Studies on the Biosynthesis, Assembly and Secretion of Vitellogenin, an Oestrogen-Induced Multicomponent Protein

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1. The process by which the egg-yolk protein precursor vitellogenin is biosynthesized, assembled and secreted by *Xenopus laevis* (South African clawed toad) liver was studied. It was previously shown in other laboratories that vitellogenin contains the two egg-yolk proteins lipovitellin (mol.wt. 140000) and phosvitin (mol.wt. 35000). 2. Evidence is presented which shows that *Xenopus* liver microsomal fractions synthesize precursors of vitellogenin. These precursors were solubilized from the membranes with detergent and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This analysis indicated that there is only one precursor polypeptide, and this has mol.wt. approx. $200\,000 \pm 20\,000$. This demonstrates that the egg-yolk proteins are translated as part of this larger polypeptide. 3. Experiments also demonstrate the existence of a microsomal proteinase which is able to cleave the precursor into smaller fragments. The nature of these fragments provided some indirect evidence that phosvitin and lipovitellin light chains are situated together within the precursor molecule. 4. These precursor data fit in well with structural studies on serum vitellogenin, since it has been shown that the latter protein consists of two identical subunits each with a mobility on sodium dodecyl sulphate/polyacrylamide gels identical with that shown by the microsomal precursor. This indicates that both the intracellular precursor and subunit of vitellogenin have similar (but not necessarily identical) molecular weights. 5. It was also shown that trypsin or chymotrypsin can cleave the serum vitellogenin into leucine- and serine-rich fragments which resemble lipovitellin and phosvitin respectively. Attention is, however, drawn to the fact that the serine-rich fragment is not identical with phosvitin, since it contains eight times more leucine than that expected for the authentic phosvitin molecule [Penning (1976) Ph.D. Thesis, University of Southampton].

Many proteins contain, in addition to the polypeptide backbone, several peripheral moieties such as phosphate, carbohydrate and lipids. Although in recent years much progress has been made on delineation of the processes which participate in the formation of peptide bonds, the mechanism involved in the biosynthesis and assembly of multi-component proteins are not fully understood.

The livers of oestrogen-induced *Xenopus laevis* (South African clawed toad) provide a convenient system *in vitro* for studying the biosynthesis and assembly of vitellogenin (Follett & Redshaw, 1968; Munday *et al.*, 1968; Wallace & Dumont, 1968; Follett *et al.*, 1968; Dolphin *et al.*, 1971), a Ca^{2+} -binding glycolipophosphoprotein with mol.wt. 450000 (Ansari *et al.*, 1971; Redshaw & Follett, 1971; Wallace, 1970). Elegant studies by Wallace & Jared (1968, 1969) have shown that, under the influence of gonadotropins, vitellogenin is selectively

taken up from the serum by the ovaries, where it is converted into its two component egg-yolk proteins, phosvitin and lipovitellin. Phosvitin (mol.wt. 35000) is unique in that 56% of its constituent amino acids are serine residues (Redshaw & Follett, 1971), many of which are contained in blocks of phosphoserine, whereas lipovitellin (140000 mol.wt.) is more hydrophobic than phosvitin and is therefore correspondingly richer in leucine (Redshaw & Follett, 1971).

The present paper is aimed at the identification of the intracellular precursors that are assembled to give the final *Xenopus* vitellogenin molecule and sheds light on the arrangement of the phosvitin and lipovitellin moieties within the precursors and secreted vitellogenin. The reader's attention is drawn to the work of Deeley *et al.* (1975) and Jost & Pheling (1976) which related hen's-egg-yolk phosvitin and lipovitellin to the structure of hen vitellogenin.

Experimental

Animals

Female *Xenopus* weighing approx. 100 g were used throughout this work. These animals were kept in fresh water maintained at a temperature of 22°C and were fed on diced ox liver at weekly intervals.

Injections

Animals for biosynthetic experiments were injected intramuscularly with 1 mg of oestradiol-17 β in 0.2 ml of olive oil 9–16 days before use.

Buffers

All incubations *in vitro* were performed with a 'phosphate/saline' buffer, pH 7.3 (Dulbecco & Vogt, 1954). This contained 4.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na₂HPO₄ (anhydrous), 0.2 g of KH₂PO₄ (anhydrous), 0.1 g of MgCl₂·6H₂O and 0.1 g of CaCl₂·6H₂O in 1 litre of water. This buffer was supplemented with 20 mM-sodium pyruvate as an energy source. Tissue homogenization and cellular fractionations were performed in 0.25 M-sucrose in 0.05 M-Tris/HCl buffer, pH 7.5, containing 25 mM-KCl and 5 mM-MgCl₂.

Radiochemicals

L-[4,5-³H]Leucine (59 Ci/mmol) and L-[3-³H]serine (500 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Special chemicals

Phenylmethanesulphonyl fluoride, trypsin, chymotrypsin and protein standards for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were purchased from the Sigma Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.

Incubation of liver slices

Animals were pithed and cut open from the ventral surface. The livers were washed twice in chilled phosphate/saline buffer and cut into approx. 2–3 mm² slices. For continuous labelling experiments 1 g portions of the liver were weighed into 25 ml Erlenmeyer flasks, containing 10 ml of the buffer. In pulse-labelling experiments 0.5 g portions of the liver were weighed into 25 ml flasks containing 5 ml of the buffer. A known amount of radioactive amino acid was then added and incubation was carried out at 22°C with constant shaking for various time-periods. Samples of the incubation medium were retained for the determination of the incorporation of radioactivity into vitellogenin.

Pulse-labelling experiments

At the end of the pulse period, further incorporation of radioactive amino acid into protein was inhibited by the addition of cycloheximide; the

incubation was then allowed to continue in the same buffer. In a 2 h pulse-labelling experiment, cycloheximide was added to a final concentration of 100 μ g/ml at both 2 h and 3½ h. In a 1 h pulse-labelling experiment, cycloheximide was added to a final concentration of 100 μ g/ml at both 1 h and 2½ h. This final concentration of 100 μ g/ml was previously shown to inhibit protein biosynthesis in *Xenopus laevis* liver slices (Dolphin *et al.*, 1971).

