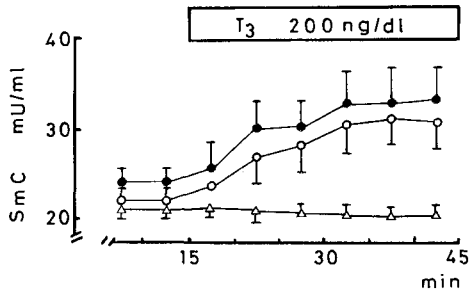

SmC release from perfused rat liver. The bars represent SD (n = 6); ○, liver of control rats perfused with T_3; ●, liver of T_4-treated rats perfused with T_3; △, liver of control rats perfused with vehicle; T_3 infusion significantly increased SmC concentration in the effluent from livers of control and T_4-treated rats.

and stored until the assay for SmC. Using this method, the efficiency of extraction of added SmC was 75 ± 5%.

Statistical analysis. Statistical differences were determined by Student's unpaired two-tailed t-test.

Results. Body weight, liver weight, plasma levels of T_4, T_3 and SmC and liver SmC. As shown in the table, body weight (111 ± 7 g, mean ± SD) and liver weight (4.8 ± 0.3 g) in T_4-treated rats were significantly lower than those (121 ± 5 g and 5.6 ± 0.3 g) in controls. Plasma levels of T_4 (18.0 ± 3.0 µg/dl), T_3 (359 ± 80 ng/dl), SmC (3.3 ± 0.3 U/ml) and liver tissue SmC (10.3 ± 0.5 U/liver) in T_4-treated rats were significantly higher than those (4.8 ± 0.5 µg/dl, 104 ± 20 ng/dl, 2.4 ± 0.2 U/ml and 8.3 ± 0.4 U/liver) in controls.

SmC release from perfused rat liver and liver tissue SmC. As shown in the figure, basal SmC release from perfused rat liver was 22–24 mU/ml. And SmC release was increased to 30–33 mU/ml by the addition of T_3 (200 ng/dl) to the perfusing medium. Control vehicle infusion through a side arm to the basic perfusion medium did not increase SmC concentration in the effluent.

Liver tissue SmC concentration in T_4-treated (6.2 ± 0.5 U/liver) and control rats (5.6 ± 0.5 U/liver) perfused with 200 ng/dl T_3 was significantly higher ($p < 0.005$) than that (3.3 ± 0.4 U/liver) in control rats perfused with control vehicle.

Discussion. In the present study, plasma SmC levels were significantly increased in T_4-treated rats. This result was in agreement with the report of Cavaliere et al.[14] that T_4 administration to normal man increased plasma SmC levels. Liver tissue SmC concentration was also significantly increased in T_4-treated rats. These observations indicate that thyroid hormone administration may increase SmC synthesis in the liver. According to previous reports[3–5], thyroid hormone may act independently from growth hormone on SmC synthesis and/or release. Therefore, in the present study, the direct effect of triiodothyronine on SmC release and synthesis in the liver was investigated. An addition of physiological doses of T_3 (200 ng/dl) significantly increased SmC release and SmC concentration in the perfused rat liver. The present results suggest that T_3 may directly enhance the release and synthesis of SmC in the rat liver. Increased plasma levels of SmC in T_4-treated rats may partly be due to increased release of SmC from the liver. The mechanism by which T_3 directly enhances the synthesis and release of SmC in the perfused liver remains to be elucidated.

In summary, we conclude that T_3 directly enhances the synthesis and release of SmC in the liver and increases the plasma level of SmC in the rat.

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The effect of homogenization temperature upon the apparent cellular compartmentalization of unoccupied estrogen receptor[1]

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Summary. Homogenization of rat uterus at elevated temperatures results in an increased nuclear localization of unoccupied estrogen receptor. This is a nonlinear effect which is accounted for by an increased population of KCl-resistant nuclear binding sites at the elevated homogenization temperatures.
Key words. Estrogen receptor; homogenization; temperature; nucleus; uterus.

