J. Biosci., Vol. 7, Number 2, March 1985, pp. 77–94. © Printed in India.

Hormonal modulation of reproduction–specific thiamin carrier protein in the rat

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MS received 14 December 1984

Abstract. The hormonal modulation of thiamin carrier protein in the plasma and uterine luminal secretion during the normal reproductive phases of the animal (estrous cycle and pregnancy) as well as during experimental estrogenisation was investigated in the rat using a specific and sensitive homologous radioimmunoassay procedure developed for this purpose. Following a single injection of estrogen to immature male rats, thiamin carrier protein rapidly accumulated in plasma attaining peak concentration at 48 h and declining thereafter. A 1·5-fold amplification of the inductive response was observed on secondary stimulation with the hormone. The magnitude of the response exhibited a clear dependency on the dose of the steroid hormone, whereas the time at which peak levels of thiamin carrier protein production was remained unaltered in the concentration range of the steroid tested. The inductive effect of estrogen was severely curtailed by the antiestrogens, *viz.,* En- and Zu-clomiphene citrates, while progesterone was incapable of either modulating the estrogen-induced response or eliciting an induction by itself. Cycloheximide drastically blocked the response to estrogen. Evidence for the ability of uterus to serve as yet another independent site of thiamin carrier protein synthesis was obtained by *in vitro* incorporation of radioactive amino acids into immunoprecipitable thiamin carrier protein in the tissue explants of estrogenised female rats. The levels of thiamin carrier protein in uterine luminal fluid measured during estrous cycle, pregnancy and experimental estrogenisation exhibited remarkable similarity to the plasma thiamin carrier protein profiles.

Keywords. Thiamin carrier protein; radioimmunoassay; estrogen induction; specificity; kinetics; antiestrogens; secondary stimulation; physiological modulation; uterine synthesis.

Introduction

Earlier investigations in this laboratory (Adiga and Murty 1983) have revealed that estradiol-17β (E_2) administration to immature chicks can elicit *de novo* biosynthesis of riboflavin carrier protein (RCP) and thiamin carrier protein (TCP); the kinetics of these inductive responses exhibit an overall similarity to those governing the hormonally-induced elaboration of vitellogenin (Gruber *et al.,* 1976; Tata and Smith, 1979; Tata, 1970). With regard to RCP, evidence has been recently adduced for a dual site of biogenesis, *viz.,* the estrogenised liver and the oviduct in the birds, which respond to the same hormonal signal to elaborate the vitamin carrier, but with certain subtle

Abbreviations used: E_2, Estradiol 17β; RCP, riboflavin carrier protein; TCP, thiamine carrier protein; RIA, radioimmunoassay; AH; aminohexyl; PBS, sodium phosphate buffer, pH 7·2 containing 0·9 % NaCl; BSA, bovine serum albumin; PMSF, phenylmethylsulphonyl fluoride; IgG, immunoglobulin G.

differences in terms of kinetic details and hormonal specificity during subsequent hormonal stimulations (Durga Kumari, 1985). With the discovery for the first time of the evolutionary conservation of RCP in terms of structure and function in mammals (Adiga and Murty, 1983), recent researches have shown that in the female rat RCP levels fluctuate in consonance with changing levels of E_2 during estrous cycle and pregnancy (Muniyappa and Adiga, 1980a) and that administration of E_2 can induce *de novo* this specific protein in male rats with kinetic patterns closely resembling those encountered in birds (Murty and Adiga, 1982). These findings suggest the possibility that other similarly conserved gene products might have retained certain basic aspects of their hormonal inducibility and responsiveness in the mammalian liver in addition to their structural and functional characteristics.

We have recently (Adiga and Muniyappa, 1978) reported on the occurrence as well as isolation and physicochemical characterisation of TCP from the pregnant rat for the first time (Malathy, 1985). Since preliminary data revealed that this specific vitamin carrier can also be detected immunologically in E_2-treated rodent sera, it was of interest to estimate the amount of this protein by developing a specific and sensitive homologous radioimmunoassay (RIA) so that the details of its physiological modulation and endocrine basis could be examined.

The data presented in this paper clearly establish E_2 as the primary endocrine signal responsible for TCP induction in the rodent liver. In addition, they reveal that the hormone stimulated uterus is an additional site of biosynthesis of this vitamin carrier.

Materials and methods

Thirty five-day-old male and 21-day old female rats (Wistar) from our Institute Colony were fed on a pelleted diet (Hindustan Lever Products, Bombay) and water *ad libitum* and exposed to a 14–10 h light-dark schedule. TCP was purified from pregnant or estrogenised rat sera by affinity chromatography on TPP-aminohexyl-(AH)-Sepharose (Malathy, 1985). The antiserum against the purified rat TCP was raised in rabbits by subcutaneous injections of the protein (100 μg) emulsified with Freund's complete adjuvant, once a week, for 3 weeks followed by a booster dose of the protein (100 μg) in saline. A week after the booster injection, venous blood was collected from the ear, serum separated and kept frozen at – 20°C. $Na^{125}I$ for iodination of the protein was purchased from the Radiochemical Center, Amersham, Buckinghamshire, UK. [^{14}C]-Labelled Chlorella hydrolysate was procured from Bhaba Atomic Research Centre, Bombay and served as a source of [^{14}C] -amino acids. The sources of other chemicals and reagents were the same as described earlier (Muniyappa and Adiga, 1980b). Hormones and antiestrogens in propane-1, 2-diol were administered intramuscularly, whereas cycloheximide in 0·01 M sodium phosphate buffer, pH 7·2 /0·15 M NaCl was injected intraperitoneally. Blood samples were collected by cardiac puncture and uterine secretions were collected by flushing the uteri with 0·01 M sodium phosphate buffer, pH 7·2/0·15 M NaCl.

