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Ontogeny of the Vitellogenic Response to Oestradiol and of the Soluble Nuclear Oestrogen Receptor in Embryonic-Chick Liver

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A specific high-affinity oestradiol-binding protein was characterized in salt extracts of liver nuclei of the developing chick embryo. It is present in very small amounts at day 10 of development and is marginally stimulated by oestradiol injection into the yolk sac on day 8. Injection of oestradiol on day 10 evokes a substantial increase in the nuclear oestradiol-binding activity measured on day 12 of development. This oestradiol-binding protein has properties of sedimentation, hormone specificity and high-affinity binding very similar to those of the soluble nuclear receptor in hatched chicks. Livers from the 12-day embryos injected 48h earlier with oestradiol do not synthesize vitellogenin, as judged by a specific immunochemical and electrophoretic assay for this oestrogen-induced protein. Traces of vitellogenin synthesis can be induced in 13-day-embryo liver, and a substantial response, equivalent to that in hatched chicks, is seen in liver from 15-day embryos injected on day 13. The development of the ability of oestradiol to increase the concentration of the soluble nuclear receptor appears to be one, but not the only, critical factor involved in the development of the ability of chick liver to synthesize vitellogenin.

Oestradiol induction of vitellogenin synthesis in the liver of oviparous vertebrates provides an excellent system for the study of the control of gene expression in a primary hormone response (Clemens, 1974; Tata, 1976). Vitellogenin is a large phospholipoglycoprotein, the soluble serum precursor of the egg-yolk proteins phosvitin and lipovitellin (Ansari *et al.*, 1971; Deeley *et al.*, 1975). Until recently, there was considerable confusion about the primary gene product of oestradiol action in chick liver. Many workers thought that phosvitin and lipovitellin were present in serum as separate entities, and there were some studies on their biosynthesis as discrete proteins (Heald & McLachlan, 1965; Jost *et al.*, 1975). We now know that vitellogenin (mol.wt. approx. 240000) is the only serum phosphoprotein induced by oestradiol and that its extreme susceptibility to specific proteolytic cleavage probably gave rise to the earlier findings (Deeley *et al.*, 1975; Bergink & Wallace, 1974).

In the first study on the development of the ability of the chick embryo to produce 'phosvitin and lipovitellin' in response to injected oestradiol, Schjeide *et al.* (1960) found that competence was achieved only on day 19–20 of embryonic development. Carinci *et al.* (1976), however, reported that cultured liver from the 14-day chick embryo produced an uncharacterized phosphoprotein in response to oestradiol. In the present work, I have examined the ontogeny of the vitellogenic response, using a specific and sensitive assay for vitellogenin biosynthesis in which proteolysis is controlled.

The study of steroid receptors during development has provided some useful insights into possible mechanisms by which organs become responsive to hormones (Teng & Teng, 1975*a*,*b*, 1976; Kato *et al.*, 1974; Somjen *et al.*, 1973). Liver of immature chicks and of 15-day chick embryos contains a salt-soluble nuclear oestrogen receptor, and oestradiol injection results in a substantial and prolonged increase in its activity (Mester & Baulieu, 1972; Lazier, 1975; Joss *et al.*, 1976; Gschwendt, 1977*a*). I have investigated the ontogeny of this oestrogen receptor in the chick-embryo liver with particular regard to its possible relation to the ontogeny of the vitellogenic response.

Materials and Methods

Chemicals

[2,4,6,7-^{3}H]Oestradiol-17β (98.5Ci/mmol), [6,7-^{3}H]oestradiol-17β (47.9Ci/mmol), L-[4,5-^{3}H]leucine (42.6Ci/mmol) and [^{14}C]formaldehyde (50mCi/mmol) were from New England Nuclear Corp., Montreal, Quebec, Canada. The labelled oestradiol, supplied in solution in benzene/ethanol (9:1, v/v), was evaporated to dryness and redissolved in ethanol. Dilutions were made in buffer (10mM-Tris/HCl/1.5mM-EDTA, pH7.4). Radiochemical purity was monitored by t.l.c., and purification on LH-Sephadex columns (Pharmacia, Montreal, Quebec, Canada)

Abbreviation used: SDS, sodium dodecyl sulphate.

was carried out when contaminants exceeded 3%. Unlabelled steroids were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., as were the marker proteins used in gel electrophoresis and sucrose-gradient centrifugation. Materials for liquid-scintillation counting were from New England Nuclear Corp. The charcoal/dextran suspension was prepared from Norit A (Fisher Chemical Co., Fairlawn, NJ, U.S.A.) and Dextran T70 (Pharmacia). DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K. Phenylmethanesulphonyl fluoride was from Sigma, and benzamidine from Calbiochem (San Diego, CA, U.S.A.).

Animals and injections

Fertile white-Leghorn chicken eggs obtained from a local hatchery were kept in a humidified incubator at 38°C. Injections of oestradiol-17β in propylene glycol (25mg/ml) or of propylene glycol alone were given into the yolk sac. Solutions were sterilized by membrane filtration. The dose used was 0.05ml and exposure time was 48 h unless otherwise indicated.

