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Oestrogen-Induced Synthesis of Thiamin-Binding Protein in Immature Chicks

KINETICS OF INDUCTION, HORMONAL SPECIFICITY AND MODULATION

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A specific radioimmunoassay procedure was developed to monitor the plasma concentrations of thiamin-binding protein, a minor yolk constituent of the chicken egg. By using this sensitive assay, the kinetics of oestrogen-induced elaboration of this specific protein in immature chicks was investigated. After a single injection of the steroid hormone, with an initial lag period of 4–5 h the thiamin-binding protein rapidly accumulated in the plasma, attaining peak concentrations around 75 h and declining thereafter. A 4-fold amplification of the response was noticed during the secondary stimulation, and this increased to 9-fold during the tertiary stimulation with the steroid hormone. The magnitude of the response was dependent on the hormone dose, and the initial latent period and the duration of the ascending phase of induction were unchanged for the hormonal doses tested during both the primary and secondary stimulations. The circulatory half-life of the protein was 6 h as calculated from the measurement of the rate of disappearance of the exogenously administered ^{125}I-labelled protein. Simultaneous administration of progesterone, dihydrotestosterone or corticosterone did not alter the pattern of induction. On the other hand, hyperthyroidism markedly decreased the oestrogenic response, whereas propylthiouracil-induced hypothyroidism had the opposite effect. The anti-oestrogen *E*- and *Z*-clomiphene citrates, administered 30 min before oestrogen, effectively blocked the hormonal induction. α-Amanitin and cycloheximide administered along with or shortly after the sex steroid severely curtailed the protein elaboration. A comparison of the kinetics of induction of thiamin- and riboflavin-binding proteins by oestrogen revealed that, beneath an apparent similarity, a clear-cut difference exists between the two vitamin-binding proteins, particularly with regard to hormonal dose-dependent sensitivity of induction and the half-life in circulation. The steroid-mediated elaboration of the two yolk proteins thus appears to be not strictly co-ordinated, despite several common regulatory features underlying their induction.

Oestrogen-induced synthesis of specific egg-yolk proteins continues to be one of the intensively pursued areas of gene expression in animal cells (Gruber *et al.*, 1976; Tata, 1976; Palmiter *et al.*, 1976). The observations that the female sex hormone induces these proteins *in vivo* (Murthy & Adiga, 1978; Wittliff & Kenney, 1972) and *in vitro* (Ventling, 1978; Tata, 1976) in the livers of adult male or immature birds and of amphibians of either sex have greatly aided an understanding of the underlying mechanisms. In the past, these investigations were largely concerned with the major yolk protein, vitellogenin (Deeley *et al.*, 1975). More recently, we demonstrated (Murthy & Adiga, 1978) that the hormone-induced elaboration of another functionally important, albeit minor, yolk constituent, namely riboflavin-binding protein, shares several common features with vitellogenin biogenesis in terms of kinetics of induction and hormonal specificity. This raised the possibility that the steroid hormone may be eliciting the enhanced expression of all other yolk proteins by a common regulatory mechanism.

More recently, we reported for the first time the

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isolation and physico-chemical characterization of another vitamin carrier, namely thiamin-binding protein from egg white, and showed that this protein is similar to riboflavin-binding protein in terms of molecular size and affinity to bind to the respective vitamins. Furthermore, the two proteins exhibit great affinity to interact specifically with each other, and, unlike the vitellogenin split-products (lipovitellin and phosvitin), are present in both the yolk and white compartments of the egg. However, they can be easily distinguished chemically and immunologically and appear to be different gene products (Muniyappa & Adiga, 1979). In view of the preliminary evidence for early oestrogenic induction of thiamin-binding protein in immature male chicks (Muniyappa *et al.*, 1978), it was decided to investigate the details of the kinetics of elaboration and hormonal modulation of this protein and compare them with those of riboflavin-binding protein (Murthy & Adiga, 1978). To facilitate these studies, a highly sensitive and specific radioimmunoassay procedure to monitor this protein in the blood was developed.

Materials and Methods

The source of male chicks and their maintenance have been reported (Murthy & Adiga, 1978). Blood samples were collected by cardiac puncture directly into heparinized tubes and the prepared plasma was stored frozen until used. Thiamin-binding protein was isolated and the specific antiserum was raised in rabbits as detailed elsewhere (Muniyappa & Adiga, 1979). Riboflavin-binding protein was purified and the highly specific antibodies to the protein were raised in rabbits as described by Murthy *et al.* (1976). Immunoglobulin fraction of normal rabbit serum was isolated (Campbell *et al.*, 1970) and used to raise antibodies in goats (double antibody; Murthy & Adiga, 1977*a*). $Na^{125}I$ for protein iodination was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Sephadex G-75, bovine serum albumin, oestradiol-17β, progesterone, dihydrotestosterone, cycloheximide, corticosterone, L-thyroxine, propylthiouracil and α-amanitin were products of Sigma Chemical Co., St. Louis, MO, U.S.A. Chloramine-T was the product of Aldrich Chemical Co., Milwaukee, WI, U.S.A. The anti-oestrogens *E*- and *Z*-clomiphene {1-chloro-2-[4-(2-diethylaminoethoxy)phenyl]-1,2-diphenylethylene} citrates were gifts from Merrell National Laboratories, Cincinnati, OH, U.S.A. Other chemicals and reagents were of analytical grade. Hormones and anti-oestrogens in propane-1,2-diol were injected intramuscularly, whereas cycloheximide and α-amanitin dissolved in 0.15 M-NaCl were administered intraperitoneally.