Homologous radioimmunoassay for thiamin carrier protein

The iodination of TCP was performed at room temperature in the presence of iodogen (Fraker and Speck, 1978). Briefly, 5 μg of TCP in 50 μl of 0·1 M sodium phosphate

buffer, pH 7·5 was introduced into an iodogen coated tube and exposed to 0·25 mCi of carrier-free $Na^{125}I$ for 1 min. The iodinated protein was resolved from free iodine by gel filtration chromatography on a Sephacryl S-200 column (1·5 cm × 42 cm) pre-equilibrated with sodium phosphate, 0·01 M pH 7·2 containing 0·9 % NaCl (PBS) containing 0·2 % (w/v) bovine serum albumin (BSA). The presence of BSA ensured complete recovery of the protein from the column. Aliquots (100 μl) from each fraction (0·5 ml) were counted for radioactivity in a mini gamma counter (LKB-Model 1275). The iodinated protein was stored frozen in small batches.

Immunoassay procedure

It may be mentioned that the isolated rat TCP even when iodinated using the relatively milder iodogen method, resolved by gelfiltration on Sephacryl-200 into fractions of both higher and lower molecular sizes presumably representing aggregation and fragmentation respectively. These fractions were able to bind to the antiserum non-specifically. Therefore, only those labelled protein fractions which exhibited the specific binding to the antiserum *i.e.,* displaceable by a 100-fold excess uniodinated protein, were pooled and used for RIA. An antiserum dilution capable of binding 25–30% of the labelled protein was selected. The assay was performed at 37°C (Moudgal and Madhwaraj, 1974). The diluted antiserum (0·1 ml) was incubated with 0·5-1000 ng of rat TCP for the standard in duplicates and 10–25 μl of 1:100 diluted plasma samples and 10–50 μl of uterine flushings with appropriate controls (samples from untreated animals) for 10–12 h. Labelled protein (*Ca* 25,000 cpm) was added to all the tubes and incubation was continued for another 12 h and the antigen-antibody complex was precipitated by the double antibody method (Murthy and Adiga, 1978) in the presence of a carrier (0·1 ml of 1:100 diluted normal rabbit serum). The bound radioactivity in the immunoprecipitates was determined after centrifugation at 5000 *g* in a Sorval RC 5B centrifuge for 15 min. The amounts of TCP in the unknown samples were calculated by extrapolation from the standard curve (figure 1) that was constructed by adding various known amounts of the unlabelled

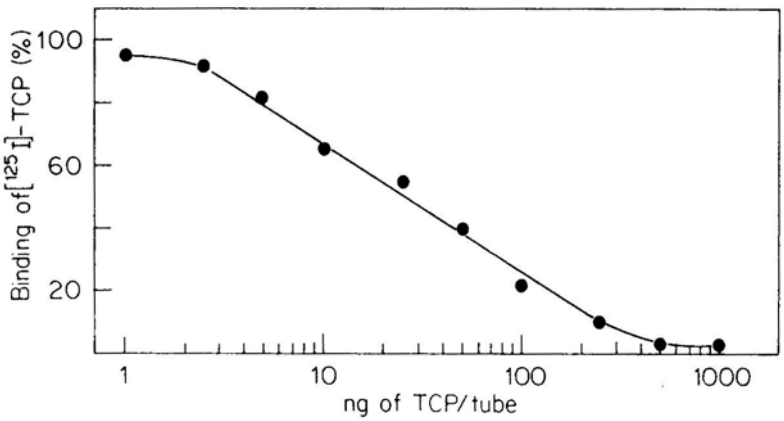

Figure 1. Standard curve for the radioimmunoassay for rat thiamin carrier protein. The dilution of the antiserum used was 1:10000. Details in the text.

protein to the antiserum. The total bound radioactivity in the protein refers to the amount of specifically bound radioactivity in the absence of the unlabelled protein. The non-specific binding (to non-immunised rabbit serum) was 3–8 % of the total input radio activity and this was always subtracted.

Interassay and intrassay variations

Aliquots from a single pool of serum sample were analysed 4–8 times for interassay and intraassay variations and the data were analysed statistically (Mulholland and Jones, 1968). Coefficients of intra and inter assay variations were 3–6 % and the assay method had a sensitivity of 1 ng.

Determination of half-life of TCP

Freshly [^{125}I]-labelled TCP (~5×10^5 cpm) was injected to estrogenised and untreated immature male rats and day 14 pregnant rats. The labelled protein was diluted with unlabelled purified rat TCP (100 μg) when administered to the control or untreated animals. Blood samples withdrawn at suitable intervals were counted for radioactivity and subsequently treated with antiserum to rat TCP (1:1000) for 6 h at 37°C to ensure that the blood radioactivity represented that due to injected TCP. A least square fit of the data was computed.

In vitro incorporation of [^{14}*C*]*-amino acids into uterine tissue explants*

The uterine tissue slices (150–200 mg) from estrogen-primed (5mg/kg body wt.) and untreated (control) rats were transferred quickly into 25 ml conical flasks containing 2 ml of Krebs-Ringer bicarbonate buffer, pH 7·4. 5 μCi of [^{14}C]-labelled chlorella hydrolysate was added to each flask, flushed with oxygen for 20 sec, tightly corked and incubated at 37°C with shaking in a water bath for 4 h. The incubation was terminated by placing the flasks in ice for 5 min. A homogenate (10% w/v) of the tissue along with the incubation medium was prepared in 25 mM Tris-HCl (pH 7·6) containing 25 mM NaCl, 5mM $MgCl_2$, 1% Triton X-100 and 0·1 mM phenylmethylsulphonyl fluoride (PMSF) with a motor-driven glass-teflon homogeniser. The homogenate was centrifuged at 105,000 *g* for 60 min in a Spinco L5-ultracentrifuge and the supernatant stored at – 20°C was used for the immunoprecipitation of labelled TCP employing excess of rat TCP antiserum. The amount of radioactivity incorporated into 10 % trichloroacetic acid-insoluble proteins was determined by placing on Whatman No. 3 filter paper discs, 50 μl aliquots of uterine cytosols prepared from control and estrogenised immature rats (Palmiter, 1972). To determine the radioactivity specifically associated with TCP, 0·5ml aliquots of [^{14}C]-labelled cytosol preparations were incubated with 100 μl of rat TCP antiserum in the presence of rat cold TCP (10 μg) as a carrier. Following immunoprecipitation 10 μg of rat cold TCP was added to the clarified supernatants and incubated with 100 μl of the TCP antiserum. Radioactivity in these immunoprecipitates was taken to represent non-specific radioactivity. The immunoprecipitates obtained were electrophoresed on 10% Polyacrylamide gels under both reducing and non-reducing conditions (Weber and Osborn, 1968).At the end of the run ,the gels were stained to locate the protein bands on the gel. The gels were then sliced into pieces of

0·5 cm dimensions, digested with 30% H_2O_2 and counted in toluene: 2-methoxy ethanol (1:1, v/v) containing 0·5% 2,5-diphenyl oxazole (w/v) by liquid scintillation spectrometry.