Chicks were maintained in quarters lighted for 12h daily and were used for experiments when body weight reached 100g. Oestradiol-17β (2.5mg) or the propylene glycol vehicle was injected intraperitoneally. In the secondary response chicks were re-injected 1 week after the primary injection. Liver incubations were carried out 48h after the last injection.

Preparation of nuclear extract and assay of [^{3}H]-oestradiol-binding activity

Liver removed from chick embryos was placed in cold 0.9% NaCl and washed twice. Homogenization and preparation of the washed crude nuclear pellet was essentially as described earlier (Lazier, 1975), except that the sucrose-containing buffers were supplemented with thioglycerol (10mM). The pellet was homogenized in high-salt buffer (0.5M-KCl/1.5mM-EDTA/10mM-Tris/HCl/10mM-thioglycerol, pH7.4) (1g/ml of buffer), frozen for 1h at −20°C, thawed and centrifuged at 37000g for 30min. DNA was determined in the homogenized pellet by the method of Burton (1970).

For determination of [^{3}H]oestradiol-binding activity the nuclear extracts were first preincubated for 15min at 37°C with an equal volume of charcoal/dextran (0.25% Norit A, 0.0025% dextran in 10mM-Tris/HCl/1.5mM-EDTA, pH7.4) to remove endogenous bound and unbound steroids (Mester & Baulieu, 1972; Lazier, 1975). The 'stripped' nuclear extracts (0.3ml) were then incubated for 16h at 2°C with a series of tubes containing [^{3}H]oestradiol in final concentrations of 0.25–20nM unless otherwise indicated in the text. Unlabelled diethylstilboestrol (100-fold excess) was included in parallel tubes for determination of non-specific binding. Specificially bound [^{3}H]oestradiol was determined as previously described (Lazier & Alford, 1977). The concentration of binding sites and the apparent equilibrium dissociation constants were calculated from Scatchard (1949) analysis of the binding data.

Sucrose gradient centrifugation

The embryonic-chick liver nuclear oestrogen receptor was not reproducibly stable on conventional sucrose gradient centrifugation, similar to the nuclear oestrogen receptor from hatched chicks (Lazier & Alford, 1977). The method of Harrison & Toft (1975) in which receptor was centrifuged through a 5–20% sucrose gradient containing an even distribution of [^{3}H]oestradiol was therefore used. The procedure was exactly as described by Lazier & Alford (1977), except that the concentration of [^{3}H]oestradiol was 2nM, the KCl concentration was 0.25M and 0.18ml of nuclear extract (mixed with 0.02ml of ^{14}C-labelled bovine serum albumin) was applied. The labelled albumin was prepared by the method of Rice & Means (1971). Non-specific binding of [^{3}H]oestradiol in the sucrose gradients was determined by including parallel tubes containing an even distribution of 100nM-diethylstilboestrol as well as 2nM-[^{3}H]oestradiol.

Purification of vitellogenin and production of antibody

Vitellogenin was purified from the serum of oestrogen-treated chicks by the method of Bergink *et al.* (1973) with some modifications by Deeley *et al.* (1975). Blood was collected from decapitated chicks into chilled centrifuge tubes containing phenylmethanesulphonyl fluoride (0.3mM). After centrifugation at 150000g for 2h, the subnatant fluid was chromatographed on DEAE-cellulose as described by Bergink *et al.* (1973), except that all buffers contained phenylmethanesulphonyl fluoride (0.3mM). Protein eluted in the position expected for vitellogenin (a single peak) was pooled and examined for homogeneity by polyacrylamide-gel electrophoresis (5% acrylamide) under denaturing conditions (Weber & Osborn, 1969). ^{3}H-labelled vitellogenin was prepared as described above from serum of oestrogen-treated chicks which had been injected with 2mCi of [^{3}H]-leucine 4h before death.

To elicit antibody production 1ml of vitellogenin (2mg) was emulsified with an equal volume of complete Freund's adjuvant and injected into rabbits subcutaneously on five occasions each 2 weeks apart. Blood was collected by heart puncture and the globulin fraction prepared from serum by $(NH_4)_2SO_4$ precipitation, dialysis, heat treatment and

centrifugation as described by Luskey *et al.* (1974). Antibody was stored in portions at −20 °C. Ouchterlony double-diffusion analysis of the anti-vitellogenin antibody (Kabat & Meyer, 1961) showed a single line of precipitation with purified vitellogenin and with serum for oestrogen-treated chicks. Control serum gave no precipitate.

