Nuclear Synthesis of Egg White Protein Messenger Ribonucleic Acids in Chick Oviduct: Effects of the Anti-Estrogen Tamoxifen on Estrogen-, Progesterone-, and Dexamethasone-Induced Synthesis†

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ABSTRACT: Tamoxifen is a potent anti-estrogen in the chicken oviduct [Sutherland, R., Mester, J., & Baulieu, E. E. (1977) *Nature (London) 267*, 434-435]. Its action on egg white protein gene transcription was studied in isolated nuclei under various hormonal conditions. Injected alone to estrogen-primed and then withdrawn chickens, tamoxifen was unable to trigger gene transcription. After its administration together with or 6 h after diethylstilbestrol (a synthetic estrogen), tamoxifen stopped or suppressed the estrogen-dependent increase of ovalbumin and conalbumin gene transcription. On the contrary, when tamoxifen was given with progesterone or with dexamethasone (a synthetic glucocorticosteroid), two steroids that also increased specific transcription of the ovalbumin and conalbumin genes, there was an amplification effect that lasted up to 24-30 h. These results demonstrate that tamoxifen is active at the transcriptional level when inhibiting estrogen action and when increasing progesterone and dexamethasone effects on protein synthesis [Catelli, M. G., Binart, N., Elkik, F., & Baulieu, E. E. (1980) *Eur. J. Biochem. 107*, 165-172; Le Bouc, Y. (1983) Thèse de 3ème cycle, Université Paris VII]. The complexity of hormone-anti-estrogen interactions on transcriptional efficiency was also illustrated by the greater amplifying effect of tamoxifen on conalbumin than on ovalbumin gene transcription and by the lack of potentiation by the anti-estrogen of dexamethasone-dependent ovomucoid gene transcription. The role of tamoxifen-estrogen receptor complexes in these responses is discussed in view of their differential amount in chromatin in the presence of estrogen or of progesterone.

Tamoxifen (TAM)[1] is a potent nonsteroidal anti-estrogen in the chick oviduct system. It shows no significant estrogenic activity although it binds to the estrogen receptor and although TAM-estrogen receptor complexes accumulate in the nuclear fraction of homogenates (Sutherland et al., 1977a). TAM inhibits the estrogen-mediated effects, particularly the egg white protein synthesis, when injected simultaneously or after estrogen (Sutherland et al., 1977a; Catelli et al., 1980).

While it has been already reported that the induction of ovalbumin and conalbumin synthesis by estrogen is well correlated with an increase of the corresponding gene transcription (Schütz et al., 1977; Swaneck et al., 1979; McKnight & Palmiter, 1979), the action of TAM on the estrogen effect has never been investigated. Only a report from Palmiter et al. (1977) shows that the concentration of cellular ovalbumin messenger ($mRNA_{ov}$) is decreased after TAM injection to estrogen-stimulated birds; however, this effect can be produced by a decrease of either the synthesis or the half-life of the $mRNA_{ov}$.

As opposed to its inhibitory action on estrogenic effects, TAM remarkably enhances the relative rate of synthesis of egg white proteins when injected simultaneously with progesterone (PRO) or dexamethasone (DEX) (Catelli et al., 1980; Binart et al., 1982; Y. Le Bouc and A. Groyer, personal communication). Neither mRNA accumulation nor mRNA synthesis has been measured under these conditions.

Altogether, these observations raise the intriguing question whether TAM acts at the gene transcription level when it antagonizes estrogen action and when it increases protein synthesis when given with PRO or DEX. To answer this question, we measured the transcriptional activities under different hormonal situations. The in vitro transcription in isolated nuclei used in this study allows steady-state measurement of specific gene transcription. Endogenous RNA polymerase II elongates RNA chains initiated in vivo (Swaneck et al., 1979; McKnight & Palmiter, 1979). Newly synthesized structural sequences are not significantly degraded during transcription and RNA purification (McKnight & Palmiter, 1979; Tsai et al., 1980). Therefore, the incorporation of ^{32}P-labeled precursor into RNA in vitro is proportional to the number of RNA polymerase II molecules engaged in the transcription of a given gene in vivo, at the time of killing.

We examined the transcription of egg white protein genes in chicken oviduct cells after in vivo TAM administration and after injection of each hormone (estrogen, PRO, and DEX) alone or in combination with TAM. Early effects on ovalbumin gene transcription by estrogen (Schütz et al., 1977; Swaneck et al., 1979) or by estrogen and PRO (McKnight & Palmiter, 1979; LeMeur et al., 1981) or DEX (Compere et al., 1981) have been already reported. In addition, data have been presented on the transcription of conalbumin gene by McKnight & Palmiter (1979) after estrogen and PRO injection and on ovomucoid gene transcription by Compere

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[1] Abbreviations: DES, diethylstilbestrol; DEX, dexamethasone; DNA, deoxyribonucleic acid; cDNA, single-stranded DNA complementary to mRNA; DNase, deoxyribonuclease; ds, double stranded; E_2b, estradiol benzoate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; ATP, adenosine 5'-triphosphate; GTP, guanosine 5'-triphosphate; CTP, cytidine 5'-triphosphate; UTP, uridine 5'-triphosphate; ppm, part per million; PRO, progesterone; RNA, ribonucleic acid; mRNA, messenger RNA; $mRNA_{ov}$, $mRNA_{con}$, or $mRNA_{omu}$, ovalbumin, conalbumin, or ovomucoid mRNA; RNase, ribonuclease; SET, 1% sodium dodecyl sulfate, 5 mM EDTA, and 10 mM Tris-HCl, pH 7.5; SSC, 0.15 M NaCl and 0.015 M trisodium citrate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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et al. (1981) after estrogen, PRO, and DEX administration. Our data demonstrate that even when acting in opposite directions on either estrogen or PRO and DEX action, TAM always modifies hormone-induced transcription rates of ovalbumin and conalbumin genes. However, TAM does not act uniformly on egg white protein genes: conalbumin PRO- or DEX-stimulated gene transcription was more increased by TAM than ovalbumin gene transcription, whereas DEX-augmented ovomucoid gene transcription was unaffected by the antiestrogen.

