Oestrogen-Induced Cholesterol and Fatty Acid Biosynthesis in *Xenopus laevis* Liver during Vitellogenic Response

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1. Oestradiol-17 β induces livers of *Xenopus laevis* (South African clawed toad) to synthesize and secrete into the serum large quantities of the egg-yolk-protein precursor, vitellogenin. The peak of this response occurs 9–16 days after hormone treatment [Dolphin, Ansari, Lazier, Munday & Akhtar (1971) *Biochem. J.* **124**, 751–758]. It is now shown that 6 days after hormone treatment a 120–160-fold stimulation of the synthesis of cholesterol and fatty acid compared with control values occurred. 2. A cell-free system, derived from *Xenopus* liver, which synthesizes squalene and fatty acid is described. By using this system, several hundredfold stimulation of incorporation of [^{14}C]acetate into squalene was recorded 6 days after the administration of oestradiol-17 β , compared with a 3–4-fold stimulation of incorporation of [^3H]mevalonate compared with control values. It is argued that oestradiol-17 β must affect enzyme(s) catalysing step(s) between acetate and mevalonate in the biosynthetic pathway to cholesterol. 3. In incubation of liver slices *in vitro*, most of the lipid and cholesterol synthesized in response to the steroid hormone was associated with those subcellular fractions that contained membranes. Moreover, pulse-labelling experiments *in vivo* showed that 70% of this lipid and cholesterol was retained in the liver. The remainder appeared in the serum, where it was equally distributed between vitellogenin and vitellogenin-free serum. 4. G.l.c. analyses of the cholesterol content of liver microsomal fractions of *Xenopus laevis* indicated that the cholesterol content was at least 50% higher in microsomal fractions obtained from livers that had been exposed to oestradiol-17 β . Meanwhile, g.l.c. analysis of the lipid moiety of secreted vitellogenin showed that up to 35% of its lipid was cholesterol.

Oestradiol-17 β induces the liver of *Xenopus laevis* (South African clawed toad) to biosynthesize and secrete into the serum the egg-yolk-protein precursor vitellogenin (Follett & Redshaw, 1968; Wallace & Dumont, 1968; Munday *et al.*, 1968), which has a mol.wt. of 450000–500000 (Ansari *et al.*, 1971; Redshaw & Follett, 1971). Although the protein can be detected in the serum as early as 12 h after hormone treatment, maximal vitellogenin synthesis occurs much later, at 9–16 days after oestrogen injection (Dolphin *et al.*, 1971; Clemens, 1974). At the peak of the response, at least 40–60% of the total protein made in the liver is destined to become vitellogenin (Merry *et al.*, 1973a; Clemens *et al.*, 1975; Penning *et al.*, 1977). Since this protein is both a glycolipophosphoprotein (Ansari *et al.*, 1971; Redshaw & Follett, 1971) and is secreted, it is envisaged that the processing and transport of this molecule through the cell would require the production of a well-developed endoplasmic reticulum. It is thus of considerable importance to see if the lag that exists between the first detection of serum vitellogenin and the maximal production of this protein can be explained by a requirement for membrane synthesis.

In the light of these considerations we have now studied the oestrogen-induced biosynthesis in *Xenopus laevis* liver of two essential membrane components, fatty acids and cholesterol, and have shown that the maximum stimulation of the pathways for their biosynthesis occurs before the maximal production of vitellogenin.

Materials and Methods

Radiochemicals

[^3H]Acetate (sp. radioactivity 500 mCi/mmol), [U- ^{14}C]acetate (sp. radioactivity 59 mCi/mmol) and DL-[2- ^3H]mevalonic acid lactone (sp. radioactivity 382 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [^3H]Mevalonate was prepared immediately before use by alkaline hydrolysis of the lactone.

Special chemicals

β -Amyrin was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Animals

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

Injections

Experimental animals were injected via the dorsal lymph sac with 1mg of oestradiol-17 β in 0.2ml of olive oil. Control animals received olive oil only. In pulse-labelling experiments *in vivo*, both experimental and control animals received injections of 100 μ Ci of [³H]acetate (sp. radioactivity 500mCi/mmol) in 0.2ml of water, via the dorsal lymph sac.