Incubation of liver and preparation of supernatant

Liver from embryos or hatched chicks was cut into 3 mm cubes and incubated in culture medium at 37 °C for 4 h under O_2/CO_2 (19:1) (1 g of tissue/5 ml of medium). The medium was the same as that used by Luskey *et al.* (1974), except that one-fifth the usual concentration of amino acids was used. The concentration of [^{3}H]leucine was 10 μCi/ml. After 4 h the incubation mixtures were homogenized in 1 M-NaCl, centrifuged at 30000 *g* for 30 min and dialysed exactly as described by Luskey *et al.* (1974), except that phenylmethanesulphonyl fluoride (0.3 mM) and benzamidine (1 mM) were added to the mixtures on termination of the incubation, and were added to all dialysis buffers as well as to the immunoprecipitation tubes. In early experiments phenylmethanesulphonyl fluoride alone was used; however, following the suggestion of R. Deeley (personal communication) benzamidine was also included (Geratz *et al.*, 1976). Incorporation of label into total protein was determined by precipitation with trichloroacetic acid and delipidation as described for determination of serum phosphoprotein P by Lazier (1975). The delipidated pellets were dissolved in Protosol (1 ml) and counted for radioactivity in 10 ml of Omnifluor (8 g/l) in toluene. Incorporation of [^{3}H]leucine into total protein was linear with time over the 4 h incubation period.

Immunoprecipitation of vitellogenin

The standard assay mixture contained the following ingredients in a total volume of 350 μl: 10 mM-sodium phosphate buffer, pH 7.5, 15 mM-NaCl, 1 % Triton X-100, 5 μg of vitellogenin carrier, 100 μl of anti-vitellogenin and 50 μl of liver supernatant. Incubation was for 2 h at 37 °C, followed by 16 h at 2 °C. Precipitates were sedimented by centrifugation at 5000 *g* for 20 min, washed twice with 0.15 M-NaCl and dissolved in 0.5 ml of Protosol. Each tube was washed with an additional 0.5 ml of Protosol and the combined fractions were counted for radioactivity in 10 ml of toluene/Omnifluor in an Isocap 300 liquid-scintillation spectrometer at an efficiency of approx. 40 %. Immunoprecipitation curves were carried out by using increasing concentrations of purified ^{3}H-labelled vitellogenin in the standard assay mixture containing supernatant from livers cultured in the absence of [^{3}H]leucine. Complete precipitation of all concentrations of the labelled antigen up to about 40 μg/tube was obtained.

SDS/polyacrylamide-gel electrophoresis

The immunoprecipitates were dissolved in 100 μl of 10 mM-sodium phosphate buffer, pH 7.0, containing 1 % SDS and 1 % mercaptoethanol. After boiling for 5 min, electrophoresis was carried out as described by Weber & Osborn (1969), except that gels containing 5 % acrylamide were used. After electrophoresis the gels were frozen on solid CO_2, sliced into 2 mm sections with a Mickle slicer, incubated in 5 % (v/v) Protosol in toluene/Omnifluor overnight with gentle shaking at 37 °C, cooled and counted for radioactivity. Recovery of immunoprecipitated purified ^{3}H-labelled vitellogenin labelled *in vivo* was approx. 93 %. Marker proteins in companion gels were stained with Coomassie Blue (Weber & Osborn, 1969).

Results

[^{3}H]Oestradiol-binding activity in salt extracts of embryonic liver nuclei: effect of oestradiol injection

Depot injection of oestradiol into 1-week-old (Joss *et al.*, 1976) or newly hatched chicks (C. B. Lazier, unpublished work) results in a substantial increase in the salt-soluble nuclear oestrogen receptor, which is maintained for over 48 h. Investigation of the same response to oestradiol in the developing chick embryo necessitated consideration of dose, route of administration and time of exposure to the hormone. Subsequent to completion of the present work, Gschwendt (1977*a*) reported that injection of oestradiol into the 15-day embryonated egg provoked an increase in the liver nuclear oestrogen receptor. It had earlier been shown that injection of high doses of oestrogen into the yolk sac gave an increased serum concentration of a light lipoprotein at early stages of development (Schjeide *et al.*, 1960). For the purpose of this study large doses of hormone, which would be likely to give maximal responses at each stage, were necessary. The exposure time had to be long enough to ensure that vitellogenin synthesis would be well established in those livers from oestrogen-treated embryos which were sufficiently developed. A preliminary experiment with 15-day embryos treated for 48 h with oestradiol showed that 100 μg of oestradiol injected into the yolk sac gave a sub-maximal increase in the salt-soluble nuclear [^{3}H]oestradiol-binding activity, and 500 μg and 2.5 mg gave an equally great response. The oestradiol would obviously be diluted in the yolk sac and assimilated by the embryo at an unknown rate. With the higher dose of oestradiol an increase in nuclear [^{3}H]oestradiol

binding was obvious 5h after treatment, and was maximal at 24–48h (results not shown). Accordingly, embryonated eggs at days 8, 10, 13, 17 and 19 of development were injected with oestradiol (1.25mg in 0.05ml of propylene glycol) in the yolk sac, and after a further 48h incubation salt extracts of liver nuclei were prepared and the concentrations of specific binding sites for oestradiol determined. Fig. 1 shows that yolk-sac injection of oestradiol results in a 3–4-fold increase in nuclear binding activity at every stage except day 10. The response at day 12 is not significantly different from that at later stages. The number of binding sites in the control liver preparations does not change during development, and approximates to that found in immature chicks (Mester & Baulieu, 1972; Lazier, 1975).