Homologous radioimmunoassay for thiamin-binding protein

Thiamin-binding protein was radioiodinated at 4°C by the procedure of Greenwood *et al.* (1963). Briefly, 0.5–1 mCi of carrier-free $Na^{125}I$ was introduced directly into a tube containing 5 μg of thiamin-binding protein in 50 μl of 100 mM-sodium phosphate buffer, pH 7.5. The reaction was initiated by the addition of 25 μl of chloramine-T (1 mg/ml). The protein was exposed to $Na^{125}I$ just for 30 s and the reaction was terminated by the addition of 25 μl of sodium metabisulphite (10 mg/ml). The reaction mixture was transferred to a Sephadex G-75 column (0.7 cm × 18 cm) pre-equilibrated with 0.15 M-NaCl in 10 mM-sodium phosphate buffer, pH 7.5 (phosphate-buffered saline), containing 0.5% (w/v) gelatin. The presence of gelatin ensured complete recovery of the protein from the column. Fractions (1 ml) were collected and small portions (25 μl) from each fraction were counted for radioactivity in a gamma counter (Packard Autogamma, model 2002). The iodinated protein was stored frozen in small batches in the presence of 2% (w/v) bovine serum albumin. Since repeated freezing and thawing led to immunological inactivation, when once thawed the labelled protein was used immediately.

Immunoassay procedure

A dilution of the antiserum that was capable of binding 25–40% of the labelled protein was selected. The assay was performed in glass tubes (Moudgal & Madhwaraj, 1974) at 37°C in a water bath. The antiserum (0.1 ml) was incubated with 0.5–100 ng of thiamin-binding protein for the standards in duplicates, and 0.1–0.2 ml portions of unknown samples were incubated with appropriate controls, for 12–14 h. A portion of the labelled protein (40000 c.p.m.) was added to all the tubes and the incubation continued for another 12–14 h. At the end of

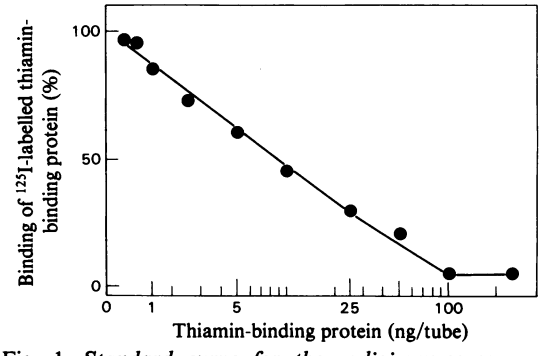

Fig. 1. *Standard curve for the radioimmunoassay of thiamin-binding protein*
The dilution of the antiserum used was 1:15000. For other details see the text.

this period 0.1 ml of 1:50-diluted normal rabbit serum and 0.1 ml of goat antiserum (double antibody) were added and incubated for another 12–14 h. The total input and bound radioactivities were determined in the immunoprecipitates after centrifugation at 2500 rev./min. A standard graph was constructed (Fig. 1) by using various known amounts of unlabelled thiamin-binding protein to inhibit the binding of labelled protein, and the results were expressed as percentage of total radioactivity bound to protein against log of input radioactivity. The total bound radioactivity in the protein refers to the amount of specifically bound radioactivity in the absence of unlabelled protein. This forms the basis for all the measurements of plasma thiamin-binding-protein concentrations under various conditions. The non-specific binding (to non-immune rabbit serum; Murthy & Adiga, 1977*a*) was 3–7%, and this was always subtracted. Radioimmunoassays for riboflavin-binding protein in the chicken plasma were conducted as described by Murthy & Adiga (1977*a*).

Inter-assay and intra-assay variations

A serum pool was analysed four to eight times for the intra-assay and four times for inter-assay variation, and data were analysed statistically (paired '*t*' test; Mulholland & Jones, 1968). Coefficients of intra- and inter-assay variations were 2–6% and 3–5% respectively and the assay method had a sensitivity of 0.5 ng/ml.

Half-life of thiamin-binding protein

Freshly prepared ^{125}I-labelled thiamine-binding protein was diluted with non-iodinated thiamin-binding protein (1 mg/ml) and injected into control (untreated) or oestrogen-treated birds. Blood samples (0.5 ml) withdrawn at suitable intervals were counted for radioactivity. Portions (0.2 ml) of the plasma samples were treated with antiserum (diluted 1:50) against thiamin-binding protein for 6 h at 37°C. The double antibody was added and the precipitate counted for radioactivity to ensure that the blood radioactivity primarily represented that corresponding to injected thiamin-binding protein. A least-squares fit of the data was computed.

Production of hypothyroid and hyperthyroid conditions

The chickens (2 months old) were rendered hyperthyroid by six daily intraperitoneal injections of L-thyroxine (100 μg/kg body wt.) in 0.15 M-NaCl in 10 mM-phosphate buffer. Propylthiouracil (100 mg/kg body wt.) in 0.5% gelatin in 0.15 M-NaCl (Murthy & Adiga, 1977*b*) was similarly administered to induce hypothyroid conditions. The control birds received 0.15 M-NaCl in 10 mM-phosphate buffer only. After 6 days, a single dose (10 mg/kg body wt.) of oestradiol-17β in propane-1,2-diol was administered intramuscularly to individual birds at the indicated doses.