Materials and Methods

Chemicals. Diethylstilbestrol (DES) and DEX were given by Roussel-Uclaf (Romainville, France), and TAM was given by ICI (England). PRO was obtained from Industrie Biologique Française. Unlabeled nucleotides were purchased from Sigma Chemical Co. Radioactive [5,6-^{3}H]UTP (43 Ci/mmol) was purchased from Amersham France, and [α-^{32}P]UTP (600 Ci/mmol) was supplied by New England Nuclear (France).

Hormonal Treatment. Chickens of age 5–15 days of the Warren breed received a series of 12–15 intramuscular injections of DES (2.5 mg/day per chicken) in sesame oil (primary stimulation). They were subsequently withdrawn from the hormone for 4 days. The chickens were reinjected with either DES (2.5 mg/day per chicken) or TAM (10 mg/day per chicken or 25 mg/chicken) or a mixture of both compounds. Similarly, PRO (3 mg/day per chicken) or DEX (2 mg/chicken) was injected with or without TAM (10 mg/chicken).

Preparation of Oviduct Nuclei. Chickens were killed by decapitation at the times indicated. The magnum portion of the oviducts was excised and extensively minced with razor blades (six to eight chicken oviducts were pooled per experimental point). The nuclei were prepared essentially as described by Marshall & Burgoyne (1976). Briefly, the oviducts were homogenized in 10 volumes of buffer A (60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.4–7.5. 15 mM β-mercaptoethanol, 0.5 mM spermidine, 0.15 mM spermine) containing 0.34 M sucrose, 2 mM EDTA, and 0.5 mM EGTA. Phenylmethanesulfonyl fluoride was added to 0.2 mM final concentration. Crude nuclei were obtained by 15-min centrifugation at 27000*g* in an HB-4 rotor (Sorvall), through a 1.22 M sucrose cushion in buffer A containing 1 mM EDTA and 0.15 mM EGTA. The nuclear pellet was resuspended in buffer A containing 2.1 M sucrose, 0.1 mM EDTA, and 0.1 mM EGTA. Nuclei were further purified by 40-min centrifugation at 150000*g* in an SW 41 rotor (Spinco) and washed with buffer A containing 0.34 M sucrose. They were resuspended in 40% glycerol, 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, and stored in liquid nitrogen.

Transcription in Nuclei and Isolation of RNA. We have adapted the method of Evans et al. (1981) to our experimental conditions. Nuclei (about 6×10^7) were incubated at 26 °C for 20 min in a reaction mixture (100 μL) containing 0.5 mM ATP, 0.25 mM GTP, 0.25 mM CTP, 7 units/mL nucleosidediphosphate kinase, 2 mM creatine phosphate, 35 μg/mL creatine phosphokinase, 40 mM Tris-HCl, pH 8.3, 0.1 mM EDTA, 2.5 mM dithiothreitol, 5 mM $MgCl_2$, 70 mM KCl, 32% glycerol, and 1 mCi/mL [α-^{32}P]UTP (~600 Ci/mmol).

The reaction mixture was then treated with DNase I (30 μg/mL) in 740 μM $CaCl_2$ for 5 min at 26 °C and deproteinized for 30 min at 45 °C by proteinase K (50 μg/mL final concentration) in 500 μL of SET buffer (1% SDS, 5 mM EDTA, 10 mM Tris–HCl, pH 7.5). After extraction with phenol–chloroform (1/1) in the presence of 0.12 M NaCl and 100 μg of yeast tRNA as carrier, the RNA was precipitated by adding 1/20 volume of a saturated pyrophosphate solution and 1/20 volume of trichloroacetic acid solution (100% w/v). The precipitate was collected on a cellulose acetate filter (0.45 μM, Millipore, HAWP), extensively washed with a trichloroacetic acid (5%) and sodium pyrophosphate (1% w/v) mixture. After a second treatment with DNase I (by incubating the filters in 1 mL of 100 mM Tris-HCl, pH 8.0, 25 mM Hepes, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, containing 10 μg/mL DNase I at 37 °C for 30 min), RNA was eluted by heating at 65 °C with SET. After extraction with phenol–chloroform, the aqueous phase was precipitated with 2.5 volumes of ethanol.

DNA Probes and [^{3}H]RNA Standards. Plasmids pCRI-ov 2.1 (Humphries et al., 1977) and pBR 322-con-1 (Cochet et al., 1979) were generously provided by P. Chambon (Strasbourg, France), and plasmid pom-48 (Lindenmaier et al., 1979) was provided by G. Schütz (Heidelberg, GFR).

Plasmid pCRI-ov 2.1 contains a 1857-nucleotide insert of the ovalbumin cDNA corresponding to 99% of the total messenger sequence without poly(A) tail (O'Hare et al., 1979). In order to prepare standard ovalbumin [^{3}H]RNA, pCRI-ov 2.1 was digested with the restriction enzyme *Hha*I. A major DNA band of 2.4 kb containing the ovalbumin ds cDNA insert was eluted from an agarose gel and used as template for *Escherichia coli* RNA polymerase as described by McKnight & Palmiter (1979).

Plasmid pBR 322-con-1 contains a 2350-nucleotide insert of the ds cDNA conalbumin, which represents 99% of the total messenger sequence without poly(A) tail (Cochet et al., 1979; Jeltsch & Chambon, 1982). The ds cDNA conalbumin insert cut in three fragments was obtained after digestion of the plasmid by Taq restriction endonuclease and gel electrophoresis; [^{3}H]RNA conalbumin messenger copies were obtained as for ovalbumin sequence.

Plasmid pom-48 includes 50% of the ds cDNA sequence of the ovomucoid messenger ($mRNA_{omu}$) (Lindenmaier et al., 1979). Standard [^{3}H]RNA_{omu} was synthesized from the purified *Bam*HI insert as described above.

Preparation of DNA Filters. The DNA quantities deposited on filters were calculated to represent an identical number of DNA sequences, irrespective of the different plasmids lengths. Filters containing either 2.3 μg of pCRI-ov 2.1 or 1 μg of pBR 322-con-1 or 0.7 μg of pom-48 or 0.65 μg of pBR 322 were prepared according to Kafatos et al. (1979) with minor modifications. DNA was denatured in 0.4 M NaOH, 10 min at room temperature, and then cooled and diluted with an equal volume of 2 M ammonium acetate to a final concentration of 20 μg of DNA/mL. DNA drops were spotted on nitrocellulose filters (diameter 0.7 cm), placed on the top of a platform consisting of moist nitrocellulose paper, moist 3MM paper, and paper towels. Before and after the DNA solution was sucked through by capillary action, filters were washed by a drop of 1 M ammonium acetate. After DNA application, the filters were rinsed in 4× SSC (1× SSC contained 0.15 M NaCl and 0.015 M trisodium citrate), air-dried, shaken in 2× Denhardt's solution (1× Denhardt's contained 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) for 2 h, air-dried, and baked under vacuum at 80 °C for 4 h.