Characterization of the embryonic oestradiol-binding activity

The apparent equilibrium dissociation constants (K_d) for the embryonic nuclear oestradiol-binding activity at different stages are 1–2nM. Fig. 2 shows representative Scatchard plots for individual extracts prepared from oestrogen-treated 12-, 15- and 21-day embryos and from 12-day control embryos. The small variation is not thought to be significant, and is similar to that seen for different preparations from livers of hatched chicks (Lazier & Alford, 1977).

The hormone specificity of the embryonic oestradiol-binding activity is also very similar to that in immature chicks (Lazier & Alford, 1977). Table 1 shows that diethylstilboestrol competes with [^{3}H]-oestradiol as effectively as unlabelled oestradiol-17β. Progesterone, dihydrotestosterone and cortisol do not compete, whereas oestradiol-17α exhibits partial competition.

Sedimentation of embryonic-liver preparations in sucrose gradients containing an even distribution of [^{3}H]oestradiol shows that significant amounts of specific binding are present only in extracts from oestrogen-treated embryos from day 12 of development onwards (Fig. 3). The sedimentation coefficients (4.3–4.5S) are similar to those of hatched chicks (Lazier & Alford, 1977). The properties of the salt-soluble oestradiol-binding activity in oestrogen-treated embryonic liver nuclei therefore closely resemble those of the salt-soluble nuclear receptor in immature chicks.

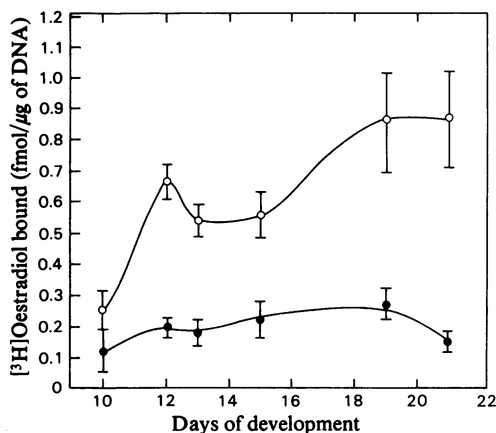

Fig. 1. *Effect of oestradiol on [^{3}H]oestradiol binding by salt extracts of embryonic liver nuclei*

Embryonated eggs were injected with oestradiol in the yolk sac and after 48h the [^{3}H]oestradiol binding in extracts of liver nuclei was determined as described in the Materials and Methods section. Each point represents the mean ± S.E.M. for at least three different liver preparations. ○, Oestradiol-treated embryos; ●, propylene glycol-treated control embryos.

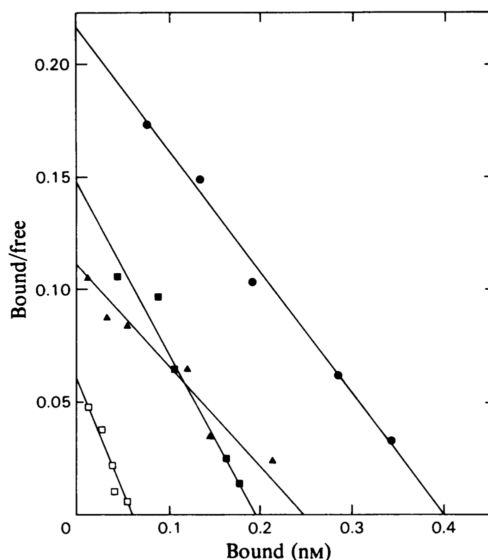

Fig. 2. *Scatchard plots of specific [^{3}H]oestradiol binding by single preparations of nuclear extract from control and oestrogen-treated embryos*

Specific binding was determined as described in the Materials and Methods section. Each point is the mean of duplicate determinations. ●, 21-day, oestradiol-treated, DNA 500µg/ml, K_d 1.9nM; ▲, 15-day, oestradiol-treated, DNA 416µg/ml, K_d 2.1nM; ■, 12-day, oestradiol-treated, DNA 303µg/ml, K_d 1.4nM; □, 12-day, control, DNA 350µg/ml, K_d 1.1nM. The DNA values refer to the concentration in the equivalent homogenate from which the nuclear extract was prepared.

Table 1. *Specificity of [^{3}H]oestradiol binding by salt extracts of embryonic nuclei*

Binding of 2nM-[^{3}H]oestradiol was determined in liver extracts of oestrogen-treated chicks at three stages of development. Potential competitors were added in a concentration of 0.2 μM. Data were calculated from the means of duplicate determinations. The variation of the duplicates was less than 5%. Binding in the presence of [^{3}H]oestradiol alone was 3240, 3700 and 4330 c.p.m. for 13-, 15- and 21-day preparations respectively.