Buffers

Liver-slice and liver-mince incubations *in vitro* were performed in a phosphate/saline buffer, pH 7.3 (Dulbecco & Vogt, 1954), containing 4.0g of NaCl, 0.2g of KCl, 1.15g of Na₂HPO₄ (anhydrous), 0.2g of KH₂PO₄ (anhydrous), 0.1g of MgCl₂·6H₂O and 0.1g of CaCl₂ in 1 litre of water. Sodium pyruvate (20mM) was added as an energy source.

Tissue homogenization and subcellular fractionations were performed in 0.25M-sucrose in 0.05M-Tris buffer, adjusted to pH 7.5 with HCl and containing 25mM-KCl and 5mM-MgCl₂ (TKMS buffer). Homogenization and fractionation of livers for the production of cell-free fractions were performed in 0.1M-potassium phosphate buffer, pH 7.4, containing 30mM-nicotinamide and 4mM-MgCl₂.

Incubation of liver slices and liver mince

Animals were pithed, the livers removed and washed twice in phosphate/saline buffer (10ml/g of liver) and then either cut into approx. 2–3mm² slices (for the measurement of incorporation of [³H]-leucine into protein and [³H,¹⁴C]acetate into lipid products) or finely minced on filter paper (for the measurement of incorporation of [³H]mevalonate and [¹⁴C]acetate into lipid products).

Portions of the liver were weighed into 25ml Erlenmeyer flasks to give a tissue/buffer ratio of 1g/10ml. After addition of a known amount of radioactivity, incubation was carried out at 22°C with constant shaking.

Subcellular fractionation of liver slices

After incubation, liver slices (0.5g) were washed twice in 10vol. of TKMS buffer and then homogenized and fractionated to give material sedimenting at 4000g, a microsomal fraction and a post-microsomal supernatant, as described by Penning *et al.* (1977).

Cell-free system for the biosynthesis of fatty acids and squalene

Livers from oestrogen-treated and control animals were removed, weighed and samples (4g) finely minced, before transfer to the tube of a glass/Teflon homogenizer, containing 10ml of fresh TKMS buffer. The mince was homogenized by using two full strokes only of a loose-fitting pestle (1mm clearance) rotated by a Tri-R homogenizer (Tri-R Instruments, Jamaica 35, NY, U.S.A.) at speed setting 3.5–4. The homogenate was then centrifuged at 4000g for 15min and the supernatant (S₄) filtered through cotton wool.

Portions (1ml) of supernatant S₄ were incubated for 1h at 22°C, with constant shaking, with the following additions: 3mM-ATP, 5mM-GTP, 0.5mM-NADP⁺, 10mM-KHCO₃, 0.7mM-glutathione (reduced), 2.5 μ Ci of [³H]mevalonate and 5 μ Ci of [¹⁴C]acetate.

Measurement of *Xenopus* plasma volume

Xenopus plasma volume was measured via a dye-dilution method with Evans Blue (Jones, 1970), as described by Penning (1976). By this technique, it was found that the plasma volume for female *Xenopus* weighing 100g was 4.19 $\pm$ 0.85 (mean $\pm$ S.D.; *n* = 12).

Preparation of serum vitellogenin

Vitellogenin was precipitated from the serum by the dimethylformamide technique as described by Ansari *et al.* (1971).

Determination of microsomal protein content

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Determination of the incorporation of radioactivity into secreted protein

This was done by the trichloroacetic acid-precipitation procedure, as described by Penning *et al.* (1977).

Determination of the incorporation of radioactive precursors into total lipids

The incorporation of radioactivity into (1) total lipids, by liver slices derived from labelling experiments both *in vivo* and *in vitro* together with their cellular fractions, and (2) the lipid moiety of vitellogenin, was determined by the method of chloroform/methanol extraction (Folch *et al.*, 1957).

All samples were homogenized in 5ml of chloroform/methanol (2:1, v/v), the non-aqueous layer was removed and the aqueous fraction re-extracted with 2 $\times$ 2ml of the same solvent system. The pooled non-aqueous extracts (9ml) were washed with 2.25ml of 0.58% NaCl and 0.2ml of lower phase, and the resulting upper layer was washed by the addition of 3 $\times$ 1ml

of upper phase [upper and lower phases were prepared from a mixture of chloroform/methanol/0.58% NaCl (8:4:3, by vol.)]. The washed extracts were then evaporated to dryness and radioactivity incorporation was measured.

