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Studies on the Induction and Biosynthesis of Vitellogenin, an Oestrogen-Induced Glycolipophosphoprotein

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1. Oestrogen treatment induces the formation of a Ca^{2+}-binding glycolipophosphoprotein, vitellogenin, in *Xenopus laevis*. 2. The incorporation of L-[4,5-^{3}H]-leucine into vitellogenin *in vivo* and *in vitro* was observed 12–24h after hormone treatment and increased progressively up to 21 days after treatment. 3. Vitellogenin is shown to be the major protein component biosynthesized and released into the incubation medium *in vitro* by livers from oestrogen-treated animals. 4. The biosynthesis *in vitro* of vitellogenin was inhibited by cycloheximide and carbonyl cyanide *m*-chlorophenylhydrazone, stimulated by increased Ca^{2+} concentrations and decreased by raising the incubation temperature from 22 to 37°C. 5. Incorporation of labelled amino acids into vitellogenin began after approx. 2h. No lag phase was noted for the incorporation of labelled amino acids into total tissue proteins. 6. The incorporation of label from [^{32}P]phosphate and [2-^{14}C]acetate into the protein as well as into the lipid moiety of vitellogenin showed a lag phase similar to that noted for the incorporation of amino acids. 7. These results suggest that the release of vitellogenin into the incubation medium occurs about 2h after the initiation of its biosynthesis.

Several groups have noted that the South African clawed toad, *Xenopus laevis*, responds dramatically to oestrogen treatment by forming a serum protein (Follett & Redshaw, 1967; Follett, Nicholls & Redshaw, 1968; Rudack & Wallace, 1968; Wallace & Dumont, 1968; Munday, Ansari, Oldroyd & Akhtar, 1968; Wallace & Jared, 1969). This protein, which is referred to as vitellogenin, binds Ca^{2+} and contains 1.65% of phosphorus, 12% of lipid and 1.35% of carbohydrate (Ansari, Dolphin, Lazier, Munday & Akhtar, 1971). Vitellogenin is synthesized by the liver and accumulates in the serum, from which it may be isolated in preparative quantities (Ansari *et al.* 1971). Electrophoretic analysis was used by Munday *et al.* (1968) to study the time-course of the appearance of vitellogenin in the serum after a single 1mg dose of oestradiol, and vitellogenin first appeared about 96h after hormone treatment. We have now focused attention on the earliest response to hormone treatment by studying the biosynthesis of vitellogenin as estimated by the incorporation of radioactive amino acids.

The present paper reports on the capacity of *Xenopus* liver to incorporate labelled precursors into vitellogenin as a function of time after hormone treatment and describes experiments pertinent to the biosynthesis and release of this multicomponent protein.

MATERIALS AND METHODS

Animals. *Xenopus laevis* of medium size (70–80g) were imported directly from South Africa and were kept in large tanks at 22°C with constant running water. The toads were fed every 7 days on chopped ox liver. In all cases, except in the tissue-culture experiments, female *Xenopus laevis* were used.

Injections. Animals were injected intramuscularly or via the dorsal lymph sac with 1mg of oestradiol 17β in 0.2ml of olive oil. Control animals received the medium only.

Buffer. All incubations *in vitro*, except the tissue-culture experiments, were performed with a phosphate–saline buffer, pH 7.3 (Dulbecco & Vogt, 1954), containing 4.09g of NaCl, 0.20g of KCl, 1.15g of Na_2HPO_4 (anhydrous), 0.20g of KH_2PO_4 (anhydrous), 0.10g of $CaCl_2,6H_2O$ and 0.10g of $MgCl_2,6H_2O$ in 1 litre of water. The buffer was supplemented with 20mM-sodium pyruvate as an energy source.

Radiochemicals. Sodium [2-^{14}C]acetate (55.1mCi/mmol), L-[U-^{14}C]glycine (11.3mCi/mmol), L-[2,5-^{3}H]-histidine (500mCi/mmol), L-[4,5-^{3}H]leucine (19.0Ci/mmol), L-[2,5-^{3}H]phenylalanine (100mCi/mmol), L-[*Me*-^{14}C]methionine (60mCi/mmol), DL-[1-^{14}C]valine

(30mCi/mmol) and [^{32}P]orthophosphate in dilute HCl (44Ci/mg) were all obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Special chemicals. Hyamine hydroxide (1.0M in methanol) and 5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. AnalaR trichloroacetic acid and 100-volume (30%, w/v) hydrogen peroxide were from BDH Chemicals Ltd., Poole, Dorset, U.K. NCS solubilizer was from Nuclear–Chicago Corp. via G. D. Searle, High Wycombe, Bucks., U.K.

