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Assay of Total Estradiol Receptor in Tissue Homogenate and Tissue Fractions by Exchange with Sodium Thiocyanate at Low Temperature†

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ABSTRACT: After injection of radioactive estradiol to ovariectomized rats, the [^{3}H]estradiol–receptor complex transferred to the nuclei can be solubilized by low concentrations of NaSCN. The extraction by NaSCN is significantly more efficient than that obtained by KCl and is, in fact, complete; i.e., no radioactivity can be found in the nuclei after extraction. Since NaSCN also induces the exchange of receptor-bound estradiol with free hormone [Sica, V., Puca, G. A., Molinari, A. M., Buonaguro, F. M., & Bresciani, F. (1980) *Biochemistry 19*, 83], a simple assay method has been set up which measures receptor in tissue and tissue fractions, including nuclei and whole homogenate, at 0–4 °C, irrespective of whether the receptor is or is not interacting with endogenous hormone. The procedure consists of a simple incubation step at 0–4 °C overnight (16 h) of the nuclear fraction, cytosol, and a total homogenate in the presence of excess radioactive estradiol and 0.5 M NaSCN. This method is very easy to carry out, accurate, and precise and avoids the loss of binding sites which results from the heating procedures utilized in other methods. The ability to measure the binding in both the soluble and the particulate fractions of rat uterus permits the determination of the rate of the cytoplasmic to nuclear transfer of estrogen after injection of various hormone concentrations. No nuclear transfer was observed after administration of other nonestrogen hormones such as progesterone, testosterone, or hydrocortisone while a nonsteroid antiestrogen, tamoxifen, was able to translocate the receptor. It was found that 2 h after injection of estradiol into ovariectomized rats total receptor content of uterus shows a decrease which is proportional to the amount of hormone injected. After injection of a hyperphysiological dose of 17β-estradiol, a certain amount of the receptor–hormone complex remains in the cytosol for at least 4 h. The nuclear turnover of estradiol receptor related to the progesterone receptor induction has been studied. Actinomycin D and cycloheximide prevent nuclear processing.

When estradiol receptor is assayed in normal or neoplastic tissues of postpuberal animals, [^{3}H]estradiol[1] binding assays conducted at 0 °C do not measure preformed receptor–estrogen endogenous complexes because at temperatures close to 0 °C the dissociation rate of the estrogen–receptor complex is so low that only minimal exchange occurs during incubation time (Truong & Baulieu, 1971; Sanborn et al., 1971; Katzenellenbogen et al., 1973).

Because of the obvious importance of being able to assess total receptor content of the cell, and specifically the receptor complexed to endogenous hormone in the nucleus, there have been numerous attempts to optimize conditions for increasing

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[1] Abbreviations used: [^{3}H]estradiol, 17β-estradiol-6,7-t_2; DTT, dithiothreitol; DCC, dextran-coated charcoal; TED buffer, 10^{-2} M Tris-HCl, 10^{-3} M EDTA, and 10^{-3} M dithiothreitol, pH 7.4.

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the rate of dissociation of the estrogen–receptor complex without losing binding activity. All these attempts are based on careful and controlled temperature increases. The first demonstration on the feasibility of an exchange between receptor-bound estradiol with excess [^{3}H]estradiol was reported in the rat uterus nuclear receptor system (Puca & Bresciani, 1968).

In a subsequent work, Anderson et al. (1972) carried out an estradiol exchange assay of the nuclear receptor by directly incubating nuclear fractions at 37 °C in the presence of a large excess of [^{3}H]estradiol. Zava et al. (1976) have proposed a nuclear protamine assay that can be used to quantitate estrogen binding sites in mature rat uterus.

It has been shown that NaSCN, a chaotropic salt used to inhibit receptor aggregation (Sica et al., 1976), increases the rate of dissociation of the estradiol–receptor complex at 4 °C at concentrations (0.5 M) compatible with the stability of the complex (Anand Kumar et al., 1978; Sica et al., 1980). On the basis of these properties, a simple method has been developed by which receptor containing unlabeled hormone can be assayed in uterine cytosol by incubation with saturating amounts of [^{3}H]estradiol and 0.5 M NaSCN (Sica et al., 1980). In this paper, we show that thiocyanate solubilizes the nuclear receptor from nuclei more efficiently than KCl. The results have been utilized to set up a method which measures estradiol receptor in whole tissue homogenate and tissue fractions (cytosol and nuclei), independent of whether or not the receptor is complexed with hormone.

