PROTEINS AND LIPOPROTEINS IN YOLK FROM EGGS OF THE ESTUARINE CROCODILE *(CROCODYLUS POROSUS)*; A COMPARISON WITH EGG YOLK OF THE HEN *(GALLUS DOMESTICUS)*

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Abstract-1. The livetin patterns of the two species *Crocodylus porosus* and *Gallus domesticus* were very different and it was difficult to find a correspondence.

2. In *C. porosus* the granules were a larger proportion of the total yolk but they contained very little low-density lipoprotein when compared with granules from hens' eggs.

3. The low-density lipoprotein was a lower proportion of the yolk of *C. porosus:* 30% of the dry weight compared with 60% for the hen. Furthermore, the former apparently contained an unstable fraction consisting of large particles.

4. The apoprotein patterns of the low-density lipoprotein of *C. porosus* eggs had a higher proportion of high-molecular weight constituents than that of the hen.

5. A glycoprotein with *M*, approximately 2 x 10^4 has been isolated that does not correspond to a known avian protein.

6. The characteristic protein of avian egg yolk, apovitellenin I, if present, was a minor constituent.

INTRODUCTION

We have recently compared the albumen and vitelline membrane of eggs of *C. porosus* with those of the hen (Burley et *al., 1987).* This work was part of a study of the water retention of *C. porosus* eggs in relation to the environment (Grigg and Beard, 1985; Grigg, 1987*).* Very large chemical differences were found, particularly in the proteins of the albumen; and these may be relevant to the evolutionary history of the species. We have now extended this work to the egg yolk by comparing the composition, proteins, and lipoproteins of yolk from *C. porosus* and the hen. There have been few chemical studies on crocodiles' eggs. In one of these the egg shells of another species (C. *novaeguineae*) were compared with those of the hen (Jenkins, *1975).*

MATERIALS AND METHODS

Materials

As in previous experiments (Burley *et al.,* 1987) eggs of the estuarine crocodile (*C. porosus)* came from the Edward River Crocodile Farm, Cairns, Queensland. They were stored at 2°C. Hens eggs were from Australorp hens.

Methods

Egg yolk fractions. After the eggs had been broken open (those of *C. porosus* were opened with the help of scissors) the albumen was removed on a yolk-white separator. The yolk was rolled on tissue paper to remove adhering albumen, pierced with a glass rod, and the contents squeezed out to avoid contamination with albumen.

The yolk fractions were separated as follows.

Granules and lipovitellins

The yolk was diluted with an equal volume of 0.16 M sodium chloride and homogenized briefly (20 sec in an Ultraturrax mixer at low speed). The mixture was then centrifuged for 60 min at 20,000 rpm; 10°C *(*80,000 ***g*** maximum force) to sediment the yolk granules. An SW 65 or SW 28 rotor was used in a Beckman L8 preparative ultracentrifuge. The granules were purified by dispersion in 0.16 M sodium chloride followed by recentrifuging - a procedure that was repeated three times (Burley and Cook, 1961*).* For most tests the granules were dissolved in M sodium chloride. In order to separate the granules' low-density lipoprotein from

the lipovitellin fraction, a solution of the granules in 2 M sodium chloride was centrifuged at high speed as described below for the yolk low-density lipoprotein. Phosvitin was isolated from the lipovitellin fraction by the new procedure of Wallace and Morgan (1986), using precipitation with ammonium sulphate. Salt was removed by dialysis.

Yolk low-density lipoprotein and livetins

To obtain the yolk low-density lipoprotein, the yolk supernatant solution, after removal of the granules, was diluted with an equal volume of 4 M sodium chloride and centrifuged for 1.4 x 10^6 hr x ***g***, 10°C (approximately 20 hr in the SW 28 rotor at 20,000 rpm). The lipoprotein floated and if necessary was dispersed in 2 M sodium chloride and recentrifuged. As the lipoprotein of *C. porosus* was apparently less stable, after preliminary experiments this step was omitted. The subnatant solution contained the *livetin fraction,* which was dialyzed against 0.16 M sodium chloride.