Cellular fractionation

At the end of the incubation time, liver slices (0.5 g) were washed twice in 5 ml of 0.25 M-sucrose in 0.05 M-Tris/HCl buffer, pH 7.5, containing 25 mM-KCl and 5 mM-MgCl₂.

The slices were then transferred in a fresh 5 ml of this buffer to a tight-fitting Potter-Elvehjem homogenizer in which they were homogenized by rotating the pestle at 4000 rev./min with a Tri-Stir tissue homogenizer (speed setting 3–4); 12 passes of the pestle were made in all. One-fifth of the total homogenate was then retained for the determination of incorporation of radioactivity into total tissue protein. Cell debris, nuclei and mitochondria were sedimented at 4°C by centrifugation at 4000 g for 10 min in the 16×15 ml fixed-angle rotor of an MSE High-Speed 18 centrifuge. The postmitochondrial supernatant was filtered through cottonwool and microsomal fractions were sedimented at 4°C by centrifugation at 105 000 g for 60 min in the 10×10 ml titanium fixed-angle rotor of an MSE Superspeed 65 centrifuge.

Solubilization of microsomal protein

The microsomal pellet (3 mg) was solubilized in the presence or absence of the proteinase inhibitor phenylmethanesulphonyl fluoride. In the former case the microsomal pellet was resuspended in 1 ml of 0.25 M-sucrose in 0.05 M-Tris/HCl buffer, pH 7.5, containing 25 mM-KCl and 5 mM-MgCl₂ and then incubated at 18°C for 30 min in the presence of 50 μ l of a solution containing 6 mg of phenylmethanesulphonyl fluoride/ml in 95% (v/v) ethanol (Weber *et al.*, 1972). At the end of the incubation, sodium dodecyl sulphate, 2-mercaptoethanol and Na₂CO₃ were added to give final concentrations of 2% (w/v), 10% (v/v) and 50 mM respectively. The suspension was then solubilized by boiling at 100°C for 5 min.

In the absence of the proteinase inhibitor the microsomal pellet was resuspended in 2 ml of 2% (w/v) sodium dodecyl sulphate containing 10% (v/v) 2-mercaptoethanol and 50 mM-Na₂CO₃. The suspension was then solubilized by incubating at 37°C overnight.

In both methods insoluble material was sedimented at room temperature by centrifugation at 60 000 g for 2 h in the 10×10 ml titanium fixed-angle rotor of an MSE Superspeed 65 centrifuge; a clear brown

supernatant was obtained which was designated 'solubilized microsomal protein'.

Determination of incorporation of radioactively labelled amino acid into protein

(a) *Determination of incorporation into vitellogenin.* Since it has been shown (Merry *et al.*, 1973) that 95% of the labelled protein in the incubation medium is vitellogenin, precipitation of the medium with trichloroacetic acid to a final concentration of 10% (w/v) is an acceptable method of isolating this protein for incorporation studies. The protein precipitate was then worked up by using Method C as described by Dolphin *et al.* (1971).

(b) *Determination of incorporation into total tissue protein.* One-fifth of the total tissue homogenate was diluted with 3 vol. of 0.1M unlabelled amino acid; proteins were then precipitated by the addition of trichloroacetic acid to a final concentration of 10% (w/v), and worked up by the method described by Dolphin *et al.* (1971). Bleaching in H_2O_2 was found to take 2–3 h.

(c) *Determination of incorporation of radioactive amino acid into either microsomal protein, solubilized microsomal protein or supernatant protein from a 105000g centrifugation.* If incorporation into microsomal protein was followed, the microsomal pellet (3 mg) was resuspended in 2 ml of 0.25M-sucrose in 0.05M-Tris/HCl buffer, pH 7.5, containing 25 mM-KCl and 5 mM-MgCl₂. A 1 ml portion of the resuspension was then mixed with 3 ml of 0.1M unlabelled amino acid; portions (1 ml) of either solubilized microsomal protein or of the supernatant from a 105000g centrifugation obtained in the cellular fractionation were also mixed with 3 ml of 0.1M unlabelled amino acid. Proteins in these three different samples were then precipitated by the addition of trichloroacetic acid to a final concentration of 10% (w/v). These protein precipitates were washed five times with 5 × 5 ml of 5% (w/v) trichloroacetic acid containing 0.1M unlabelled amino acid. The precipitates were then given a hot wash at 90°C in 5% (w/v) trichloroacetic acid for 15 min. The protein precipitates were then prepared for counting radioactivity as described by Dolphin *et al.* (1971).

Dissociation of delipidated vitellogenin with sodium dodecyl sulphate

A portion (1.0 mg) of either [³H]leucine- or [³H]serine-labelled vitellogenin obtained from a liver-slice incubation *in vitro* by precipitation of the medium with dimethylformamide (Ansari *et al.*, 1971) was delipidated in 1 ml of chloroform/methanol (2:1, v/v) at 37°C for 1 h. The resulting precipitate was air-dried and treated with 0.4 ml of 2% (w/v) sodium dodecyl sulphate containing 10% (v/v) 2-mercaptoethanol and 50 mM-Na₂CO₃ at 100°C for 5 min. Then 40 µg of the solubilized protein was

subjected to analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Trypsin or chymotrypsin digestion of vitellogenin

Vitellogenin labelled with either [³H]leucine or [³H]serine, obtained by the method described above, was dissolved in 0.2M-NaHCO₃ to give a final concentration of 2.5 mg/ml. Trypsin or chymotrypsin was then added to the solution to give a substrate/enzyme ratio of 500:1 (w/w) and the mixture was incubated at 37°C for 30 min. The incubation was stopped by boiling the mixture in a final concentration of 2% (w/v) sodium dodecyl sulphate containing 10% (v/v) 2-mercaptoethanol and 50 mM-Na₂CO₃ for 5 min. After cooling, 100 µg of material was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Protein determination of vitellogenin in the incubation medium

A sample of the medium obtained at the end of a liver-slice incubation *in vitro* was centrifuged, and the supernatant had its vitellogenin concentration (mg/ml) determined by either the method of Lowry *et al.* (1951) or the modified biuret method of Gasbarro *et al.* (1972).