Incubation of liver tissue. Animals were pithed and cut open from the ventral side. The livers were obtained under sterile conditions, and were washed twice in chilled buffer and cut into approx. 3mm cubes, 1g portions of which were weighed out into 25ml conical flasks. A known volume of buffer was added and the tissue incubated in the presence of radioactive precursors at 22°C with constant shaking for various time-periods (indicated in the Results and Discussion section).

Tissue-culture experiments. Basal Eagle's medium, pH7.3 (Eagle, Oyama, Levy & Freeman, 1957), was obtained from Flow Laboratories, Irvine, Ayrshire, U.K., and supplemented with chicken serum (10%, v/v), penicillin G (100 units/ml) and streptomycin (0.5mg/ml). This medium was then used for the culture of *Xenopus* liver. Male toads were killed at various time-intervals after injection of 1mg of oestradiol via the dorsal lymph sac. The livers were removed under aseptic conditions and sliced, and portions (0.4g) were incubated in 5ml of the supplemented Eagle's medium which also contained 10μCi of L-[4,5-^{3}H]leucine (0.5nmol) for 24h or 100μCi of [^{32}P]P_i (2ng) for 10h at 22°C with constant shaking. At the end of the incubation period the tissue was sedimented by centrifugation and 0.1ml samples of the clear medium were prepared for counting of the trichloroacetic acid-insoluble radioactivity by the filter-paper-disc method of Mans & Novelli (1961).

Determination of the incorporation of radioactive precursors into vitellogenin in vivo *and* in vitro. Method A. The results shown in Table 1, Table 2 (Expts. 1 and 2) and Figs. 1 and 2 were obtained by using the following method. After incubation *in vitro* for 3h the liver tissue was removed by centrifugation and 50mg of carrier vitellogenin (prepared by the method of Ansari *et al.* 1971) was dissolved in the clear supernatant. The mixture was then cooled in an ice bath and diluted with chilled dimethylformamide (3.7ml/10ml of sample). The pH of the resulting solution was adjusted to 7.0–7.5 with 0.2M-acetic acid, and the precipitate that formed was collected by centrifugation and dissolved in 2ml of 0.3M-NaCl. This solution was dialysed against four changes of water for 12h at 5°C. The non-diffusible material was then freeze-dried, dissolved in Hyamine hydroxide and the radioactivity of a sample counted. When the incorporation *in vivo* of radioactive precursors into vitellogenin was investigated (Table 1), 50mg of carrier vitellogenin was added to 1ml samples of *Xenopus* serum. The vitellogenin was then reprecipitated, dialysed and counted for radioactivity, as described above.

Method B. The results shown in Table 2 (Expt. 3) and Figs. 4 and 6 were obtained using this method. At suitable times during the incubation 1ml samples of the medium were centrifuged to remove cell debris. Vitellogenin (10mg, or less in some cases) was added to the clear supernatant as carrier. The vitellogenin was then precipitated with dimethylformamide and redissolved in 0.3M-NaCl as in method A. The protein was reprecipitated by adding 2ml of 20% (w/v) trichloroacetic acid containing 0.1M non-radioactive precursor (i.e. 0.1M-leucine, or other amino acid, sodium acetate or NaH_2PO_4). The precipitate was collected by centrifugation and washed four times in all with 5% (w/v) trichloroacetic acid, including two times at a temperature of 90°C for 15min. Lipids were removed from the precipitate by washing it twice with 3ml of acetone and once with 3ml of ether. The protein was dissolved in Hyamine hydroxide and the radioactivity of a sample counted.

Method C. The results shown in Fig. 5 were obtained by using this method. This method was identical with method B except that the protein contained in the 1ml portions of the incubation medium was initially precipitated by the addition of 1ml of 20% (w/v) trichloroacetic acid containing 0.1M non-radioactive precursors and not with dimethylformamide. The protein thus obtained was then washed with 5% (w/v) trichloroacetic acid at 90°C as described in method A.

It should be noted that, when the incorporation of labelled amino acids into vitellogenin was studied by using the above techniques in which lipid-removing reagents were used, the actual incorporation of radioactivity measured was that into the lipid-free protein moiety and not into native vitellogenin, which contains about 12% (w/w) of lipid (Ansari *et al.* 1971). The pattern of incorporation observed was consistent in 20 experiments.

Incorporation of [2-^{14}C]*acetate into the lipid and protein moieties of vitellogenin.* Oestrogen-treated animals were killed 16 days after hormone treatment and 1g of the sliced liver was incubated in 12ml of buffer in the presence of 10μCi of [2-^{14}C]acetate. At 2h intervals 1ml samples of the incubation medium were removed and added to 3mg of carrier vitellogenin. The vitellogenin was then reprecipitated and washed by method B except that in this case lipids were removed from the precipitate with 3ml of ethanol–ether (1:1, v/v), 3ml of ethanol–acetone (1:1, v/v) and finally 3ml of ether (Hechter, Yoshinaga, Halkerston & Birchall, 1967). The extracts were pooled and their radioactivities counted as described below.

