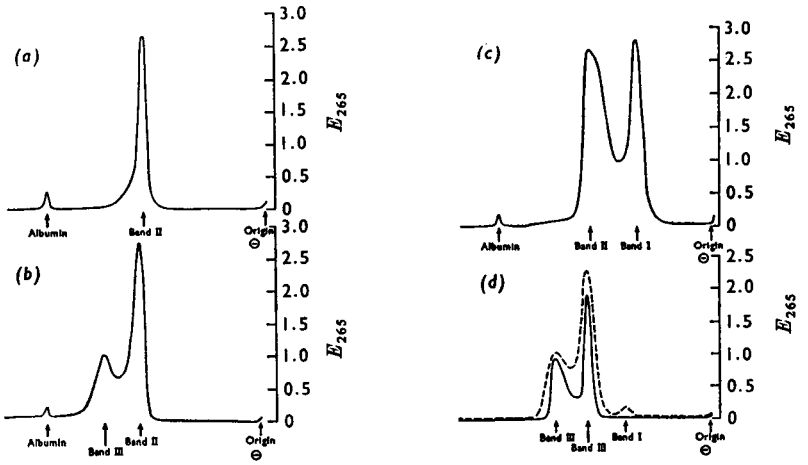
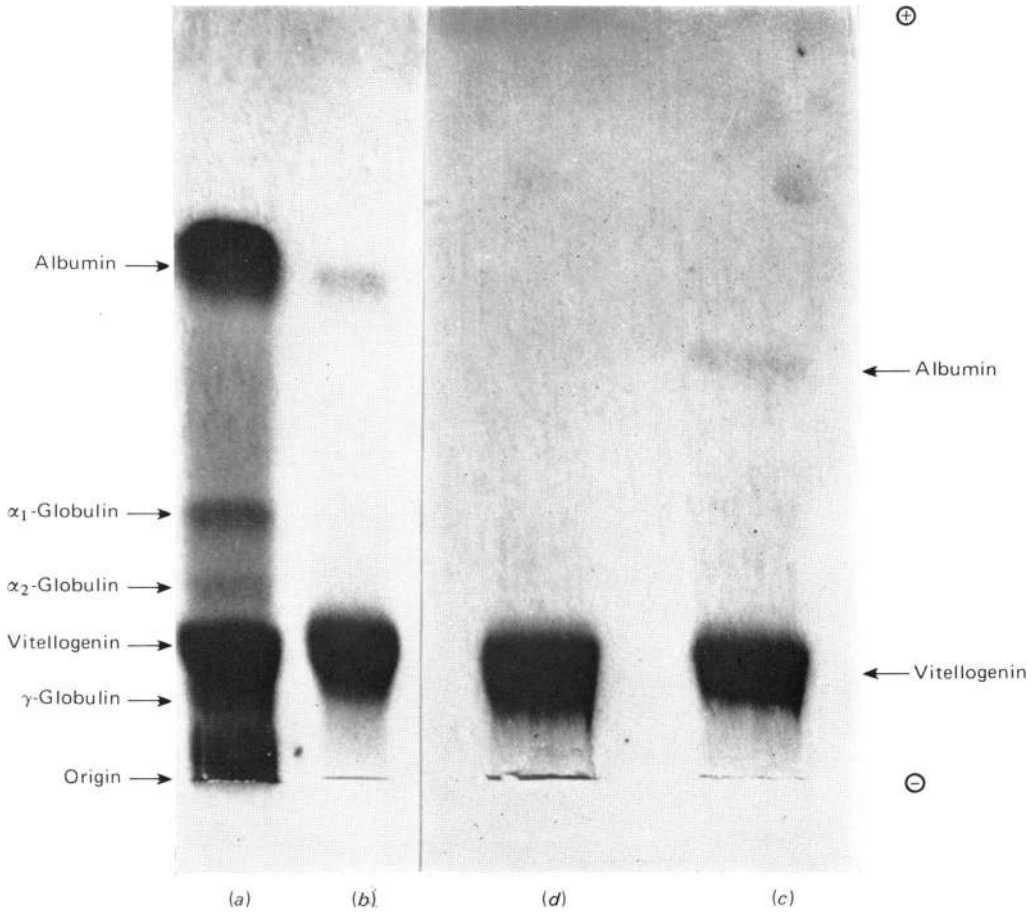