Polyacrylamide-disc-gel electrophoresis

Protein from the incubation medium was analysed on 5% (w/v) polyacrylamide gels as described by Dolphin *et al.* (1971). For the analysis of sodium dodecyl sulphate-treated protein, a polyacrylamide-gel-electrophoretic system was used which would give a high resolution. This was essentially the discontinuous gel system reported by Neville (1971); 3%/7.5% and 3%/11% double-stack gels were used for the present work.

Calibration of sodium dodecyl sulphate/polyacrylamide-gel system

Portions (1 mg) of proteins of known subunit molecular weight were dissociated into their polypeptides in 2 ml of 2% (w/v) sodium dodecyl sulphate, containing 10% (v/v) 2-mercaptoethanol and 50 mM-Na₂CO₃, by incubating at 100°C for 5 min. Samples (40 µl) of these proteins were then run on the gel systems described above. To obtain these proteins in their polymeric forms, the same treatment was used except that the solubilization medium was 0.1% (w/v) sodium dodecyl sulphate containing 50 mM-Na₂CO₃.

Preparation of polyacrylamide gels for scintillation counting

After destaining, polyacrylamide gels were frozen in crushed solid CO₂ and then sliced on a bank of razor blades. Each 2 mm slice was put into a capped vial which contained 0.8 ml of NCS solubilizer. Vials were heated to 50°C overnight; after cooling, 15 ml

of toluene containing 5-(4-biphenyl)-2-(4-t-butyl-phenyl)-1-oxa-3,4-diazole was added. The radioactivity of all samples was counted at 12°C (see below).

Recovery of radioactivity in a polyacrylamide gel

The percentage recovery of radioactivity in a polyacrylamide gel was calculated on the basis that the trichloroacetic acid-precipitable radioactivity present in a sample used for electrophoretic analysis was 100%.

Measurement of radioactivity

The radioactivity of all samples was counted at 12°C in either an Intertechnique ABAC SL 40 or a Philips liquid-scintillation counter. Automatic correction was made for quenching from quench curves by using chloroform/methanol (2:1, v/v) as a quenching agent. The accuracy of the quench correction was $\pm 5\%$ (S.D.). All results were calculated as d.p.m. by reference to a standard which was counted at an efficiency of 58% for ^3H .

Results

Demonstration of incorporation of [^3H]leucine and [^3H]serine into vitellogenin

Fig. 1 shows polyacrylamide-gel-electrophoretic analysis of the incubation medium obtained by incubating liver slices from oestrogen-treated *Xenopus* with [^3H]leucine for 6h. An identical result was obtained when [^3H]serine was used as the labelled amino acid. Therefore it appeared that with both radioactively labelled amino acids, 95% of the protein-bound radioactivity extruded in the incubation medium was associated with a single protein peak that corresponded to the position of authentic vitellogenin, thus confirming the previous results of Dolphin *et al.* (1971), Merry *et al.* (1973) and Clemens *et al.* (1975). In a time-course study it was found that the incorporation of labelled amino acid into the incubation-medium protein had a 2h lag phase (Dolphin *et al.*, 1971). Merry *et al.* (1973) showed that this lag phase is the time taken to biosynthesize, assemble and secrete the final vitellogenin molecule. A typical profile showing the lag phase is illustrated in Fig. 2(b).

Determination of the percentage of total tissue protein biosynthesis directed towards vitellogenin production; demonstration that the microsomal fraction is a source of precursor molecules

The successful isolation of vitellogenin precursors depended on a significant proportion of total tissue protein biosynthesis being directed towards vitellogenin production, and this feature was evaluated as follows. Liver slices from oestrogen-treated *Xenopus* were allowed to incorporate radioactively labelled amino acid for the length of the lag phase. At the end of this 2h period, new peptide-bond

Fig. 1. Analysis of incubation medium on a 5% (w/v) polyacrylamide gel

Liver slices (1g) were incubated for 6h in 10ml of phosphate/saline buffer (see the Experimental section) containing 10 μCi of [^3H]leucine. At the end of this time a 100 μl portion of the incubation medium was applied to a 5% (w/v) polyacrylamide gel and was electrophoresed towards the anode. The gel was then scanned at 265nm (—) and the radioactivity in 2mm slices was determined (· · ·).

formation was inhibited by the addition of cycloheximide. A subsequent 3h 'chase' period in the presence of the antibiotic was then followed. The pattern of incorporation from these pulse-chase experiments using either [^3H]leucine or [^3H]serine showed that during the chase period there was an increase in protein-bound radioactivity in the incubation medium which was accompanied by a concomitant decrease of an equal amount of label from the total tissue protein. This decrease represented a decrease of 40% in protein-bound radioactivity (see Merry *et al.*, 1973).

Polyacrylamide-gel analysis showed that the labelled protein secreted into the medium after the addition of cycloheximide was in fact vitellogenin. These experiments highlight two aspects; first, that the molecular events occurring in the further elaboration of vitellogenin precursors during the cycloheximide chase period involve neither peptide-bond synthesis nor another molecular process which is susceptible to cycloheximide inhibition; secondly, taken together these results suggest that at least 40% of the total tissue protein biosynthesis is directed towards vitellogenin production and therefore indicates that the isolation of leucine- and serine-

rich intracellular precursors of vitellogenin is a feasible task.

In another experiment, liver slices were incubated as described above and then processed for the incorporation of either [^3H]leucine or [^3H]serine into the microsomal fraction. The results of these experiments showed that with both these labelled amino acids the specific radioactivity of the microsomal fraction decreased by 40% during the chase period; Fig. 2(a) shows the result obtained with [^3H]leucine. The kinetics of the decay of radioactivity from the microsomal fraction correlates well with the appearance of radioactive vitellogenin

in the medium, thus suggesting that 40% of microsomal protein biosynthesis is directed towards vitellogenin production and that the microsomal fractions are an appropriate source of precursors.