Removal of lipid from lipoproteins and yolk. To a solution (approximately 4% w/v of low-density lipoprotein or 1 % w/v of lipovitellin) that had been dialyzed at 2°C against water, EDTA was added to 1.5% (w/v), the pH being adjusted to 7. To this solution was then added two volumes each of chloroform and methanol. The precipitated apoprotein was removed by filtering or, for small amounts, by centrifuging at 2000 rpm, and washed well with chloroform-methanol, with water, and with methanol. While still damp, the solid was dissolved in 4% sodium dodecyl sulphate, 6 M urea, pH 8; or in 6 M urea, 0.02M hydrochloric acid, pH 3.3.

For fatty-acid analyses, lipid was separated from the whole yolk by the method of Bligh and Dyer (1959). *Isolation of a substance of low molecular weight from the lipoprotein apoprotein mixture.* Lipid was removed from the low-density lipoprotein as described above. The protein was dissolved in 4% sodium dodecyl sulphate and applied to a column (1.5 x 80 cm) of Sephadex G-150 eluting with 0.5% sodium dodecyl sulphate. After most of the protein had been eluted, as measured by the absorbance at 280 nm, a small fraction was eluted close to the void volume. The macromolecules in this fraction were concentrated by ultrafiltration and dialysed for 14 days at 2°C against water. The unknown substance remained in solution after a small precipitate had been centrifuged off. It was freeze dried. Less than one mg was isolated from 5 g of yolk. The amino-acid composition was determined by the procedure used previously (Burley *et al.,* 1987).

Physical measurements. Sedimentation patterns were determined on an analytical ultracentrifuge with schlieren optics (Spinco Model E, Beckman Instruments, CA, USA), the phase-plate angle being 60°.

Analytical methods

The concentrations of protein solutions and the weights of yolk fractions were determined gravimetrically after a sample had been dialyzed against water and dried at 105°C.

High-pressure liquid chromatography (HPLC) was performed on an instrument made by ETP-Kortec Pty Ltd (Sydney, NSW), as described previously (Burley *et al.,* 1987).

For gel electrophoresis, polyacrylamide gels (7%, 1.5% cross linking) containing sodium dodecyl sulphate were used in modifications to the method of Weber and Osborn, as described previously (Burley *et al.,* 1987). To stain the gels, a mixture of Coomassie Blue and Amido Black each 0.5% in ethanol: water: acetic acid (5:4:1 by volume) was used. Phosvitin was stained under our conditions. It has been reported that this protein is not stained by Coomassie Blue (Hegenauer *et al.,* 1977), unless trivalent metal ions are present, which were presumably provided by iron electrodes used during electrolytic destaining.

Fatty-acid concentrations in yolk lipids were estimated by gas-liquid chromatography of the methyl esters prepared by the method of Glass and Christopherson (1969). A coiled glass column (4 m x 2 mm) containing 10% Silar on 100/120 Gas-Chrom Q (Applied Science Laboratories, PA, USA) was used for gas chromatography.

RESULTS

The composition of yolk

Table 1 gives data on the compositions of egg yolk from *C. porosus* and the hen. The former has more water and the solids have less lipid. Results of centrifuging diluted yolk are shown in Fig. 1. Tubes A and B refer to *C. porosus* and C and D to the hen. There is clearly a much higher proportion of granules and a lower proportion of low-density lipoprotein in *C. porosus* eggs. This difference was confirmed by separating and weighing the fractions (see Methods) as shown in Table l.