Early investigations on the mechanism of action of estrogen in reproductive tissues suggested a two-step process of intracellular hormone binding and subsequent translocation of the hormone-receptor complex to the nucleus[2–4]. This proposed mechanism was based upon the observation that estrogen binding was primarily localized to the cytoplasmic

compartment of tissue homogenates (cytosol) in the absence of prior tissue or animal exposure to hormone. Specific estrogen binding sites after estrogen exposure, however, were predominantly found in the nuclear fraction of the tissue homogenate. Furthermore, the cytosolic/nuclear distribution of estrogen binding sites after hormone exposure exhibited an inverse stoichiometric temporal pattern[5,6]. This pattern of estrogen-dependent nuclear distribution of the receptor is also observed in vivo as a function of endogenous estrogen titers during the rat estrous cycle[7].

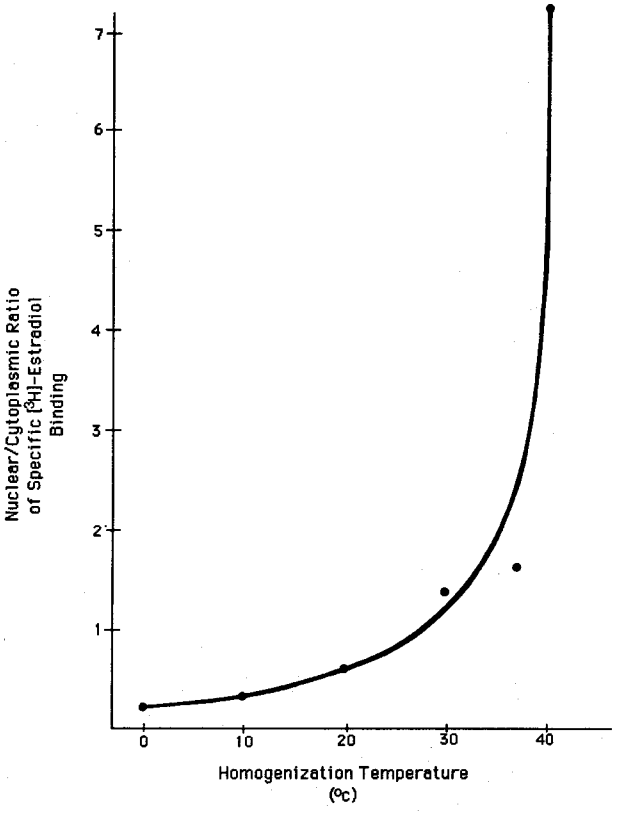
More recently, questions and uncertainty have developed regarding the translocation process and the prominent cellular domain of the estrogen receptor. Several laboratories, using polyclonal or monoclonal antibodies to steroid hormone receptors, report both a cytoplasmic (unoccupied) and nuclear (occupied) distribution of receptor which is consistent with the initially proposed two-step mechanism[8-11]. However, others, using similar techniques, have reported only a nuclear presence for the steroid hormone receptor regardless of physiological state[12-16]. Cytochalasin B enucleation in two different cell lines has also indicated the presence of unoccupied estrogen receptor largely in the cell nucleus[17-19]. Furthermore, Callard and Mak[20] have demonstrated an exclusive nuclear occupancy of estrogen receptor in tissue from a marine elasmobranch. This latter observation is relevant to an hypothesis originally proposed by Sheridan et al.[21] and Martin and Sheridan[22]. These authors postulated that the cytoplasmic presence of the estrogen receptor is an artifact caused by the extraction of loosely bound receptor protein from the nucleus during tissue homogenization in hypotonic buffers. According to this proposal, the increased affinity that charged (occupied) receptor exhibits for DNA prevents such extraction from tissue which has been exposed to estrogenic hormone.