Materials and Methods

Materials. All reagents were of analytical grade. NaSCN (ACS) was purchased from C. Erba. 17β-Estradiol-*6,7*-t_2 (60 Ci/mmol specific activity) and progesterone-*1,2,6,7*-t_4 (93 Ci/mmol specific activity) were from Amersham. Dithiothreitol was from Calbiochem. Tris (Trizma base, reagent grade) and EDTA (disodium salt) were purchased from Sigma. 17β-Estradiol, testosterone, progesterone, and hydrocortisone were from Calbiochem; tamoxifen [*trans*-1-[*p*-[(dimethylamino)ethoxy]phenyl]-1,2-diphenylbut-1-ene, ICI 46474] was obtained from ICI, Ltd; charcoal (Norit A) was from Matheson Coleman and Bell, Norwood, OH; Dextran T 70 was from Pharmacia; actinomycin D (grade I) and cycloheximide were from Sigma.

Radioactivity Assays. Aqueous samples (0.1–1 mL) were added to 5 mL of Insta Gel liquid scintillation cocktail (Packard) in glass scintillation vials, and the radioactivity was measured in Beckman LS-3150 T-counter with 30% efficiency. Radioactivity of ether-extracted samples (see below) was measured by addition of 10 mL of toluene–phosphor solution containing 3.92 g/L 2,5-diphenyloxazole and 0.18 g/L *p*-bis(*o*-methylstyryl)benzene to the vials.

Protein Assays. Protein determinations were performed by the Bio-Rad protein assay based on the work of Bradford (1976). Thiol groups, Tris, and EDTA do not interfere with this assay.

Preparation of Rats. Mature female Sprague-Dawley rats (age 45–60 days) were used in this study. When required, animals were ovariectomized under light ether anesthesia 7 days prior to use. Rats were given food and water ad lib. Estradiol dissolved in 10% ethanol–0.9% NaCl was injected intraperitoneally. Where indicated, [^{3}H]estradiol, progesterone, testosterone, hydrocortisone, tamoxifen, or 0.9% NaCl were used in place of the estradiol. At an indicated time after injection, rats were sacrificed by cervical dislocation. The uteri were rapidly removed and cleaned of adhering fat and mesentery.

Preparation of Homogenate and Cytoplasmic and Nuclear Fractions. Uteri were weighed, chopped into fine pieces with scissors, and homogenized in 4 volumes (w/v) of TED buffer by means of an Ultraturrax homogenizer (Janke and Kunkel, Model TP 18/2) in four runs of 15 s each with 60-s intervals. The homogenate was filtered through a nylon filter (Satisfil Nylon NY 43.80, 110 mesh/in.) in order to eliminate gross residue and then centrifuged at 1000*g* for 20 min. The pellet was resuspended twice in TED buffer and centrifuged (1000*g*, 20 min). The final pellet (crude nuclear fraction) was resuspended in TED buffer in a final volume equal to the original volume of homogenate. The supernatant was centrifuged for 45 min at 150000*g* (Beckman Spinco L2-65 centrifuge, Ti 50 rotor).

Assay of Estrogen Binding Activity of Cytosol. Aliquots of cytosol (0.1–0.3 mL) to be tested were brought to 1 mL with TED buffer containing 3×10^5 dpm of [^{3}H]estradiol. The mixture was incubated at 4 °C for 3 h, a sufficient time to reach equilibrium. After incubation, separation of free from bound estradiol was accomplished by addition of 1 mL of Dextran-coated charcoal (1% charcoal, 0.05% Dextran). The suspension was allowed to stand for 15 min and centrifuged at 1000*g* for 15 min. A 1-mL aliquot of supernatant was assayed for radioactivity.

Parallel tests were performed to measure the nonspecific binding in the presence of a 500-fold excess of unlabeled estradiol.