Hybridization of RNA to DNA Filters. Hybridizations were performed at 42 °C for 18 h in a 40-μL reaction mixture containing the transcribed [^{32}P]RNA [(1–2) $\times 10^6$ cpm] and the appropriate tritiated internal standard (about 2000 cpm) in 50% formamide and 1.4× SSC, pH 7.0. Before the filters were added, the ds sequences were denatured by heating the solution at 80 °C, and then the solution was ice cooled.

Evaporation of the reaction mixture during hybridization was prevented by adding a layer of paraffin oil. At the end of the incubation, the filters were washed as described by Roop et al. (1978). Briefly, they were incubated at 30 °C twice for 60 min with 1.4× SSC, then twice for 20 min with 0.1× SSC before being treated with pancreatic RNase A (20 μg/mL) in 2× SSC for 1 h at room temperature to digest the nonhybridized sequences. Finally, they were washed with 2× SSC for 15 min at 30 °C.

The RNA on the filters was solubilized by 0.25 mL of 0.04 M NaOH, neutralized by 0.1 mL of 0.1 M CH_3COOH, and then counted in a scintillation mixture containing 75% xylol, 25% Triton X-100, and 4 g/L omnifluor.

Relative rates of transcription, expressed in parts per million (ppm), were obtained from the ratio of the specific ^{32}P bound radioactivity (corrected for unspecific binding to pBR 322 filter and for the efficiency of each hybridization) over total input of [^{32}P]RNA. The efficiency of transcription was calculated from the recovery of standard [^3H]RNA bound to DNA filters and was between 30 and 50%. The inhibition or activation of specific gene transcription was calculated from the measured transcription rates after substraction of withdrawn chicken values. A total of 8–20 separate hybridization measurements were performed on pooled oviduct nuclei for each group of chickens.

Protein Synthesis Measurements. Labeling of oviduct proteins and measurement of relative rates of protein synthesis (ovalbumin and conalbumin) are carried out as described by Palmiter & Wrenn (1971), Palmiter (1972), and Y. Le Bouc and A. Groyer (personal communication).

Results

Transcription of Ovalbumin and Conalbumin Genes during Estrogen Withdrawal. Chickens were primarily stimulated for 2 weeks by daily injection of 2.5 mg of DES. Nine hours after the last injection, relative rates of transcription for ovalbumin and conalbumin genes were ~800 and ~600 ppm, respectively. As early as 48 h after withdrawal, the transcription rates had already decreased to ~140 and ~110 for these genes. After 3 days of withdrawal, ovalbumin and conalbumin transcription rates were the same as those in untreated chickens, that is, 16 ppm for the ovalbumin gene and 45 ppm for the conalbumin gene. These results were in agreement with the data of McKnight & Palmiter (1979), McKnight et al. (1980), and Evans et al. (1981).

The fall of the transcription rates during the 4 days of withdrawal is coincident with other changes. The estrogen receptor concentration found in the nuclear extract decreases to unstimulated level while the estrogen receptor sites increase in the cytosol (Taylor et al., 1980; Palmiter et al., 1981). Similarly, the transcriptionally active conformation of the ovalbumin gene assessed by micrococcal nuclease sensitivity is largely lost (Bloom & Anderson, 1982).

This return to the unstimulated state is not accompanied by either cellular population changes (75–85% remain tubular gland cells) (Shepherd et al., 1980; Bloom & Anderson, 1979, 1982) or an important loss of oviduct tissue: after 4-day DES withdrawal, the magnum portion of the oviduct weighed about 0.5 g compared to 0.8 g before withdrawal.

Comparative experiments carried out with chickens primarily stimulated with estradiol benzoate (E_2b, 1 mg/day per chicken) instead of DES showed that the return to basal transcription rates required at least 3 weeks withdrawal. By that time, oviduct involution is dramatic: each magnum weighs 0.1 g and contains only 15% of tubular gland cells (Palmiter, 1973; Harris et al., 1975). Therefore, the choice of 4-day DES withdrawn oviducts has the advantage of a low transcriptional activity in a relatively abundant tissue composed predominantly of glandular cells.

TAM Inhibition of DES-Induced Transcription. Secondary stimulation by a 2.5-mg injection of DES was followed by a progressive increase in ovalbumin and conalbumin transcription rates during the first 9 h. At this time, relative rates of transcription were 500 ppm for ovalbumin gene and 382 ppm for conalbumin gene. When E_2b (2 mg/chicken) was injected instead of DES, a less marked increase was observed: by 9 h after the injection, relative transcription rates of ovalbumin and conalbumin genes were only 217 and 197 ppm, respectively. Therefore, we chose to inject DES rather than E_2b in the following experiments.

When TAM (10 or 25 mg) was injected simultaneously with DES, a slight diminution of the induced transcription of specific genes was observed as soon as 3 h after the injection (Figure 1). Conalbumin gene transcription was more affected by 25 mg of TAM than ovalbumin gene transcription. After 3 h, the inhibition of specific transcription by TAM became dramatic and was correlated to the TAM dose administered.

In order to confirm the limited inhibition produced at 3 h by TAM injected together with DES, ovalbumin and conalbumin rates of synthesis were measured under the same physiological conditions as the transcription studies (Figure 2). A good correlation was found between protein synthesis and transcriptional activities even if at 3 h the inhibition of ovalbumin synthesis was more pronounced than that of its gene transcription.

When TAM (10 mg/chicken) was administered 6 h after DES (2.5 mg/chicken), a rapid and intense inhibition of the two genes' transcription was observed (Figure 1). An arrest (with TAM, 10 mg) or a decrease (with TAM, 25 mg) of the synthesis of the corresponding proteins was observed (Figure 2).