Addition	[^{3}H]Oestradiol binding (% of control)		
Days of development ...	13	15	21
None (control)	100	100	100
Oestradiol-17β	25	29	25
Oestradiol-17α	36	40	39
Diethylstilboestrol	24	25	26
Progesterone	99	108	99
Dihydrotestosterone	93	105	101
Cortisol	98	106	93

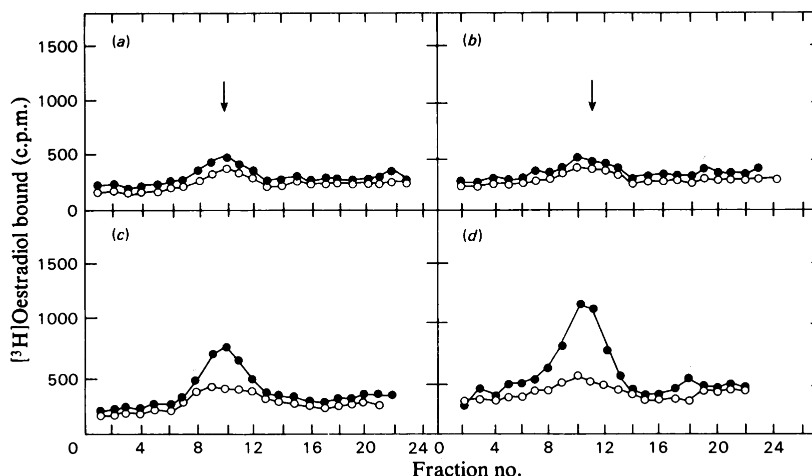

Fig. 3. *Sucrose gradient centrifugation of salt extracts of liver nuclei of oestradiol-treated and control embryos*

Centrifugation in gradients containing an even distribution of [^{3}H]oestradiol was carried out as described in the Materials and Methods section ●, Bound [^{3}H]oestradiol; ○, bound [^{3}H]oestradiol in gradients run in the presence of 0.1 μM-diethylstilboestrol. (*a*), 10-day, oestradiol-treated, DNA 340 μg/ml; (*b*), 10-day, control, DNA 330 μg/ml; (*c*), 12-day, oestradiol-treated, DNA 335 μg/ml; (*d*), 21-day, oestradiol-treated, DNA 405 μg/ml. The arrow represents the position of marker ^{14}C-labelled bovine serum albumin. Sedimentation was from the left to the right-hand side of the graph.

Vitellogenin synthesis in chick liver in vitro

The only previous reported investigations into the capacity of embryonic liver to synthesize egg-yolk phosphoprotein were carried out by using ultracentrifugal analysis of serum after oestrogen treatment (Schjeide *et al.*, 1960) or phosphoprotein P analysis of secreted protein (Carinci *et al.*, 1976). An assay based on radiolabelling of vitellogenin synthesized by embryonic livers *in vitro* would be preferable. It was first necessary to determine that such a technique would be satisfactory for liver from oestrogen-treated immature chicks, in which vitellogenin synthesis was known to be taking place (Lazier, 1975).

The protocol used was similar to that developed by Luskey *et al.* (1974) for oestrogen-induced very-low-density lipoprotein synthesis in rooster liver. Liver mince from control chicks and from chicks given a single injection of oestradiol (primary response) or two injections one week apart (secondary response) was incubated with [^{3}H]leucine-containing culture medium for 4h and the supernatants were analysed as described in the Materials and Methods section. Table 2 shows the stimulatory effect of the hormone

Table 2. *[^{3}H]Leucine incorporation into total supernatant proteins, vitellogenin immunoprecipitate and vitellogenin band on SDS/polyacrylamide-gel electrophoresis*

Liver from control or oestradiol-treated chicks was incubated with [^{3}H]leucine, and 50μl samples of liver supernatant were treated with trichloroacetic acid or with anti-vitellogenin antibody and electrophoresed on SDS/polyacrylamide gels as described in the Materials and Methods section. Data represent the mean ± range for duplicate determinations on two preparations.

	10^{-2} × Incorporation (c.p.m.)		
	Oestrogen-treated chicks		Control chicks
	Primary response	Secondary response	
(*a*) Trichloroacetic acid precipitate	174 ± 39	215 ± 25	120 ± 1.2
(*b*) Immunoprecipitate	22.1 ± 1.1	51.5 ± 8.9	8.1 ± 2.3
(*c*) Vitellogenin band on electrophoresis	11.5 ± 0.5	38.8 ± 5.1	2.8 ± 0.5
Percentage of total protein (*c*/*a*)	7.1 ± 1.9	18.0 ± 0.4	2.3 ± 0.5

on the incorporation of [^{3}H]leucine into supernatant trichloroacetic acid-precipitable material, into the vitellogenin–anti-vitellogenin immunoprecipitate and into the area corresponding to marker vitellogenin on electrophoresis on SDS/polyacrylamide gels. The stimulation of total protein synthesis by oestrogen in rooster liver has been shown earlier (Chan *et al.*, 1976). As might have been expected from serum phosphoprotein P measurements (Jailkhani & Talwar, 1972), about twice as much vitellogenin is synthesized in the secondary response to oestradiol as in the primary response.

The SDS/polyacrylamide-gel-electrophoresis profiles are shown in Fig. 4. Immunoprecipitates from control liver supernatant show no discrete peaks of radioactivity. Immunoprecipitates prepared from livers of the oestrogen-treated chicks and processed in the presence of proteinase inhibitors reveal a single peak of radioactive protein which co-migrates with authentic vitellogenin. Omission of the proteinase inhibitors results in degradation of the vitellogenin. The pattern of degradation, giving a major peak corresponding to a protein of mol.wt. 135000, is very similar to that observed by others (Bergink *et al.*, 1974; Jost & Pehling, 1976).