Fig. 2. Electrophoretograms of 4% polyacrylamide gels stained with Amido Black. (*a*) Dimethylformamide-precipitated vitellogenin from the serum of oestrogen-treated animals 18 days after injection shows the presence of band II only and 4% albumin. (*b*) Appearance of band III on exposure of vitellogenin to low pH. (*c*) Appearance of band I on storage of originally homogeneous vitellogenin for 14 days at 4°C. (*d*) Removal of albumin from dimethylformamide-precipitated vitellogenin by gel filtration on Sepharose 4B and the concomitant appearance of band III. The broken line shows the appearance of band I after the column effluent had been dialysed and freeze-dried.

conversion into band III material. Vitellogenin, when subjected to gel filtration on Sepharose 4B, Bio-Gel A1.5m or Sephadex G-200, was eluted as a single peak. However, polyacrylamide-gel electrophoresis showed that bands II and III were present in all fractions of the effluent peak corresponding to vitellogenin (Fig. 3, also see Fig. 2*d*). The partial conversion of band II into band III after gel filtration coincides with the loss of bound calcium (Table 2).

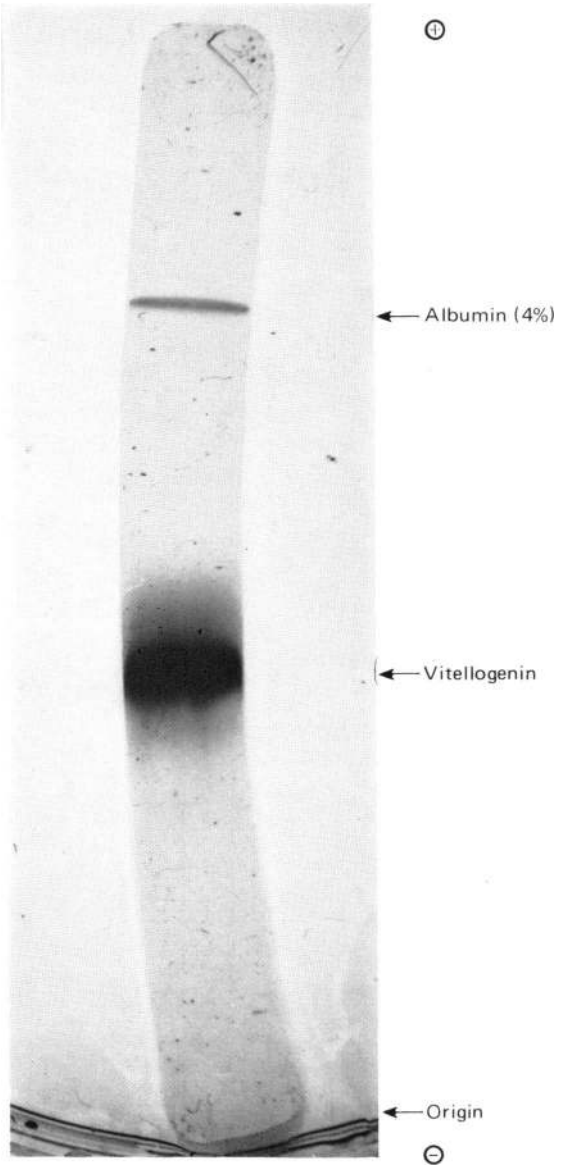
That these apparently simple manipulations cause the decomposition or rearrangement of vitellogenin illustrates the mildness and specificity of the dimethylformamide precipitation technique used to isolate the protein from serum.