Table 1. Properties of egg yolk. For yolk of *C. porosus* the values are averages for up to nine eggs

	C. porosus		Hen
Property	Range	Mean ± SE	Average
Weight of yolk (g)	30-32	31	20
Water (%)	58.3-59.4	59.1	51
Yolk lipid (% of dry weight)	45.4-50.2	47.1 ±0.9	60
Livetins (% of dry yolk)		13.1	17
Granules (% of dry yolk)	36-43	41 ± 2	23
Floating fraction* (% of dry yolk)	28.3-31.3	30.3±1.5	60
Lipid in floating fraction (%)		87	86
Phospholipid (% of lipid)	20.4-23.1	22.4±0.7	27
Protein in floating fraction (% dry)	11.93-12.8	12.1±0.3	12

*Mainly low-density lipoprotein.

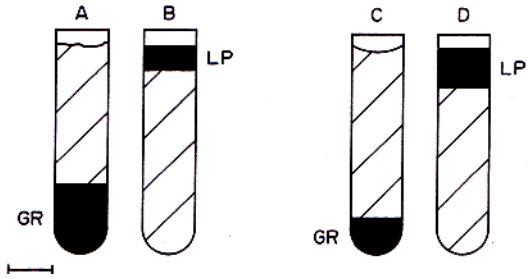

Fig. 1. Drawing of the results of centrifuging yolk in sodium chloride solutions: Tubes A and B were centrifuged for 1 hr, 30,000 rpm, in a Beckman SW 65 rotor, and tubes C and D for 19 hr at 35,000 rpm, 5°C. A and C contained dispersions of yolk (each 50% w/v) in 0.16 M sodium chloride. B and D contained the supernatants from A and C diluted 1: l with 4 M sodium chloride. A and B refer to *C. porosus*, and C and D to the hen. LP = yolk low density lipoprotein; GR = yolk granules. The scale refers to 1 cm.

In Table 2 the fatty-acid compositions of the lipid from yolk of the two species are compared. In general they are similar. The differences in highly unsaturated residues probably reflect the difference in diet.

Proteins in the three fractions of yolk, livetins, granules and low-density lipoprotein, are discussed separately.

1. *Livetins.* The livetin fractions from eggs of *C. porosus* and the hen were compared by gelelectrophoresis in detergent which separates proteins according to molecular size (Fig. 2a and b). The two livetin fractions were also compared by HPLC using an ion-exchange column (Fig. 3). Each of these methods indicated that there are large differences in the livetin fractions of the two species. The individual proteins of *C. porosus* were not isolated.

Table 2. Fatty-acid composition of total lipids from egg yolk

Residue	C. porosus	Hen
C16:0	26.7	24.0
C16:lw7	6.6	3.3
C18:0	7.3	8.9
C18:1w9	42.7	44.1
C18:2w6 plus		
C20:0	5.8	11.7
C18:3w3	2.3	
C20:4w6	2.1	2.2

The values are percentages of the total residues. Coefficients of variation were 3% or less.

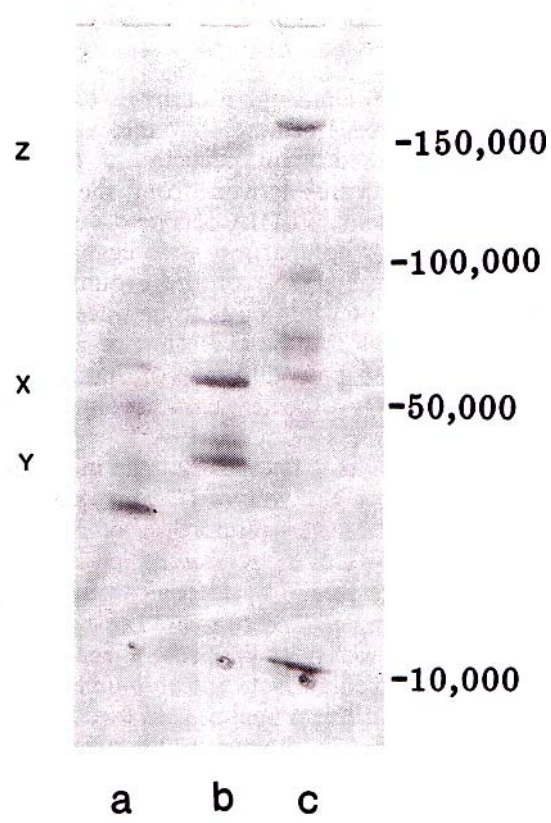

Fig. 2. Gel electrophoresis of yolk livetins in detergent. (a) Livetin fraction from *C. porosus*, (b) livetin fraction from the hen, (c) control. Apoproteins of hens' egg low-density lipoprotein. *x*, y, and *z* refer to alpha-, beta-, and gamma livetins and the hen (b). The right-hand scale gives M_r values from standards. Lower dots indicate positions of marker dye.