Vitellogenin synthesis by chick-embryo liver

To study the development of the capacity to synthesize vitellogenin, embryonated eggs were injected in the yolk sac with oestradiol (1.25mg) on days 8, 10, 11, 13, 17 and 19 of incubation, and after a further 48h incubation the liver was cultured for 4h. Incorporation of [^{3}H]leucine into the total trichloroacetic acid-precipitable protein (Fig. 5) and into the vitellogenin band on electrophoresis of the vitellogenin–anti-vitellogenin immunoprecipitates was determined (Fig. 6). Total protein synthesis is variable, particularly between otherwise identical preparations cultured on different days. In contrast with the observations with hatched chicks, there is no stimulatory effect of oestradiol on total protein labelling, nor are there any pronounced changes during embryonic development. Nevertheless, when labelling of the vitellogenin band on SDS/polyacrylamide gels is calculated as a percentage of total protein labelling in a given preparation, a very distinct oestradiol induction of vitellogenin synthesis is seen in embryonic livers cultured on or after day 15 of development (Fig. 7). A small amount of vitellogenin synthesis is detected when livers from oestrogen-treated embryos are cultured on day 13, but no discrete high-molecular-weight proteins immunologically related to vitellogenin are synthesized in the control livers at any stage. An experiment with ^{32}P-labelling of protein by livers from oestradiol-treated and control 15-day embryos showed that the embryonic vitellogenin is phosphorylated. No ^{32}P incorporation was found in the gel slices corresponding to vitellogenin in the controls (results not shown).

The capacity to synthesize vitellogenin appears to be as fully developed in the 15-day embryo as in the 2-week-old chick (primary response, Table 2).

Discussion

The oestradiol-binding activity in salt extracts of liver nuclei from oestradiol-treated chick embryos is very similar to the soluble nuclear oestrogen receptor in hatched chicks (Mester & Baulieu, 1972; Lazier & Alford, 1977). Both preparations exhibit high-affinity binding of oestradiol with a K_d of 1–2nM, the same hormone specificity and the same behaviour on sedimentation in sucrose gradients containing an even distribution of [^{3}H]oestradiol.

Small amounts of the receptor (0.1–0.2fmol/μg of DNA) are present in livers of untreated embryos, but yolk-sac injection of oestradiol on day 10 of egg

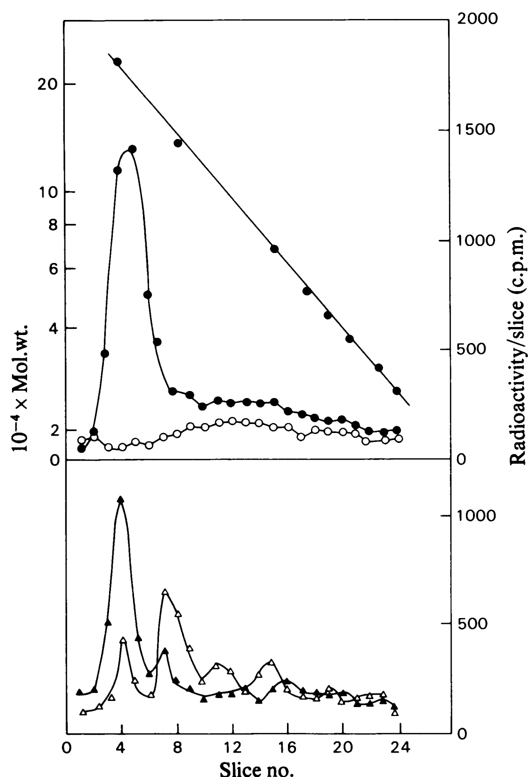

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of immunoprecipitates from liver supernatants of control and oestradiol-treated chicks*

Immunoprecipitates from 50 μl of liver supernatant were subjected to electrophoresis as described in the Materials and Methods section. The upper line is a plot of molecular weight (log scale) versus slice no. for marker proteins run in a companion gel: vitellogenin, 240000; β-galactosidase, 131000; bovine serum albumin, 67000; heavy chain of immunoglobulin, 50000; ovalbumin, 43000; pepsin, 35000; carbonic anhydrase, 29500; light chain of immunoglobulin, 24000. ●, Secondary response, oestradiol-treated chicks; ○, control chicks; ▲, primary response, oestradiol-treated chicks; △, primary response, oestradiol-treated chicks, supernatant processed in the absence of proteinase inhibitors.

