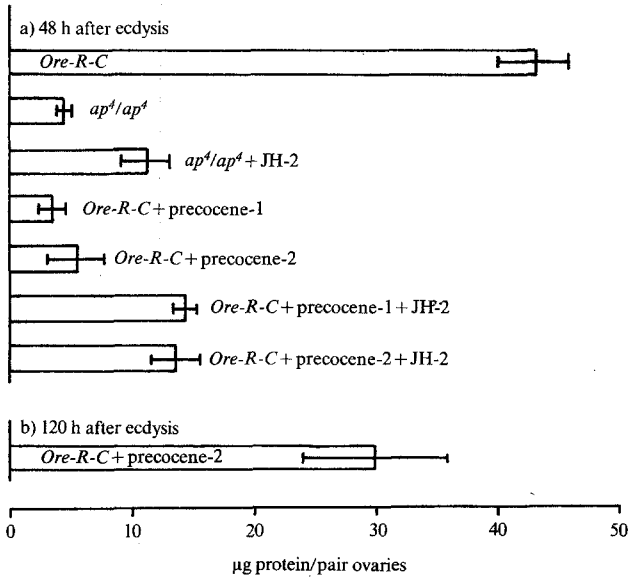

Fig. 2. Protein content of *Drosophila melanogaster* ovaries. Lowry test[13] with bovine serum albumin standard. 0.2 µl acetone, sometimes containing 4 µg precocene-1 or -2 (CalBiochem) and/or 0.5 µg JH-2, was applied to the abdomen of a female 0–4 h after adult ecdysis. Samples were taken 48 or 120 h after treatment. Mean protein data based on at least 5 determinations on 10 pooled ovaries. Error bars indicate 2 SD.

(figure 1, d). Quantitatively similar rescue occurred when juvenile hormone was administered to ap^4 homozygotes (figure 1, e). Thus, the major effects of precocene on vitellogenesis apparently stem from interference with the normal functioning of the juvenile hormone system.

Total ovarian protein was measured in all of the experimental groups. This quantitative data on protein content (figure 2, a) supports the conclusions already drawn from inspection of ovaries (figure 1).

Several laboratories have reported that precocene-treatment produces abnormal morphology or function of the corpora allata in *Oncopeltis*[8,9], *Locusta*[10,11] and *Periplaneta*[12]. Ultrastructural evidence of massive autophagy and cellular degradation or collapse suggested that the chemical allatectomy is irreversible[9,11]. Our results with *Drosophila* differ; the flies can significantly recover from precocene effects. *Ore-R-C* females, treated with precocene at ecdysis, show very little vitellogenesis at 2 days (figures 1, b and 2, a) but significant vitellogenesis at 6 days (figures 1, f and 2, b). In addition, the fact that precocene-treated flies do not show the premature death characteristic of adult ap^4 homozygotes argues for reversibility of the precocene effects.

Our demonstration that precocene inhibits vitellogenesis in *Drosophila* allows many new experimental approaches to studies of vitellogenesis and juvenile hormone action in a defined genetic context. In addition, our demonstration that precocene inhibition is reversible suggests that the hypothesis of precocene action by its selective cytotoxic attack on the corpora allata deserves further critical investigation, at least for *Drosophila*.

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Intracellular distribution of estrogen receptors: A function of preparation[1]

P.M. Martin and P.J. Sheridan

Laboratoire Récepteurs Hormonaux, Institut Paoli Calmettes, F-13009 Marseille (France), and Department of Anatomy, The University of Texas, Health Science Center at San Antonio, San Antonio (Texas 78284, USA), 27 July 1979

Summary. The intracellular distribution of unbound estrogen receptor was estimated using nonaqueous and aqueous isolation of nuclei and autoradiography. The estimated amount of nuclear receptor varied greatly with the procedure used and thus caution is urged in approximating the in vivo intracellular distribution of receptors.