Results

Time course of induction of thiamin-binding protein by oestrogen

It is now well established (Gruber *et al.*, 1976) that the avian liver has little capacity to store secretory proteins, which are released into circulation soon after synthesis. Therefore the production of thiamin-binding protein in the liver was monitored by direct measurement of plasma concentrations of the protein by using the sensitive and specific radioimmunoassay developed for this purpose (see the Materials and Methods section). The profile of plasma accumulation and disappearance of the protein during primary, secondary and tertiary stimulations with oestradiol-17β (10 mg/kg body wt. each time) is illustrated in Fig. 2. The plasma concentrations analysed before hormone administrations served as the respective baseline values, which were in the range 25–130 ng/ml, depending on the batch of the birds and/or the feed. After each hormone treatment, a lag period of 4–5 h during which there was no detectable enhancement in the plasma concentration of this specific protein was observed in several experiments.

During primary stimulation with the steroid, the maximum accumulation of thiamin-binding protein was around 1400 ng/ml at 75 h, although as early as 6 h a significant increase in the concentration of the protein (200–250 ng/ml) was repeatedly observed. After reaching a peak at 75 h, the concentrations gradually declined, returning to the baseline value by day 6. A second hormonal dose administered after this time elicited the typical 'memory' effect noticed earlier with vitellogenin (Gruber *et al.*, 1976) and riboflavin-binding protein (Murthy & Adiga, 1978) in oestrogen-treated chickens. Moreover, the response was amplified roughly 4-fold, which was further magnified 9-fold (relative to that during primary stimulation) during the tertiary stimulation, as estimated by the measurement of the areas under the curves (Fig. 2). The corresponding maximum concentrations of the protein were also increased by similar orders of magnitude. However, the time-courses of accumulation and disappearance after the three hormonal stimuli remained qualitatively the same with regard to not only the initial latent phases, but also the periods around which maximum concentrations were recorded after each hormonal dosing.

A comparison of these data with those obtained with riboflavin-binding protein (Murthy & Adiga, 1978) clearly showed distinct qualitative and quantitative differences in finer details of the kinetics of the

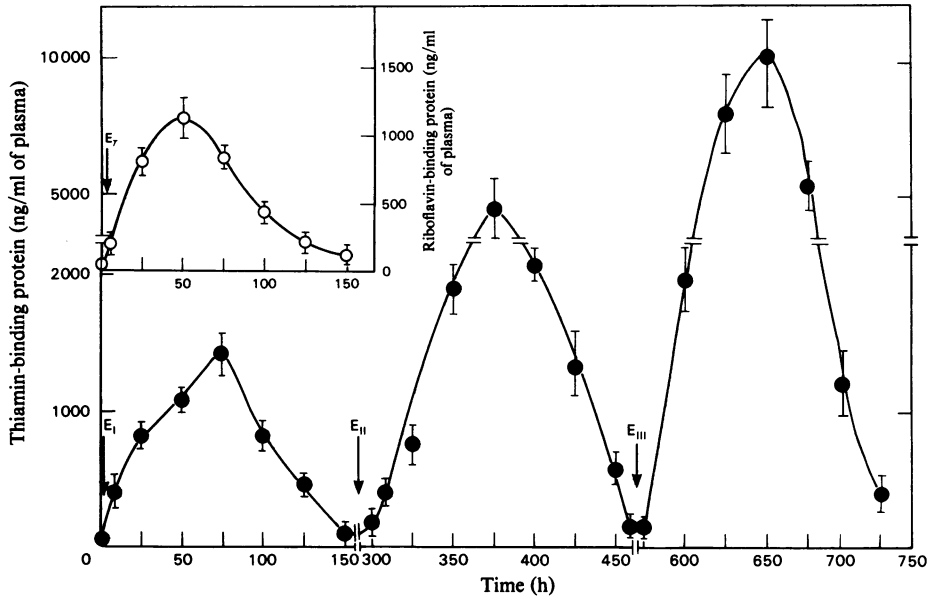

Fig. 2. *Time course of accumulation of thiamin-binding protein in the plasma after sequential injections (E_I, E_{II}, E_{III}) of oestradiol-17β* in propane-1,2-*diol* (10 *mg/kg body wt.*)
The values represent means ± S.D. ($n = 4$). The inset shows the measurement of riboflavin-binding protein in the same sample during primary stimulation (E_I).

hormonal induction of the two specific proteins. Confirmatory evidence for this premise was sought by simultaneous measurement of the plasma concentrations of the two proteins by using the respective specific radioimmunoassay procedure (Murthy & Adiga, 1977*a*). The data depicted in Fig. 2 (inset) show that, although initial lag phases and the overall rates of accumulation of the two proteins are grossly similar, the time of maximum protein accumulation (i.e. 48 h for riboflavin-binding protein, 75 h for thiamin-binding protein) as well as the rates of decay during the primary stimulation were clearly different. Furthermore, the amplification of the hormonal response during secondary stimulation was higher with thiamin-binding protein by an order of magnitude.