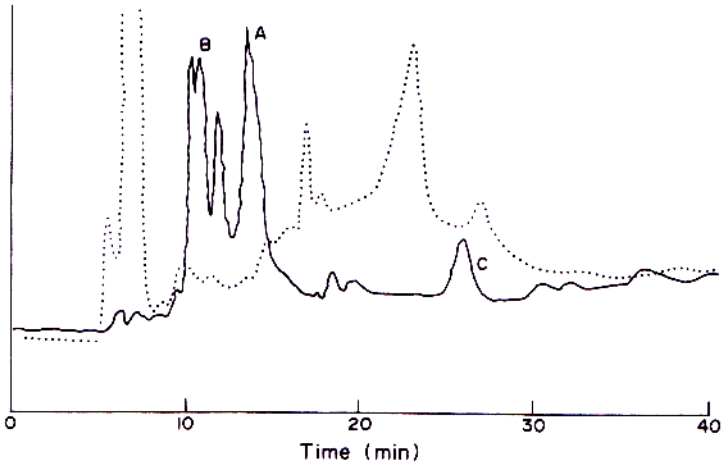

Fig. 3. HPLC of livetin fraction from *C. porosus* and the hen. An ion-exchange column (Ultropac TSK DEAE-SPW, from LKB-Produkter AB, Sweden) was used, eluting with a gradient of from 0.05 to 1.0 M ammonium acetate pH 7.5. Dotted line, *C. porosus*; full line, hen. A, B, and C refer to alpha-, beta-, and gamma-livetins in hens' egg yolk. The y-axis represents optical absorbance at 280 nm.

2. *The yolk granules.* Some properties of the yolk granules of *C. porosus* are given in Table 3 for comparison with those of the hen. Evidently there are important differences. In particular the granules of *C. porosus* do not contain a large amount of low-density lipoprotein. Furthermore the proportions of alpha and beta-lipovitellin are quite different. These proportions were determined by weight after separating the lipovitellins on a column of hydroxyapatite (Burley and Cook, 1961).

The gel-electrophoretic patterns for proteins in yolk granules of the two species are compared in the gels shown in Fig. 4. They are clearly related. The upper bands "x" probably represent the large apoprotein (M_r 130,000) of lipovitellin and the lower bands ("y") the phosvitins, which were not resolved. According to Dr R. A. Wallace (private communication) a sample *of* the phosvitin *of C. porosus* contained a series of subfractions analogous to those of the hen (Wallace and Morgan, 1986). Phosvitin has previously been isolated from eggs of C. *niloticus* (Clark and van Zyl, 1976).

Table 3. Properties of yolk granules

Property	C. Porosus	Hen
Low-density lipoprotein (%)	1.9	12
Lipid (%)	21.7+2.7	30
Phospholipid (% lipid)	34.7+0.9	25
Lipid in lipovitellins (%) ~	24	22
Ratio alpha/beta lipovitellin	1.6	0.6