Enhancement by TAM of PRO-Induced Transcription. (*a*) *Effect of a Single Injection.* In a first set of experiments, withdrawn chickens (six to eight per group) received a single injection of TAM (10 mg/chicken) or PRO (3 mg/chicken) or a combination of both compounds. The relative concentrations of these compounds were chosen such as to elicit a maximal potentiation of the PRO-induced ovalbumin and conalbumin synthesis (Binart et al., 1982). The transcriptional rates of ovalbumin and conalbumin genes were measured in isolated nuclei during the following 24 h.

TAM itself did not stimulate $mRNA_{ov}$ or $mRNA_{con}$ synthesis (Figure 3). Transcription rates remained similar to those determined for 4-day-withdrawn chickens.

When chickens were stimulated by PRO, a rapid and intense increase of specific gene transcription took place. The $mRNA_{ov}$ synthesis increased sharply and reached a maximum 6 h after the injection (Figure 3a). The maximal value (~450 ppm) represented more than 20-fold the control level obtained for the withdrawn chickens. The transcriptional rate decreased after 6 h but remained much greater than the control level during the 24-h period studied.

The time course of $mRNA_{con}$ synthesis followed a similar pattern, but the maximum value reached 6 h after the injection (~200 ppm) corresponded to 4-fold stimulation compared with the control level (45 ppm) (Figure 3b). In contrast with $mRNA_{ov}$ synthesis, $mRNA_{con}$ synthesis dropped to the control level between 12 and 18 h after PRO injection.

When chickens were stimulated by the combined injection of PRO and TAM, the increase of $mRNA_{ov}$ and $mRNA_{con}$ synthesis followed a similar kinetic pattern to that previously

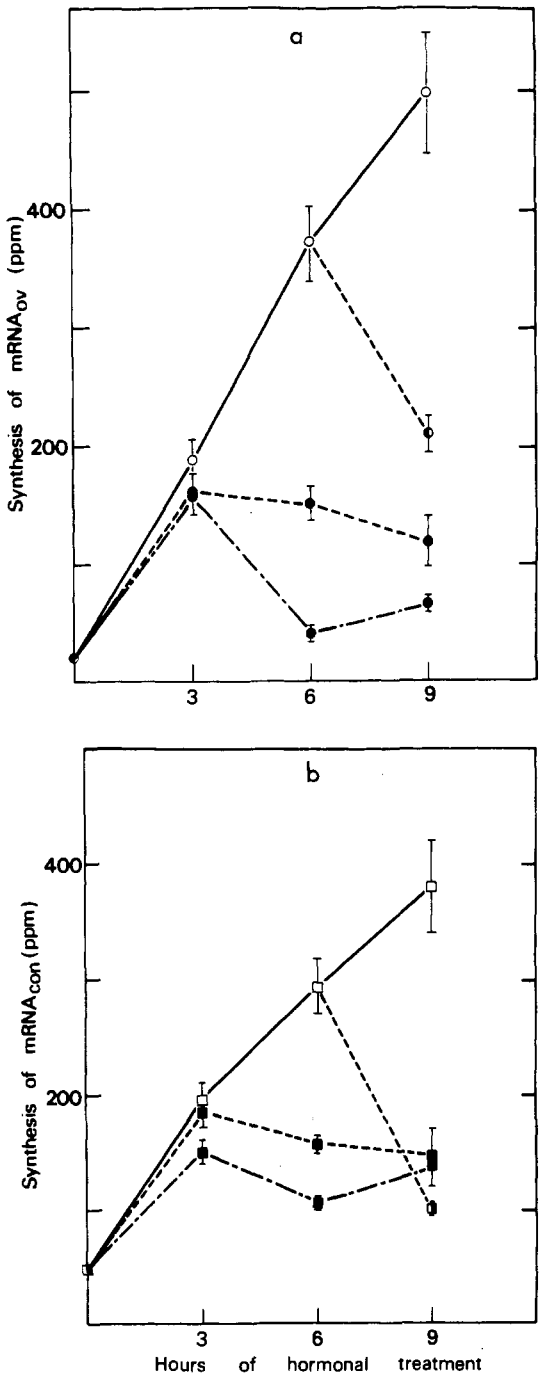

FIGURE 1: Effect of tamoxifen on the diethylstilbestrol-induced ovalbumin and conalbumin gene transcription. Four days after diethylstilbestrol (DES) withdrawal, chickens were injected either with DES [2.5 mg/chicken (○, □)] or with DES and tamoxifen [TAM at 10 mg/chicken (●–●, ■–■) or 25 mg/ chicken (●–·–●, ■–·–■)]. Some of the chickens injected with DES alone were reinjected at 6 h with 10 mg of TAM/chicken (◐, right solid box). Chickens were killed at various time intervals after DES injection. Oviduct nuclei were isolated and allowed to incorporate [α-^{32}P]UTP. The radioactive RNAs were hybridized to DNA probes fixed on nitrocellulose filters. The rates of mRNA$_{ov}$ and mRNA$_{con}$ synthesis were determined by substracting the nonspecific radioactivity bound to the pBR 322 filter from the counts bound to the pCRI-ov 2.1 DNA filter or the pBR 332-con-1 DNA filter and dividing the obtained specific counts by the input radioactivity. Results were corrected for the efficiency of filter hybridizations as measured by [^{3}H]RNA standards. The rates of mRNA synthesis were expressed in parts per million (ppm). The values shown represent the means of 8–20 determinations ± SEM. The input ^{32}P counts in each hybridization was (1–2) × 10^6 cpm. (a) mRNA$_{ov}$ synthesis (○, ●, ◐); (b) mRNA$_{con}$ synthesis (□, ■, right solid box).