Determination of the incorporation of radioactive precursors into cholesterol and squalene

The incorporation of radioactivity into cholesterol was determined, in all cases, by the extraction of non-saponifiable lipids followed by the formation of cholesterol dibromides.

Samples of 10% (w/v) liver-slice homogenates or their subcellular fractions, cell-free liver fractions, serum vitellogenin or vitellogenin-free serum were saponified with an equal volume of 10% (w/v) KOH in methanol. After cooling, non-saponifiable lipids were extracted by 2×5ml of light petroleum (b.p. 40–60°C). Methanol (1 ml) was added to the remaining aqueous layer, which was then further extracted by 2×5ml of light petroleum. The pooled extracts were used for the preparation of cholesterol dibromides.

Carrier cholesterol (100mg) was added to non-saponifiable lipid extracts, which were subsequently evaporated to dryness under vacuum and the resulting precipitate was redissolved in 1.5ml of diethyl ether. Cholesterol dibromides were then prepared by the method of Wilton *et al.* (1968), recrystallized at least twice, weighed and radioactivity incorporation was measured. Correction was made for 100% yield (d.p.m. per mg of the dibromide × theoretical yield of the dibromide).

The incorporation of radioactivity into squalene by cell-free liver fractions was determined by the extraction of non-saponifiable lipids, as described previously, followed by the formation of squalene hexachlorides.

Non-saponifiable-lipid extracts were evaporated to dryness under vacuum, 3ml of acetone was added and the flasks were cooled on solid CO₂. After saturation with HCl gas, 100mg (0.12ml) of carrier squalene was added. Formation of the hexachloride as a whitish precipitate occurred within 15–30min, and this precipitate was then filtered and washed with ice-cold methanol. At least two recrystallizations from hot acetone were made before weighing and measurement of radioactivity incorporation. Correction for a 100% yield of hexachloride was made (d.p.m. per mg of the hexachloride × theoretical yield of the hexachloride).

Determination of the incorporation of radioactive precursors into non-esterified fatty acids

The aqueous layer remaining after extraction of non-saponifiable lipids (see the previous section) was

acidified by addition of 10M-HCl and further extracted with 4×5ml of light petroleum (b.p. 40–60°C). Pooled extracts were evaporated under vacuum and the residue was redissolved in 10ml of diethyl ether. Samples (5ml) of this fraction were evaporated to dryness and radioactivity incorporation was measured.

Determination by g.l.c. of the amount of cholesterol in vitellogenin and in Xenopus laevis liver microsomal fractions

Samples of serum vitellogenin or liver microsomal fraction were saponified in the presence of β -amyirin, as internal standard, and extracted for non-saponifiable lipids, which were evaporated to dryness and redissolved in ethyl acetate. These fractions were analysed by a Pye series 104 g.l.c. instrument, set at 290–300°C, linked to an Infotronics digital integrator. The analytical system was precalibrated with standard cholesterol and β -amyirin solutions, and cholesterol concentration in test samples was corrected for 100% recovery of β -amyirin. The retention times for cholesterol and β -amyirin were 3.44 and 5.57min respectively.

Measurement of radioactivity

All samples were dissolved in 10ml of toluene containing 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole, and the radioactivity was counted at 12°C with a Philips liquid-scintillation counter. Automatic correction was made for quenching from quench curves constructed with chloroform/methanol (2:1, v/v) as quenching agent [accuracy of correction was $\pm 5\%$ (s.d.)].

All results were calculated as d.p.m. by reference to standards, which were counted for radioactivity at an efficiency of 58% for ³H and 98% for ¹⁴C.

Results

Time-course study of the incorporation of labelled precursors into Xenopus liver cholesterol, fatty acids and secreted protein after administration of oestradiol-17 β

When livers were removed from *Xenopus laevis* at different time intervals after the administration of oestradiol-17 β and liver slices incubated *in vitro* with [¹⁴C]acetate, cholesterol and fatty acid synthesis increased with time (Fig. 1). At about 6 days after hormone treatment there was maximal synthesis of both lipid products, and this represented a 120–160-fold increase in the production of these compounds

Fig. 1. Incorporation of $[^3\text{H}]$ leucine and $[^{14}\text{C}]$ acetate *in vitro* into vitellogenin, cholesterol and fatty acids in *Xenopus* liver slices after the administration of oestradiol- 17β *in vivo*