In this report, we demonstrate that the protocol of tissue preparation for estrogen binding assays can, indeed, affect the distribution of unoccupied estrogen receptors between the cytoplasm and the nucleus.

Materials and methods. Sprague-Dawley rats were obtained from Holtzman Laboratory Animals (Madison, WI). The animals were maintained in the UAH controlled environment animal rooms on a 13-h light/11-h dark cycle and were provided commercially pelleted rodent feed and water ad libitum. The adult rats (150–200 g) were bilaterally ovariectomized under ether anesthesia. Two to four weeks later uteri were obtained for estrogen receptor assay. Radioactive estradiol (SA 41 Ci/mmol; 98% radiochemical purity) was obtained from Amersham (Arlington Heights, IL). Diethylstilbestrol (DES, Sigma Chemical Co.) was used as the competitor in the estrogen receptor assays. All radioactive samples were counted in a Beckman LS 100C scintillation counter using minivials and Bio-Verse (Fisher Scientific Co.) as the cocktail.

In one set of experiments, uteri were quickly obtained from animals killed by cervical disarticulation, stripped of adhering connective tissue and homogenized at 0, 10, 20, 30, 37 and 40 °C in 10 mM Tris-1.5 mM EDTA-1 mM dithiothreitol (TED) buffer (pH 7.4) using a motor-driven Kontes duall glass homogenizer. All tissues were homogenized at 40 mg/ml. The homogenates were centrifuged in a Beckman J2-21 centrifuge at 1000 × g and at appropriate homogenization temperature for 10 min. The supernatant (cytosol) was decanted and centrifuged at 48,000 × g for 20 min at 4 °C. The nuclear myofibrillar pellet was washed twice with 3 ml volumes of buffer with each wash consisting of thorough resuspension of the pellet and subsequent centrifugation. Both the cytosol and washed nuclear fraction of the uterus were assayed at 20-mg tissue equivalents. The cytosolic estrogen receptor analysis was performed using the charcoal-dextran assay protocol of Korenman[23]. A single point assay which used 0.5 ml cytosol from each temperature treatment and which contained 18 nM 6,7[^{3}H]-estradiol plus or minus 100-fold excess DES was employed. The tubes were incubated at ambient temperature (22–24 °C) for 1 h before adding 1.0%–0.05% charcoal-dextran to adsorb free steroid. The nuclear pellet fraction (0.5 ml) was incubated at 37 °C for 30 min with 18 nM labeled hormone. This hormone concentration is sufficient to occupy at least 95% of the specific uterine binding sites[23]. At the end of their respective incubation periods, a 0.5-ml aliquot of the cytosol was added to scintillation vials for counting after the charcoal-dextran particles had been pelleted by centrifugation at 1000 × g, while the nuclear fraction was washed three times in 3 ml volumes of TED buffer and extracted overnight in absolute ethanol prior to counting. Specific estrogen binding is determined by subtracting the radioactivity in the assay tubes containing the labeled estrogen and excess DES (nonspecifically bound estradiol) from the radioactivity associated with the assay tube containing only [^{3}H]-estradiol (total bound hormone).

In another experiment, uteri were homogenized in buffer at 0 and 30 °C, centrifuged at 1000 × g and the resulting nuclear pellet washed twice in 3 ml volumes of buffer. The washed nuclear pellet was resuspended at 40 mg/ml in buffer containing 0.4 M KCl and incubated on ice for 1 h with frequent vortexing. The suspension was then centrifuged at 1000 × g to repellet the nuclear myofibrillar fraction. The supernatant was incubated in 0.5 ml volumes (20 mg tissue equivalent) with 18 nM [^{3}H]-estradiol plus or minus 100-fold excess DES at ambient temperature for 2 h with frequent vortexing. At the end of incubation, the solution was charcoal-stripped, centrifuged and counted to determine the quantity of KCl-extractable estrogen binding sites. The nuclear pellet was resuspended in Tris buffer at 40 mg/ml and 0.5 ml of this was



The effect of homogenization temperature upon the cellular distribution of the estrogen receptor. Each point is the mean of 4–5 experiments with each experiment involving 2–4 animals per data point.