Incorporation of radioactive precursors into liver lipids. Samples of sliced liver (0.5g) taken from animals 15 days after oestrogen treatment and from control animals were incubated in 5ml of buffer in the presence of 10μCi of [2-^{14}C]acetate or 100μCi of [^{32}P]P_i. At the end of the incubation (0, 2, 4 or 6h) the medium was removed and the liver slices were blotted dry. Then two 200mg samples of liver from each flask were homogenized in 5ml of chloroform–methanol (2:1, v/v) and the lipids extracted by the method of Folch, Lees & Sloane-Stanley (1957).

Incorporation of labelled precursors into liver proteins. The incubation was performed as described above, the 5ml of incubation medium being supplemented with 10μCi of L-[4,5-^{3}H]leucine, 10μCi of [2-^{14}C]acetate or 100μCi of [^{32}P]P_i. At the end of the incubation the lipids were first extracted from the 200mg samples of liver by the method of Folch *et al.* (1957). The tissue protein was then rehomogenized in 5ml of 5% (w/v) trichloroacetic acid and centrifuged, and the supernatant was discarded.

Then 5ml of 5% (w/v) trichloroacetic acid containing 0.1M non-radioactive precursor was added and the mixture heated at 90°C for 15min. This treatment was repeated after centrifugation of the sample. Then 5ml of 100-volume (30%, w/v) hydrogen peroxide was added to the protein precipitate and the mixture heated at 60°C until the protein had turned white (15min). The solution was cooled and 5ml of 20% (w/v) trichloroacetic acid added. The precipitate was washed with trichloroacetic acid, twice with 10% (w/v) and twice with 5% (w/v), twice with acetone and once with ether. The protein was dissolved by adding 2ml of 1.0M-Hyamine hydroxide and the radioactivity of a sample was counted.

Cellulose acetate electrophoresis. This was performed as previously described (Ansari *et al.* 1971).

Polyacrylamide-gel electrophoresis. The 4% polyacrylamide gels were prepared and run as previously described (Ansari *et al.* 1971). Samples of the incubation media were centrifuged and sucrose was added to a final concentration of 10% (v/v) before electrophoresis. The gels were stained in 1% (w/v) Amido Black and electrophoretically destained in 7% (v/v) acetic acid. After scanning of the gels at 265nm in a Joyce–Loebl U.V. Polyfrac either bands I and II were removed and processed for radioactivity or the whole gel was frozen on solid CO_2 and cut into 2mm slices, then 0.5ml of NCS solubilizer was added to each 2mm slice and the mixture maintained at 65°C for 5h.

Measurement of radioactivity. (*a*) Proteins. Protein samples were dissolved in a known volume (1–2ml) of 1.0M-Hyamine hydroxide at 60°C. Samples of the solution were added to 8ml of scintillation fluid containing 0.8g of 5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole in 100ml of sulphur-free toluene. The solution was made acidic to minimize phosphorescence induced by the Hyamine–protein complex by adding 0.1ml of acetic acid (Herberg, 1958, 1960). NCS-solubilized proteins from sliced polyacrylamide gels were added to 8ml of the above scintillation fluid and maintained at 12°C in the dark for 12h to allow the leaching process to reach completion before counting of the radioactivity (Basch, 1968). The radioactivity of all samples was counted at 12°C as described below.

(*b*) Lipids. All lipid extracts were evaporated to dryness on a water bath and redissolved in a known volume of scintillation fluid and a sample of each was added to 8ml of scintillation fluid. The radioactivity of all samples was counted at 12°C in an Intertechnique ABAC SL40 liquid-scintillation spectrometer, programmed for computerized quench correction to less than 2% standard deviation.

Table 1. *Appearance of vitellogenin in the serum of* Xenopus laevis *as measured by the incorporation of* [^{3}H]*leucine*

The animals were injected with 10μCi of L-[4,5-^{3}H]-leucine at various times after hormone treatment. Then 12h after the injection of [^{3}H]leucine the animals were killed, their serum was obtained and vitellogenin was precipitated as described in the Materials and Methods section. Each value is the mean obtained from three animals.