Solubilization and identification of microsomal precursors of vitellogenin

Liver slices from oestrogen-treated *Xenopus* were pulsed for 2h with labelled amino acid; at the end of the pulse period, cycloheximide was added and the incubation was continued for a subsequent 3h chase period. Microsomal fractions were harvested at 2h and 5h and solubilized in 2% sodium dodecyl sulphate containing 10% (v/v) 2-mercaptoethanol. As a precaution, solubilization was carried out at 100°C in the presence of a proteinase inhibitor, phenylmethanesulphonyl fluoride, since it has been reported that solubilization of membrane systems may release proteinases active in the presence of detergent (Lansman *et al.*, 1974). The solubilized microsomal protein fractions were then divided into two portions. One was used to determine the protein-bound radioactivity by precipitation with trichloroacetic acid, and the other was kept for analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

The incorporation patterns observed in these experiments with either [^3H]leucine or [^3H]serine as

Fig. 2. Incorporation of [^3H]leucine into secreted vitellogenin, total tissue protein, microsomal protein, solubilized microsomal protein and supernatant protein from a 105 000g centrifugation, with and without the addition of cycloheximide at 2h

Flasks containing liver slices (0.5g), 5ml of phosphate/saline buffer (see the Experimental section) and 10 μCi of [^3H]leucine were incubated over 5h. In one set of experiments (a) cycloheximide was added at 2h to give a final concentration of 100 $\mu\text{g}/\text{ml}$ (marked by an arrow) and a similar amount was added at 3½h (also marked by an arrow). In another set of experiments (b) no addition of cycloheximide was made. During the course of the incubation, flasks were removed and their contents were analysed for the incorporation of radioactivity into the various protein fractions shown. For detailed methodology see the Experimental section. In (a) incorporation at 2h into total tissue protein was taken to be 100% and was $(9.57 \pm 0.56) \times 10^5 \text{ d.p.m.}/\text{g}$ of liver. In (b) incorporation at 5h into total tissue protein was taken to be 100% and was $(1.29 \pm 0.121) \times 10^6 \text{ d.p.m.}/\text{g}$ of liver. In both (a) and (b) the mean $\pm$ s.d. for three experiments is shown. ●, Incorporation into secreted vitellogenin; ○, incorporation into total tissue protein; ■, incorporation into microsomal protein; □, incorporation into microsomal protein solubilized in the presence of a proteinase inhibitor; ▼, incorporation into microsomal protein solubilized in the absence of a proteinase inhibitor; △, incorporation into the supernatant protein from a 105 000g centrifugation.

labelled amino acids were the same. Fig. 2(a) shows the pattern observed with [^3H]leucine, and demonstrates that radioactivity present in the microsomal protein fraction can be quantitatively solubilized by sodium dodecyl sulphate. Since the loss of label from the solubilized microsomal protein fraction followed a time-course similar to that of the appearance of radioactive vitellogenin in the medium, the polypeptides which disappear from this fraction during the chase period must represent those molecules

which are destined for incorporation into the secreted protein.

Meanwhile, analysis of [^3H]leucine- or [^3H]serine-labelled solubilized microsomal protein by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gave identical results, i.e. only one major radioactive polypeptide is present at 2h and the radioactivity associated with this region of the gel is almost completely lost at 5h (Fig. 3). This polypeptide can be assigned a mol.wt. of approx. 200000 from the calibration curves shown in Fig. 4. The patterns of incorporation into secreted vitellogenin, total tissue

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel analysis of [^3H]leucine-labelled microsomal protein obtained at the end of a 2h pulse period and 3h after the addition of cycloheximide, and solubilized in the presence of a proteinase inhibitor

Samples ($100\mu\text{l}$) of solubilized microsomal protein obtained from the experiments described in Fig. 2(a) were applied to 11% (w/v) gels and electrophoresed towards the anode. The radioactivity in 2mm slices was then determined. 'X' marks the position of a polypeptide with a mol.wt. of approx. 200000 which disappears during the chase period. Recovery of radioactivity on the gels was found to be 90% at 2h (a) and 82% at 5h (b). For detailed methodology, see the Experimental section.

Fig. 4. Calibration of 3%/7.5% (w/v) and 3%/11% (w/v) double-stack sodium dodecyl sulphate/polyacrylamide-gel systems

Gels were prepared and run as described in the Experimental section. The mobility relative to Bromophenol Blue was determined for each protein. Standard proteins (mol.wts. in parentheses) used were: insulin (4600), haemoglobin (16000), trypsin (23300), lactate dehydrogenase (36000), pyruvate kinase (57000), catalase (60000), serum albumin (68000), catalase dimer (120000), albumin dimer (136000), albumin trimer (204000) and albumin tetramer (272000). ○, 7.5% (w/v) gel; ▲, 11% (w/v) gel.

protein and solubilized microsomal protein obtained from another series of experiments, in which liver slices were pulsed for only 60 min with [^3H]leucine, are shown in Fig. 5. Similar patterns are observed when [^3H]serine is used as the labelled amino acid (not shown). When the solubilized microsomal protein from these short-term pulse-chase experiments was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, a radioactive polypeptide of mol.wt. approx. 200000 and which contains both leucine and serine was found to be present at 60 min and at 2 h, but was lost at 4 h.

Fig. 5. Incorporation of [^3H]leucine into secreted vitellogenin, total tissue protein and solubilized microsomal protein in 1 h-pulse-3 h-cycloheximide-chase experiments
Flasks containing liver slices (0.5 g), 5 ml of phosphate/saline buffer (see the Experimental section) and 20 μCi of [^3H]leucine were incubated over 4 h. Cycloheximide was added at 1 h to give a final concentration of 100 $\mu\text{g}/\text{ml}$ (marked by an arrow) and a similar amount was added at 2½ h (also marked by an arrow). During the course of the incubation, flasks were removed and their contents were analysed for the incorporation of radioactivity into the various protein fractions shown. For detailed methodology, see the Experimental section. The incorporation at 2 h into total tissue protein was taken to be 100% and was found to be 1.40×10^6 d.p.m./g of liver. ●, Incorporation into secreted vitellogenin; ○, incorporation into total tissue protein; □, incorporation into microsomal protein solubilized in the presence of a proteinase inhibitor.