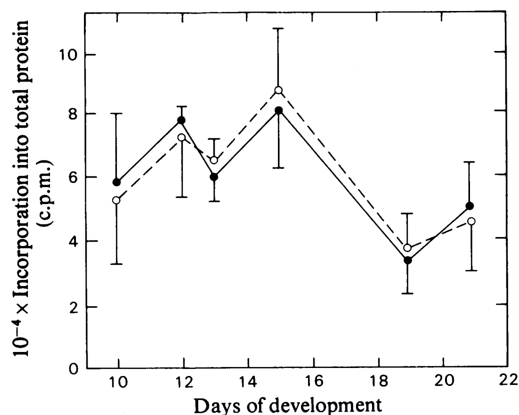

Fig. 5. *Incorporation of [³H]leucine into total supernatant proteins by liver of oestrogen-treated and control chick embryos at different stages of development*

Incubation of liver and trichloroacetic acid precipitation of protein from 50 μl of supernatant was carried out as described in the Materials and Methods section. Each point is the mean ± S.E.M. or range for duplicate determinations on two or three separate preparations. ●, Oestradiol-treated embryos; ○, propylene glycol-treated control embryos.

incubation results in a 3–4-fold increase in nuclear receptor concentrations measured on day 12. Exposure to the hormone on day 8, however, does not give a corresponding increase in receptor on day 10. It is noteworthy that in the chick embryonic Müllerian duct, the concentration of cytosol oestrogen receptor gradually increases from day 8 to day 12 of development, and at the latter stage has reached the value in the newborn (Teng & Teng, 1975*a*,*b*). Exogenous oestradiol provokes an increase in the Müllerian-duct nuclear oestrogen receptor which can be entirely accounted for by translocation of the cytosol receptor (Teng & Teng, 1976). The capacity for translocation is fully developed at day 12.

For the embryonic chick liver it has been difficult to demonstrate that the increase in the soluble nuclear receptor after administration of oestradiol is due to translocation. Many workers have been unable to measure high-affinity cytosol oestrogen receptor, either in embryonic (Teng & Teng, 1975*a*) or in hatched chick livers (Mester & Baulieu, 1972; Ozon & Bellé, 1973; Gschwendt, 1975; Lazier & Alford, 1977). This appears to be due to lability of the cytosol receptor in liver preparations. Gschwendt (1977*b*) has reported that specific high-affinity binding of oestradiol is detectable in an $(NH_4)_2SO_4$ precipitate from embryonic-chick liver. I have found similar results, provided that the liver is processed in the presence of proteinase inhibitors (C. B. Lazier, unpublished work). Gschwendt (1977*b*) showed that a single injection of oestradiol into the 19-day embryonated egg provoked an apparent translocation of about 50% of the cytoplasmic oestradiol-binding sites to the nuclear fraction. Other stages were not studied. The developmental pattern for the cytoplasmic oestradiol-binding protein showed an increase in the number of binding sites up to day 19, followed by a decline. Eggs from a fattening chicken strain were used instead of the white Leghorn (laying hen) strain

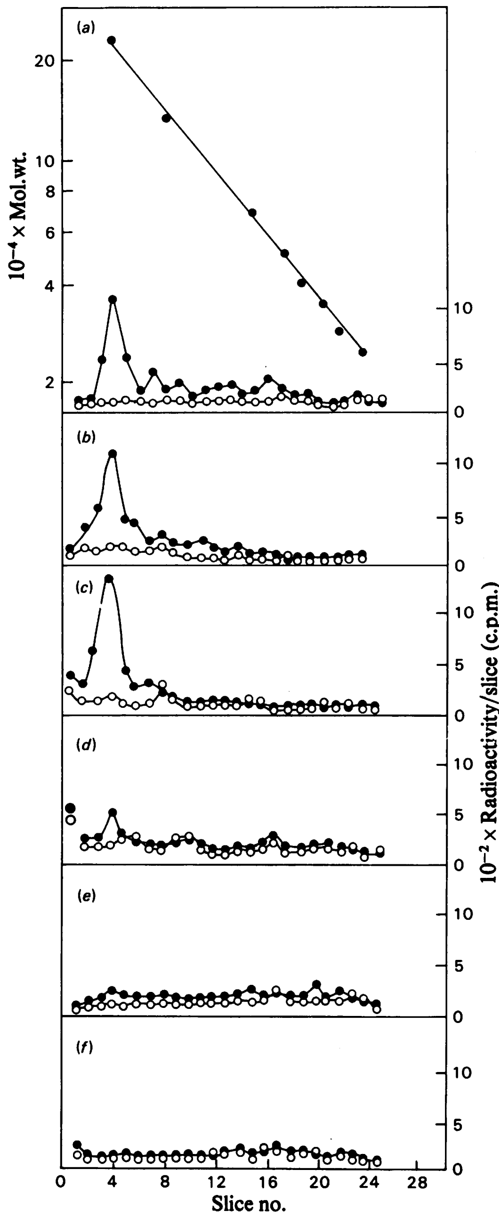

Fig. 6. *SDS/polyacrylamide-gel electrophoresis of immunoprecipitates from liver supernatants of control and oestradiol-treated chick embryos at different stages of development*

Immunoprecipitates from 50μl samples of incubated liver supernatants were subjected to electrophoresis on SDS/polyacrylamide-gels. The upper line shows the mobility of standard marker proteins (listed in Fig. 4). ●, Immunoprecipitates from oestradiol-treated embryos; ○, propylene glycol-treated control embryos. (*a*) 21 days, (*b*) 19 days, (*c*) 15 days, (*d*) 13 days, (*e*) 12 days, (*f*) 10 days of egg incubation at the time of analysis.