The most widely accepted model for the mechanism of action of steroids was developed from a plethora of studies concerning the uptake and retention of estradiol by the female reproductive tract. According to this model, estradiol enters the uterine cells, binds to a specific high-affinity cytoplasmic receptor and then is translocated to the nucleus by a temperature-sensitive process[2,3]. However, there are data in the literature which question this model[4] and which suggest that the amount of unbound receptor found in a given preparation might be more a function of the isolation procedure rather than a true representation of the in vivo intracellular distribution of receptors[5–8]. In the experiments described below, different methods used to isolate nuclei were compared in 2 different tumor systems.

Methods. Cells from a breast tumor line, designated MCF-7 (kindly supplied by Dr W. McGuire, Department of Medicine, The University of Texas Health Science Center at San Antonio) and cells obtained from the pleural cavity of a patient with advanced breast cancer were used in these experiments. Labeled estradiol was purchased from Amersham/Searle (106 Ci/mM) and unlabeled estradiol was purchased from Sigma.

Standard preparation of nuclei: Cells were incubated in MEM (Grant Island Biological Co.) for various periods of time in the presence of various concentrations of steroids [^{3}H-estradiol ($1-5\times10^{-9}$ M) or ^{3}H-estradiol ($1-5\times10^{-9}$ M) + unlabeled estradiol ($1-5\times10^{-7}$M)]. The cells were then washed in TRIS buffer (50 mM TRIS, 5 mM Mg,

25 mM KCl, 10% glycerol, 0.01% monthioglycerol, pH 7.4 at 0 °C) or phosphate buffer (0.1 mM PO_4, 5 mM Mg, 25 mM KCl, 10% glycerol, 0.01% monthioglycerol, pH 7.4 at 0 °C), homogenized and spun at 800 × g for 10 min. The resultant pellet was defined as the crude nuclear pellet. The crude supernatant was centrifuged at 105,000 × g for 1 h and the resultant supernatant decanted off and used as the cytosol. The nuclei were extracted with 0.4 M KCl and aliquots of both the nuclear extract and the cytosol were then assayed using Sephadex LH-20 columns[9].

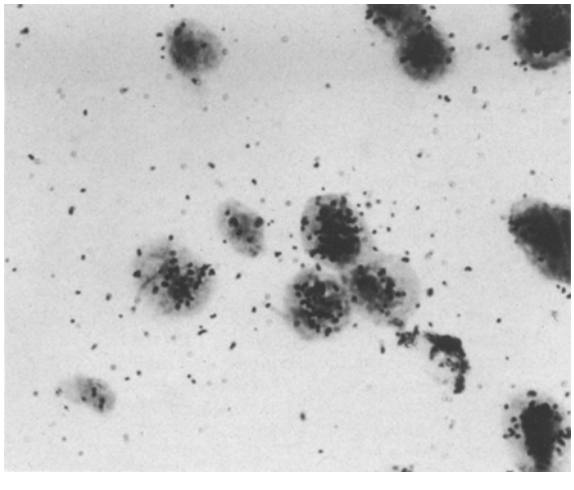
Nonaqueous preparation of nuclei: A nonaqueous isolation of nuclei was accomplished using 100% glycerol[10]. Briefly, after incubation and washing, the cell pellets were frozen, freeze-dried and then homogenized in 100% glycerol at 0 °C. The homogenates were then centrifuged through an additional volume of 100% glycerol at 100,000 × g for 1 h at 4 °C. The pellets were then extracted in 0.4 M KCl and assayed using Sephadex LH-20 columns.

Sucrose preparation of nuclei: After a crude nuclear pellet was prepared as indicated above, the pellet was resuspended in one of the above buffers containing 0.5 M sucrose and then centrifuged at 6000 × g for 10 min. The supernatants were decanted off, the pellets resuspended in 2.0 M sucrose and centrifuged at 25,000 × g for 20 min. The resultant pellet was then extracted with 0.4 M KCl and assayed using Sephadex LH-20 columns.