Oestrogen-dose-dependence of induction

The effects of various doses of oestradiol during primary stimulation on the rates of plasma accumulation and disappearance of thiamin-binding protein are depicted in Fig. 3. It is clear that as low a hormonal dose as 100 μg/kg body wt. elicited a measurable response, which progressively increased with the steroid dose. However, the time course of accumulation and disappearance of the protein, including the initial lag phase, was unaltered throughout the hormonal doses administered; the only perceptible difference is related to the rates of accumulation and clearance as well as the magnitudes of the protein concentrations at the time of

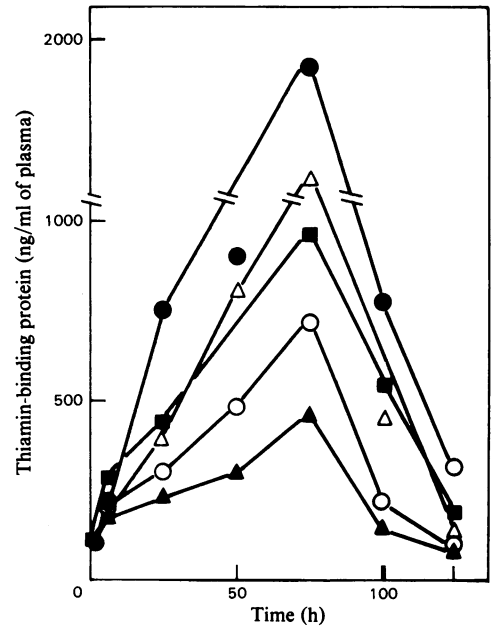

Fig. 3. *Hormonal dose–response of the plasma thiamin-binding protein during primary stimulation*
Time course of plasma thiamin-binding protein accumulation with increasing concentrations of oestradiol-17β (mg/kg body wt.): ▲, 0.1; ○, 0.5; ■, 1.0; △, 5.0; ●, 10.0. Results are means of four experiments and S.D. values were less than 10% of the mean.

maximum accumulation, namely 75h (Fig. 3). Furthermore, the results obtained (not shown) have demonstrated that this dose-dependency of the induction phenomenon persisted during the secondary stimulation also, with the expected difference, namely that the amplification of the response was proportional to the hormonal dose. Again the time course and the time of maximum accumulation in the plasma remained the same throughout.

It was considered desirable at this stage to compare the sensitivity of the induction of thiamin- and riboflavin-binding proteins to different hormonal doses at early periods of primary stimulation during which the plasma accumulations of the proteins reflect primarily the respective biosynthetic rates. The data of Fig. 4(*a*) show that, when measured at 6h after steroid dosing, the plasma concentration of the thiamin-binding protein increased linearly and steeply with the hormonal dose throughout the concentration range tested. By contrast, a minimum dose of approx. 1mg of oestrogen/kg body wt. was necessary to elicit any significant production of riboflavin-binding protein at this early time of induction. Even with the maximum dose of the steroid hormone administered, the absolute amount of riboflavin-binding protein elaborated was around 35% of that of thiamin-binding protein. The sensitivities of the induction processes as represented by the slopes of the dose–response curves were clearly different for the two proteins. At 24h (Fig. 4*b*) this differential sensitivity persisted, particularly at lower doses of oestrogen. Thus it is clear that the relative proportions of the two proteins would change with both the hormonal dose and the time after hormonal dosing, indicating a non-co-ordinate elaboration of the two proteins.

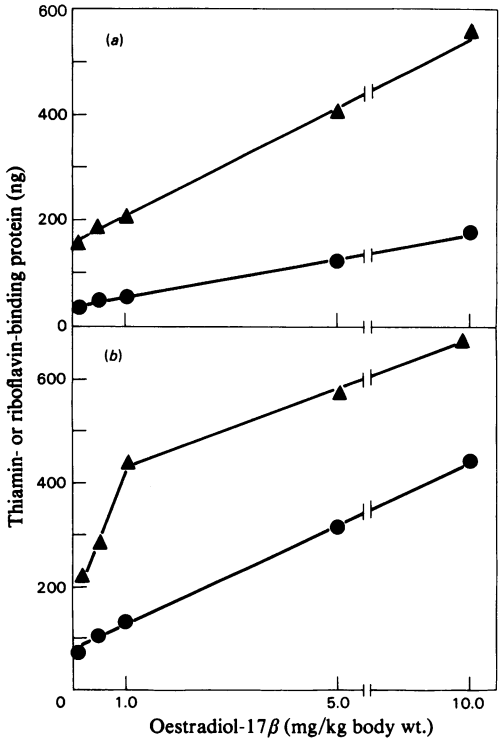

Fig. 4. *Dose–response of synthesis of thiamin-binding protein* (▲) *and riboflavin-binding protein* (●) *with oestrogen at* (*a*) 6*h and* (*b*) 24*h*
For details see the text.