Analysis of vitellogenin. Preparations of vitellogenin containing over 95% of band II and 2–4% of albumin were used for the analysis of carbohydrate, lipid, calcium and phosphorus reported in Table 2. The analysis for carbohydrate was repeated with vitellogenin from which all the albumin had been removed by gel filtration (Fig. 2*d*). As mentioned above, this procedure causes the conversion of some of band II into band III. It does not, however, significantly affect the carbohydrate content, but does result in the loss of bound calcium (Table 2). The analysis by g.l.c. of the saponifiable fatty acids in the lipid fraction of vitellogenin is shown in Table 3. Approx. 75% of the chloroform–methanol-extractable lipid was saponifiable. Recovery of palmitic acid after saponification and methylation of known amounts of recrystallized tripalmitate



EXPLANATION OF PLATE 1

Starch-gel electrophoresis. This was performed as described by Smith (1968*b*) with 0.027 M-borate buffer, pH 8.7, at a constant current of 10 mA per tray applied for 6 h. The gels were then fixed in 5% (v/v) acetic acid and stained in Amido Black. (*a*) Serum from oestrogen-treated animals 18 days after injection. (*b*) Vitellogenin precipitated from the serum of oestrogen-treated animals by the dimethylformamide method. (*c*) Dimethylformamide-precipitated vitellogenin showing the presence of a small albumin impurity before gel filtration. (*d*) Dimethylformamide-precipitated vitellogenin after gel filtration on Sephadex G-200 showing the removal of albumin.



EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of vitellogenin precipitated from the serum of oestrogen-treated animals, 18 days after injection, by the dimethylformamide method.

was 75%. The values quoted in Table 3 are corrected for a 25% loss of fatty acid during the procedure.

Determination of molecular weight. The approximate molecular weight of vitellogenin was determined by comparing its mobility with that of known standards on columns of Bio-Gel A1.5m and Sepharose 4B. From a plot of log molecular weight versus elution volume the molecular weight of vitellogenin was estimated to be 5.9×10^5 on the former column and 6.7×10^5 on the latter. The estimate given by Wallace & Jared (1968) for the approximate molecular weight of vitellogenin determined on agarose columns was 7×10^5, although more recent ultracentrifugal analysis gave a value of 4.6×10^5 (Wallace, 1970).

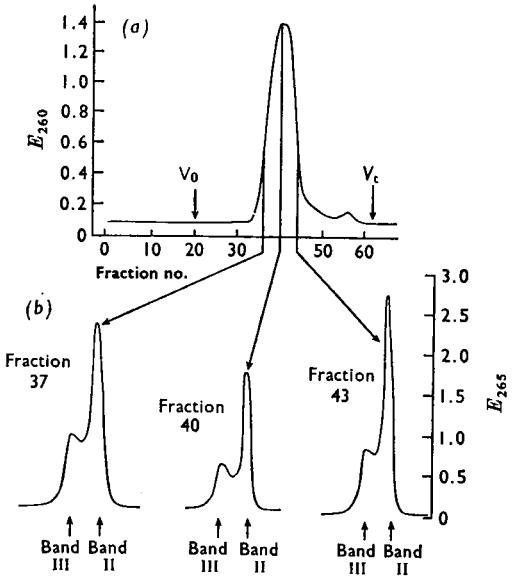


Fig. 3. Gel filtration on Sepharose 4B (*a*) of vitellogenin previously purified from the serum of oestrogen-treated animals by dimethylformamide precipitation and containing over 95% of band II and 2–4% of albumin when analysed by polyacrylamide-gel electrophoresis. The eluent was 0.5M-NaCl. The protein eluted from fractions 53–57 corresponds to albumin. Fractions 37, 40 and 43 were analysed by polyacrylamide-gel electrophoresis (*b*). The patterns obtained on scanning the stained gels are shown in the lower half of the figure. All three fractions showed the presence of bands II and III. (Only a partial scan is shown.) V_0, Void volume; V_t, total volume.