Results

Physiological modulation of circulatory levels of TCP

Following the detection of TCP-like protein with cross-reactivity to the chicken vitamin carrier in sera of rats during gestation (Adiga and Muniyappa, 1978), the modulation of this specific protein in the rodent sera during estrous cycle and different stages of pregnancy were investigated (figure 2) using the sensitive and specific

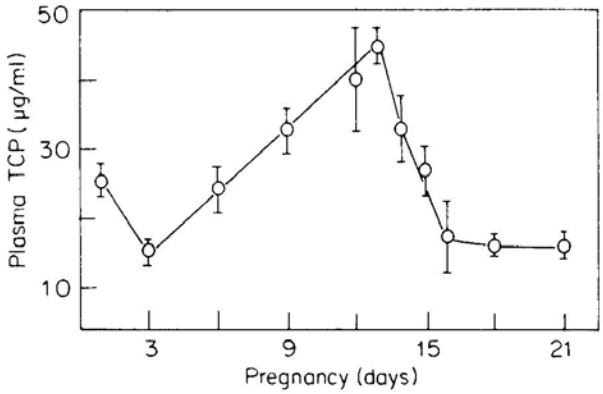

Figure 2. Plasma levels of thiamin carrier protein during pregnancy. The values represent mean and vertical bars represent S.D. (n = 5).

homologous RIA (figure 1). During gestation, a significant amount of TCP could be measured as early as day 4 of pregnancy *i.e.,* even before implantation, suggesting that the protein is of maternal origin (figure 2). The concentration of TCP progressively increased to reach peak values on day 13 and thereafter steadily declined to base-line values (*i.e.*, of day 1) by day 16 to maintain a steady state level thereafter. It is noteworthy that high concentrations of the protein in circulation were encountered between days 11 to 14 which coincide with the critical period of rapid embryonic growth and organogenesis (Guha and Janne, 1976). Levels of TCP in serum more or less correlated with the reported estrogen levels (Shaikh, 1971) upto day 14 of pregnancy, but not later on. In regularly cycling rats with a 4-day estrous cycle, a good parallelism between serum concentrations of the steroid hormone (Shaikh, 1971) and TCP levels was clearly evident, with the proestrous estrogen surge coinciding with peak concentration of TCP (figure 3). This pattern of TCP modulation by estrogen in cycling rats was analogous to that observed with RCP during the estrous cycle (Muniyappa and Adiga, 1980a).

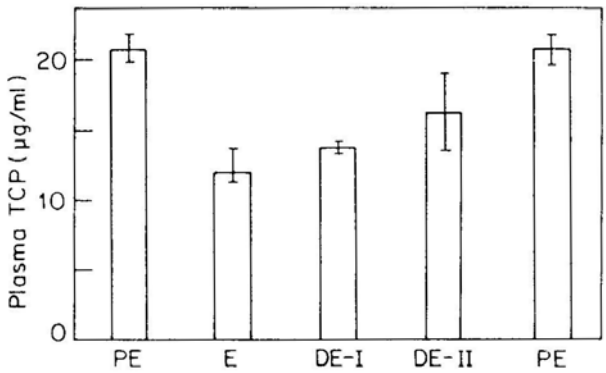

Figure 3. Plasma levels of thiamin carrier protein during estrous cycle. The values represent mean and vertical bars represent S.D. (n = 5). PE, Proestrous; E, estrous; DE-I, diestrous I; DE-II, diestrous II of the cycle.

Induction of thiamin carrier protein in male rats

Since preliminary observations suggested that the sera from estrogen-treated male rats exhibited a clear-cut immunological cross-reactivity with antiserum to purified chicken and rat TCPs, the time-course of accumulation of this protein following estrogenisation was investigated. Figure 4 depicts the kinetics of induction and disappearance of TCP during both primary and secondary stimulation with the steroid (10 mg/kg body

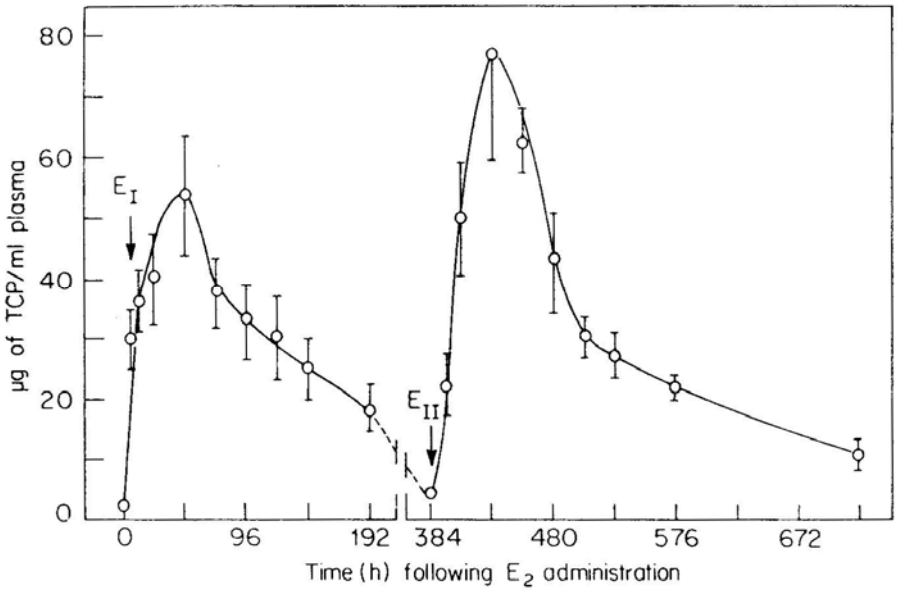

Figure 4. Time course of accumulation of thiamin carrier protein in the plasma after sequential injections (E_I, E_{II}) of estradiol- 17β in propane- 1, 2-diol (10 mg/kg body wt:). The values represent mean and vertical bars represent S.D . (n = 5).

wt.) in 35-day old male rats. The plasma TCP levels measured prior to estrogen dosing represent base-line values which varied from 0·25–2·5 μg/ml depending on the batch of animals and/or feed. Significant increments in TCP levels were observed in the rat as early as 6 h following a single estrogen dose. Thereafter, the level increased gradually with time reaching peak values of approximately 50 μg/ml by about 48 h. This was followed by a steady decline and by 15 days, the levels returned to the base-line values.