Rate of disappearance of thiamin-binding protein

The data of Fig. 2 show that, despite the clear difference between the times of maximum accumulation during primary stimulation, the two proteins apparently were cleared from the circulation to attain their respective pre-stimulus values at around the same time (150h) after hormone administration. In other words, the rates of disappearance represented by the slopes of the curves during the late descending phase (representing primarily metabolic degradation) were different in the two cases, with the thiamin-binding protein apparently having a faster turnover rate. This premise was substantiated by measuring the rate of disappearance of exogenously administered ^{125}I-labelled thiamin-binding protein. Fig. 5 depicts the kinetics of disappearance of the injected ^{125}I-labelled thiamin-binding protein (1mg/bird) from the blood of the control and oestrogen-treated 2-month-old male chicks. By

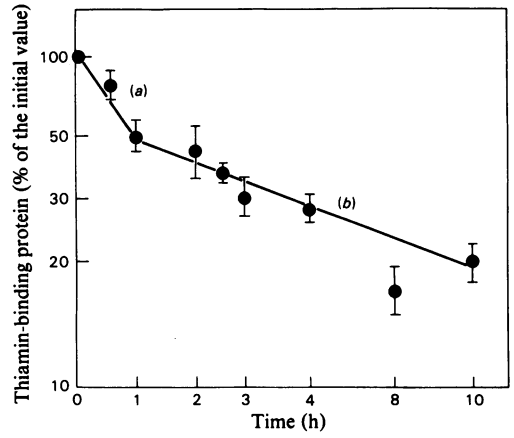

Fig. 5. *Rate of disappearance of* ^{125}*I-labelled thiamin-binding protein from the plasma*
Freshly labelled ^{125}I-thiamin-binding protein (1mg, approx. 10^5c.p.m.) was injected into 2-month-old birds, and at the indicated intervals plasma was collected and radioactivity measured. Values are means ± S.D. ($n = 5$). An identical pattern was obtained with oestrogen-treated chicks. (a) Phase of rapid disappearance; (b) phase of catabolism (degradation).

analogy with the riboflavin-binding protein (Murthy & Adiga, 1978), exogenously administered thiamin-binding protein disappeared from the circulation in a biphasic manner with an initial rapid phase (*a*), presumably representing dilution with extravascular space, and tissue binding, to be followed by a relatively slower phase (*b*), predominated by metabolic clearance (McFarlane, 1964). The disappearance during phase (*b*) followed first-order kinetics, and the least-squares fit of the data gave a slope of 0.059, corresponding to a half-life of 6 h in both control and oestrogen-treated chickens. This value is much lower than that calculated for riboflavin-binding protein (10 h) and may be related to the fact that it is not a glycoprotein, unlike riboflavin-binding protein (Murthy & Adiga, 1977*a*). This estimate, initially arrived at by measuring the whole blood radioactivity, was later confirmed by immunoprecipitation of the blood ^{125}I-labelled thiamin-binding protein in the samples, which uniformly corresponded to around 70% of the total blood radioactivity.

Modulation of thiamin-binding protein induction by thyroid status

Our earlier studies with the riboflavin-binding protein in chickens have shown that the oestrogen induction of synthesis of this specific protein is markedly influenced by the thyroid status of the birds (Murthy & Adiga, 1977*b*). In view of the gross similarities in the kinetics of elaboration of the two vitamin-binding proteins, the modulation of the steroid-hormone-induced production of the thiamin-binding protein was investigated under experimentally induced hypothyroid and hyperthyroid conditions. The concentrations of this specific protein have been monitored during both the ascending (48 h) and the descending (96 h) phases of primary stimulation to ensure that the observed values reflect the changes throughout the time-course of induction, which was dependent on the thyroid status of the birds. As shown by the data presented in Table 1, under conditions of hyperthyroidism the elaboration of thiamin-binding protein was drastically curtailed, whereas this oestrogen response was significantly augmented when hypothyroidism was produced by propylthiouracil administration. The finding that the marked decrease in the hormonal response during hyperthyroidism could be annulled by doubling the hormonal dose lends support to the earlier conclusion (Murthy & Adiga, 1977*b*) that the enhanced catabolism of the inducer hormone was one of the causative factors for diminishing protein accumulation under hyperthyroid conditions.

Table 1. *Influence of hypothyroidism and hyperthyroidism on oestrogen-induced plasma concentrations of thiamin-binding protein in male chicks*

Experimental details are given in the text. Pre-injection control values are means of those obtained with 12 chicks. Experimental values are means ± S.D. for four chicks.

Treatment (mg/kg body wt.)	Plasma concentration of thiamin-binding protein (ng/ml) At 48 h	At 96 h
None (control)	61 ± 2.8	76 ± 10.6
Oestrogen (10)	820 ± 7.25	786 ± 6.0
Propylthiouracil (100) + oestrogen (10)	1820 ± 53.3	1653 ± 160
Thyroxine (0.1) + oestrogen (10)	426 ± 30.6	213 ± 47.0
Thyroxine (0.1) + oestrogen (20)	1076 ± 46.6	626 ± 84.6

Effect of progesterone, other steroid hormones and anti-oestrogens

It has been reported earlier (Palmiter & Wrenn, 1971) that oestrogen-induced growth, cytodifferentiation and synthesis of specific egg-white proteins by the magnum portion of the immature chick oviduct is effectively antagonized by progesterone during primary stimulation, whereas during subsequent stimulation this steroid could substitute for oestrogen in terms of the production of egg-white proteins. However, such a phenomenon could not be observed in the oestrogen-induced synthesis of a specific yolk protein such as riboflavin-binding protein (Murthy & Adiga, 1978) and very-low-density