Groups of three experimental animals were killed at various time intervals after the injection of oestrogen. Their livers were removed, sliced and pooled for duplicate incubation. Control experiments were performed identically except that liver slices from a group of three untreated animals were used. Liver slices were incubated with $5\mu\text{Ci}$ of $[^{14}\text{C}]$ acetate and $2.5\mu\text{Ci}$ of $[^3\text{H}]$ leucine for 4 or 6 h, for incorporation into lipid and protein respectively. At the end of the incubation period, liver slices were homogenized and the radioactivity incorporation into cholesterol ($\circ$) and fatty acids ($\bullet$) was determined. Radioactivity incorporation into secreted vitellogenin ($\blacksquare$) was determined in the incubation medium. Detailed methodology is described in the Materials and Methods section.

over control values. Simultaneous measurements of the incorporation of $[^3\text{H}]$ leucine into vitellogenin showed that maximum synthesis of this protein occurred at about 8–10 days after hormone treatment and was maintained at this high rate up until at least 15 days, whereas fatty acid and cholesterol biosynthesis attained a maximum somewhat earlier and then rapidly declined during the period when vitellogenin synthesis was still high. The heavy expense involved, in terms of the number of animals required for such experiments, precluded repeating the time course in which all the three parameters were measured in the same incubation. It should, however, be noted that the temporal relationships mentioned earlier have been confirmed in experiments in which the parameters were individually measured. Thus the time course of vitellogenin biosynthesis emerging

from Fig. 1 is similar to that obtained on several occasions in our laboratory (for example, Ansari, 1970) and reported by Shapiro *et al.* (1976). The incorporation pattern into lipid has been confirmed by the experiments described in Fig. 2.

The present findings extend our original observations (Dolphin *et al.*, 1972; Merry *et al.*, 1973b) and show that the oestrogen-induced increase in lipid biosynthesis previously reported not merely reflects a rise in cholesterol production but is due in part to an increase in fatty acid biosynthesis. In addition, the results recorded in Fig. 1 show that the stimulation of cholesterol and fatty acid synthesis is coincident. The possibility that the enhanced incorporation of $[^{14}\text{C}]$ acetate into cholesterol in oestrogen-treated liver is due to a diminution of the acetate-pool size is made less likely by previous experiments (Dolphin *et al.*, 1972) showing that the incorporation of radioactivity from $^3\text{H}_2\text{O}$ into cholesterol was also dramatically stimulated in the livers of oestrogen-treated animals compared with that of control animals. Further support for the hypothesis that the enhanced incorporation of labelled acetate into lipids, as shown by the experiments of Fig. 1, reflect a genuine increase in the activity of the biosynthetic pathways is provided by the cell-free studies described in the next section. Despite these supporting experiments, the possibility cannot be ignored that the intra-cellular specific activities of both acetate and leucine may be affected by oestrogen.

Development of Xenopus liver cell-free fraction for the biosynthesis of squalene: indirect evidence that oestradiol- 17β affects the activity of 3-hydroxy-3-methylglutaryl-CoA reductase

To investigate the mechanism by which oestradiol- 17β affects cholesterol synthesis in *Xenopus* liver, it was necessary to conduct radioactivity-incorporation experiments by using intermediates from the cholesterol-biosynthetic pathway. One of the intermediates of choice was mevalonate, but the extent of the permeability of the cell membrane to this compound is not known and so a prerequisite for experiments was the development of a cell-free system for the biosynthesis of cholesterol. However, in initial attempts, supernatants obtained from *Xenopus* liver homogenates did not incorporate $[^{14}\text{C}]$ acetate into sterols. This failure was attributed to the relatively severe homogenization technique used to break up the 'tough' liver of *Xenopus laevis*, compared with the much gentler procedure needed to produce a homogenate from rat liver capable of the synthesis *de novo* of cholesterol. Maintenance of an intact membrane system is essential for the complete synthesis of sterols. By the milder homogenization and centrifugation procedures described in the present paper, a cell-free fraction was obtained

Table 1. Comparison of the sterol biosynthetic ability of cellular and cell-free fractions derived from oestrogen-treated *Xenopus* liver

Groups of three animals were killed 5 days after oestrogen treatment and their livers pooled and used in the preparation of both a cellular system (liver mince) and a cell-free system. Control experiments were performed identically except that livers from a group of three untreated animals were used. The incorporation of $5\mu\text{Ci}$ of $[^{14}\text{C}]$ acetate and of $2.5\mu\text{Ci}$ of $[^3\text{H}]$ mevalonate into non-saponifiable lipids, cholesterol and squalene was then determined, as described in the Materials and Methods section. Results are expressed as means $\pm$ s.e.m. for five independent determinations. For comparison it should be noted that the incorporation of acetate into cholesterol in a rat liver cellular preparation, incubated at 37°C , is $1.74\text{--}3.60 \times 10^5$ d.p.m./h per g of liver.