Time after hormone treatment at which leucine was injected (h)	Radioactivity in vitellogenin (c.p.m./ml of serum)	
	Control	Oestrogen-treated
12	3000	8150
36	4500	30500
60	4000	60150
84	5500	66750
108	5000	70600

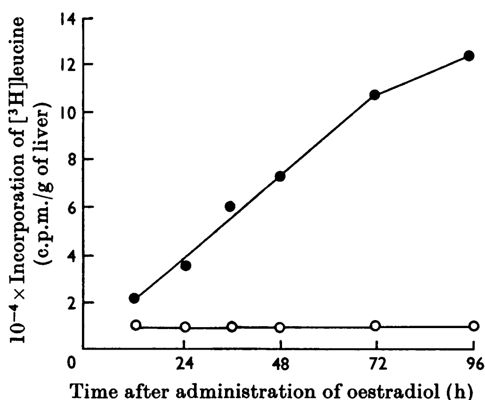


Fig. 1. Oestrogen-stimulated vitellogenin biosynthesis. Animals were killed at various times after receiving 1mg of oestradiol via the dorsal lymph sac. The sliced livers were incubated in 5ml of buffer containing 10μCi of L-[4,5-^{3}H]leucine for 3h. The incorporation of [^{3}H]leucine into vitellogenin was estimated by method A as described in the Materials and Methods section. ●, Oestrogen-treated; ○, control.

RESULTS AND DISCUSSION

Incorporation of L-[4,5-^{3}H]*leucine into vitellogenin* in vivo. Animals were injected intramuscularly with oestradiol. At 12, 36, 60, 84 and 108h after hormone treatment the animals were injected intraperitoneally with 50μg (10μCi) of [^{3}H]leucine. The animals were killed 12h after the latter injection and vitellogenin was precipitated from their serum by the dimethylformamide method. The specificity of this method for the precipitation of vitellogenin from the serum of oestrogen-treated *Xenopus* has been described by Ansari *et al.* (1971). The results in Table 1 show that incorporation of [^{3}H]leucine into vitellogenin occurred within 12h of hormone treatment.

Incorporation of L-[4,5-^{3}H]*leucine into vitellogenin* in vitro. The capacity of *Xenopus* liver to incorporate [^{3}H]leucine *in vitro* into the vitellogenin released into the incubation medium after hormone treatment was also studied. In a typical experiment a number of toads were injected with 1mg of

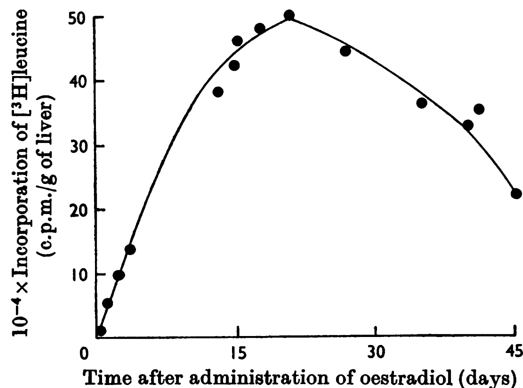


Fig. 2. Vitellogenin biosynthesis after oestrogen treatment. Animals were killed at various times after receiving a single 1 mg dose of oestradiol. The conditions of incubation and the protein precipitation method were as described in Fig. 1. The ordinate shows the total incorporation of [^{3}H]leucine into vitellogenin released into the incubation medium during the 3h incubation.

oestradiol via the dorsal lymph sac and killed at suitable times after injection. The livers were removed, and a 1g sample of the sliced liver was incubated at 22°C for 3h in 5ml of buffer containing 2μCi of L-[4,5-^{3}H]leucine/ml. The results in Fig. 1 show that a definite incorporation of [^{3}H]leucine had started 12h after the initial injection of oestradiol. Synthesis of vitellogenin by the liver increased linearly during 72h after hormone treatment. A systematic investigation of the rate of biosynthesis by livers from oestrogen-treated animals was not carried out beyond 96h; however, observations showed that the liver synthesized vitellogenin up to 2 months after the animal had been given a single 1mg dose of oestradiol. The maximum incorporation of [^{3}H]leucine was observed between days 21 and 24. After that time the incorporation of [^{3}H]leucine into vitellogenin decreased considerably (Fig. 2).

Specificity of the incorporation of radioactive precursors into vitellogenin in vitro. This was evaluated by using the more critical analytical techniques of cellulose acetate and polyacrylamide-gel electrophoresis. Thus 1g of liver taken from female toads 28 days after hormone treatment was incubated at 22°C in 5ml of buffer for 6h in the presence of 25μCi of [1-^{14}C]valine. The secreted vitellogenin synthesized during this time was precipitated from the incubation medium by the dimethylformamide method. The resulting precipitate was redissolved in 0.3M-sodium chloride and dialysed against 0.3M-sodium chloride at 4°C for 18h. A 5μl sample of the non-diffusible material containing 6600c.p.m. was subjected to cellulose acetate electrophoresis. After being stained with Ponceau S the strips were sliced into 1cm portions and their radioactivities counted. Nearly all (94%) of the applied radioactivity was associated with the vitellogenin band.