Calcium-binding nature of vitellogenin. Previous work on the calcium-binding property of vitellogenin (Munday *et al.* 1968) was extended. First 20μCi of $^{45}CaCl_2$ solution was injected into the dorsal lymph sac of control and oestrogen-treated animals (18 days after hormone treatment) and serum samples were collected after 24h. Serum from oestrogen-treated animals contained 9.7 times (15.8×10^3 c.p.m./10μl) the amount of ^{45}Ca radioactivity found in the serum of control animals (1.6×10^3 c.p.m./10μl). Vitellogenin, when precipitated from the serum of oestrogen-treated animals with dimethylformamide, contained 60% of the total ^{45}Ca radioactivity present (9.5×10^3 c.p.m. from 10μl of serum).

Samples of serum from oestrogen-treated and control animals were dialysed against 0.15M-sodium chloride for 6h at 5°C to remove unbound ^{45}Ca. Samples (10μl) of the dialysed serum from control and oestrogen-treated animals had 88c.p.m. and 11.4×10^3 c.p.m. respectively, thus showing that most of the ^{45}Ca radioactivity in the serum of control animals was present in an unbound form. The electrophoretic pattern of the dialysed serum from oestrogen-treated animals showed that 65.4% (7.4×10^3 c.p.m.) of the total radioactivity present after dialysis was recovered bound to the vitellogenin band (Table 4). This represents 47% of the original ^{45}Ca radioactivity of whole (undialysed) serum. In similar experiments (Munday *et al.* 1968), only 23% of the ^{45}Ca radioactivity was associated with the vitellogenin band on electrophoresis. The improvement in the present work was achieved

Table 2. *Analysis of vitellogenin*

The lipid, calcium, phosphorus and carbohydrate moieties were determined as described in the Materials and Methods section. The results are given as means ±S.D. of the number of determinations in parentheses.

Moiety	Dimethylformamide precipitate containing over 95% of band II and 2–4% of albumin (g/100g of native vitellogenin)	Dimethylformamide precipitate containing over 95% of band II and 2–4% of albumin (g/100g of delipidated vitellogenin)	After gel filtration on Sepharose 4B (g/100g of vitellogenin)
Lipid (extractable)	11.9 ± 1.1 (4)	—	—
Calcium	0.85 ± 0.03 (3)	—	<0.1 (2)
Phosphorus	1.65 ± 0.05 (3)	—	—
Hexose	0.40 ± 0.10 (20)	0.60 ± 0.10 (10)	0.40 (2)
Hexosamine	0.77 ± 0.04 (5)	0.80 ± 0.03 (5)	0.72 (2)
Sialic acid	0.18 ± 0.02 (20)	0.23 ± 0.03 (10)	0.22 (2)

Table 3. *Identification and determination of the fatty acids present in the lipid moiety of vitellogenin*

The extraction of the lipid moiety, its saponification and methylation of the fatty acids were as described in the Materials and Methods section. Values for fatty acids are corrected for 100% recovery.

Standard methyl ester	Retention time (min) of standard methyl ester	Retention time (min) of methyl ester from vitellogenin lipid	Fatty acid		
			(mg/g of lipid)	(mg/g of vitellogenin)	(% of the total fatty acid isolated)
Methyl palmitate	21.0	22.5	216.3	27.5	45.9
Methyl palmitoleate	23.4	25.2	46.5	5.9	9.9
Methyl stearate	44.0	45.2	35.6	4.5	7.5
Methyl oleate	47.0	47.5	126.1	16.0	26.5
Methyl linoleate	54.5	55.6	47.6	6.0	10.1

Table 4. *Electrophoretic pattern of* ^{45}Ca *binding in the dialysed serum*

Dialysed samples of serum (10 μl) from control and oestrogen-treated animals having 88 and 11380 c.p.m. respectively were subjected to cellulose acetate electrophoresis. Various protein bands were cut, dissolved and their radioactivities counted as described in the Materials and Methods section.