This molecule had the same mobility as that shown by the precursor polypeptide identified in Fig. 3.

It should be pointed out that the analysis of solubilized microsomal protein from these short-term pulse-chase experiments was carried out on both 11% (w/v) and 7.5% (w/v) polyacrylamide gels so that reliable molecular-weight determinations for the precursor molecule could be obtained. It must be emphasized, however, that even when this precaution was taken, the molecular weight of the precursor migrating in the 200000-mol.wt. region of the gel can only be estimated to an accuracy of 10%.

Reliability of pulse-chase experiments

It was established in the experiments of the type described above that addition of cycloheximide caused a cessation of total tissue- and microsomal-protein biosynthesis (Fig. 5), whereas in the absence of the inhibitor the incorporation of labelled amino acids into total tissue protein and microsomal protein occurred for up to 5 h (Fig. 2b).

As the percentage decrease of radioactivity from the tissue or microsomal protein in the chase period is a measure of the amount of total protein in the fraction which is directed towards vitellogenin production, it is not surprising to find that the decrease observed depended on how well the animals were sensitized to oestrogen. This decrease varied from 20 to 60%, and a decrease of 40% could be obtained as a routine if animals 9–16 days after injection were used (see the Experimental section).

It is also apparent that at all time-intervals the microsomal protein fractions contain only 30–60% of the total radioactivity present in the tissue used for their preparation. This meant that during the chase period the microsomal fractions lost less label than that found in the secreted vitellogenin. This discrepancy could not be explained by the incorporation of label into the soluble protein fraction, i.e. the supernatant from a 105000g centrifugation, since the amount of radioactivity in this fraction was low and showed no decay during the chase period (Fig. 2b). That the radioactivity missing from the microsomal fractions was present in the 4000g pellet was suggested by another series of experiments.

In 60 min pulse-chase experiments similar to those described in Fig. 5, in which, instead of cycloheximide, unlabelled amino acid was used to chase the pulse of radioactivity, incorporation into the 4000g pellet was followed. It was shown that the missing radioactivity could be accounted for by that present in the 4000g pellet and may be attributed to a low yield of the microsomal fraction owing to either the incomplete disruption of the tissue or the sedimentation of some of the microsomal fraction with the mitochondria. It is now well established from other studies that only a portion of the microsomal fraction can be isolated by conventional homogenization and centri-

fugation procedures (Howell *et al.*, 1964; Blobel & Potter, 1967; Lewis & Tata, 1973).

Solubilization of microsomal precursors of vitellogenin in the absence of proteinase inhibitor at 37°C

When the microsomal fraction obtained from 2h-pulse/3h-chase experiments were solubilized without taking the precaution of adding a proteinase inhibitor and the resulting proteins analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, entirely different results were obtained. With both [^3H]leucine and [^3H]serine, there was a noticeable absence of a radioactive peak corresponding to a polypeptide of 200000 mol.wt. Analysis of [^3H]leucine-labelled protein solubilized at the end of a 2h pulse period showed that the protein-bound radioactivity was distributed among low-molecular-weight polypeptides. During the chase period the loss of radioactivity was associated with those regions of the gel corresponding to polypeptides with mol.wts. 10000, 25000 and 50000. Meanwhile, analysis of [^3H]serine-labelled material obtained at the end of a 2h pulse period showed that most of the protein-bound radioactivity was

associated with a single peak which corresponded to mol.wt. 80000–90000. This serine-rich polypeptide was subsequently lost during the chase period. The loss of these leucine- and serine-rich fragments in the chase period suggests that they are related to the 200000-mol.wt. precursor polypeptide identified in Fig. 3.

To discover whether the 80000–90000-mol.wt. serine-rich fragment was homogeneous, the material was re-analysed on a larger-pore-size gel. It was found that the material in the serine-rich peak was slightly heterogeneous and contained at least two polypeptides, of mol.wts. 65000 and 89000. It is noteworthy that a common feature of the polyacrylamide-gel analysis of serine-labelled solubilized microsomal protein was the presence of a considerable amount of radioactivity associated with the dye front. Although the nature of this radioactivity is uncertain, the mobility of the label suggests that the label is associated with compounds of mol.wts. lower than 4000, it is also apparent that this radioactivity is acid-soluble, since it cannot be detected in a trichloroacetic acid precipitate of the solubilized microsomal protein fraction.

Fig. 6. Polyacrylamide-gel analysis of vitellogenin labelled with [^3H]leucine after delipidation and dissociation in sodium dodecyl sulphate

Vitellogenin labelled with [^3H]leucine, treated as described in the Experimental section, was applied to a 7.5% (w/v) gel and was electrophoresed towards the anode. The gel was then scanned at 265 nm (—) and the radioactivity in 2mm slices was determined (· · · ·). 'Y' marks the position of the sole polypeptide product, which from the calibration curve in Fig. 4 has a mol.wt. of approx. 200000. Recovery of radioactivity on the gel was calculated to be 82%.

Fig. 7. Analysis of the fragments produced from the trypsin digestion of (a) [^3H]leucine- or (b) [^3H]serine-labelled vitellogenin by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. [^3H]Leucine- or [^3H]serine-labelled vitellogenin treated as described in the Experimental section was applied to 7.5 (w/v) gels and electrophoresed towards the anode. The gels were scanned at 265 nm (—) and the radioactivity in 2 mm slices was determined (·····). Numbers in parentheses indicate the molecular weights $\times 10^3$ of the various polypeptide fragments identified. 'Vt' marks the position of the vitellogenin subunit.

Fig. 8. Analysis of the fragments produced from the chymotrypsin digestion of (a) [^3H]leucine- or (b) [^3H]serine-labelled vitellogenin by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

[^3H]Leucine- or [^3H]serine-labelled vitellogenin treated as described in the Experimental section was applied to 7.5% (w/v) gels and electrophoresed towards the anode. The gels were scanned at 265 nm (—) and the radioactivity in 2 mm slices was determined (·····). Numbers in parentheses indicate the molecular weights $\times 10^3$ of the various polypeptide fragments identified. 'Vt' marks the position of the vitellogenin subunit.