To investigate the pattern of incorporation of labelled amino acids into all the proteins secreted into the incubation medium by *Xenopus* liver *in vitro*, 1g of sliced liver taken from oestrogen-treated animals 18 days after hormone injection was incubated for 6h in the presence of 10μCi of L-[4,5-^{3}H]leucine as described above. A 30μl sample of the incubation medium (estimated to contain 6880c.p.m. of dimethylformamide-precipitable radioactivity) was analysed by polyacrylamide-gel electrophoresis. A total of 6515c.p.m. was localized in the vitellogenin band II. Thus the dimethylformamide-precipitable radioactivity (6880c.p.m.) in the incubation medium compares well with the radioactivity (6515c.p.m.) recovered from band II of vitellogenin on electrophoresis. There was no significant incorporation of [^{3}H]leucine into albumin and only a small amount into band I, which has been shown to be produced on storage of band II material (Ansari *et al.* 1971). Liver from control animals, when incubated under the above conditions for 12h instead of 6h, showed no significant incorporation of [^{3}H]leucine into the vitellogenin region, and an extremely low incorporation into albumin. These experiments therefore demonstrate, by using two electrophoretic techniques, one (cellulose acetate) based on the charge of the protein molecule and the other (polyacrylamide gel) on the charge as well as the molecular weight, that livers from oestrogen-treated animals incorporate radioactive amino acids *in vitro* almost exclusively into the vitellogenin band II region. The possibility that the observed incorporation into vitellogenin band II merely reflects the presence of a small contaminant of high specific radioactivity and not genuine vitellogenin biosynthesis has not been completely ruled out.

Other features of vitellogenin biosynthesis *in vitro* are shown in Table 2. The protein-synthesis inhibitor cycloheximide and the inhibitor of mitochondrial activity carbonyl cyanide *m*-chlorophenylhydrazone both inhibit the incorporation of [^{3}H]leucine into vitellogenin. Further, vitellogenin biosynthesis is stimulated by the presence of Ca^{2+} in the incubation medium, and is temperature-sensitive, being abolished at 37°C.

Incorporation of L-[4,5-^{3}H]*leucine and* [^{32}P]P_i *into vitellogenin in tissue culture.* The increasing incorporation of [^{3}H]leucine into vitellogenin with respect to time after hormone treatment (Figs. 1 and 2) could possibly have been a function of variations in the size of the intracellular amino acid pool rather than the increased capacity of the liver to biosynthesize vitellogenin. Some information

Table 2. *Effect of inhibitors, temperature and* Ca^{2+} *on the biosynthesis of vitellogenin*

In Expts. 1 and 2 samples (1g) of liver from oestrogen-treated animals were incubated in 5ml of buffer containing 10μCi of L-[4,5-^{3}H]leucine for 3h. The incorporation of radioactivity was estimated by method A as described in the Materials and Methods section. In Expt. 3 samples (1g) of liver were incubated in 8ml of buffer containing 20μCi of L-[4,5-^{3}H]leucine for 6h. Vitellogenin was then precipitated (method B) and its radioactivity counted. The additions and omissions in Expts. 1–3 are as indicated.

Expt.	Variable parameter	[^{3}H]Leucine incorporation (c.p.m./g of tissue)
(1) Addition of inhibitors	None	201700
	Cycloheximide (100μg/ml)	7570
	Carbonyl cyanide *m*-chlorophenylhydrazone (60μg/ml)	5800
(2) Incubation temperature	22°C	64000
	29°C	66500
	37°C	14800
(3) Variations in Ca^{2+} concentration	No Ca^{2+}	1015800
	0.1mg/ml (normal)	1320000
	2.5mg/ml	1500600

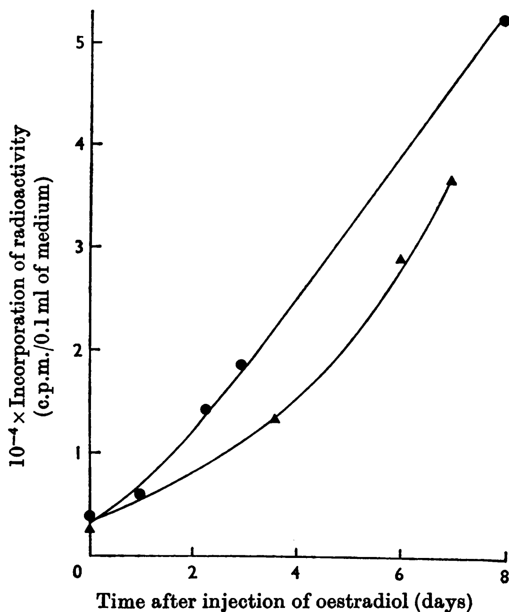


Fig. 3. Biosynthesis of vitellogenin by *Xenopus* liver cultures. The incubation conditions and technique of protein precipitation are described in the Materials and Methods section. ▲, [^{32}P]P_i incorporated into trichloroacetic acid-insoluble protein; ●, L-[^{3}H]leucine incorporated into trichloroacetic acid-insoluble protein.