Nuclear Extraction of [^{3}H]Estradiol–Receptor Complex. Ovariectomized rats were injected with [^{3}H]estradiol (30 Ci/mmol; 0.5 g/100 g of body weight) in 0.5 mL of 10% ethanol in 0.9% NaCl. After 2 h, animals were sacrificed. The uteri were homogenized and the nuclei sedimented as described.

The crude nuclear fraction was divided in three aliquots and resuspended in either 10 mL of cold (4 °C) ethyl ether or 10 mL of TED buffer containing 0.8 M KCl or 0.5 M NaSCN. The aqueous suspensions were mixed vigorously, allowed to incubate for 30 min at 4 °C and centrifuged at 150000*g* for 30 min. Aliquots (2 mL) of the supernatants were assayed for radioactivity. The ether suspension was vigorously mixed for 5 min at 4 °C, and the ether was decanted in a glass scintillation vial. The extraction was repeated twice. The pooled ether fractions were dried, and the radioactivity was measured as described. The aqueous phase and 150000*g* pellets resuspended with 2 mL of TED buffer were assayed for residual radioactivity.

Assay of Progesterone Binding Activity of Cytosol. Progesterone binding activity was measured by incubation of 0.1–0.3-mL aliquots of cytosol with 5×10^5 dpm of [^{3}H]-progesterone in the presence of 3×10^{-7} M hydrocortisone. The mixture was brought to 1 mL with TED buffer, pH 7.4, and incubated at 4 °C for 4 h. After incubation, separation of free from macromolecule-bound progesterone was accomplished by addition of 1 mL of DCC. The suspension was allowed to stand for 15 min and centrifuged at 1000*g* for 15 min. A 1-mL aliquot of supernatant was assayed for radioactivity. Parallel tests were performed to measure the nonspecific binding in the presence of a 500-fold excess of unlabeled progesterone.

Standard NaSCN Exchange Method for Assaying Estrogen Receptor in Total Homogenate and Cytosol and Nuclear Fractions. All procedures were carried out at 4 °C. Tissue homogenate and cytosol and nuclear fractions were prepared according to the standard method described above.

Table I: Salt Extraction of Nuclear Estradiol Receptor[a]

	E_2 bound (fmol/100 mg of fresh tissue)	%	residual radioactivity (fmol/100 mg of fresh tissue)
ether extraction	807 ± 14	100	12 ± 6
KCl extraction	675 ± 21	83	127 ± 13
NaSCN extraction	790 ± 31	98	18 ± 4

[a] Ovariectomized rats were injected in vivo with [^{3}H]estradiol. After 2 h, the animals were sacrificed, and the crude nuclear fraction, prepared as described under Materials and Methods, was extracted either with ethyl ether, KCl (0.4 M), or NaSCN (0.5 M). Values represent the mean ± SEM of two determinations performed in triplicate.

To 0.1–0.2 mL of homogenate, cytosol, or nuclear suspension (duplicate or triplicate samples) was added ice-cold TED buffer containing [^{3}H]estradiol of high specific activity, with or without a 500-fold excess of nonradioactive hormone, bringing the volume of sample to 0.25 mL. After 1–2 h, 50 μL of ice-cold TED buffer containing 3 M NaSCN was further added to each sample, thus achieving a final volume of 0.3 mL and a final NaSCN concentration of 0.5 M. The amount of [^{3}H]estradiol to be added to samples should be about 25-fold the estimated amount of endogenous hormone. Under usual operating conditions, 15–20 nM [^{3}H]estradiol (60 Ci/mmol) was the amount of choice. After incubation of samples for 16 h (overnight) at 4 °C, 0.3 mL of ice-cold DCC suspension was added to each sample, and, after an additional 15 min, free from bound estradiol was separated by centrifugation. Specific binding activity was computed by subtracting from the binding activity of samples with radioactive hormone only the binding activity of samples with labeled and excess unlabeled hormone (aspecific binding).