	Type of preparation	Specific radioactivity (d.p.m./h per g of liver)	
		Control	Oestrogen-treated
Acetate $\rightarrow$ total non-saponifiable lipids	Cellular	$4.70 \pm 1.52 \times 10^3$	$7.80 \pm 0.38 \times 10^5$
Acetate $\rightarrow$ cholesterol	Cellular	$1.00 \pm 0.73 \times 10^3$	$5.20 \pm 0.55 \times 10^5$
Mevalonate $\rightarrow$ total non-saponifiable lipids	Cellular	$2.40 \pm 0.70 \times 10^4$	$6.40 \pm 0.72 \times 10^4$
Mevalonate $\rightarrow$ cholesterol	Cellular	$7.91 \pm 0.01 \times 10^3$	$2.65 \pm 0.42 \times 10^4$
Acetate $\rightarrow$ total non-saponifiable lipids	Cell-free	$9.10 \pm 1.64 \times 10^3$	$5.47 \pm 0.49 \times 10^5$
Acetate $\rightarrow$ squalene	Cell-free	$1.30 \pm 0.60 \times 10^2$	$1.87 \pm 0.28 \times 10^5$
Mevalonate $\rightarrow$ total non-saponifiable lipids	Cell-free	$6.79 \pm 0.05 \times 10^5$	$1.06 \pm 0.03 \times 10^6$
Mevalonate $\rightarrow$ squalene	Cell-free	$1.99 \pm 0.02 \times 10^5$	$6.40 \pm 0.28 \times 10^5$

that could incorporate radioactive acetate into non-saponifiable lipids in amounts comparable with that incorporated in a liver whole-cell fraction (Table 1). The main radioactive compound biosynthesized in the cell-free system was identified as squalene both by g.l.c. (Smith, 1977) and by measurement of the radioactive incorporation into the hexachloride derivative. Table 1 shows typical incorporation obtained for the synthesis of non-saponifiable lipids, cholesterol and squalene in the cell-free fraction, and compares this with the incorporation measurable in a *Xenopus* liver cellular preparation.

Since squalene is an intermediate in the synthetic pathway to cholesterol, radioactivity incorporation into this compound was taken as a valid measurement of sterol biosynthesis in the cell-free system. Indeed, whole-cell cholesterol biosynthesis and cell-free squalene synthesis were shown to be maximally stimulated at the same time, i.e. 6 days after hormone treatment (Fig. 2). It is noteworthy that the incorporation data recorded in Fig. 2 (and Table 1) were obtained, at each time point, from cell-free and cellular fractions prepared from the same liver. The pattern of incorporation of precursors into squalene by the cell-free system was similar to that into cholesterol in the liver mince. The striking feature of these experiments was that in both types of preparation it was the incorporation of acetate that was dramatically stimulated by oestrogen treatment, the stimulation being several hundredfold in both systems. Since the incorporation of radioactivity from labelled acetate into cholesterol was barely detectable in the livers from control animals, even a small error in the numerical values for the control incorporation made up to 4-fold differences in calculating the extent of stimulation produced by oestrogen treatment. The

Fig. 2. Incorporation *in vitro* of $[^{14}\text{C}]$ acetate and $[^3\text{H}]$ mevalonate into cholesterol in a *Xenopus* liver cellular fraction, and into squalene in a *Xenopus* liver cell-free fraction