Two weeks after steroid withdrawal when the same animals were again challenged with a second but same dose of E_2, the circulating TCP levels exhibited a similar kinetic pattern except that the response was amplified roughly 1·5-fold.

Hormonal dose-dependency of TCP elaboration during primary stimulation

The effect of varying E_2 doses during primary stimulation on the rates of accumulation and disappearance of TCP is depicted in figure 5. It is evident that a hormonal dose as low as 0·1 mg/kg body wt. (<10 μg/rat) elicited a measurable response which progressively increased with the steroid dose. However, the time taken to reach the maximal levels remained unaltered within the ranges of E_2 dose tested (0·1 to 10 mg/kg body wt.). The rates of accumulation as well as the peak levels reached exhibited a dose-dependent phenomenon. Interestingly, however, the rates of clearance of protein

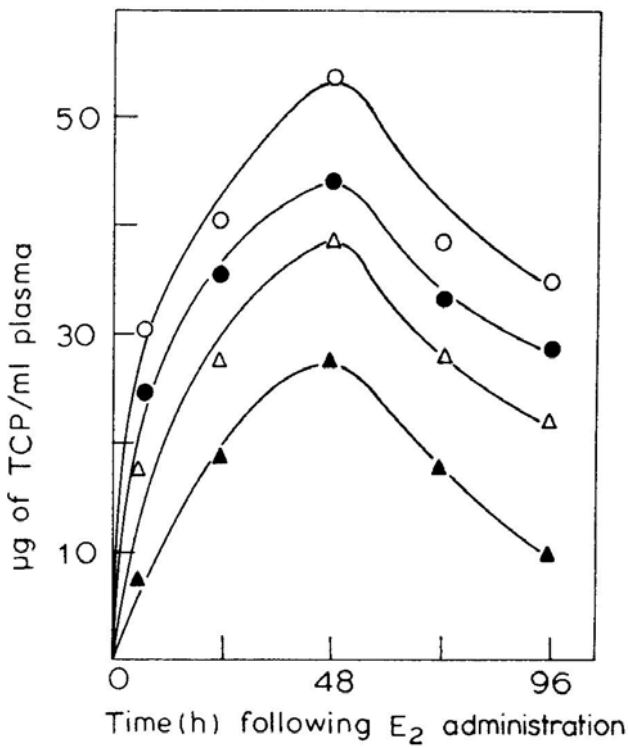

Figure 5. Hormonal dose-dependent response of plasma thiamin carrier protein during primary stimulation. Time course of plasma TCP accumulation with increasing concentrations of estradiol-17β (mg/kg body wt.). (▲), 0·1; (Δ), 1; (●), 5; (○), 10.

during the decay phase decreased with increasing E_2 dosage, *i.e.*, the protein required longer periods to reach the base-line value.

Rate of disappearance of TCP

The kinetics of disappearance of injected [^{125}I]-labelled TCP from the circulation of control, E_2-treated 35-day old male rats and day 14-pregnant rats (figure 6) revealed that the exogenously administered TCP disappeared from circulation in a biphasic manner with an initial rapid phase (A) presumably representing

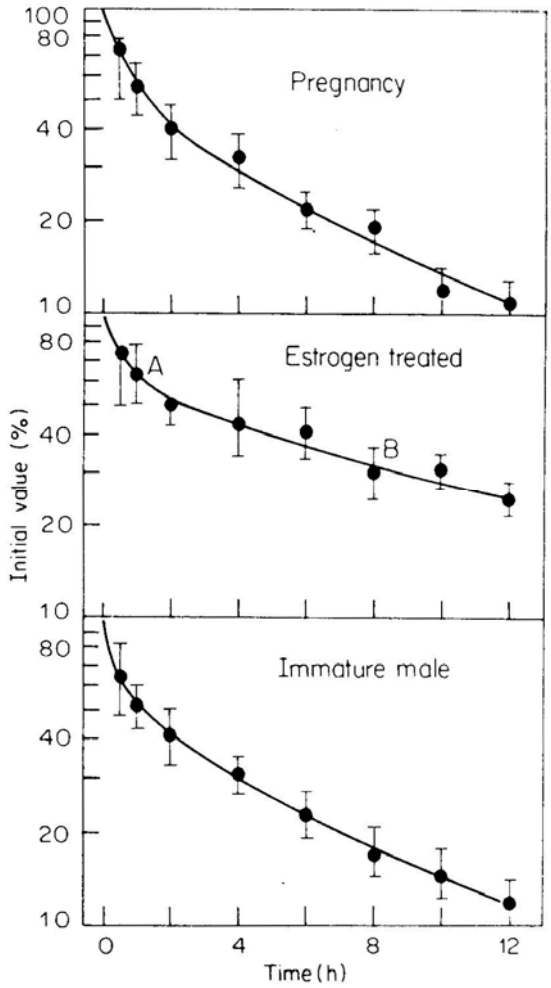

Figure 6. Rate of disappearance of [^{125}I]-labelled thiamin carrier protein from the plasma. Freshly prepared [^{125}I]-labelled TCP (*Ca.* 5×10^5 cpm) was injected into estrogenised or untreated immature male rats or day 14 pregnant rats and at the indicated time intervals blood samples were collected and the plasma prepared were monitored for radioactivity. The values represent mean and the vertical bars represent S.D. ($n = 5$). A, Phase of rapid disappearance; B, phase of catabolism.

dilution of the label with extravascular space and tissue binding followed by a slower phase (B) of disappearance, representing metabolic clearance (McFarlane, 1964). The disappearance during the catabolic phase followed first order kinetics and a least square fit of the data in control, estrogenised and pregnant rats yielded half-lives of 4·5, 8 and 5·5 h respectively. These values obtained from direct measurement of plasma radioactivity could be confirmed by immunoprecipitation of [^{125}I]-labelled TCP in these samples which uniformly corresponded to 80% of the total blood radioactivity. The half-life of TCP following estrogenisation was doubled and this 'stabilising' effect of estrogen, on the protein might account for the relatively lower clearance rates of TCP from circulation following higher steroid dosing (figure 5). During gestation, the half-life of TCP was between the half-life in untreated and estrogen-treated rats. This marginal difference in the catabolic rates in control versus pregnant animals could be due to the interplay of several hormones during gestation.