Except where stated otherwise the percentages refer to dry granules

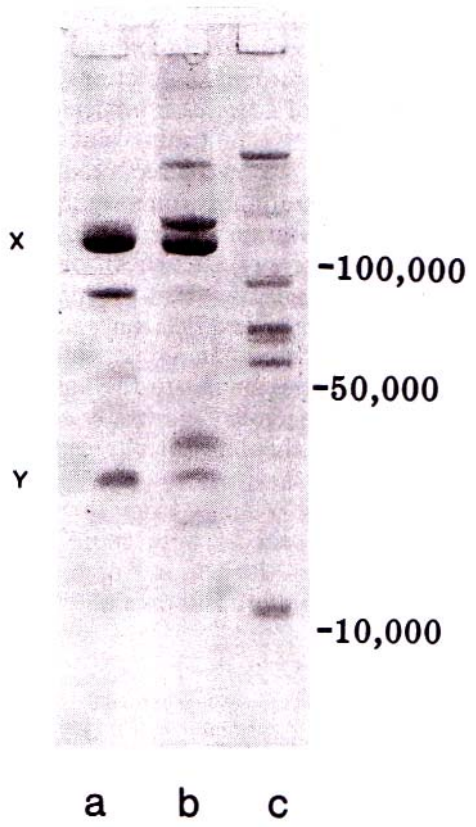

Fig. 4. Gel-electrophoresis of yolk granules' proteins in detergent. (a) Total protein from hens' egg-yolk granules; (b) total protein in granules of *C. porosus*; (c) control, apoproteins in hens' egg-yolk low-density lipoprotein. The right-hand scale gives values for M_r 'x' and 'y' probably correspond to the main apoprotein of lipovitellin and to phosvitin. The lower dots indicate positions of marker dye.

3. Yolk low-density lipoprotein. In Fig. 5 is shown the ultracentrifuge patterns of solutions of whole yolk from *C. porosus* and the hen. The low-density lipoprotein floated and is represented by inverse peaks. As the solutions had approximately the same concentration of yolk, it is clear from Fig: 5 that (i) there was much less of the low-density lipoprotein in *C. porosus* yolk, as would be expected from other results (Table 1, Fig. 1), and (ii) this lipoprotein consisted of two main fractions with some smaller fractions. Approximate flotation coefficients ($s^f_{M, NaCl}$) were 24S and 41S for *C. porosus* and 15S for the hen.

Attempts at separating the fractions of the low density lipoprotein of *C. porosus* by density gradient centrifugation and by gel-filtration chromatography were not successful. Two partly separated peaks were obtained by gel-filtration chromatography on agarose (Biogel 15 m, Biorad Laboratories, CA, USA), But rechromatography of each gave only the faster eluting peak, thus suggesting that the material in the other peak was unstable. Electron microscopy of sections of whole yolk after fixing revealed a high proportion of particles

similar to the `insoluble yolk globules' of avian egg yolk (Vadehra *et al.*, 1977) but did not account for the different ultracentrifuge patterns.