Groups of three experimental animals were killed at various time intervals after injection of oestrogen. Their livers were removed and pooled. Control experiments were performed identically except that livers from a group of three untreated animals were used. Each pool was divided into two equal portions, for the preparation of a cell-free fraction and a cellular fraction (liver mince). Both fractions were incubated independently with $5\mu\text{Ci}$ of $[^{14}\text{C}]$ acetate and $2.5\mu\text{Ci}$ of $[^3\text{H}]$ mevalonate and the incorporations into squalene ($\square$, $[^{14}\text{C}]$ acetate; $\blacksquare$, $[^3\text{H}]$ mevalonate) in the cell-free fraction, and cholesterol ($\circ$, $[^{14}\text{C}]$ acetate; $\bullet$, $[^3\text{H}]$ mevalonate) in the cellular fraction, determined as described in the Materials and Methods section.

values for stimulation emerging from the data of Table 1 and Figs. 1 and 2 therefore should not be taken literally; they merely dramatize the magnitude of the oestrogen response.

As expected, the incorporation of radioactivity from $[^3\text{H}]$ mevalonate into squalene was very much

higher in the cell-free fraction than that of this precursor into cholesterol in the mince system, but the stimulation of incorporation by oestrogen was only about 3-fold. In view of the poor utilization of mevalonate for isoprenoid biosynthesis in the mince system, the data on the cellular biosynthesis from this precursor cannot be interpreted with confidence, although the results from the cell-free study strongly suggest that oestradiol-17 β affects at least one of the enzymes catalysing steps between acetate and mevalonate. However, the activities of the enzymes responsible for the conversion of mevalonate into squalene are probably similar in both experimental and control animals. It is suggested that one of the enzymes stimulated may well be 3-hydroxy-3-methylglutaryl-CoA reductase, which is a well-defined control point in rat liver cholesterol biosynthesis (Higgins & Rudney, 1973; Rodwell *et al.*, 1973).

Fatty acid synthesis in the Xenopus cell-free system in vitro

In view of the successful development of a squalene-synthesizing cell-free system from *Xenopus* liver, it was also decided to measure fatty acid synthesis in this system, for comparison with the observed increase in fatty acid synthesis in *Xenopus* liver slices.

To measure the incorporation of [^{14}C]acetate into fatty acids, the cell-free incubations used for the determination of incorporation into non-saponifiable lipids (Fig. 2) were further extracted under acidic conditions, as described in the Materials and Methods section. The time-course data obtained from these measurements showed that the cell-free system was relatively efficient in the synthesis of fatty acids from acetate, compared with its inability to synthesize cholesterol. Maximum incorporation of [^{14}C]acetate was recorded at 7 days after oestrogen treatment, 1 day later than that recorded in Fig. 1 for the cellular biosynthesis (but this comparison is being made between two sets of experiments carried out on different occasions). Stimulation over control incorporation at this stage was in excess of 200-fold. The control incorporation in the cell-free system was

424–1169 d.p.m./h per g of liver compared with 1680–2560 d.p.m./h per g in the liver slices, and the incorporations into oestrogen-treated liver samples at the maximum stimulation were about 2.5×10^5 and 7×10^5 d.p.m./h per g of liver for the cell-free and cellular preparations respectively.

Subcellular distribution of total lipid and cholesterol in Xenopus laevis liver

Initial studies were performed to determine whether the biosyntheses of cholesterol and lipid induced by oestrogen were associated with the synthesis of new membranes. The subcellular distribution of the radioactive cholesterol and lipid synthesized *in vitro* by liver slices from oestrogen-treated animals was measured. The results of these experiments are shown in Table 2. Incorporation data into homogenates from liver slices clearly show that the livers were maximally induced to produce lipid and cholesterol, since the stimulation of [^3H]acetate incorporation into total lipid was 52-fold whereas the stimulation of [^3H]acetate into cholesterol was 250-fold over control values. Of more interest, however, was that there was a preponderance of the newly synthesized lipid and cholesterol in those subcellular fractions that contained membranes, i.e. the 4000g pellet and microsomal fraction. Although these results could be taken as evidence that the oestrogen-induced biosynthesis of lipid and cholesterol was required for the synthesis of new membranes, it had to be considered that the increase in labelled cholesterol and lipid in these subcellular fractions could merely reflect an increase in the synthesis of precursor lipids for the lipid moiety of vitellogenin.