Influence of cycloheximide

To rule out the possibility that the rapid TCP accumulation in the plasma after hormone administration merely reflected an interference with the altered metabolic clearance rate, especially at lower hormonal doses, evidence for *de novo* synthesis of the specific protein was sought by administering cycloheximide, a powerful translation inhibitor. When this drug was administered intraperitoneally (0·8 mg/kg body wt.) 30 min prior to steroid administration, a drastic decrease in TCP levels was observed when measured at 48 h, *i.e.*, at a time when maximal concentration of TCP was otherwise encountered (table 1). It was evident that induction of the specific protein above the base-line was totally abolished.

Table 1. Effect of the administration of cycloheximide on estrogen-induced TCP accumulation.

Treatment (mg/kg body wt.)	Plasma TCP (μg/ml)
Control*	2·5 ± 0·11
Estrogen (10)	50·0 ± 10
Estrogen (10) + Cycloheximide (0·8)	3·6 ± 0·71

* Pre-injection value.
Cycloheximide (0·8 mg/kg body wt.) was administered intraperitoneally 30 min prior to estradiol-17β injection and plasma TCP levels estimated at 48 h after estrogenisation. Data are expressed as mean ± S.D. (n = 4).

Effect of progesterone and antiestrogens

Progesterone administration did not have any measurable influence on the kinetics of estrogen-induced TCP production in the rodent with regard to the rates of TCP

accumulation and decay (table 2). In other words, the level of plasma TCP was unaffected when progesterone was administered in combination with E_2 during primary stimulation. Furthermore, progesterone when administered singly also failed to induce the protein above the base-line value.

Table 2. Effect of progesterone on the estradiol-stimulated plasma TCP content.

Treatment (mg/kg body wt.)	Plasma TCP (μg/ml)
None (control*)	3·0 ± 0·10
Estrogen (10)	50.0 ± 10
Progesterone (10)	2·4 ± 0·2
Estrogen (10) + Progesterone (10)	48·1 ± 14

* Pre-injection value.
Estradiol-17β + progesterone (10 mg of each/kg body wt.) or progesterone (10mg/kg body wt.) were administered intramuscularly and plasma TCP levels estimated at 48 h after the treatment. Data are expressed as mean ± S.D. (n = 4).

The absolute hormonal specificity of induction of TCP could also be shown by exposing the animals to potent synthetic antiestrogens, *viz.,* En- and Zu-clomiphene citrates, 30 min prior to E_2 treatment. Both the antiestrogens severely curtailed the estrogenic response of TCP and were effective by themselves to elicit only weak estrogen-like activity with regard to TCP production (table 3).

Table 3. Influence of antiestrogens on the estradiol-stimulated plasma TCP content.

Anti-estrogens (mg/kg body wt.)	Plasma TCP (μg/ml)	
	− Estradiol	+ Estradiol
None (control)	2·5 ± 0·11	50·0 ± 10
En-Clomiphene citrate (10)	9·2 ± 3·0	15·0 ± 0·9
Zu-Clomiphene citrate (10)	6·4 ± 2·2	12·3 ± 3·1

En-and Zu-clomiphene citrates (100 mg/kg body wt.) were administered 30 min prior to estradiol-17β administration and plasma TCP was estimated at 48 h after estrogenisation. Data are expressed as mean ± S.D. (n = 4).

Uterine expression of TCP and hormonal modulation

The presence of TCP in the uterine flushings of immature rats primed with E_2 was detected by using the homologous RIA. While the uterine luminal fluid from

estrogenised rats was able to compete with [^{125}I]-labelled TCP for binding to its antiserum in a dose-dependent manner, the corresponding samples from control animals failed to show such competition (figure 7). It would therefore appear that E_2 was capable of inducing uterine synthesis besides hepatic modulation. It is also possible that TCP in the uterine flushings may have been released from the serum into the uterine lumen by a transudative process due to increased capillary permeability brought about E_2 (Daniel, 1971).

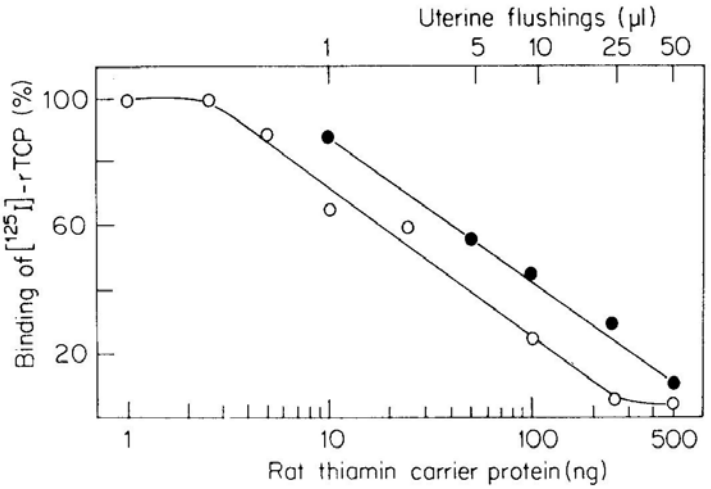

Figure 7. Displacement of [^{125}I]-labelled thiamin carrier protein from its homologous antiserum by uterine flushings. The dilution of the specific anti-serum used was 1:10,000. Details in the text, (○), Rat TCP standard; (●), uterine flushings.