Demonstration of microsomal proteinase activity in sodium dodecyl sulphate solution

To see if the microsomal fractions contained a proteinase which was active in sodium dodecyl sulphate solution, the fraction was resuspended and used as a possible source of crude enzyme. The presence of a proteinase was demonstrated by assaying its ability to degrade bovine serum albumin in a solution of 2% sodium dodecyl sulphate containing 10% (v/v) 2-mercaptoethanol, either in the absence of phenylmethanesulphonyl fluoride or in its presence at 100°C. The products of these reactions were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. It was shown that a proteinase can completely degrade bovine serum albumin in the absence of phenylmethanesulphonyl fluoride at 37°C, but this degradation does not take place in the presence of the proteinase inhibitor.

Demonstration of the polypeptide content of vitellogenin

By using [³H]leucine-labelled vitellogenin obtained from a liver-slice incubation *in vitro*, Merry (1974) attempted to dissociate this molecule into its individual polypeptides by treatment with 2% (w/v) sodium dodecyl sulphate containing 10% (v/v) 2-mercaptoethanol at 37°C. Analysis of the products by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that one major radioactive band was usually produced, and this was assigned a mol.wt. of 150000–200000. Subsequently Clemens (1974) and Clemens *et al.* (1975) subjected [³H]serine-labelled vitellogenin produced by liver explants to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and found that the mol.wt. of the major radioactive product was 180000. However, some radioactivity was also found in regions of the gel corresponding to smaller polypeptides. Bergink & Wallace (1974) have confirmed that the dissociation product of vitellogenin labelled with [³H]leucine and ³²P_i treated with sodium dodecyl sulphate and 2-mercaptoethanol is a single fragment of mol.wt. 200000. They also found that delipidation of vitellogenin before detergent treatment did not decrease the size of this 200000-mol.wt. species.

These results implied that the polypeptides of lipovitellin and phosvitin cannot be dissociated from vitellogenin. As this was pertinent to the identification of any microsomal precursor of vitellogenin, we decided to check this observation by conducting the following experiment. Samples of vitellogenin labelled with [³H]serine or [³H]leucine were dissociated after prior delipidation and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; the results obtained showed that only one radioactive peak was indeed present. For the sake of economy only the data for [³H]leucine-labelled

vitellogenin are shown (Fig. 6). In experiments where solubilized microsomal protein fractions were analysed on these same pore-size gels (i.e. 7.5% w/v, polyacrylamide), it was found that the microsomal precursor had the same mobility as the radioactive species dissociated from vitellogenin. This implies that the precursor polypeptide of vitellogenin and the subunit of vitellogenin have similar, but not necessarily identical, molecular weights.

Bergink & Wallace (1974) have shown that treatment with proteolytic enzyme of vitellogenin labelled with [³H]leucine and ³²P_i results in the formation of leucine-rich peptides of mol.wts. about 120000 and a phosphoprotein with a mol.wt. of 30000–40000. We have now found that radiochemically pure [³H]serine-labelled vitellogenin when treated with trypsin gives, on sodium dodecyl sulphate/polyacrylamide-gel analysis, radioactive peaks corresponding to mol.wts. of 30000 and 110000 (Fig. 7). However, when [³H]leucine-labelled vitellogenin was used as substrate, only one predominant radioactive peak resulted, and this had a mol.wt. of 110000. Chymotrypsin digestion of either [³H]leucine- or [³H]serine-labelled vitellogenin showed similar patterns (Fig. 8).

The cumulative results in the last two sections confirm previous work by Bergink & Wallace (1974) suggesting that vitellogenin may consist of two subunits each with mol.wt. approx. 200000. In addition, each polypeptide contains leucine- and serine-rich fragments which resemble lipovitellin and phosvitin in their molecular weights. It should be borne in mind that Penning (1976) has shown that the serine-rich fragment (about 30000 mol.wt., Figs. 7 and 8) produced by controlled proteolytic cleavage of native vitellogenin is not identical with phosvitin, since it contains eight times more leucine than that expected for the authentic phosvitin molecule. Finally, as the mol.wt. of serum vitellogenin is approx. 450000–550000, there appears to be a discrepancy of 50000–150000 between the mol.wt. of two subunits and that of the native molecule. Part of this discrepancy can be explained by the fact that vitellogenin contains about 12% lipid (Dolphin *et al.*, 1971; Redshaw & Follett, 1971), which would mean that two 200000-mol.wt. subunits with their lipid content would yield a molecule of mol.wt. 450000.

Discussion

Follett & Redshaw (1968) showed that on treatment with oestradiol-17 β the livers of *Xenopus laevis* synthesize a new serum protein. During the same period we made similar observations and in addition showed that this new protein could be precipitated from the serum of oestrogen-treated *Xenopus* with dimethylformamide (Munday *et al.*,

1968). Independently, Wallace and co-workers demonstrated that the serum from oestrogen-treated *Xenopus* when subjected to TEAE-cellulose chromatography gave a new protein fraction rich in phosphate (Wallace & Dumont, 1968; Wallace & Jared, 1968). As the more critical technique of polyacrylamide-gel electrophoresis for assessing the purity of proteins became available, it was established that the serum protein purified by a single-step procedure involving dimethylformamide was in fact completely homogeneous (Ansari *et al.*, 1971). Further, the dimethylformamide precipitation method was the only one which allowed the isolation of the protein with its associated Ca^{2+} (Ansari *et al.*, 1971). The easy availability of large amounts of this serum protein, vitellogenin, enabled its characterization as a Ca^{2+} -binding glycolipophosphoprotein with mol.wt. 450000–550000 (Ansari *et al.*, 1971; Redshaw & Follett, 1971; Wallace, 1970). The more recent work of Merry (1974), Clemens (1974), Clemens *et al.* (1975) and Bergink & Wallace (1974) (showing that vitellogenin may be dissociated by sodium dodecyl sulphate and 2-mercaptoethanol into one major polypeptide with mol.wt. 180000–200000) when interpreted in the light of the conventional dogma of protein chemistry suggests that vitellogenin consists of two polypeptide chains of mol.wt. 200000. This result has been confirmed in this present paper.