about this is given by experiments studying the incorporation of labelled precursors into vitellogenin by the liver in tissue culture. The culture medium contained an excess of amino acids. Thus, provided that the size of the intracellular pool is kept constant by the excess of extracellular amino acids, variations of pool size may be prevented. Fig. 3 shows that when the livers from male toads killed at various times after hormone treatment were cultured in the presence of either [^{3}H]leucine or [^{32}P]P_i the pattern of incorporation observed was essentially similar to that shown in Fig. 1 for the livers incubated in the presence of tracer amounts of L-[4,5-^{3}H]leucine.

Time-course of the incorporation of labelled amino acids into vitellogenin in vitro. The above experiments demonstrate that livers obtained from animals 2–3 weeks after hormone treatment incorporate an impressive amount of radioactivity from labelled precursors *in vitro*, almost exclusively into vitellogenin, a Ca^{2+}-binding glycolipophosphoprotein. To study the biosynthesis of this multicomponent protein, liver slices from oestrogen-treated *Xenopus* were incubated with [^{3}H]leucine and the time-course of the incorporation of radioactivity into the vitellogenin released into the incubation medium was determined. Fig. 4 shows that a definite incorporation of L-[4,5-^{3}H]leucine, as measured by the co-precipitation of radioactivity with carrier vitellogenin, began after a lag period of 2h. No significant co-precipitation of radioactivity with vitellogenin occurred in the parallel incubation with livers from non-oestrogen-treated control animals. These biosynthetic experiments were also performed by using the more reliable, though laborious, technique of polyacrylamide-gel electrophoresis. Liver slices from oestrogen-treated animals were incubated with [^{3}H]leucine and 30μl samples of the incubation medium were removed at various time-intervals and subjected to electrophoresis (Plate 1). The gels were stained and

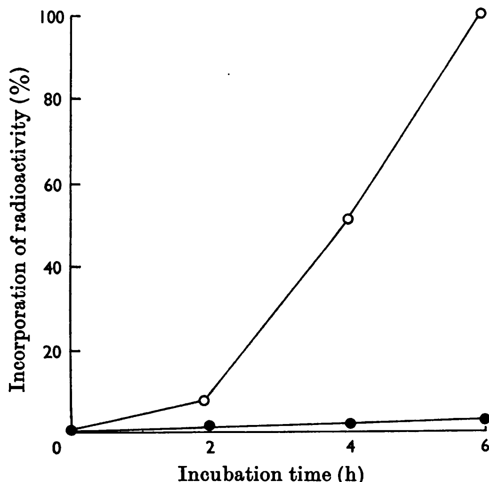


Fig. 4. Incorporation of L-[4,5-^{3}H]leucine into vitellogenin released into the incubation medium. Sliced liver (1 g) taken from animals 15 days after hormone treatment was incubated in 12 ml of buffer containing 1 μCi of L-[4,5-^{3}H]leucine. At 2 h intervals 1 ml portions of the incubation medium were removed and, after the addition of 10 mg of carrier vitellogenin, were processed by method B as described in the Materials and Methods section. The total incorporation after 6 h of incubation is taken to be 100% and was 1×10^4 c.p.m./g of liver. ○, Liver from oestrogen-treated animals; ●, liver from control animals.