Destiny of oestrogen-induced lipid and cholesterol

To evaluate the proportion of the newly synthesized cholesterol and lipid that was retained within the liver and was non-mobile, experiments *in vivo* were conducted in which *Xenopus* that had received

Table 2. Subcellular distribution of cholesterol synthesized in response to oestradiol-17 β in *Xenopus laevis* liver
Liver slices from oestrogen-treated and control animals were incubated with 50 μCi of [^3H]acetate for 4 h, and the amount of radioactivity incorporated into cholesterol determined as described in the Materials and Methods section. In another experiment with animals that had been treated with hormone 8 days previously, the distribution of the total lipid radioactivity in the subcellular fractions was identical with that for cholesterol below.

Treatment	Stimulation over control (fold)	Incorporation (d.p.m./4 h per g of liver)			
		Total homogenate	4000g pellet	105 000g pellet	105 000g supernatant
Control	—	2.8×10^3	1.7×10^3	5.2×10^2	3.7×10^2
4 days after hormone treatment	250	7.3×10^5	5.3×10^5	1.6×10^5	2.9×10^4

an injection of oestradiol-17 β 4 days earlier were treated with [3 H]acetate and the radioactivity incorporated into total lipid and cholesterol from liver, serum vitellogenin and vitellogenin-free serum was determined over a 30h period. The percentage distribution of newly synthesized lipid and cholesterol among these fractions was then calculated. After the animals had been labelled for 5h, the percentage distribution of radioactive lipid was constant for the remaining 25h (Fig. 3), and 70–80% of the total radioactivity remained associated with liver lipid, whereas only 10–15% of the radioactivity was present in the lipid moiety of vitellogenin. In

Fig. 3. Distribution of lipid and cholesterol synthesized in response to oestradiol-17 β during a 30h pulse-labelling period *in vivo*

Specimens of *Xenopus* 4 days after oestrogen treatment were given injections *in vivo* of 100 μ Ci of [3 H]acetate and groups of four animals were killed at time intervals over the subsequent 30h, their blood was collected and their livers were excised. Pooled serum and liver were then divided into equal portions for the independent determination of radioactive incorporation into either lipid or cholesterol in total liver, serum vitellogenin and vitellogenin-free serum. Detailed methodology is described in the Materials and Methods section. The total radioactivity present in liver and serum lipids was taken as 100% and was 5.6×10^6 , 7.9×10^6 , 5.4×10^6 and 4.6×10^6 d.p.m. at 5, 10, 15 and 30h respectively. $\bullet$, $\blacksquare$ and $\triangle$ represent percentages of total radioactivity in liver lipids, serum vitellogenin and vitellogenin-free serum respectively. The total radioactivity present in liver and serum cholesterol was taken as 100% and was 2.1×10^6 , 2.0×10^6 , 2.4×10^6 and 2.3×10^6 d.p.m. at 5, 10, 15 and 30h respectively. $\circ$, $\square$ and $\triangle$ represent percentages of total radioactivity in liver cholesterol, serum vitellogenin and vitellogenin-free serum respectively.

another series of experiments three groups of animals that had been treated with oestrogen 5 days previously were given an injection of [3 H]acetate (100 μ Ci) and 12h later the animals were killed and the distribution of radioactivity between the three components was shown to be as follows: liver lipids $69.2 \pm 3.3\%$ (mean $\pm$ s.e.m.), vitellogenin lipids $13.8 \pm 0.8\%$, vitellogenin-free serum lipids $17.2 \pm 1.0\%$. The results confirm the salient features emerging from the data of Fig. 3.

Essentially similar results were obtained for the percentage distribution of radioactive cholesterol (Fig. 3). From an experiment of the type described in Fig. 3 vitellogenin was purified, and it was shown that 40–50% of the radioactivity present in the lipid moiety of vitellogenin was associated with cholesterol. A quantitative determination of the cholesterol content of purified vitellogenin was therefore undertaken, showing that 4.5% of the weight of vitellogenin was cholesterol. As the total lipid content of vitellogenin is 12%, cholesterol accounts for 35% of the lipid constituents of vitellogenin. Whether cholesterol in vitellogenin is present in a non-esterified or esterified form is not shown by these experiments.