Strips from different regions	^{45}Ca incorporated into various protein bands (c.p.m.)	
	Control	Oestrogen-treated
Origin	10	472
Globulin	8	568
Vitellogenin	12	7452
Albumin	6	282
Background	0	0

primarily by avoiding delay in processing the serum after its removal from the animals. The cumulative evidence above suggests that about half of the ^{45}Ca present in the serum of oestrogen-treated animals is bound to vitellogenin in a form which is stable to dialysis and electrophoresis.

DISCUSSION

The work described in this paper shows that the toad *Xenopus laevis* synthesized large amounts of a high-molecular-weight serum calcium-binding glycolipophosphoprotein in response to a single 1 mg dose of oestradiol (Munday *et al.* 1968). Wallace & Dumont (1968) and Follett & Redshaw (1968) also reported the formation of the oestrogen-induced serum protein in *Xenopus*. The partial chemical composition given by these workers suggests that their protein may be identical with that described in the present work. The analysis of the lipid moiety and the presence of bound hexoses, hexosamines and sialic acids in vitellogenin has not hitherto been reported. Our finding of a total carbohydrate content of 1.35% could, admittedly, be accounted for by contamination of vitellogenin by a glycoprotein of very high carbohydrate content. For example, a contaminating glycoprotein representing 2.5% of the purified vitellogenin and having a carbohydrate content of 50% would account for the observed total carbohydrate content of vitellogenin. Such a possibility seems less likely in view of the homogeneity of vitellogenin (qualifications about the presence of albumin have been made above) on different types of electrophoresis and in view of the reproducibility of the assay on different batches of protein.

The dimethylformamide method used in the present study made available vitellogenin which contained its associated calcium attached to the protein in a form stable to dialysis and electrophoresis. This observation is to be contrasted with the report that triethylaminoethylcellulose-purified vitellogenin contains no calcium (apparently lost during dialysis before chromatography) (Wallace, 1970). Our work revealed that the process of gel filtration could cause the removal of calcium from vitellogenin. This result is compatible with that of Wallace (1970), who found that serum from [^{45}Ca]calcium chloride-injected oestrogen-treated male toads gave no ^{45}Ca radioactivity in the vitellogenin peak after gel filtration on agarose.

The atomic ratio of calcium to protein-bound phosphorus in the whole serum of oestrogen-treated toads has been estimated to be 1.01 for males (Wallace, 1970) and 0.83 for females (Table 2 of Follett & Redshaw, 1968). The ratio for the dimethylformamide-purified vitellogenin is 0.46. Whether the 50% decrease in the ratio is due to loss of calcium during purification or to some other factor cannot be assessed. This consideration, however, should not detract from the fact that the present work constitutes an example of the isolation of a protein that has retained much of the originally bound calcium during purification.

The formation and biosynthesis of new serum

proteins in response to oestrogen treatment have in the past been extensively studied in avian species (Heald & McLachlan, 1965). Several types of protein have been isolated from the serum of oestrogen-treated fowl. Phosvitin, mol.wt. 4×10^4 (Mok, Martin & Common, 1961), was isolated by Heald & McLachlan (1964). Component X_2, a lipoprotein of mol.wt. 4×10^5, was isolated by Schjeide & Urist (1958) and a protein of mol.wt. 1.83×10^5 (component 1) by Clegg *et al.* (1951) and De Guzman & Clegg (1968).