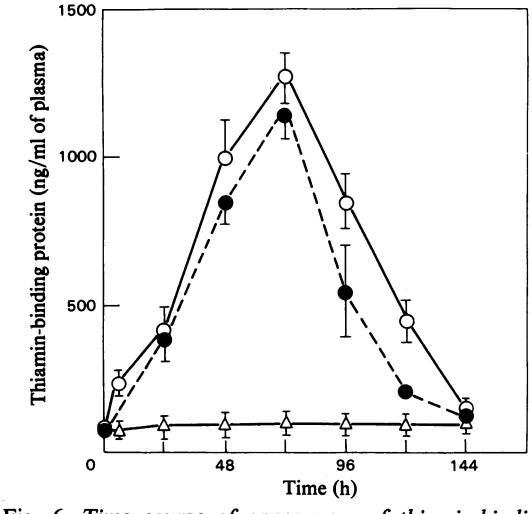

Fig. 6. *Time course of appearance of thiamin-binding protein in the plasma on treatment with oestrogen* (10 *mg/kg body wt.*) (●), *oestrogen* (10 *mg/kg body wt.*) + *progesterone* (10 *mg/kg body wt.*) (○), *and progesterone alone* (△)

For details see the text.

lipoprotein (Chan *et al.*, 1977). The data on the elaboration of thiamin-binding protein by the chicken liver are also in accordance with these earlier findings (Fig. 6). In fact, the ineffectiveness of progesterone during primary stimulation extended from the initial lag phase, throughout the duration of accumulation and decay to the extent of the maximum protein accumulation. Furthermore, even during secondary stimulation, progesterone was without any influence either by itself or in combination with the oestrogen. A similar phenomenon was encountered when either dihydrotestosterone or corticosterone (both 10mg/kg body wt.) was administered either individually or in combination with oestrogen during both primary and secondary stimulation (results not shown).

The absolute hormonal specificity of induction of thiamin-binding protein was tested by exposing the animals to various concentrations of potent synthetic anti-oestrogens, namely *E*- and *Z*-clomiphene citrates, 30min before the hormonal dosing. When the plasma thiamin-binding protein concentrations were measured after 48h, the anti-oestrogen appeared to have severely inhibited the hormone-specific response at all the doses tested (Table 2). However, it is significant that both the anti-oestrogens by themselves were completely devoid of even low oestrogen-like activity in terms of induction of this specific yolk protein.

Table 2. *Effect of anti-oestrogens on the oestradiol-stimulated plasma thiamin-binding-protein content*

Anti-oestrogen in propane-1,2-diol was injected 30min before oestrogen (10mg/kg body wt.) administration and plasma thiamin-binding protein was analysed by radioimmunoassay 48h after oestrogen treatment. The results are means of closely agreeing values from two animals assayed in duplicate.

Anti-oestrogens (mg/kg body wt.)	Thiamin-binding protein (ng/ml)	
	− Oestradiol	+ Oestradiol
None (control)	112	627
E-Clomiphene citrate (10)	96	120
(25)	112	128
Z-Clomiphene citrate (10)	156	175
(20)	136	180

Effects of protein-synthesis inhibitors on the induction

In attempts to establish whether new mRNA synthesis is an essential prerequisite for the induction, α-amanitin, a specific inhibitor of nucleoplasmic DNA-dependent RNA polymerase (Tata *et al.*, 1972), was administered to immature male chicks at different times after both primary and secondary stimulation with oestrogen. A uniform dose of 200μg of α-amanitin/kg body wt. was chosen in these experiments, although higher doses (>500μg/kg body wt.) were required for eliciting maximum inhibition of RNA synthesis, which, however, often proved lethal to the experimental birds within 24h after the drug administration. When the plasma thiamin-binding-protein concentrations were analysed at 30h after oestrogen administration, it could be shown (Table 3) that the earlier the drug was injected, the more pronounced was the inhibition. Furthermore, although the extents of inhibition during early times were comparable during the two stimulations, the induction phenomenon was relatively refractory during the secondary stimulation to the transcriptional inhibitor given later than 4h after the hormonal dosing.

When cycloheximide, a powerful translation inhibitor, was given along with the steroid hor-

Table 3. *Effect of α-amanitin on induction of thiamin-binding protein during primary and secondary stimulation with oestradiol-17β*

All chicks (1 month old) except the controls received 10mg of oestradiol/kg body wt. Groups of four to six chicks were used, and each bird received α-amanitin (0.02mg/kg body wt.) at the time indicated. All chicks were killed 30h after oestrogen administration. Data are expressed as means ± S.D.

Treatment	Primary stimulation		Secondary stimulation	
	Thiamin-binding protein (ng/ml of plasma)	Inhibition (%)	Thiamin-binding protein (ng/ml of plasma)	Inhibition (%)
None (control)	98 ± 27		76 ± 11.0	
Oestrogen	678 ± 2.2		968 ± 33	
Oestrogen-treated chicks receiving α-amanitin at time:				
Zero	88 ± 24	87	92 ± 18	90.5
+4h	140 ± 13.3	80	216 ± 62	78
+10h	246 ± 58	64	670 ± 14	31
+14h	328 ± 8.6	52	872 ± 10.8	10

Table 4. *Effect of simultaneous administration of cycloheximide on oestrogen-induced thiamin-binding-protein accumulation*

Data are expressed as means ± S.D. ($n = 3$). Oestradiol in 0.5 ml of propane-1,2-diol was injected into 1-month-old male chicks (10 mg/kg body wt.). Cycloheximide was administered intraperitoneally and the plasma concentrations of thiamin-binding protein were analysed 48 h thereafter by radio-immunoassay.