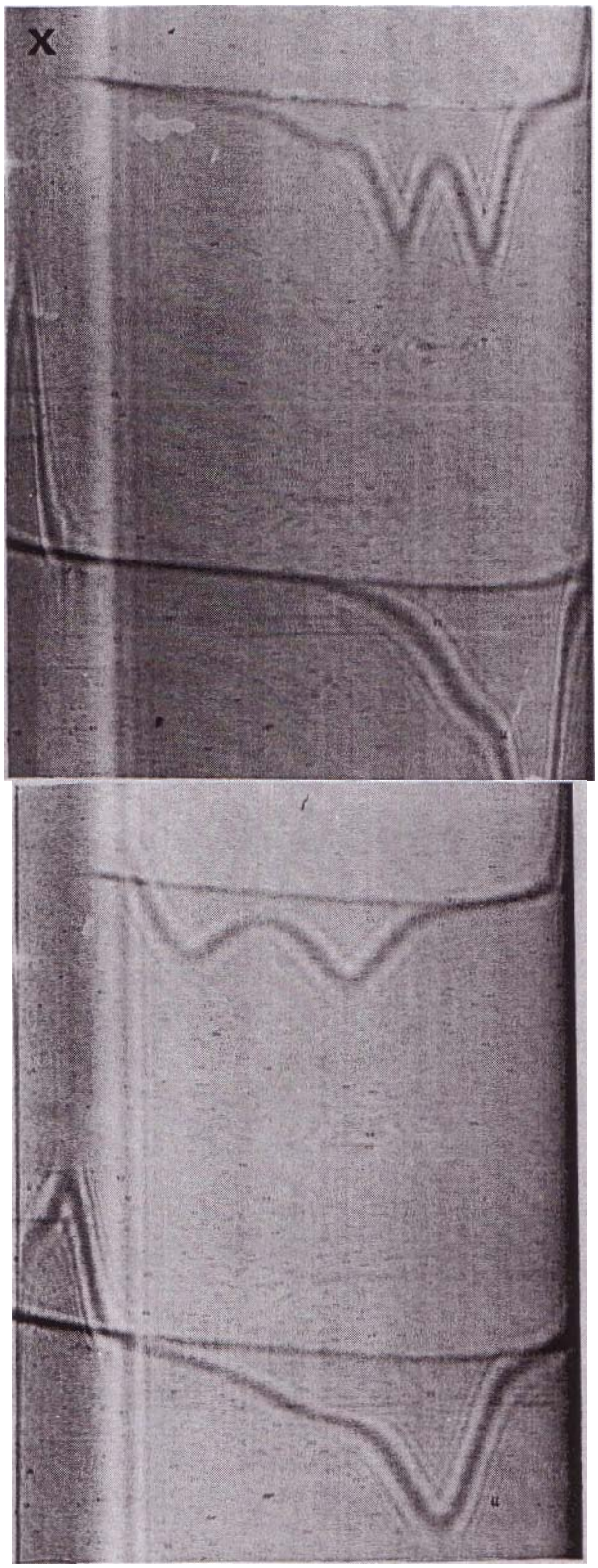

Fig. 5. Ultracentrifuge schlieren patterns (52,000 rpm, 20.0°C) of egg yolk in 1 M sodium chloride. Photographs were taken after 32 min (top) and 64 min (bottom). Flotation was from right to left. The inverse peaks indicate the low-density lipoprotein. Upper patterns: yolk of *C. porosus* (2.3% w/v); infinite schlieren at the meniscus (X) indicates the high concentration of yolk granules. Lower patterns: hen's egg yolk (20% w/v); the upper peaks on the left represent the lipovitellins plus gamma livetin.

The lipid-free apoproteins from the low-density lipoprotein of *C. porosus* were more difficult to dissolve than those of the hen. In Fig. 6, gels (a)-(c), the electrophoretic pattern is shown at different loadings. It differs from that of the hen in the following ways. (1) There is a higher proportion of relatively slowly moving bands. In fact the pattern was distorted by material that did not penetrate the gel, in the presence or absence of mercaptoethanol (a disulphide bond breaker). (2) The fast-moving band, corresponding in the hen to the protein, apovitellenin I, was just noticeable. An attempt was made at isolating the low-molecular-weight material. It was not eluted separately by gel-filtration chromatography in 6 M urea, pH 3.3, because in this' solvent it was apparently present as an aggregate. A very small amount was isolated by chromatography in 0.5% SDS on columns of Sephadex G-100. Attempts at finding an N-terminus in this material were not successful.

During attempts at isolating a protein corresponding to apovitellenin I from the apoprotein mixture, a substance was obtained (see Methods) with the amino-acid composition shown in Table 4. Amino acids accounted for less than half the weight after hydrolysis. The remainder was probably carbohydrate from the appearance of the residue, so the unknown substance may have been a glycoprotein. Its apparent molecular weight from gelelectrophoresis in sodium dodecyl sulphate was about 20,000. In its composition, which is notable for a high proportion of glutamic acid, it does not resemble any known egg protein.