In initial studies we have shown (Dolphin *et al.*, 1971; Merry *et al.*, 1973) that incubation of liver slices from oestrogen-treated animals with labelled amino acids results in the incorporation of radioactivity into vitellogenin extruded into the medium and that this process has a lag phase of 120 min. Merry *et al.* (1973) have shown that this lag phase is the time taken for the biosynthesis, assembly and secretion of the final vitellogenin molecule. In the present paper we have explored the nature of the events that occur during the lag phase, and have attempted to identify precursors of vitellogenin.

It was shown in a series of pulse-chase experiments that the secretion of vitellogenin into the incubation medium after a 2 h lag phase is accompanied by the disappearance of a polypeptide from the microsomal fraction; this polypeptide is both leucine- and serine-rich, and has a mol.wt. of approx. 200000 (Figs. 2a and 3). Since this molecule is the sole polypeptide species that can be characterized as a precursor of vitellogenin, it follows that all polypeptides destined to become a part of the final molecule must have similar molecular weights from the initial stages of the biosynthetic process until the end of the lag phase. This was borne out to some extent by short-term pulse-chase experiments that showed that a 200000 mol.wt. precursor was present in the microsomal fraction 60 min after the addition of labelled amino acid. These results

indicate that the polypeptides of phosvitin and lipovitellin must be biosynthesized as part of a polypeptide precursor of mol.wt. about 200000, which during the lag phase does not undergo any gross alteration in the length of its chain but may undergo subtle molecular alterations, e.g. addition of lipid, phosphate and carbohydrate. It should also be borne in mind that since this precursor polypeptide is similar in molecular weight to the subunit of native vitellogenin, it would appear that vitellogenin is synthesized differently from some other secretory proteins. For example the α chains of collagen are translated as pro-(α chains), which are approximately 50% larger than the α chains present in the secreted molecule (Byers *et al.*, 1975).

Support for the view that the 200000-mol.wt. precursor polypeptide must be the translation product for vitellogenin comes from the work of Tata & Baker (1975), who studied the oestrogen-induced changes in *Xenopus laevis* nuclear RNA. These workers showed that after oestrogen treatment, a new poly(A)-rich species of RNA (mol.wt. 2.2×10^6) appeared in the nucleus. This poly(A)-rich RNA would thus be of the correct size to code for a product of mol.wt. 200000.

After submission of this paper for publication Shapiro *et al.* (1976) reported the isolation from oestrogen-treated *Xenopus* of the mRNA for vitellogenin. When this mRNA was translated in a reticulocyte-lysate system, a product with a mol.wt. of 200000 was obtained. Since it is well established that translation of a mRNA species in a heterologous system may be unfaithful in that products of the incorrect size may be obtained [e.g. translation of total mRNA from oestrogen-treated *Xenopus* in a reticulocyte-lysate system gave polypeptides which were immunoprecipitable with anti-vitellogenin varying from 40000 to 200000 in mol.wt. (Berridge *et al.*, 1976)], it is comforting to note that the present paper for the first time shows that in the homologous whole-cell system the polypeptide translated and destined to become the subunit of vitellogenin has a mol.wt. of 200000.

It should be emphasized that the 200000-mol.wt. polypeptide precursor of *Xenopus* vitellogenin could only be detected by us in the course of a pulse-chase experiment if the microsomal fraction of the liver was solubilized in the presence of a proteinase inhibitor (phenylmethanesulphonyl fluoride). In the absence of this inhibitor, leucine-rich polypeptides of mol.wts. 10000, 25000 and 50000, and a predominantly serine-rich polypeptide of mol.wt. 80000–90000 that contained an impressive amount of radioactivity, were identified as precursors of vitellogenin. To explain these results it is proposed that during these experiments *in vitro* a proteinase that is associated with the microsomal fraction and

is active in sodium dodecyl sulphate digests the 200000-mol.wt. precursor into these leucine- and serine-rich fragments. Although the peptide bonds cleaved by this proteinase are not the same as those hydrolysed in the developing oocyte, the detection of the 80000-mol.wt. serine-rich fragment provides valuable information on the arrangement of the egg-yolk proteins phosvitin and lipovitellin within the 200000-mol.wt. precursor.

First, the size of this serine-rich fragment forcefully emphasizes that the serine-rich species destined to become phosvitin (mol.wt. 35000) in the ovary does not have an independent existence within the microsomal fraction of *Xenopus* liver and that the phosvitin moiety of vitellogenin is translated in a peptide sequence which is serine-rich up to a chain-length of mol.wt. 80000. Further, the formation of the 80000-mol.wt. serine-rich fragment must by necessity involve the removal of a 120000-mol.wt. leucine-rich portion from the 200000-mol.wt. precursor. It is therefore not unreasonable to hypothesize that this leucine-rich portion may represent that part of the precursor molecule which contains the 'heavy' chain of lipovitellin, whereas the 80000-mol.wt. serine-rich fragment may contain both phosvitin and lipovitellin light chain (Scheme 1).

In deducing these conclusions we have used the information arising from the work of Bergink & Wallace (1974), which showed that lipovitellin isolated from *Xenopus* ovary contained two polypeptide chains, a 'heavy' chain (mol.wt. 110000–120000) which was leucine-rich, and a 'light' chain (mol.wt. 30000) which was a phosphopeptide and correspondingly rich in serine; it seems that both

these chains can be derived from vitellogenin after its cleavage in the ovary (Wallace *et al.*, 1972). Bergink & Wallace (1974) also showed that mild chymotrypsin digestion of vitellogenin labelled with [^3H]leucine and $^{32}\text{P}_i$ produced a 90000-mol.wt. fragment rich in phosphorus which could be subsequently completely cleaved into smaller phosphopeptides of mol.wt. 30000–40000. It was thus speculated that the 90000-mol.wt. fragment was related to phosvitin and lipovitellin 'light' chain. By comparing these results of Bergink & Wallace (1974) with those on the proteolytic cleavage *in vitro* of the microsomal precursor, it can be concluded that the intracellular and secreted forms of vitellogenin share a similar protein chemistry.