Treatment (mg/kg body wt.)	Plasma thiamin-binding protein (ng/ml)
None (control)*	94 ± 10.0
Oestrogen (10)	780 ± 80.0
Oestrogen (10) + cycloheximide (1)	90 ± 12.0
Cycloheximide	70 ± 23.3

* Pre-injection values.

mone and the circulatory concentration of the induced protein was measured at 48 h, it was found that the induction of the specific protein was totally abolished, but the baseline values were relatively unaffected (Table 4).

Discussion

One of the intriguing questions concerning the regulation of protein synthesis in eukaryotic cells elaborating in concert multiple inducible proteins is whether there are common regulatory factors co-ordinating such a phenomenon, in analogy with the functioning of bacterial operons (Calhoun, 1975). Attempts have been made to find an answer to this question for oestrogen-induced production of some of the egg-white proteins by the oviduct magnum of hormone-stimulated immature chicks (Palmiter, 1972; McKnight, 1978). The chicken liver, responding with massive synthesis of several yolk proteins within a brief period after a single dose of oestrogen (Gruber *et al.*, 1976), should also be an attractive model system to investigate the above phenomenon of co-ordinate synthesis of several proteins. However, no concerted efforts have been made so far, presumably owing to paucity of well-characterized proteins that are amenable to such investigations. Our earlier studies (Murthy & Adiga, 1978) on the hormone-induced synthesis of riboflavin-binding protein (a minor yolk constituent) in immature chicken liver did reveal close qualitative similarities between the kinetics of induction of this vitamin carrier and those of vitellogenin (Bergink *et al.*, 1973), but a strict quantitative comparison was not feasible in view of the differential sensitivities of the methods used to monitor the two proteins produced in vastly different quantities. The recent isolation and characterization of thiamin-binding protein and the demonstration that two vitamin-carrier proteins have in common several qualitative and quantitative characteristics (including dual sites of synthesis, unlike vitellogenin), but are different gene products (Muniyappa & Adiga, 1979), prompted a more meaningful comparison of their induction by oestrogen. Their immunological non-identity, leading to the development of specific radioimmunoassays having comparable sensitivities (Murthy & Adiga, 1977*a*), has been profitably exploited to quantify the concentrations of the two proteins in the plasma to understand the dynamics of the induction phenomena. Our data on the kinetics of induction of thiamin-binding protein during primary and secondary stimulations are in general comparable quantitatively with those for riboflavin-binding protein (Murthy & Adiga, 1978), and qualitatively for vitellogenin (Bergink *et al.*, 1973), particularly with regard to initial lag periods and overall rates of accumulation during ascending phases. The similar duration of the latent periods suggests that the molecular events preceding the protein induction may be common for the two vitamin-binding proteins as well as for vitellogenin. The data of Joss *et al.* (1976) clearly rule out the possibility that nuclear translocation of the hormone is the limiting factor responsible for this delay in the induction phenomena. Studies on vitellogenin synthesis (Jost *et al.*, 1978; Burns *et al.*, 1978), demonstrating that the rapid transcription and accumulation of specific protein mRNA molecules start becoming manifest during the latent period itself, are relevant in this context. Since α-amanitin, a specific inhibitor of RNA polymerase II (Tata *et al.*, 1972), administered during this time severely curtailed subsequent accumulation both of vitellogenin (Jost *et al.*, 1973) as well as of thiamin-binding protein during primary and secondary stimulation (Table 3), it would appear that an analogous situation prevails during induction of the vitamin-binding proteins also.

However, it is clear from the data of Fig. 2 that, beneath these apparent similarities, there exist definite qualitative and quantitative differences between the induction kinetics of the two vitamin-binding proteins, particularly with regard to the duration of ascending phases and the kinetics of decay from circulation. One possible explanation for the difference in the times of maximum protein accumulation is suggested by the results of Fig. 4 concerning the differential sensitivities of the responses of the two proteins to various hormonal doses at early times of induction, with thiamin-binding protein requiring much lower concentrations of oestradiol-17β to initiate and sustain protein accumulation compared with riboflavin-binding protein. It is conceivable that, after an initial rapid rise at the sites of action in the hepatocytes, the administered steroid hormone would slowly decrease in concentration (Joss *et al.*,