Results

Extraction of Estradiol Receptor from Nuclei by NaSCN vs. KCl. After administration of radiolabeled estradiol to ovariectomized female rats, the estrogen receptor binds hormone, and the complex migrates into the nuclei. This complex can be directly measured by extracting the radioactive hormone with ether. The estradiol–receptor complex can be extracted also with KCl-containing buffer (Jensen et al., 1967; Puca & Bresciani, 1968; Korenman & Rao, 1968; Mester & Baulieu, 1975; Zava et al., 1976; Clark & Peck, 1976; Ruh & Baudendistel, 1977; Muller et al., 1977; Barrack et al., 1977).

Preliminary experiments (Puca et al., 1975) have shown that NaSCN inhibits the estradiol receptor–nuclear acceptor interaction at concentrations as low as 50 nM. On the basis of these findings, the ability of NaSCN to solubilize nuclear receptor compared with ether and KCl extraction was investigated.

Ether solubilizes all estradiol from nuclei, since practically no radioactivity can be recovered in the aqueous phase after the extraction. A comparison of NaSCN and KCl with ether extraction (Table I) indicates that the chaotropic salt is more efficient than KCl and no residual receptor remains in the nuclear pellet while after exposure to KCl there are approximately 100 fmol/100 mg of fresh tissue as established by measuring residual radioactivity in the sediments after salt extraction. As shown in Table I, NaSCN releases 790 ± 31 fmol of bound [^{3}H]estradiol from nuclei of 100 mg of uterus, compared to 807 ± 14 fmol of bound [^{3}H]estradiol extracted by ethyl ether; that is, NaSCN solubilizes 98% of total nuclear radioactivity. KCl, on the other hand, solubilizes 675 ± 21 fmol or only 83% of total.

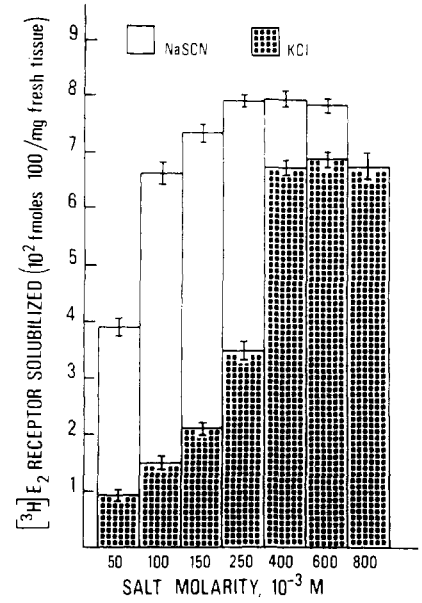

FIGURE 1: Solubilization by NaSCN vs. KCl of receptor from nuclear fraction of uteri from rats injected intraperitoneally with 17β-estradiol 2 h before sacrifice. Rats were injected with [^{3}H]estradiol (0.5 μg/100 g of body weight, sp act. 30 Ci/mmol). Nuclear pellets were resuspended in TED buffer, pH 7.4, containing either KCl or NaSCN at the given molarities. The final volumes of nuclear suspension were equal to the volume of corresponding amounts of homogenate. After incubation with shaking for 30 min at 4 °C, the suspensions were centrifuged at high speed for 30 min, and radioactivity in the supernatant was measured. NaSCN (0.25 M) solubilizes over 98% of nuclear receptor vs. 83% by KCl (0.4 M) (see Table I). The error bars represent the mean ± SEM of three determinations performed in triplicate.

In order to determine the optimal NaSCN concentration for extraction, ovariectomized animals were injected with [^{3}H]estradiol (30 Ci/mmol; 0.5 μg/100 g of body weight). After 2 h, animals were sacrificed, and the crude nuclear pellets were extracted with different NaSCN or KCl concentrations (Figure 1). The most effective KCl concentration was 0.6 M, but only approximately 80% of estradiol–receptor complex can be solubilized with even higher salt concentrations.

The presence of salt-resistant estradiol binding sites in uterine nuclei has been already described by several authors (Zava et al., 1976; Clark & Peck, 1976; Baudendistel & Ruh, 1976; Ruh & Baudendistel, 1977; Barrack et al., 1977). When NaSCN is used instead of KCl, the optimal concentration able to extract estradiol–receptor complex was 0.25 M, and the total amount of receptor extracted is higher.