Conclusive proof for the *de novo* synthesis of TCP by the estrogenised rat uterus was obtained from *in vitro* experiments with [^{14}C]-labelled amino acids. Both the tissue and the medium were processed simultaneously to recover both the tissue and secreted TCP. It is evident from table 4, that on treatment of the cytosol fractions with TCP antiserum (in the presence of unlabelled TCP as a carrier), about 0·8–1·0% of the total labelled protein could be immunoprecipitated from the estrogenised rodent uterus whereas only < 0·04 % of the radioactivity could be recovered from the cytosols of the control tissue. The identity of this "*in vitro* synthesised TCP" was confirmed by subjecting the immunoprecipitate to sodium dodecyl sulphate polyacrylamidegel electrophoresis (SDS-PAGE) (Weber and Osborn, 1968). Electrophoresis of the samples both in the presence and in the absence of β-mercaptoethanol was necessary to rule out the possibility of radioactivity being associated with other protein components, like H-chain of IgG which has a molecular weight very similar to that of TCP. The unambiguous evidence for the elaboration of TCP by the uterus upon E_2 stimulation is presented in figure 8. All the radioactivity in the immunoprecipitate could be located in the region of.TCP, while no measurable radioactivity was associated with any protein fraction on the gel in the case of control animals.

Table 4. *In vitro* incorporation of [^{14}C]-amino acids into uterine tissue explants.

Group	Total radioactivity incorporated (cpm × 10^4)	Specific activity (cpm × 10^3/mg protein)	cpm/mg tissue × 10^2	Immunoprecipitable radioactivity (cpm)	TCP (%)
Control	104·7	168·9	52·3	502	0·046
Estrogen-primed	182·8	200·9	91·4	15,125	0·83

The total amount of radioactivity associated with TCP precipitable proteins were measured in uterine cytosols of estrogen-stimulated (5 mg/kg body wt.) and untreated (control) 21-day old female rats. Radioactivity specifically associated with TCP was estimated by immunoprecipitation of cytosols with monospecific rat TCP antiserum.

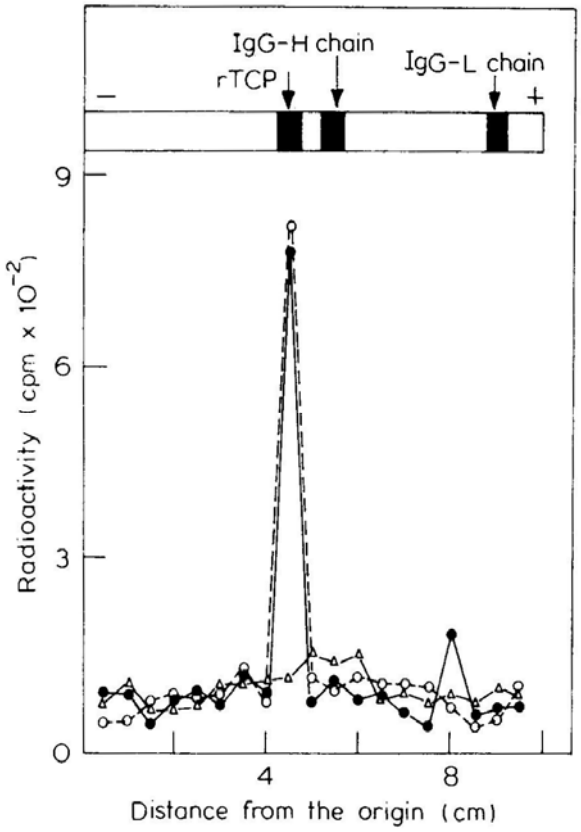

Figure 8. Distribution of radioactivity in the immunoprecipitates by SDS-PAGE. Aliquots (0·5 ml) of [^{14}C]-labelled uterine cytosol preparations were immunoprecipitated with 0·1 ml of rat TCP a/s in the presence of cold rat TCP (10 μg). Details in the text.
(●), SDS-PAGE of immunoprecipitate in absence of β-mercaptoethanol; (○), SDS-PAGE in presence of β-mercaptoethanol; (Δ), SDS-PAGE of immunoprecipitate from uterine cytosol of control animals.

Hormonal modulation of uterine TCP content

Experimental estrogenisation (various doses of E_2 in the range 0·1–5·0 mg E_2/rat) resulted in an increase in the concentration of TCP in the uterine flushings (figure 9) in a manner similar to the dose response observed in the plasma. The content of TCP in the uterine fluid is expressed per unit protein, since the total volume of uterine fluid could not be measured. Such a measurement also avoids possible errors arising from changes in uterine fluid volume following estrogenisation. Beyond a certain concentration of the steroid, the stimulatory effect observed on tissue weight and TCP concentration plateaued of, the increase in protein response being no longer proportional to the steroid dosage. Simultaneous measurement of TCP in both plasma and uterine flushings revealed qualitatively similar patterns. The uterine contents of TCP show an absolute specificity for E_2 (like hepatic synthesis) since clomiphene citrate administrated 30 min prior to E_2-dosing, severely inhibited the hormone specific response (figure 9). Progesterone administered at a dose of 10 mg/kg body wt. was ineffective in inducing this response.

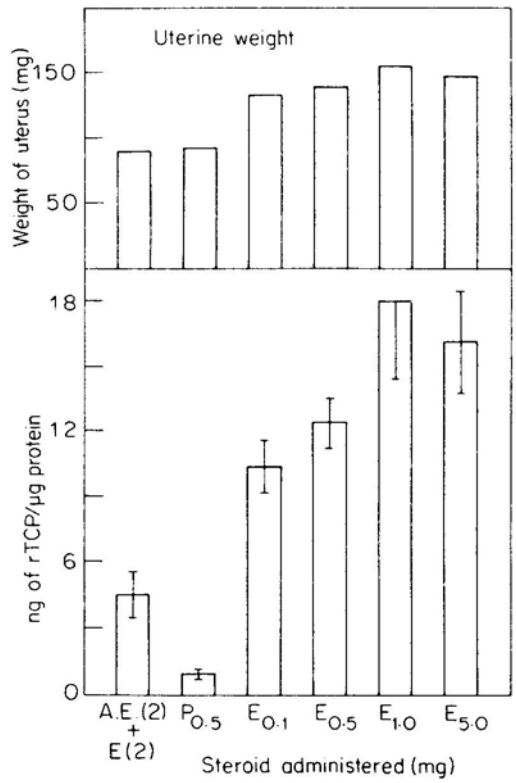

Figure 9. Effect of estrogen on uterine weight and thiamin carrier protein content in the uterine flushings. 21-day old female rats were administered clomiphene citrate, progesterone and estradiol-17β at the indicated concentration in propane-l,2-diol (mg/rat). Details in the text. The values represent mean and vertical bars represent S.D. (n = 5).
AE, The antiestrogen clomiphene citrate.