Finally, as lipovitellin 'heavy' chain (mol.wt. 120000), lipovitellin 'light' chain (mol.wt. 30000) and phosvitin (mol.wt. 35000) give a total mol.wt. approaching 200000, it would seem that there are two possible structures for the precursor molecule (see Scheme 1). The future problem in this field involves the determination of the terminus which houses the heavy lipovitellin moiety and the arrangement of the phosvitin and light lipovitellin moieties within the precursor molecule.

In the last decade intracellular precursors of some of the multicomponent secretory proteins have been identified. Thyroglobulin and vitellogenin are the two largest proteins which are being subjected to detailed biosynthetic studies. Whereas for thyroglobulin the chemistry and sequence of addition of carbohydrate and iodide moieties to the protein backbone is at an advanced state of understanding, the precise correlation of its intracellular polypeptide precursors to the final secreted molecule is not yet unambiguously resolved (Spiro & Spiro, 1966; Vecchio *et al.*, 1972; Spiro, 1973; De Nayer *et al.*, 1974; Di Lavro *et al.*, 1975; Vassart *et al.*, 1975). It is therefore gratifying that some progress towards such an end has been possible with the second-largest multicomponent protein, vitellogenin.

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References

- Ansari, A. Q., Dolphin, P. J., Lazier, C. B., Munday, K. A. & Akhtar, M. (1971) *Biochem. J.* **122**, 107–113
- Bergink, E. W. & Wallace, R. A. (1974) *J. Biol. Chem.* **249**, 2897–2903
- Berridge, M. V., Farmer, S. R., Green, C. D., Henshaw, E. C. & Tata, J. R. (1976) *Eur. J. Biochem.* **62**, 161–171
- Blobel, G. & Potter, V. R. (1967) *J. Mol. Biol.* **26**, 279–292
- Byers, P. H., Click, E. M., Harper, E. & Bornstein, P. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3009–3013
- Clemens, M. J. (1974) *Prog. Biophys. Mol. Biol.* **28**, 69–108
- Clemens, M. J., Lofthouse, R. A. & Tata, J. R. (1975) *J. Biol. Chem.* **250**, 2213–2218

Scheme 1. Two possible arrangements of phosvitin and lipovitellin in the 200000-mol.wt. precursor molecule of vitellogenin

In (i) the lipovitellin light chain is located in the B region of the molecule and links a heavy lipovitellin moiety with a phosvitin moiety, whereas in (ii) the lipovitellin light chain is located at a terminus, and the lipovitellin heavy chain is directly linked to phosvitin.

- Deeley, R. G., Mullinix, K. P., Weteekan, W., Kronenberg, M. M., Meyers, M., Eldridge, J. D. & Goldberger, R. F. (1975) *J. Biol. Chem.* **250**, 9060-9066
- De Nayer, Ph., Durieux, B., Nyssen-Gautier, Fr. & De Visscher, M. (1974) *Life Sci.* **14**, 625-630
- Di Lavro, R., Metafora, S., Consiglo, E., Gentile, G. & Vavrone, S. (1975) *J. Biol. Chem.* **250**, 3267-3272
- Dolphin, P. J., Ansari, A. Q., Lazier, C. B., Munday, K. A. & Akhtar, M. (1971) *Biochem. J.* **124**, 751-758
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167-182
- Follett, B. K. & Redshaw, M. R. (1968) *J. Endocrinol.* **40**, 439-456
- Follett, B. K., Nichols, T. J. & Redshaw, M. R. (1968) *J. Cell. Physiol.* **72**, 91-102
- Gasbarro, L., Bandinelli, R. & Tomassini, G. (1972) *Clin. Chim. Acta* **36**, 275
- Howell, R., Loeb, J. N. & Tomkins, G. M. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **52**, 1241-1248
- Jost, J. P. & Pheling, G. (1976) *Eur. J. Biochem.* **62**, 299-306
- Lansman, R. A., Rowe, M. J. & Woodward, D. O. (1974) *Eur. J. Biochem.* **41**, 15-23
- Lewis, J. A. & Tata, J. R. (1973) *J. Cell Sci.* **13**, 447-459
- Lowry, O. H., Rosebrough, N. J., Farr, R. J. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Merry, A. H. (1974) Ph.D. Thesis, University of Southampton
- Merry, A. H., Dolphin, P. J., Munday, K. A. & Akhtar, M. (1973) *Biochem. J.* **132**, 459-463
- Munday, K. A., Ansari, A. Q., Oldroyd, D. & Akhtar, M. (1968) *Biochim. Biophys. Acta* **166**, 748-751
- Neville, D. M. (1971) *J. Biol. Chem.* **246**, 6328-6334
- Penning, T. M. (1976) Ph.D. Thesis, University of Southampton
- Redshaw, M. R. & Follett, B. K. (1971) *Biochem. J.* **124**, 759-766
- Shapiro, D. J., Baker, H. J. & Stitt, D. T. (1976) *J. Biol. Chem.* **251**, 3105-3111
- Spiro, M. J. (1973) *J. Biol. Chem.* **248**, 4446-4460
- Spiro, R. G. & Spiro, M. J. (1966) *J. Biol. Chem.* **241**, 1271-1282
- Tata, J. R. & Baker, B. (1975) *Biochem. J.* **150**, 345-355
- Vassart, G., Refetoff, S., Brocos, M., Dinsart, C. & Dumont, J. E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3839-3843
- Vecchio, G., Clarr, G. M. & Salvatore, G. (1972) *J. Biol. Chem.* **247**, 4908-4913
- Wallace, R. A. (1970) *Biochim. Biophys. Acta* **215**, 176-183
- Wallace, R. A. & Dumont, J. N. (1968) *J. Cell. Physiol.* **72**, 73-89
- Wallace, R. A. & Jared, D. W. (1968) *Can. J. Biochem.* **46**, 953-959
- Wallace, R. A. & Jared, D. W. (1969) *Dev. Biol.* **19**, 498-526
- Wallace, R. A., Nickol, J. M., Ho, T. & Jared, D. W. (1972) *Dev. Biol.* **29**, 255-272
- Weber, K., Pringle, J. R. & Osborn, M. (1972) *Methods Enzymol.* **26**, 1-27