Extraction and Exchange of Nuclear Receptor in a Single Step. Recently (Sica et al., 1980), a simple exchange assay has been developed to measure cytoplasmic estrogen receptor in uterine cytosol preincubated with the unlabeled hormone. This assay is based on the fact that NaSCN increases both the dissociation and the association rates of estradiol to the receptor. On the basis of the above results, the extraction and exchange steps for assay of nuclear receptor were unified. Nuclei were directly incubated with 0.5 M NaSCN at 4 °C for 16 h (overnight) in the presence of increasing amounts of [^{3}H]estradiol (Figure 2).

The experiment was carried out by using the uterine nuclear fraction from ovariectomized rats injected with cold estradiol (1 μg/100 g of rat weight) 1 h before. Parallel in vitro tests were carried without and with addition of a 500-fold excess of unlabeled hormone in order to measure total and aspecific binding and to derive specific binding by subtraction. Ex-

Table II: Accuracy and Precision of the NaSCN Exchange Method in Uterine Nuclei[a]

	intact rats			ovariectomized rats		
		17β-E_2 treated			17β-E_2 treated	
assay	control	60 min	120 min	control	60 min	120 min
1	204 ± 9	1018 ± 35	652 ± 18	129 ± 7	1120 ± 31	661 ± 15
2	249 ± 11	1058 ± 22	618 ± 23	66 ± 2	1181 ± 27	612 ± 12
3	323 ± 16	998 ± 37	637 ± 21	84 ± 5	1190 ± 28	648 ± 31
4	363 ± 8	1097 ± 18	651 ± 41	98 ± 7	1208 ± 27	608 ± 29
5	324 ± 15	1087 ± 36	590 ± 17	91 ± 8	1103 ± 19	516 ± 26

[a] Specific binding of [^{3}H]E_2 by uterine nuclei incubated with [^{3}H]E_2 in the presence of NaSCN for 16 h at 4 °C (fmol/100 mg of fresh tissue). Intact or ovariectomized rats were injected with saline alone or with 17β-estradiol (17β-E_2) (1 μg/100 g of body weight) 1 and 2 h before sacrifice. Nuclear pellets were resuspended in TED buffer, pH 7.4. Aliquots (0.2 mL) were incubated with [^{3}H]estradiol (15 nM) in the presence of 0.5 M NaSCN according to the standard exchange method (see Materials and Methods). Values represent the mean ± SEM of three determinations.

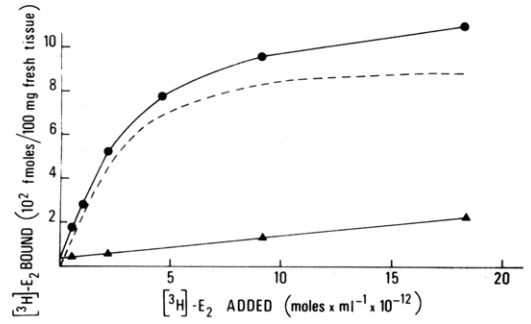

FIGURE 2: Exchange of [^{3}H]estradiol for 17β-estradiol at 4 °C in rat uterus nuclear fraction in the presence of 0.5 M NaSCN as a function of [^{3}H]estradiol concentration. The nuclear fractions from homogenates of uteri from rats injected with 1 μg/100 g of body weight of 17β-estradiol were incubated with 0.5 M NaSCN and [^{3}H]estradiol according to the standard NaSCN exchange method (see Materials and Methods) except that the concentration of radioactive hormone was varied. Experimental points refer to total binding (●), aspecific binding (▲), and specific binding (---), i.e., total minus aspecific binding.