It is generally considered that these oestrogen-induced serum proteins in avian and amphibian species are precursors of the egg-yolk proteins. Credibility for this view is provided by the fact that a protein which is similar to or identical with serum phosvitin has been isolated from hen's egg-yolk (Joubert & Cook, 1958). Further, Wallace & Jared (1969) demonstrated that vitellogenin in the serum of *Xenopus* is accumulated by the ovary on treatment with human chorionic gonadotrophin. It is thus of considerable interest to investigate the mechanism through which the high-molecular-weight oestrogen-induced calcium-binding glycolipophosphoprotein is transformed by the ovary of *Xenopus* into the two major yolk-platelet proteins, amphibian phosvitin (mol. wt. 3.2×10^4) and amphibian lipovitellin (mol. wt. 4.2×10^5) (Wallace, 1963, 1970).

A.Q.A. thanks the Government of Pakistan for a Central Overseas Scholarship, P.J.D. the Medical Research Council for a postgraduate research studentship, and C.B.L. the Medical Research Council of Canada for a fellowship.

REFERENCES

Clegg, R. E., Sanford, P. E., Hein, R. E., Andrews, A. C., Hughes, J. S. & Mueller, C. D. (1951). *Science, N.Y.*, **114**, 437.

DeGuzman, A. V. & Clegg, R. E. (1968). *Poult. Sci.* **47**, 1890.

Duthie, I. F. & McDonald, I. (1960). *Lab. Pract.* **9**, 705.

Follett, B. K., Nicholls, T. J. & Redshaw, M. R. (1968). *J. cell. Physiol.* **72**, 91.

Follett, B. K. & Redshaw, M. R. (1967). *J. Endocr.* **38**, v.

Follett, B. K. & Redshaw, M. R. (1968). *J. Endocr.* **40**, 439.

Gornall, A. G., Bardawill, C. S. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

Heald, P. J. & McLachlan, P. M. (1964). *Biochem. J.* **92**, 51.

Heald, P. J. & McLachlan, P. M. (1965). *Biochem. J.* **94**, 32.

Joubert, F. J. & Cook, G. H. (1958). *Can. J. Biochem. Physiol.* **36**, 399.

Kraan, J. G. & Muir, H. (1957). *Biochem. J.* **66**, 55P.

Mok, C. C., Martin, W. G. & Common, R. H. (1961). *Can. J. Biochem. Physiol.* **39**, 109.

Munday, K. A., Ansari, A. Q., Oldroyd, D. & Akhtar, M. (1968). *Biochim. biophys. Acta*, **166**, 748.

Pan, M. L., Bell, W. J. & Telfer, W. H. (1969). *Science, N.Y.*, **165**, 393.

Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.

Rudack, D. & Wallace, R. A. (1968). *Biochim. biophys. Acta*, **155**, 299.

Schjeide, O. A. & Urist, M. R. (1958). *Expl Cell. Res.* **17**, 84.

Smith, I. (1968*a*). *Chromatographic and Electrophoretic Techniques*, vol. 2, p. 84. London: William Heinemann (Medical Books) Ltd.

Smith, I. (1968*b*). *Chromatographic and Electrophoretic Techniques*, vol. 2, p. 217. London: William Heinemann (Medical Books) Ltd.

Svennerholm, L. (1957). *Biochim. biophys. Acta*, **24**, 604.

Swenson, R. & Kern, M. (1968). *Proc. natn. Acad. Sci. U.S.A.* **59**, 546.

Wallace, R. A. (1963). *Biochim. biophys. Acta*, **74**, 495.

Wallace, R. A. (1970). *Biochim. biophys. Acta*, **215**, 176.

Wallace, R. A. & Dumont, J. N. (1968). *J. cell. Physiol.* **72**, 73.

Wallace, R. A. & Jared, D. W. (1968). *Science, N.Y.*, **160**, 91.

Wallace, R. A. & Jared, D. W. (1969). *Devl Biol.* **19**, 498.

Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.

Winzler, R. J. (1955). In *Methods of Biochemical Analysis*, vol. 2, p. 279. Ed. by Glick, D. New York: Interscience Publishers Inc.