1976) to reach the respective critical threshold values essential to sustain the continued production of two proteins at different times (48h for riboflavin-binding protein, 75h for thiamin-binding protein). Beyond these stages the rates of degradation would predominate over corresponding rates of synthesis, as represented by the descending phases of the curves in Fig. 2. The finding that the concentrations of the two proteins reach their respective prestimulus values at about the same time can then be easily understood on the basis of the calculated clearance rates of the two proteins. It may be recalled that thiamin-binding protein has a much shorter half-life (6h; Fig. 5) than the riboflavin-binding protein (10h; Murthy & Adiga, 1978). This phenomenon of differential induction of specific cellular responses to steroid-hormonal doses has been observed earlier in other systems. For example, Katzenellenbogen & Gorski (1972) and Anderson *et al.* (1973) have shown that in the rat the increase in uterine weight and the induction of 'induced protein' occurs when the nuclear oestrogen receptors are 50% saturated, whereas glucose transport is maximal at rather low degrees of nuclear receptor saturation. In the chick oviduct system (Palmiter & Haines, 1973; Mulvihill & Palmiter, 1977; McKnight, 1978), the oestrogen dosage needed for half-maximal induction *in vivo* as well as *in vitro* of ovalbumin and its mRNA is 2–3 times higher than that required for induction of conalbumin and its mRNA to comparable extents. Since the corresponding percentage occupancy of the nuclear hormone receptors was also higher (80%) for ovalbumin than for conalbumin (50%), these differential responses were ascribed to either different numbers of specific chromatin sites of oestrogen-receptor binding or to different affinities of these receptors to the genomic regulatory sites corresponding to the two proteins (Mulvihill & Palmiter, 1977). The same explanations may be applicable to differential expression of the genes of the two vitamin-binding proteins referred to above.

The 'memory effect' characterized by more rapid accumulation of thiamin-binding protein with consequent amplification of the response during secondary and tertiary stimulation has been previously encountered with vitellogenin (Gruber *et al.*, 1976; Tata, 1976) and riboflavin-binding protein (Murthy & Adiga, 1978). This has been attributed to more pronounced transcription and accumulation of the corresponding mRNA species (Jost *et al.*, 1978; Burns *et al.*, 1978). The prolonged persistence of a large proportion of the nuclear oestrogen receptors (Joss *et al.*, 1976) may be one of the contributory factors to this amplification. However, a recent hypothesis implicating somatic re-arrangement of the gene leading to a stable 'micro-differentiation' of the genetic material (Jost *et al.*, 1978) appears a more attractive explanation of the 'memory effect'. Whatever may be the underlying mechanisms, the finding that during secondary stimulation oestrogen dose-dependency of the responses persists and the amplification is higher (4-fold) with thiamin-binding protein than with riboflavin-binding protein (2-fold) suggests that the phenomenon of differential sensitivity of the induction of the two vitamin carriers also extends to the duration of the 'memory' effect.

Despite these obvious differences of details the hormonal inductions of the two yolk vitamin-binding proteins appear to be inter-related and under analogous control mechanisms. For example, the modulation of induction of thiamin-binding protein by changes in the thyroid status of the birds (Table 1) is both qualitatively and quantitatively similar to the pattern obtained with riboflavin-binding protein (Murthy & Adiga, 1978). Again the complete ineffectiveness of progesterone (Fig. 6), dihydrotestosterone and corticosterone in terms of either substituting for oestrogen or modulating the induction pattern is in conformity with the findings with riboflavin-binding protein (Murthy & Adiga, 1978) and contrasts with the results reported for egg-white proteins in the chicken oviduct (Palmiter & Wrenn, 1971; McKnight, 1978).

Thus, it would appear that the induction of yolk proteins is highly oestrogen-specific. This premise finds further strong support from the data of Table 2, showing that the anti-oestrogen *E*- and *Z*-clomiphene citrates when administered before or along with the inducer hormone completely annulled the plasma accumulation of thiamin-binding protein as well as riboflavin-binding protein (Murthy & Adiga, 1978). It is significant that these anti-oestrogens did not exhibit by themselves even weak oestrogen-like activity in terms of induction of thiamin-binding protein, unlike in the rat uterus (Mohla & Prasad, 1969). These results confirm an observation that other anti-oestrogens, Nafoxidine and C-1628, were ineffective in promoting phosphoprotein production, though their affinity to bind to chicken liver chromatin was comparable with that of oestrogen (Gschwendt, 1975). The data of Wittliff & Zelson (1974) with a *Xenopus laevis* liver system agrees with the above findings.

Ample evidence exists now to support the idea that there is an obligatory requirement for synthesis of new protein and mRNA before the onset of induction of specific proteins in both the chicken liver (Murthy & Adiga, 1978; Jost *et al.*, 1973; Greengard *et al.*, 1964) and oviduct systems (Palmiter *et al.*, 1976; McKnight, 1978), presumably to initiate transcription of specific mRNA and to maintain the nuclear receptors at optimal concentrations (Joss *et al.*, 1976). Our data on the cycloheximide effect (Table 4), indicating that the inhibitory influence on thiamin-binding protein persists at a time (24h)

when general hepatic protein synthesis has recovered completely, may be indicative of an obligatory requirement for synthesis of other protein intermediates early during the induction phenomenon (Joss *et al.*, 1976; Murthy & Adiga, 1978).

Thus it is clear from the data in the present paper and those reported earlier (Murthy & Adiga, 1978) that the induction of the two vitamin-binding proteins (and by analogy, of vitellogenin also) have several common aspects, especially in terms of hormonal specificity, the initial lag phases and amplification during subsequent stimulations. However, the hormonal dose-dependent differential sensitivity of their induction and different clearance rates from circulation suggest that their elaboration, especially under conditions of limiting amounts of the inducer, may not be strictly co-ordinate and that their relative proportions are subject to experimental and physiological manipulations.

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