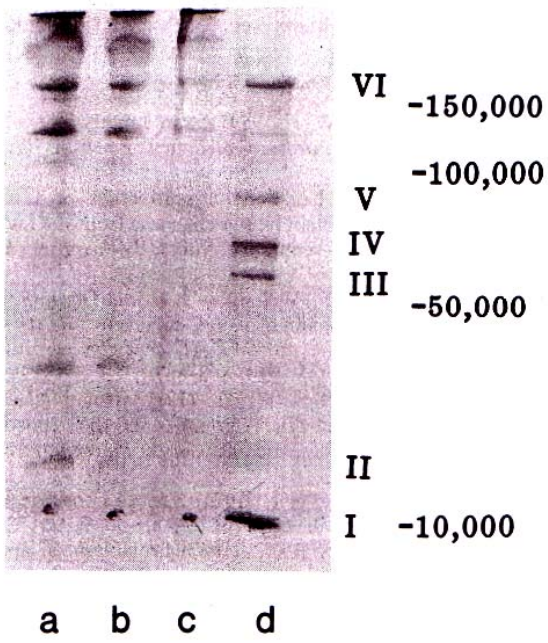

Fig. 6. Gel-electrophoretic patterns of apoproteins of yolk low-density lipoprotein. (a), (b), and (c) apoproteins from lipoprotein of *C. porosus.* Decreasing amounts were applied. (d) Apoproteins from lipoprotein of the hen. Roman numerals indicate the apoproteins (apovitellenins) of hen's lipoprotein. The right-hand numbers are M_r values from standards. The irregular dots at about 10,000 indicate the position of the marker dye.

Table 4. Amino-acid composition of an unidentified substance of low molecular weight isolated from egg yolk low-density lipoprotein of eggs of *C. porosus*

Amino-acid residue	Moles per 100 moles
Ala	4.85+0.15
Asp	5.11 +0.23
Arg	2.19+0.20
Cys/2	0
Glu	23.35+0.13
Gly	13.59+0.57
His	2.41 +_ 0.11
Ile	1.03+0.22
Leu	3.22+0.02
Lys	3.84+0.49
Met	0.72+0.20
Phe	2.04+0.01
Pro	3.52+0.04
Ser	20.66+1.20 1.20
Thr	6.37+0.03
Tyr	2.09+0.09
Val	4.93+0.42

DISCUSSION

Avian eggs contain a high proportion of lipid when compared with eggs of amphibia and invertebrates. Most of this lipid is in particles of low-density lipoprotein that are derived from the very-low-density lipoprotein (VLDL) of blood (Evans and Burley, 1987). A comparison with eggs of reptiles should enable the course of the evolution of the high-lipid lipoproteins in birds to be traced and may have implications for the evolution of mammalian lipoproteins. The crocodilia are of special interest because of their suspected close evolutionary relationship to birds.

From our results it is clear that the most notable difference between egg yolk of *C. porosus* and avian egg yolk is the lower proportion of low-density lipoprotein in yolk of *C. porosus* which is only half that of hens' egg yolk. The difference is made up by more yolk granules and water (Table 1). In its proportion of low-density lipoprotein, egg yolk of *C. porosus* is intermediate between amphibia (in which all the lipid is in the granules) and birds (in which most of the lipid is in low-density lipoprotein). Studies on other reptilia might enable the origins of the low-density lipoprotein to be traced.

The proteins in the granules are not very different in the two species. In the hens' egg yolk granules, there is much less low-density lipoprotein, as would possibly be expected from the lower proportion of this lipoprotein in the whole yolk.

The electrophoretic and chromatographic patterns of the livetins, which are essentially blood serum proteins (e.g. Williams, 1962), are quite different (Figs 2 and 3). We have not followed up this observation but the differences are presumably related to differences in the blood sera of the two species.

The pattern of the low-density lipoprotein apoproteins (Fig. 4) was also quite different in the two species. We were interested in the possible presence of
the characteristic egg-yolk protein, apovitellenin I (Burley, 1973), in eggs of *C. porosus*. So far we have been unable to detect it so it must be present in very small amounts, if at all. While searching for this protein we isolated a substance, probably a glycoprotein (Table 4), with a composition quite unlike any known egg yolk protein.

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