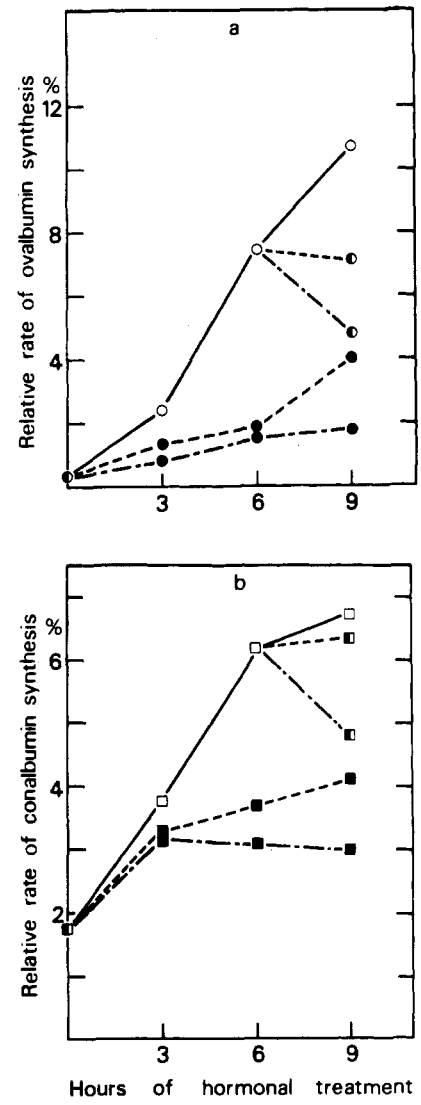

FIGURE 2: Effect of tamoxifen on diethylstilbestrol-induced synthesis of ovalbumin and conalbumin. Chickens were given a secondary injection of diethylstilbestrol [2.5 mg/chicken (○, □)] or DES with tamoxifen [TAM at 10 mg/chicken (●–●, ■–■) or 25 mg/chicken (●–·–●, ■–·–■)]. Several chickens injected with DES received TAM at 6 h [10 mg/chicken (left solid circle on dashed line, left solid box on dashed line) or 25 mg/chicken (left solid circle on dotted dashed line, left solid box on dotted dashed line)]. At the indicated times, chickens were killed and magnum pieces incubated with a radioactive amino acids mixture and then homogenized and centrifuged at 105000g; aliquots of the cytosol were immunoprecipitated with the anti-ovalbumin or anti-conalbumin serum and the relative rates of synthesis determined (Palmiter et al., 1971). (a) Ovalbumin synthesis (○, ●, ◐); (b) conalbumin synthesis (□, ■, left solid box).

observed with PRO alone (Figure 3). However, 6 h after the injection, TAM enhanced by 40 and 70% respectively the PRO-induced mRNA$_{ov}$ and mRNA$_{con}$ synthesis. Although this potentiation was maintained for conalbumin gene transcription at all the times studied, it disappeared between 9 and 18 h for ovalbumin gene transcription. In both cases, a significant second increase of transcription was observed at 18 h after hormone administration.

(*b*) *Effect of Long-Term Secondary Stimulation by TAM, PRO, or PRO and TAM.* To study transcriptional changes occurring after prolonged secondary stimulation, chickens received for 3 or 4 days daily injections of TAM (10 mg/day per chicken) or PRO (3 mg/day per chicken) or both PRO and TAM. They were killed 6 h after the last injection (Figure 4). This time was chosen in order to obtain maximal transcription rates (Figure 3).

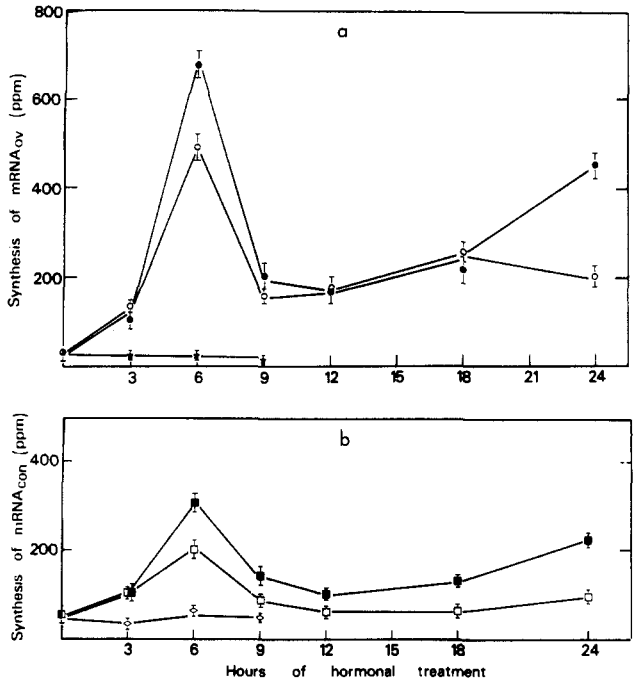

FIGURE 3: Time course of $mRNA_{ov}$ and $mRNA_{con}$ synthesis after secondary stimulation by progesterone, tamoxifen, and progesterone + tamoxifen. Chickens were secondarily injected either with progesterone [3 mg/chicken (○, □)], with tamoxifen [10 mg/chicken (*, ◊)] or with PRO + TAM (●, ■). They were killed at various time intervals after the secondary hormonal treatment, and the transcription rates were measured in oviduct nuclei as described in the legend to Figure 1. (a) $mRNA_{ov}$ synthesis (○, *, ●); (b) $mRNA_{con}$ synthesis (□, ◊, ■).