The modulation of TCP contents in uterine tissue by physiological concentrations of the hormone was evident from the changing patterns of TCP in the lumen during estrous cycle and pregnancy. Figure 10 depicts the inductive response of TCP elicited during 4-day estrous cycle. It is clear that the content of TCP was the highest during proestrous phase when the concentration of plasma estrogen is maximum.

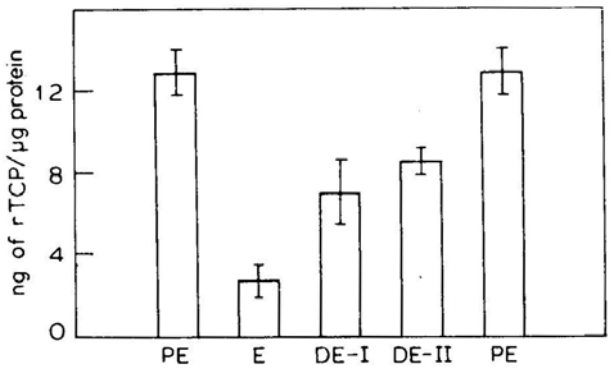

Figure 10. Content of thiamin carrier protein in uterine flushings during estrous cycle. The values represent mean and vertical bars represent S.D. ($n = 4$).
PE, proestrous; E, estrous; DE-I, diestrous I; DE-II, diestrous II of the cycle.

During gestation, the changes of TCP contents in the uterine fluid and the plasma were similar (figure 2). Significant level of TCP was detectable on day 1 of pregnancy. The hormonal signal responsible for this early triggering of TCP synthesis could be the estrogen surge occurring during proestrous phase of the fertile cycle. A similar surge in estrogen (Shaikh and Abraham, 1969) occurring at the time of implantation, *i.e.,* day 4 of pregnancy, might have brought an enhanced response as reflected in a further increase in TCP; thereafter, the protein remained at more or less constant levels (figure 11).

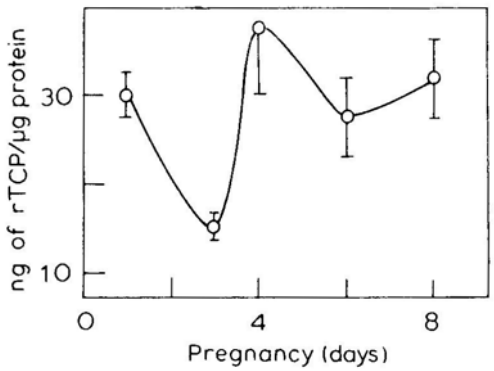

Figure 11. Thiamin carrier protein content in uterine flushings during pregnancy. The values represent mean and vertical bars represent S.D. ($n = 4$).

Discussion

The first indication that estrogen is the predominant hormonal signal modulating circulatory TCP levels during the reproductive phase of the rat (estrous cycle and pregnancy) was the changing pattern of the protein concentration in plasma in concert with parallel alterations in the steroid levels (figures 2 and 3). This suggestion was confirmed by the observation that a single injection of the steroid hormone to male rats acutely induced the vitamin carrier. In analogy with the hormone-induced TCP biogenesis in the chicken liver (Muniyappa and Adiga, 1980b), the kinetics of TCP induction in the rodent included a rapid phase of accumulation of the protein in plasma followed by a declining phase of clearance from circulation during primary stimulation. Similarly a significant amplification of the response on secondary stimulation was observed. However, when the details of the inductive responses in the avian and the rodent species are compared, certain subtle qualitative and quantitative differences became obvious beneath these overall similarities. For example, the amplification of response during secondary stimulation in the rat was only marginal (1·5-fold) compared to that in the chicken system (4–fold). Particularly striking was the absence of a detectable lag period preceding the marked rise in plasma TCP levels during primary stimulation in the rodents as in the chicken (4–5 h). Another contrasting feature was the capacity of the steroid to significantly prolong the circulatory $t_{\frac{1}{2}}$ of TCP in the rat, unlike in the birds (Muniyappa and Adiga, 1980b). The mechanism underlying these differences between the two systems may be related to such factors as species-specific differential organisation and expression of the specific gene concerned and/or the rate and pattern of metabolic clearance of the steroid from the inductive loci at the hepatocyte level.

A similar comparison of the kinetic parameters of E_2-induced TCP synthesis with those governing RCP elaboration in the rodent system (Murty and Adiga, 1982) also reveal several interesting features of similarity and differences. Particularly contrasting is the relatively prolonged (24 h) latent period attendent on RCP induction during primary stimulation. Not withstanding this difference, several gross similarities in the induction of RCP and TCP in the rodent liver were apparent; these included subsequent phases of rapid accumulation and decay in circulation and similar orders of amplification during secondary stimulation. These observations would imply that the mechanisms underlying the inductive responses of the two vitamin carriers in the mammalian and avian liver (Muniyappa and Adiga, 1980b) are very similar.