Table 1. Intracellular distribution of estrogen receptors*

Method used	Incubation temperature	Cytoplasm	Nucleus
Standard	0 °C	2.2 (Exp. I)	0.8
		1.5 (Exp. II)	0.37
		4.3 (Exp. III)	1.81
Nonaqueous	0 °C	1.2 (Exp. I)	1.8
		0.7 (Exp. II)	1.2
		2.0 (Exp. III)	2.9
Standard	37 °C	0.28 (Exp. II)	1.62

* pmoles/mg DNA: MCF-7 cells were incubated for 2 h at 0 °C with either ^{3}H-estradiol (2×10^{-9} M) or ^{3}H-estradiol (2×10^{-9} M) + unlabeled estradiol (2×10^{-7} M), washed and the nuclear pellet prepared as indicated above.



Autoradiogram of MCF-7 cells incubated for 2 h at 0 °C with ^{3}H-estradiol (2×10^{-9} M). Note the heavy concentration of grains over the nucleus. Exposure time 2 weeks. 4-µm-thick. Stained with methylgreen-pyronin. × 900.

Variable volume of buffer: Unlabeled cells were homogenized in different volumes of buffer (0.25, 0.5, 1.5, 15.0 and 25.0 mls) and a crude nuclear pellet and cytosol prepared as described above. Aliquots of the cytosol and the 0.4 M KCl extract of the nuclear pellet were incubated with ^{3}H-estradiol or ^{3}H-estradiol + unlabeled estradiol. After incubation, the incubates were assayed using Sephadex LH-20 columns.

Autoradiography: After incubation the cells were washed and pelleted. The pellet was then mounted on a stud with O.C.T. (Tissue-Tek II) and frozen in liquified propane. 4-µm frozen sections were cut in a cryostat and mounted for autoradiography on emulsion-coated slides according to the thaw-mount procedure[11]. The slides were exposed at − 15 °C for up to 6 weeks, photographically processed and stained with methylgreen-pyronin.

Results. The results of nuclear isolation using the standard procedures are compared with those using the nonaqueous isolation of nuclei in table 1. Note that only approximately 20–30% of the total receptor is nuclear at 0 °C using the standard procedures for the preparation of nuclei while 60–65% of the total is nuclear if a nonaqueous procedure is used. Grain counts of the autoradiograms (figure) made from an identical cell preparation indicated that approximately 76% of the total number of silver grains appeared over nuclei at 0 °C. Thus the autoradiographic results are in closer agreement with the results from the nonaqueous isolation of nuclei than with the results from the standard procedures used.

Table II illustrates the effect of varying the volume of buffer during the separation of the nuclear pellet from the cytosol. As the volume of the buffer is increased, the apparent quantity of nuclear receptor drops off in a non-linear fashion. Also note that preparation of the nuclear pellet by passing it through sucrose results in an apparent loss of nuclear receptor.

Conclusions. The data taken together indicate that the amount of apparent nuclear receptor varies greatly depending upon the procedure used to prepare the nuclear and cytosol fractions. The discrepancies reported in the past may be due in large part to the different procedures used to isolate nuclei[2–8]. These preliminary results suggest that the whole question of the intracellular distribution of unbound receptor needs to be reevaluated with special attention paid to the procedures used in preparing the nuclear fraction.

Table 2. Effect of buffer volume on the intracellular of estrogen receptors

Volume of buffer used (ml)	Amount of nuclear receptor (fm/mg DNA ± SD, n = 5)
0.25	580 ± 64
0.5	520 ± 58
1.5	473 ± 70
15.0	264 ± 68
25.0	163 ± 40
Sucrose	89 ± 11

Cells taken from the pleural cavity of a patient with advanced breast cancer were homogenized with different concentrations of buffer, and nuclear pellets and cytosols were prepared in the standard manner. The cytosols and the nuclear extracts were then incubated with either ^{3}H-estradiol (2×10^{-9} M) alone or with ^{3}H-estradiol (2×10^{-9} M) + unlabeled estradiol (2×10^{-7} M) for 2 h at 0 °C. The cytosols and nuclear extracts were then assayed using a Sephadex LH-20 column. Also cells were labeled and nuclei prepared by centrifugation through sucrose as described in the methods section.