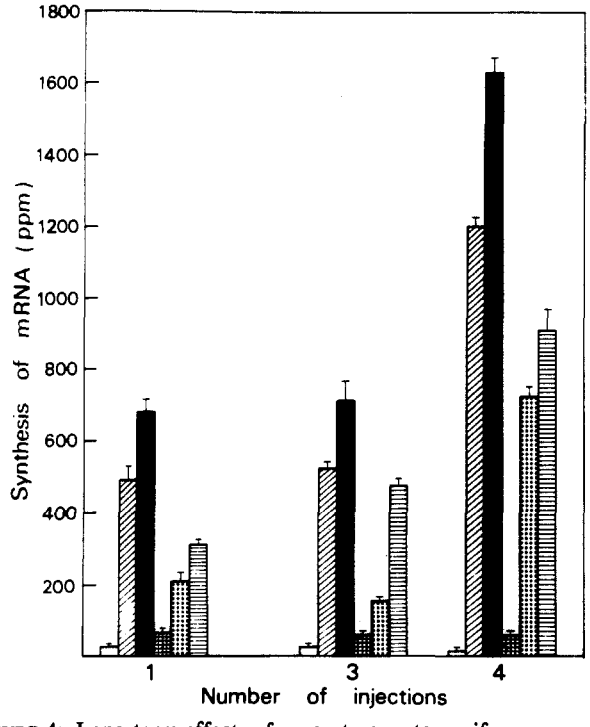

FIGURE 4: Long-term effects of progesterone, tamoxifen, or progesterone + tamoxifen on $mRNA_{ov}$ and $mRNA_{con}$ synthesis. After 4-day DES withdrawal, chickens received one, three, or four injections (one injection per day) of progesterone (3 mg/day per chicken), tamoxifen (10 mg/day per chicken), or PRO + TAM. Chickens were killed 6 h after the last injection. The syntheses of $mRNA_{ov}$ and $mRNA_{con}$ were measured in oviduct nuclei as described in the legend to Figure 1. For $mRNA_{ov}$, stimulation by TAM (open bars), PRO (diagonally striped bars), and PRO + TAM (solid bars). For $mRNA_{con}$, stimulation by TAM (checked bars), PRO (dotted bars), and PRO + TAM (horizontally striped bars).

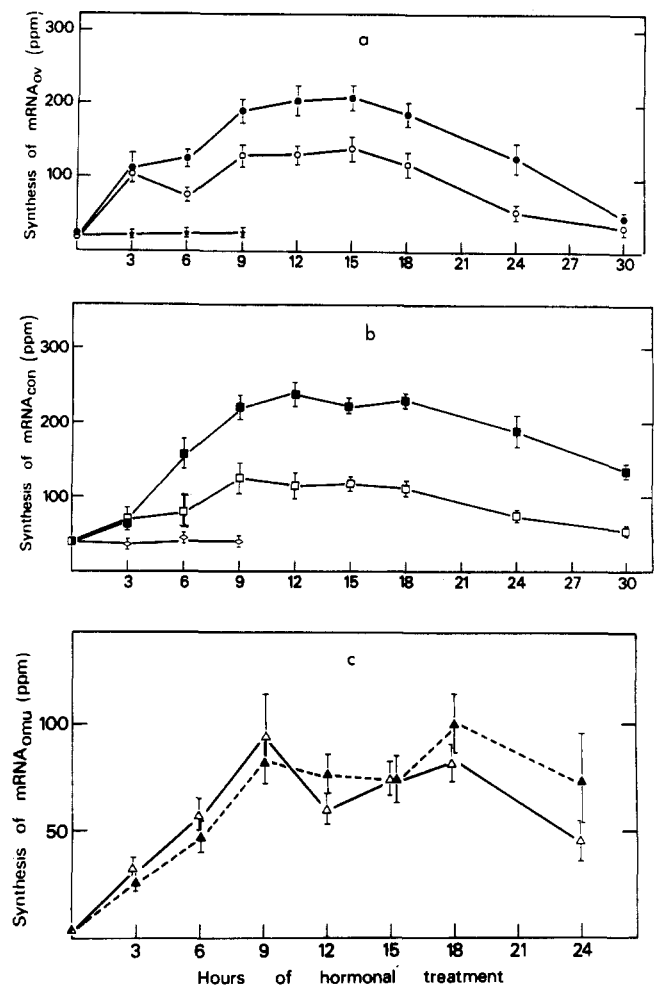

FIGURE 5: Time course of $mRNA_{ov}$, $mRNA_{con}$, $mRNA_{omu}$ synthesis after secondary stimulation by dexamethasone, tamoxifen, and dexamethasone + tamoxifen. Chickens were secondarily injected either with dexamethasone [2 mg/chicken (○, □, △)], with tamoxifen [10 mg/chicken (*, ◊)], or with DEX + TAM (●, ■, ▲). Chickens were killed at the times indicated after secondary injection. The $mRNA_{ov}$, $mRNA_{con}$, and $mRNA_{omu}$ syntheses were measured in oviduct nuclei as described in the legend to Figure 1. (a) $mRNA_{ov}$ synthesis (○, *, ●); (b) $mRNA_{con}$ synthesis (□, ◊, ■); (c) $mRNA_{omu}$ synthesis (△, ▲).

TAM alone did not induce any significant change in the transcriptional rates of ovalbumin and conalbumin genes, relative to the withdrawn chickens' level. After four daily injections of PRO, $mRNA_{ov}$ synthesis increased 2.4-fold and $mRNA_{con}$ synthesis 3.4-fold, relative to the values obtained by 6 h after a single injection. TAM, given in association with PRO, enhanced steroid-induced ovalbumin and conalbumin gene transcription. At 4 days, the transcription rates were about 1.3 times those obtained with PRO alone.

Enhancement by TAM of DEX-Induced Transcription. The effect of one DEX injection (2 mg/chicken) on transcription rates of ovalbumin, conalbumin, and ovomucoid genes was investigated. This dose was chosen because it produced maximal induction of ovalbumin synthesis when measured 6 h after DEX injection (Le Bouc, 1983).

The major increment in the rate of $mRNA_{ov}$ synthesis had occurred at 3 h, but this rate continued to increase slightly until 9 h and then remained almost constant from 9 to 18 h (Figure 5a). The transcription rate dropped back to basal level between 18 and 30 h.

Conalbumin gene transcription displayed only a weak increase at 6 h and then increased significantly until 9 h (Figure 5b). Between 9 and 15 h, a plateau of transcriptional activity

was observed; then, the transcription rate decreased, and the basal value was reached at 30 h. Maximal transcription rates for ovalbumin and conalbumin genes after DEX stimulation were 130 and 120 ppm, respectively.

The induction by DEX of the ovomucoid gene transcription was gradual until 9 h; then, a plateau of transcriptional activity (80 ppm) was recorded between 9 and 18 h, before a decrease at 24 h (Figure 5c). Since the plasmid used for measurements contains only 50% of the ovomucoid messenger sequence, it follows that the transcriptional efficiency of this gene after DEX treatment was of the same order of magnitude as that of ovalbumin and conalbumin genes.

When TAM (10 mg/chicken) was simultaneously injected with DEX to 4-day-withdrawn chickens, an increase in transcriptional activities of ovalbumin and conalbumin genes over the effect of DEX alone was observed (Figure 5b). From 9 to 15 h, conalbumin gene transcription rates were enhanced by 100–160% compared to those obtained with DEX alone. A less important increase (from 40 to 70%) was observed with ovalbumin gene transcription. At longer times, when DEX stimulation decreased, the TAM effect was relatively larger. By 24 h, for instance, $mRNA_{ov}$ and $mRNA_{con}$ syntheses were increased by about 200 and 300%, respectively.