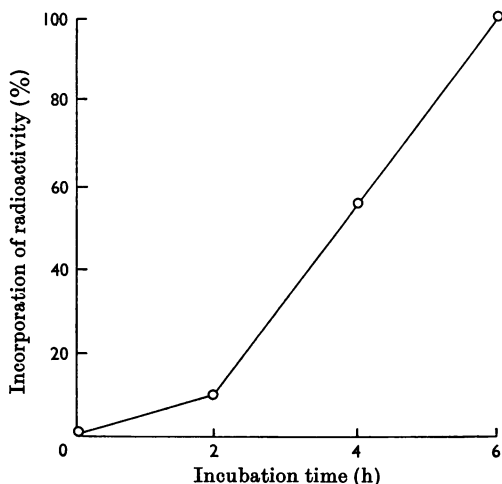
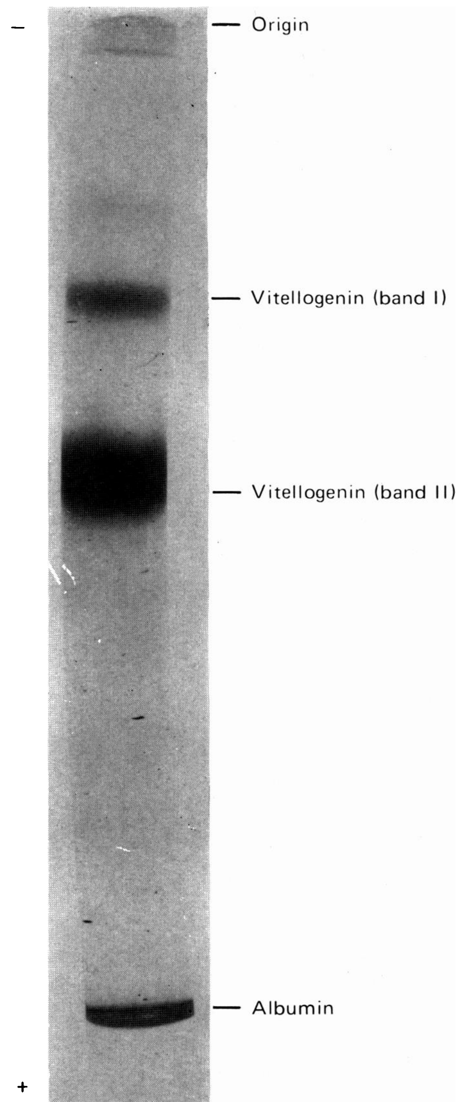


Fig. 5. Incorporation of various amino acids into vitellogenin released into the incubation medium as measured by trichloroacetic acid precipitation (method C). The conditions of incubation were as described in Fig. 4 except that the medium was supplemented with one of the following: 10 μCi of L-[2,5-^{3}H]histidine; 10 μCi of L-[2,5-^{3}H]phenylalanine; 10 μCi of [*Me*-^{14}C]methionine; or 10 μCi of L-[U-^{14}C]glycine. The total incorporation after 6 h of incubation is taken to be 100% and was 7.8×10^5, 1.14×10^6, 1.16×10^6 and 4.2×10^5 c.p.m./g of liver respectively. The percentage incorporations of radioactivity into vitellogenin from all the amino acids were identical.

scanned to assess the amount of protein present in vitellogenin band II and in the accompanying minor band I. (Band I is produced on storage of band II material and is thought to be a polymeric product of vitellogenin.) The sections of the gels containing band I and band II materials were then removed and treated with NCS solubilizer in order to determine the radioactivity associated with each band. The pattern of incorporation of radioactivity into vitellogenin band II as determined by polyacrylamide-gel electrophoresis was identical with that obtained with the dimethylformamide precipitation method. Further, the incorporation of radioactivity into band I with time paralleled that observed for vitellogenin band II, giving a lag phase of about 2 h, thus supporting the view that band I material is produced from band II material. It does not, however, establish the point beyond doubt. In less critical experiments the biosynthesis of vitellogenin was studied by incubating liver slices from oestrogen-treated animals with L-[4,5-^{3}H]leucine and precipitating the radioactivity from the incubation medium with trichloroacetic acid. The total incorporation of radioactivity into the trichloroacetic acid-precipitable proteins was found to be identical with that obtained by using the specific co-precipitation method with dimethylformamide. These results confirm the conclusion that the main protein released into the incubation medium by liver slices from oestrogen-treated animals is vitellogenin. The incorporation of other labelled amino acids into vitellogenin was also studied, by using trichloroacetic acid to precipitate the protein from portions of the incubation medium. In all cases the pattern of incorporation was identical with that observed with L-[4,5-^{3}H]-leucine (Fig. 5).

The secretory lag phase (Fig. 5) is particularly noteworthy and was studied in further detail. A similar lag phase was obtained when the [^{3}H]-leucine was added at 0, 2 or 4 h after the start of the incubation. The lag phase for the incorporation of labelled amino acids into vitellogenin contrasts with the linear incorporation of radioactivity into total liver tissue protein. The release of total proteins or vitellogenin (band II) into the incubation medium as estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) and by integration of the area under band II after scanning of the stained polyacrylamide gel was also found to be linear.

These results therefore suggest that the lag phase observed in the incorporation of ^{3}H-labelled amino



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of a 30μl portion of the incubation medium after 6h of incubation. The incubation consisted of 1g of sliced liver taken from toads 18 days after hormone treatment, 5ml of buffer and 10μCi of L-[4,5-^{3}H]leucine.