It was unequivocally demonstrated earlier that specific estrogen receptors capable of rapid nuclear translocation and functional interaction with chromatin acceptor sites are present in the hepatocytes of both male and female rats (Atew *et al.,* 1978) and that low doses (50–100 μg/kg body wt.) of the hormone markedly change the composition and functional facets of the rodent liver (Seal and Doe, 1969). Since within minutes after hormone administration, E_2-receptor complexes attained saturating concentration in the hepatic nuclei, it is quite likely that TCP induction was triggered soon thereafter, since a marked elevation in the plasma TCP levels are encountered within a short-time after E_2-dosing. It is most likely that this difference in the kinetics of induction between TCP and RCP may be related to a greater sensitivity and responsiveness of TCP gene *vis-a-vis* that of RCP to the hormone. It was shown earlier

that different threshold concentrations of E_2-receptor complex were needed at the nuclear chromatin sites to elicit induction of different inducible proteins in the chicken oviduct (Palmiter *et al.,* 1981). The magnification of response observed during secondary stimulation, culminating in greater synthetic capability has been traced to a more pronounced transcription and cytoplasmic stabilisation of the corresponding mRNAs presumably brought about by permanent changes in chromatin in the oviparous species (Jost *et al.,* 1978; Weintraub and Groudine, 1976). The data presented in figure 4 clearly reinforce the earlier finding (Murty and Adiga, 1982) that the typical 'memory effect' observed during secondary stimulation is not confined to oviparous species only, but extends to viviporous mammals. The abolute specificity of E_2 for TCP induction was supported by the finding (table 3) that both the isomers of the antiestrogen completely annulled the inductive response; progesterone alone was ineffective in this respect and also failed to modulate the estrogen influence on *de novo* TCP synthesis.

The relevance of these kinetic details and hormonal specificity of TCP induction in the male rats administered pharmacological doses of the steroid hormone to physiological situation in the adult females became evident when TCP profiles in the plasma were examined during estrous cycle and pregnancy. Since physiological concentrations of the steroid hormone bear a good correlation with the levels of the vitamin carrier protein, clearly the sex steroid is the primary inducer and modulator of the protein. The lack of a strict temporal relationship between plasma estrogen and TCP levels throughout the gestation period, particularly after day 14 might in part be ascribed to the complexity and multiplicity of hormonal interactions prevalent during this particular physiological state. As pregnancy progressed there was a steady rise in TCP content in maternal circulation which attained peak levels around day 13 and declined sharply thereafter (figure 2). The high levels of TCP in maternal circulation observed around days 9–14 clearly coincided with the critical period of rapid embryonic growth attended by marked differentiation and organogenesis (Guha and Janne, 1976). It is conceivable that the precipitous decline in maternal TCP levels after this stage is a reflection of accelerated and efficient sequestration of the vitamin-bound TCP by the fetoplacental unit from the maternal supply line; in other words, a ‘fetal sink’ may be operating which may be responsible for augmented depletion of the vitamin carrier from the maternal circulation at rates higher than its synthesis in the maternal liver. Alternatively, the vitamin carrier may be subject to rapid catabolic clearance from maternal circulation subsequent to the delivery of the vitamin at the placental site for use by the rapidly proliferating fetal tissue. Another possibility may be related to decreased rate of hepatic synthesis consequent to an apparent ‘feed-back’ signal from the fetal compartment.

The rodent uterus, yet another target organ for estrogen, also serves as an additional site of TCP elaboration during the reproductive phase of the animal. While a mere demonstration of the occurrence of TCP in high concentrations in this estrogen-stimulated reproductive tissue does not confer on it the privilege of a biosynthetic site, the data of table 4 and figure 8 clearly show that the uterine tissue can indeed synthesise TCP *in vitro* as revealed by incorporation of [^{14}C]-labelled amino acids into immunoprecipitable TCP. That the same hormonal signal influences independently the *de novo* synthesis of TCP in this extra hepatic tissue is shown by the pattern of

stimulation by physiological concentrations of the steroid hormone. However a significant proportion of TCP in the uterine lumina may originate from the circulation as a transudate, particularly since the transudation of serum proteins (IgG, albumin, transferrin) is also dependent on the hormonal status (Daniel, 1971; Wira and Sandoe, 1980). Estrogenic substances are known to influence not only the capillary permeability but also epithelial cell membrane and their junctional complexes besides affecting the basal membrane between the stroma and the epithelium thus causing selective transport of serum proteins (Beier, 1976). It is therefore conceivable that the increase in uterine luminal TCP content observed during pregnancy/estrous cycle may be attributed atleast in part to *in situ* production, although an appreciable proportion of this protein may be derived from the plasma.

However, neither the relative contribution of uterine tissue nor the functional relevance of the presence of TCP in high concentrations in the luminal fluid under physiological conditions are at present known. It is attractive to visualise that the localised synthesis of TCP may have some relevance in providing a nutritive milieu in the immediate vicinity of the implanting embryo. A number of hormone-stimulated specific proteins, such as E-induced uterine protein (IP) in the rat (Mairesse and Galand, 1982), uteroglobin in the rabbit (Beier, 1974), uteroferrin in the pig (Geisert *et al.,* 1982) and some normal serum constituents are known to be present in luminal fluid in appreciable concentrations and some of these proteins are taken up by the blastocyst for its nutrition by an endocytotic mechanism (Parr and Parr, 1974). Of relevance to this context is the finding that in the chicken oviduct, most of the high-affinity micronutrient binding proteins produced for deposition in the egg white are in the ligand-free apo-protein form (conalbumin, avidin and RCP). It has been hypothesised that their primary physiological role is related to their high capacity to avidly sequester these nutrients that may be released accidently during the development of the embryo which otherwise is rendered highly vulnerable to microbial destruction. Conceivably the uterine luminal TCP might also accomplish a similar function in terms of protective surveillance of the sensitive early embryo.

From the foregoing, it may be concluded that despite pronounced differences between the rat and the chicken in terms of the patterns of their embryonic development, the carrier-mediated thiamin delivery mechanism is conserved during the evolutionary transition. Not only conformational and ligand-binding characteristics of the TCP are retained, but also the hormonal specificity of induction and dual sites of biosynthesis during the reproductive phase of the animals are preserved. In other words, the entire synthetic apparatus including modulatory aspects governing its function are conserved from the oviparous to viviparous species apparently to fulfil a fundamental biological role, *viz.,* a carrier-mediated micronutrient delivery mechanism to ensure embryonic development and hence perpetuation of the species.

Acknowledgements

The financial assistance from Indian Council of Medical Research and the Family Planning Foundation, New Delhi is gratefully acknowledged.

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