Furthermore, we measured the $mRNA_{omu}$ synthesis after DEX + TAM compared to DEX alone (Figure 5c). The presence of TAM produced no significant enhancement of the transcriptional activity of this gene.

Discussion

We have studied transcription of egg white protein specific genes after administration of the anti-estrogen TAM alone, after administration of DES, PRO, and DEX alone, and after combination of each of these hormones with TAM.

Effect of TAM Alone. TAM did not promote any increased transcription of the ovalbumin and conalbumin genes in the DES-withdrawn chickens, in agreement with a previous report on protein synthesis (Catelli et al., 1980). Therefore, the small increase of conalbumin synthesis after TAM administration observed by Binart et al. (1982) and by Y. Le Bouc and A. Groyer (personal communication) may be due to posttranscriptional events.

Effects of Estrogen, Progesterone, and Glucocorticosteroid Alone. After *estrogen* injection (DES or E_2b), the transcription rates of ovalbumin and conalbumin genes increased progressively during the 9-h period studied. This was also found by Swaneck et al. (1979) for ovalbumin gene transcription, with higher rates due to the fact that their chickens were withdrawn for a shorter period (60 h vs. 96 h in this work) (Taylor et al., 1980).

After *progesterone* (PRO) injection, an acute increase of ovalbumin and conalbumin gene transcription was observed with a maximal value by 6 h. Our results were compatible with those of McKnight & Palmiter (1979) at the times tested by these authors. Interestingly, at the time of maximal transcription, there is a profound decrease of cytosol PRO receptor concentration not matched by an equimolecular increase of binding sites in the nuclear fraction (Mester & Baulieu, 1977b). Early work by Milgrom et al. (1973) and more recent observations (Mockus & Horwitz, 1983; Horwitz et al., 1983) show that the increase of PRO receptor in the nuclear fraction and its decrease in the cytosol are stoichiometric only for a very brief period of time and suggest that a very rapid nuclear processing of receptor takes place as indicated by loss of binding activity. The larger increase of transcription recorded after repeated stimulation by PRO as compared to a single injection is compatible with the secondary increase of PRO receptor observed by Sutherland et al. (1977b) by 24 h after PRO stimulation.

The maximal response to PRO stimulation at 6 h was followed, after a transitory fall, by a second but weaker increase at 10–18 h, which coincided with the PRO-induced appearance of estrogen binding activity in the 13–14S chromatin fraction as described by Lebeau et al. (1982). The second increase of transcription may also be due to reavailability of PRO receptor after the first period of receptor inactivation.

After administration of the synthetic *glucocorticosteroid* DEX, $mRNA_{ov}$ and $mRNA_{con}$ synthesis increased and remained high between 3 and 18 h. Our transcription rates at 8 h were lower than those reported by Compere et al. (1981) with cultures explants, as expected since they used less withdrawn oviducts.

DEX exhibited lower transcriptional efficiency than PRO under comparable conditions; this was also observed by Compere et al. (1981). In the present work, we checked with radioactive compounds that the differential efficiency of the two hormones was not due to a different time-course release of DEX and PRO from the injection site (thigh) into the circulation system (data not shown). Besides, equivalence in the binding of DEX and PRO to plasma transcortin is expected since the two hormones have the same affinity for this protein (A. Groyer and Y. Le Bouc, personal communication).

As a general rule, transcription results are in good correlation with the accumulation of $mRNA_{ov}$ and $mRNA_{con}$ measured after administration of the three hormones (Mulvihill & Palmiter, 1977, 1980; Hager et al., 1980). Since a direct proportionality between ovalbumin and conalbumin synthesis and cellular concentrations of the corresponding mRNAs has been usually observed, our results favor the hypothesis that increased specific protein synthesis under hormonal action depends on increased transcription rates of specific genes.

Effect of TAM in Combination with Various Hormones. When TAM was injected simultaneously *with estrogen* (DES), it produced an inhibition of the DES-dependent gene activation, and the decrease of mRNA synthesis became dramatic after 3 h. When TAM was injected 6 h after DES, the inhibiting effect on transcription was stronger than what was observed after simultaneous administration. Since DES has a much greater affinity for the estrogen receptor than TAM (Mulvihill & Palmiter, 1977; Mester et al., 1979), it is conceivable that the agonist activity of the former is relatively favored over that of the latter in the first hours following the simultaneous injection. Six hours later, while the DES concentration in the circulation is greatly reduced, TAM concentration remains high (Binart et al., 1979; therefore, when TAM was injected 6 h after DES, the competition effect was immediately expressed by the arrest of estrogen-induced transcription. These results agree with the hypothesis that the anti-estrogen effect of TAM results from the formation of an inactive TAM-estrogen receptor complex. Indeed, when TAM alone or TAM and estrogen are administered together, no estrogen receptor is found in the 13–14S fraction of the solubilized chromatin, although it is present in this fraction after administration of estrogen alone (Lebeau et al., 1982).

When TAM was administered *in combination with progesterone or glucocorticosteroid*, it enhanced the transcription rates of ovalbumin and conalbumin genes already increased by PRO or DEX alone. For some unknown reason, it appeared that the conalbumin gene transcription was more increased by TAM than the ovalbumin gene transcription, irrespective of the hormone (PRO or DEX) used for stimulation. In

addition, TAM increased the effect of DEX more than that of PRO. Ovomucoid gene transcription, studied only after DEX stimulation, was not modified by TAM.

A good correlation was found between our ovalbumin and conalbumin gene transcription measurements and specific protein synthesis reported by Catelli et al. (1980), Binart et al. (1982), and Y. Le Bouc and A. Groyer (personal communication) after combined injection of TAM and various hormones. The increase of PRO- or DEX-induced transcription could arise from an increased accumulation of PRO or glucocorticosteroid receptor, but we do not have any evidence for it; actually, preliminary experiments have indicated no difference in the PRO receptor content of the oviduct 20 h after PRO or PRO + TAM.