The effect of homogenization temperature upon the quantity of KCl-extractable and salt-resistant nuclear binding sites for estrogen.

Temperature of homogenization (°C)	Quantity of nuclear binding sites (CPM/mg tissue ± SEM) Salt-extractable	Salt-resistant
0	78.43 ± 16.44	33.97 ± 3.71
30	64.91 ± 6.85	65.51 ± 13.11

Each value is the mean ± SEM of 2 experiments with 3 data points each.

incubated for 2 h at ambient temperature with 18 nM labeled estradiol plus or minus 100-fold excess competitor. At the end of incubation, the suspension was rinsed 3 times with 3 ml volumes of buffer, extracted overnight in 100% ethanol and counted to measure the quantity of salt-resistant receptor sites.

Results. As illustrated in the figure, there is an increasing nuclear localization of unoccupied estrogen receptor with increasing temperature of homogenization. The increase in the nuclear/cytoplasmic ratio of specific estrogen binding is not linear. That is, the nuclear residency of estrogen binding sites as a function of homogenization temperature increases at an increasing rate, especially at temperatures above 20 °C. The table suggests an apparent cause for this relationship. As can be seen in this table, there is an increase in the proportion of estrogen binding sites that are not extracted by 0.4 M KCl from the nuclei of uterine tissue which has been homogenized at the higher temperature. At 30 °C more than 50% of total nuclear binding sites are not extracted by 0.4 M KCl, while at 0 °C less than one-third of total nuclear binding sites are resistant to salt extraction.

Discussion. The work of Sheridan et al.[21] and Martin and Sheridan[22] clearly indicates that osmolarity and volume of aqueous buffer can affect the cellular partitioning of unoccupied estrogen receptor. However, in this experiment we have used the routine hypotonic Tris buffer generally used in estrogen receptor assays for tissue disruption, maintained a constant buffer volume to tissue mass and still affected the apparent in vivo intracellular distribution of the estrogen receptor as a result of the temperature at which the tissue was homogenized. The increasing nuclear to cytoplasmic distribution of free receptor with increasing temperature of homogenization appears to be the result of an increased population of salt-resistant nuclear binding sites at the elevated preparative temperatures. These salt-resistant nuclear binding sites represent receptor protein bound to chromatin with high affinity, presumably at nuclear acceptor sites[24]. That is, at higher temperatures, a greater quantity of hormone binding sites remain in the nucleus rather than being extracted by the homogenization buffer. It is not known whether the elevated homogenization temperature results in the activation of unoccupied nuclear receptor, or whether the displacement of tissue from the normal homeothermic environment of the mammal to the aberrant thermal environment of ice-cold buffer results in a greater cytoplamic extraction of the receptor upon tissue homogenization through receptor deactivation. In either case, the apparent in vivo domain of the estrogen receptor would be artifactually altered.

It should be added that incubation of rat uteri under anaerobic conditions results in activation of the estrogen receptor and the appearance of elevated nuclear binding sites which are not dependent upon added estrogen[25]. Clearly, there are a number of conditions which can affect the in vivo residency of free receptor. Although estrogen receptor antibodies have been employed to demonstrate an exclusive nuclear occupancy of unoccupied receptor protein, there remains unresolved technical questions about specificity and interactions with other nuclear proteins which confuse the issue of interpretation. Other technical questions also confound the interpretation of the results of enucleation experiments in determining the cellular domain of unbound receptor[26,27]. Thus, while these approaches obviate the problematic procedures necessary to the biochemical characterization of the estrogen receptor, other technical constraints prevent an unequivocal answer to the enigma of the in situ cellular localization of the estrogen receptor.

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