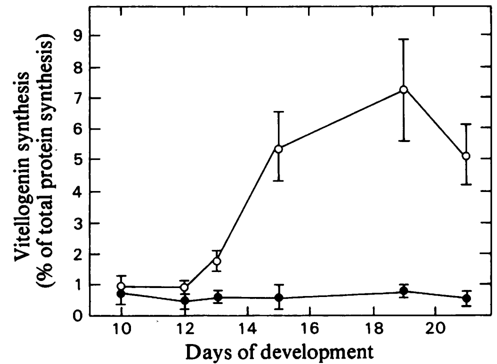

Fig. 7. *Vitellogenin synthesis by the developing chick embryo liver*

Incorporation of [^{3}H]leucine into the vitellogenin band on SDS/polyacrylamide-gel electrophoresis is expressed as percentage of total protein labelled. Each point is the mean ± S.E.M. or range for duplicate determinations on two to three separate preparations. ○, Oestradiol-treated; ●, propylene glycol-treated control embryos.

used in the present work. Further study is required to enable direct comparison of the ontogeny of the cytoplasmic receptor and of the nuclear receptor response to exogenous oestradiol. It is worth noting, however, that in white-Leghorn-chick embryos the capacity for exogenous oestradiol to increase nuclear receptor concentration is well developed by day 12 of egg incubation both in the Müllerian duct (Teng & Teng, 1976) and in the liver.

The apparently complete development of the oestrogen-translocating receptor system in the Müllerian duct at day 12, however, is somewhat dissociated from the time of rapid growth of the duct (days 14–18) (Teng & Teng, 1975*b*). Similarly, in the embryonic liver, substantial vitellogenin induction by oestradiol cannot be demonstrated until day 15, although traces are seen earlier. Receptor development is probably a critical, but not the only, event necessary for the attainment of complete hormone responsiveness. Factors limiting vitellogenin production in the oestrogen-treated embryos between days 12 and 14 could devolve around receptor interaction with chromatin and the ill-understood steps leading to transcription of the vitellogenin gene. On the other hand it is possible that the transcription processes are mature, but that elements in the translation or subsequent processing stages are missing. These possibilities could be delineated by assays for vitellogenin mRNA using a complementary DNA probe (Deeley *et al.*, 1977).

In some other systems a similar or even greater dissociation of receptor development and complete

hormone responsiveness has been seen. For example, oestrogen treatment of the post-natal rat does not evoke a complete uterotrophic response until 15–20 days after birth (Kaye *et al.*, 1972; Somjen *et al.*, 1973; Katzenellenbogen & Greger, 1974). Partial responses to oestradiol treatment are seen earlier: these include stimulation of 2-deoxyglucose metabolism at days 12–15, induction of the specific uterine 'induced protein' at days 5–10 (Katzenellenbogen & Greger, 1974), an increase in the 5S nuclear oestrogen receptor at day 5 (Somjen *et al.*, 1974), and stimulation of the activity of ornithine decarboxylase in 2-day-old rats (Kaye *et al.*, 1973). Somjen *et al.* (1976) have demonstrated the presence of a specific 8S cytoplasmic oestrogen receptor in the Müllerian duct of the 20-day rat foetus.

Glucocorticoid induction of certain hepatic enzymes occurs only after birth of the rat; however, Feldman (1974) showed that liver of the 19–20-day foetus contains an intact receptor system, capable of nuclear translocation. Thus, as in the chick, the unresponsive foetal liver appears to have a block distal to nuclear uptake of the steroid. A similar situation holds for the ontogenesis of glucocorticoid receptors in embryonic-chick neural retina (Lippman *et al.*, 1974). The appearance of receptors in development does not necessarily predicate a complete hormonal response. On the other hand, a loss of glucocorticoid receptors in developing rat lung appears to parallel the completion of glucocorticoid action in lung maturation (Giannopoulos, 1974; Solomon & Lee, 1977). These developing systems clearly give wide scope for dissecting the many contributory factors involved in steroid-hormone action.

The present study gives the first unambiguous evidence that the embryonic-chick liver is capable of the oestrogen-induced synthesis of vitellogenin. The findings of earlier workers were limited by insensitive or non-specific methods (Schjeide *et al.*, 1960; Carinci *et al.*, 1976). Direct induction of vitellogenin by physiological amounts of oestradiol added to organ cultures has not been demonstrated for chick liver, but it has been shown for liver from the toad *Xenopus laevis* (Wangh & Knowland, 1975; Green & Tata, 1976; Tata, 1976). Since embryonic tissues of warm-blooded animals often respond to culture conditions better than do adult tissues, it may be advantageous to use liver from chick embryos after day 14 of development in attempts to obtain direct induction by added oestradiol. Such a system *in vitro* would be a valuable tool in studying the regulation of the vitellogenin response in chickens.

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