On the other hand, it has been reported that PRO by 18 h after administration increases the content of estrogen receptor in the 13–14S fraction of solubilized chromatin (Lebeau et al., 1982). TAM does not decrease these estrogen binding sites specifically attached to the chromatin and even increases them slightly (20–30% by 18 h). No similar work has been done after DEX injection. In fact, it may be that the estrogen receptors present in the chromatin due to PRO action and stabilized by TAM are responsible for the recorded effects. An alternative hypothesis is that TAM may stabilize the active chromatin conformation around ovalbumin and conalbumin genes previously induced by PRO or DEX.

In conclusion, whatever are the involved mechanisms, the changes provoked by TAM to inhibit estrogen action or increase PRO or DEX stimulation of ovalbumin and conalbumin synthesis always seem to involve an effect at the transcriptional level. This study shows differential effects of each hormone (or anti-hormone) on the transcription units responsible for the synthesis of the various egg white proteins and underlines that unexpected transcriptional effects of anti-hormones may be discovered, depending on the hormonal milieu. Besides the gene specificity observed in the chick oviduct system, whether there are organ and species differences in the anti-estrogen interference with cellular steroid hormones responses remains to be elucidated.

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The Glu(B13) Carboxylates of the Insulin Hexamer Form a Cage for Cd^{2+} and Ca^{2+} Ions†

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ABSTRACT: Substitution of Cd^{2+} for Zn^{2+} yields a hexameric insulin species containing 3 mol of metal ion per hexamer. The Cd^{2+} binding loci consist of the two His(B10) sites and a new site involving the Glu(B13) residues located at the center of the hexamer [Sudmeier, J. L., Bell, S. J., Storm, M. C., & Dunn, M. F. (1981) *Science (Washington, D.C.) 212*, 560–562]. Substitution of Co^{2+} or Co^{3+} for Zn^{2+} gives hexamers containing 2 mol of metal per hexamer. Insulin solutions to which both Cd^{2+} and Co^{2+} have been added in a ratio of 6:2:1 [In]:[Co^{2+}]:[Cd^{2+}] followed by oxidation to the exchange-inert Co^{3+} state yield stable hybrid species containing both Co^{3+} and Cd^{2+} with a composition of $(In)_6(Co^{3+})_2Cd^{2+}$. The kinetics of the reaction of 2,2′,2″-terpyridine (terpy) with the exchange-labile $(In)_6(Cd^{2+})_2$ and $(In)_6(Co^{2+})_2$ derivatives are biphasic and involve the rapid formation of an intermediate with coordination of one terpy molecule to each protein-bound metal ion; then, in a rate-limiting step the terpy-coordinated metal ion dissociates from the protein, and a second molecule of terpy binds to the metal ion to form a bis complex. Reaction of the exchange-inert Co^{3+} ions of $(In)_6(Co^{3+})_2$ with terpy is a slow apparent first-order process ($t_{1/2}$ = 13.1 h). In contrast to the kinetic behavior of $(In)_6(Co^{2+})_2$ and $(In)_6(Cd^{2+})_2$, the Cd^{2+} ions bound to the hybrid $(In)_6(Co^{3+})_2Cd^{2+}$ react quite slowly with terpy ($t_{1/2}$ = 1 h at pH 8.0). We postulate that Cd^{2+} is caged within the central cavity of this hybrid hexamer at the Glu(B13) site and that the rate of reaction with terpy is determined by the slow rate of escape of Cd^{2+} from this cage. Competition studies indicate that Ca^{2+} and Cd^{2+} compete for the Glu(B13) site. Equilibrium binding studies using $^{45}Ca^{2+}$ substantiate the presence of a single high-affinity calcium binding site with k_D = 83 μM, which we propose involves the Glu(B13) carboxylates.

The elegant X-ray crystallographic work of Hodgkins, Dodson, and their co-workers [see Blundell et al. (1972) and Emdin et al. (1980)] has shown the zinc–insulin hexamer $(In)_6(Zn^{2+})_2$[1] to be a torus-shaped molecule (Chart I) with the two zinc ions separated by 17 Å and located on the 3-fold symmetry axis in a solvent-filled cavity that runs through the hexamer. The two zincs reside in octahedral ligand fields each coordinated by three histidyl imidazolyl groups [the His(B10) residues] and by three water molecules.

Previous work from this laboratory (Dunn et al., 1980) has shown that, in agreement with earlier literature (Fredericq, 1954; Tanford & Epstein, 1974a,b; Brill & Venable, 1967, 1968), the two high-affinity zinc sites observed in solution have kinetic and thermodynamic properties consistent with assignment of these sites to the crystallographically identified His(B10) sites. Our kinetic and thermodynamic evidence indicate that the assembly of the $(In)_6(Zn^{2+})_2$ species is a highly cooperative process (Dunn et al., 1980). Fourier-transform ^{113}Cd NMR experiments with $^{113}Cd^{2+}$-substituted insulin (Sudmeier et al., 1981) led to the discovery that, in concentrated solutions, Cd^{2+} substitution for Zn^{2+} results in the formation of a cadmium–insulin species presumed to be $(In)_6(Cd^{2+})_2Cd^{2+}$. The two classes of high-affinity Cd^{2+} sites were identified as (1) the two His(B10) sites and (2) a new site proposed to involve the Glu(B13) carboxylates located at the center of the hexamer (viz., Chart I). Metal ion substitution experiments with the $^{113}Cd^{2+}$-substituted species indicated that both $(In)_6(Cd^{2+})_2$ and $(In)_6(Zn^{2+})_2$ are calcium binding proteins and that Ca^{2+} can displace Cd^{2+} from the Glu(B13) site but not from the His(B10) sites (Sudmeier et al., 1981).

Crystallographic studies (Blundell et al., 1972; Emdin et al., 1980) also indicate the Glu(B13) residues form metal ion binding sites. In their review, Blundell et al. (1972) make brief mention of a $(In)_6(Cd^{2+})_2Cd^{2+}$ hexamer with cadmium bound both to the His(B10) sites and to the Glu(B13) site. Emdin

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[1] Abbreviations: In or P, insulin; $(In)_6(M^{x+})_m(M^{y+})_n$, metal-coordinated insulin hexamers where x and y designate the metal ion valencies and m and n the stoichiometries of metal ions bound to the His(B10) and Glu(B13) sites, respectively; terpy, 2,2′,2″-terpyridine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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