Determination of cholesterol concentration in microsomal fractions derived from Xenopus laevis liver after oestradiol-17 β treatment

In an effort to show more directly that the oestrogen-induced cholesterol was destined to become a component of new membranes, the cholesterol content of the microsomal fraction from oestrogen-treated and control livers was measured by g.l.c. To ensure that accumulation of cholesterol within the microsomal membranes could take place, liver slices were prepared from animals that had been pretreated 8 days earlier with oestradiol, i.e. 2 days after maximal stimulation of cholesterol biosynthesis. The liver-slice samples were then divided so that one portion could be used to measure the incorporation *in vitro* of [3 H]acetate into cholesterol, and the other portion could be used as a source of the microsomal fraction for g.l.c. determinations. The results of these incubations *in vitro* showed that the liver slices from the animals treated with oestrogen incorporated 120 times more [3 H]acetate into cholesterol than did the corresponding controls, indicating that the microsomal fraction used for g.l.c. analysis had come from *Xenopus* that were sensitized to the hormone. Meanwhile, the g.l.c. determinations showed that the microsomal fractions contained at least 50% more cholesterol 8 days after oestrogen treatment (Table 3). It is noteworthy that in another set of experiments we found that 11 days after hormone treatment the liver microsomal protein nearly doubled.

Table 3. Determination of the liver microsomal cholesterol and protein content of oestrogen-treated and control *Xenopus laevis*

Groups of experimental animals were killed either 8 (for microsomal-cholesterol content) or 11 (for microsomal-protein content) days after oestrogen treatment and their livers removed to prepare microsomal fractions. Cholesterol content of the microsomal fraction was determined by g.l.c. and protein by the method of Lowry *et al.* (1951) as described in the Materials and Methods section. Control determinations were performed identically except that microsomal fractions were obtained from untreated animals. The number of animals for each determination are shown in parentheses. The results are expressed as mean $\pm$ S.E.M.

Treatment	Microsomal cholesterol (mg/g of liver)	Microsomal protein (mg/g of liver)
Control	0.53 $\pm$ 0.04 (4)	11.8 $\pm$ 1.84 (8)
Oestrogen-treated	0.80 $\pm$ 0.03 (4)	21.5 $\pm$ 1.99 (8)

Discussion

The necessity for a cellular reorganization during the induction of vitellogenesis in *Xenopus laevis* hepatocytes has been recognized for some time. The administration of oestradiol-17 β induces a dramatic change in the function of normal liver cells, which is characterized by a gradual loss of the ability to synthesize serum albumin (Follett & Redshaw, 1968), and the cells become almost completely committed to the production of large quantities of vitellogenin. The proposal that during this reorganization process a proliferation of the membranes of the endoplasmic reticulum takes place is not a new idea, since electron micrographs obtained by Nicholls *et al.* (1968), Merry (1974) and Lewis *et al.* (1976) have shown that the proportion of *Xenopus* hepatocyte cytoplasm occupied by the endoplasmic reticulum increases in response to oestradiol-17 β , up to 6 days after administration of the hormone. The significance of the work reported in the present paper is that it provides for the first time quantitative biochemical evidence, centred on the measurement of the increased synthesis of fundamental membrane components, i.e. fatty acids and cholesterol, which supports these ultrastructural studies. In addition to this increased production of the lipid components of the microsomal membranes, an array of evidence exists that demonstrates the oestrogen-induced synthesis of the other principal membrane component, rRNA. Increases in both total RNA (Wittliff *et al.*, 1972) and in rRNA associated with the microsomal fraction (Smith, 1977) have been demonstrated, and an increase in the population of large polyribosomes that could participate in the synthesis of a high-molecular-

weight protein has also been shown (Berridge *et al.*, 1976). That these large polyribosomes actively synthesize vitellogenin has been confirmed by specific immunoprecipitation techniques. Thus the co-ordinated increases in membrane components and rRNA in vitellogenic liver are reminiscent of the changes that occur during cellular differentiation induced by growth and developmental hormones (Tata, 1973).

It has been proposed by Tata (1968, 1973, 1976) that when a cell is switched on to make a specific batch of proteins, ribosomes with mRNA for these proteins are generated and become attached to new membranes of the endoplasmic reticulum. This then allows the topological segregation of protein synthesis such that synthesis of the new proteins would not interfere with the cells' normal protein-synthetic requirements. Such a segregation within well-developed membranes would be particularly advantageous for the production of vitellogenin, since a large number of the molecular processes involved in the biosynthesis, assembly and secretion of this protein are known to be mediated by membrane-bound enzyme systems (Merry, 1974; Clemens, 1974; Penning, 1976; Penning *et al.*, 1977).

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