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Presence of thyrotropin-releasing hormone in porcine and bovine retina

E. Martino, M. Nardi, G. Vaudagna, S. Simonetti, A. Cilotti and A. Pinchera

Patologia Medica II, University of Pisa, Pisa (Italy) and Clinica Oculistica 1, University of Florence, Florence (Italy), 21 July 1979

Summary. The thyrotropin-releasing hormone (TRH) has been found in porcine and in bovine retina, and it is indistinguishable from synthetic TRH in its immunological and biological properties. The role of retinal TRH is unknown, it probably acts as a neurotransmitter.

Thyrotropin-releasing hormone (TRH) has been isolated from the hypothalamus of various mammalian species[1,2] and it regulates the secretion of pituitary thyrotropin (TSH), prolactin and in certain circumstances growth hormone[3–5]. TRH is also distributed in extrahypothalamic regions of the brain in animals[6–9] and humans[10–13], and in regions outside the central nervous system, including the gastro-intestinal tract[14], islets of Langerhans of rat pancreas[15], frog skin[16], human placenta[17], and rat and frog retina[16,18].

In the present investigation we report the presence of TRH in the porcine and in bovine retina.

Materials and methods. The porcine and bovine eyes were obtained immediately after sacrifice at a slaughter-house. The retinas were isolated under direct microscopic control, immediately frozen in dry ice and stored at −25 °C until used. TRH was extracted from frozen tissues with 90% methanol as previously described[15]. The efficiency of the extraction procedure was above 90%, as assayed by the recovery of ^{125}I-labeled TRH added to the frozen tissues; the results were thus, not corrected for the extraction losses. TRH was measured by a double antibody radioimmunoassay[19] using ^{125}I-TRH and a specific antibody obtained in rabbits by immunization with synthetic TRH coupled with bovine serum albumin according to the method of Bassiri and Utiger[20]. The minimum detectable amount of TRH was 2 pg/tube. The results were expressed as pg/mg of wet tissue weight. The proteolytic degradation of TRH was evaluated by measuring the immunoreactivity of synthetic or retinal extracted TRH before and after incubation with fresh human serum at 37 °C for 2 h[19]. The biological activity of immunoreactive TRH extracted from porcine retinas was compared to that of synthetic TRH using an in vitro bioassay based on the ability of TRH to release rat pituitary thyrotropin[19].

Results. Immunoreactive TRH was found in the retinas of both animals at a concentration of 6.5 ± 0.3 pg/mg of wet tissue wt (mean ± SE) and 1.5 ± 0.5 pg/mg in porcine and bovine tissues respectively (table). The immunoreactivity of material extracted from porcine and bovine retinas was compared to that of synthetic TRH by analyzing the displacement of ^{125}I-TRH bound to specific antibody. A parallelism between the curves was observed. The fresh human serum incubation showed that it completely inactivated the immunoreactivity of 300–500 pg of synthetic TRH as well as that of equal amounts of immunoreactive TRH extracted from retinas. Finally, the quantity of TSH released into the medium from rat pituitary tissues incubated in vitro with equal amounts of synthetic or retinal-extracted TRH was similar, showing the same biological activity.

Concentration of thyrotropin-releasing hormone in porcine and bovine retinas

Retina (number)	TRH pg/mg wet wt (mean ± SE)
Porcine (16)	6.5 ± 0.3
Bovine (4)	1.5 ± 0.5

Discussion. The present data show that TRH is present in porcine and in bovine retinas. The identity of retinal-extracted and synthetic TRH has been established; they both showed the same immunoreactivity with a specific antibody, complete inactivation after incubation with human fresh serum, and finally similar biological activity as tested by the ability to release TSH from rat pituitaries in vitro.

These findings, obtained in pigs and oxen, extend the observations of Jackson and Reichlin[16] and Schaeffer et al.[18], who found significant amounts of immunoreactive TRH-like material in the retina in the frog and rat, respectively. Recently we observed that the TRH is present in the human retina[20] at a concentration similar to that observed in the human cerebral cortex.

The exact role of retinal TRH is unknown, in animals as well as in humans. The effect of light deprivation on TRH concentration in the retina has been recently reported in adult[18] and neonatal[22] rats; the results show that dark exposure is followed by a marked decrease in retinal TRH content. On the basis of these observations it is suggested that the TRH plays a significant role in retinal function, possibly by acting as a neurotransmitter.

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