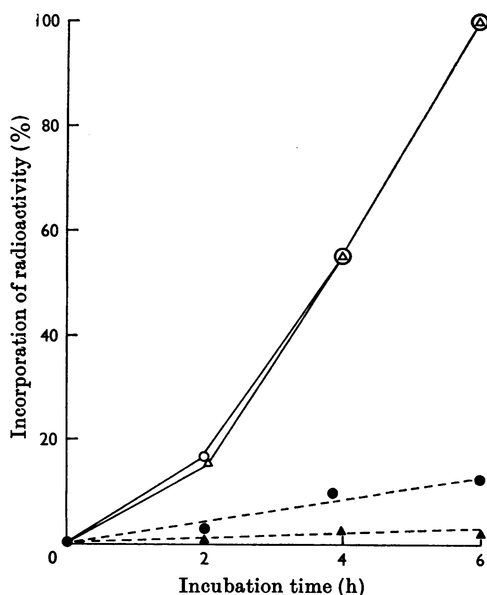


Fig. 6. Incorporation of [^{32}P]P_i and L-[4,5-^{3}H]leucine into the protein moiety of vitellogenin. Sliced liver (1g) taken from animals 18 days after hormone treatment was incubated in 12ml of buffer containing 100μCi of [^{32}P]P_i and 10μCi of L-[4,5-^{3}H]leucine. Samples (1ml) of the incubation medium were removed at 2h intervals and after the addition of 3mg of carrier vitellogenin the radioactivity was estimated by method B as described in the Materials and Methods section. The total incorporation into vitellogenin after 6h is taken to be 100% and was 7.95×10^5 and 1.92×10^5 c.p.m./g of oestrogen-treated liver for leucine and phosphate respectively. Leucine: ○, oestrogen-treated; ●, control. Phosphate: △, oestrogen-treated; ▲, control.

acids into vitellogenin may represent the total time required for the biosynthesis and release of this protein. Though this secretory lag may appear long it may be reasonable if one considers the multiplicity of biochemical processes that may be involved in the biosynthesis of this complex protein.

Time-course of the incorporation of [^{32}P]P_i *and* [2-^{14}C]*acetate into vitellogenin* in vitro. The biosynthesis of vitellogenin was also studied by using either [^{32}P]P_i or [2-^{14}C]acetate and once again a lag phase similar to that noted above for the incorporation of L-[4,5-^{3}H]leucine into vitellogenin was observed. Fig. 6 compares the lag for [^{32}P]P_i and [4,5-^{3}H]leucine incorporation. The pattern for [2-^{14}C]acetate incorporation was identical and is not shown. In theory acetate may be incorporated into either the protein or the lipid moiety of vitellogenin. Lipid was removed from labelled vitellogenin biosynthesized from [2-^{14}C]-acetate and the time-courses of the incorporation of acetate into the lipid and the protein moiety were studied separately. The incorporation of [2-^{14}C]acetate into the lipid as well as into the protein moiety of vitellogenin is accompanied by a lag phase similar to that observed for L-[4,5-^{3}H]-leucine, but there is no such lag in the incorporation of [^{32}P]P_i or [2-^{14}C]acetate into either liver lipids or total tissue proteins. It may therefore be concluded that the attachment of phosphate and lipids to vitellogenin occurs at an early stage in its biosynthesis and about 2h before its release into the incubation medium.

CONCLUSIONS

It is shown that the major protein component synthesized *in vitro* and secreted into the medium by livers of *Xenopus* pretreated *in vivo* with oestradiol is vitellogenin. The capacity of oestrogen-treated *Xenopus* liver to incorporate radioactive precursors into vitellogenin increases markedly with time after injection of the hormone. Related studies on the incorporation of labelled precursors into proteins induced by oestrogen in chicken (Heald & McLachlan, 1965), *Xenopus laevis* (Wallace & Dumont, 1968), trout (Mano, 1970) and cod (Plack, Pritchard & Fraser, 1971) have been reported. The results in the present study are in qualitative agreement with these reports, but a strict comparison of the results and conclusions from the various laboratories is prevented by the specialized approach adopted by each group.

The biosynthetic experiments show that the incorporation of labelled amino acids, [^{32}P]P_i and [2-^{14}C]acetate into vitellogenin is associated with a lag phase of about 2h. The results suggest that these precursors are incorporated at an early stage of vitellogenin biosynthesis and that the release of this protein into the incubation medium occurs about 2h after the initiation of its biosynthesis. However, the role of the intracellular pools of amino acids, P_i and acetate in contributing to the lag phase may be of significance. This problem requires further investigation.

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