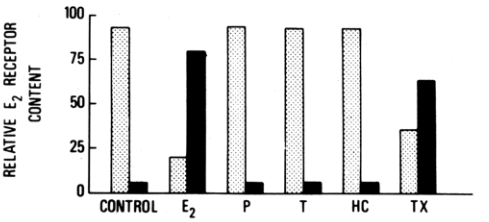

FIGURE 3: Distribution of estrogen receptor in cytosol and nuclei of uteri from ovariectomized rats injected 2 h before sacrifice with 17β-estradiol (E_2), progesterone (P), testosterone (T), hydrocortisone (HC) or tamoxifen (TX). Total receptor content of tissue fractions was assayed by the standard NaSCN exchange method (stippled box, cytosol; filled box, nucleus) (see Materials and Methods).

pectedly, the number of nuclear binding sites apparently increases as a function of the amount of [^{3}H]estradiol added, and it reaches a plateau beginning at about 10 nM [^{3}H]estradiol. At 15 nM, nonspecific binding is less than 20% of the total binding capacity. In subsequent experiments, this concentration of [^{3}H]estradiol was utilized. It is clear that the binding curve here is an isotope-dilution curve since half-maximal binding is observed at 3 nM [^{3}H]estradiol, which is at least 4-fold higher than the K_d of the estrogen receptor.

Hormonal Specificity of Nuclear Translocation. For determination of the effects of various estrogenic, antiestrogenic, and nonestrogenic hormones on the level of receptor in the uterine nuclei, ovariectomized rats were injected with 200 μg of tamoxifen, estradiol (1 μg/100 g of rat weight), and 5 μg of progesterone, testosterone, hydrocortisone, or saline alone. Uteri were homogenized 2 h after injection, and, after centrifugation, uterine cytosol and nuclear fractions were assayed by the NaSCN exchange method. The results are shown in Figure 3. Only estrogen resulted in an increase of estradiol receptor over control values. The nonsteroidal tamoxifen also binds estradiol receptor and translocates it into the nuclei (Jordan & Koerner, 1975; Horwitz & McGuire, 1978c).

However, nonestrogenic hormones failed to show any effect in the amount of nuclear receptor, thereby indicating that the NaSCN extract contains specific estrogen binding proteins.

Accuracy and Precision of the NaSCN Exchange Method in Uterine Nuclei. The NaSCN extraction–exchange assay of estradiol receptor is highly precise and accurate as shown by the low standard error of multiple determinations (Table II). 17β-Estradiol or saline was injected in intact or ovariectomized rats, and the amount of nuclear receptor was assayed in basal conditions (control) and 1 and 2 h after hormone injection.

It has been shown by several authors (Clark et al., 1972; Anderson et al., 1972, 1974; Mester & Baulieu, 1975; Juliano & Stancel, 1976; Clark & Peck, 1976; Ruh & Baudendistel, 1977) that the concentration of nuclear estradiol–receptor complex is maximal at 1 h after estradiol administration, after which time there is a rapid decrease to control levels by 10–12 h. After 120 min, the nuclear concentration of receptor is decreased to 70–50%. With the NaSCN exchange assay, we can measure concentrations of nuclear estradiol complex ranging from the very low values of ovariectomized control rats to the high levels obtained 1 h after hormonal administration. Uterine nuclei from ovariectomized animals which did not receive estrogen treatment appear to contain some estrogen receptor. This has been repeatedly observed in studies in vivo by using other exchange assays to measure content in immature rat uteri (Anderson et al., 1972, 1973, 1975; Juliano & Stancel, 1976; Clark & Peck, 1976; Clark et al., 1977; Ruh & Baudendistel, 1977).

The presence of such receptor in the nuclei may reflect either low levels of circulating estrogens of adrenal origin or distribution of unbound receptor in equilibrium, partitioned between cytoplasm and nucleus according to the equilibrium model considered in the past by Williams & Gorski (1972) and recently resumed by Sheridan et al. (1979).

Assay of Estradiol Receptor in Uterine Homogenate. The ability of NaSCN to solubilize estradiol receptor from nuclei and the possibility to perform an exchange assay in the presence of this chaotropic salt can be utilized to measure total (cytoplasmic + nuclear) receptor content in tissue homogenate, thus offering a very simple and easy assay for the screening of receptor content in normal or neoplastic tissues (for instance, in breast cancer). For this purpose, uteri of untreated ovariectomized rats were used. The binding as a function of [^{3}H]estradiol concentration is shown in Figure 4. Since the

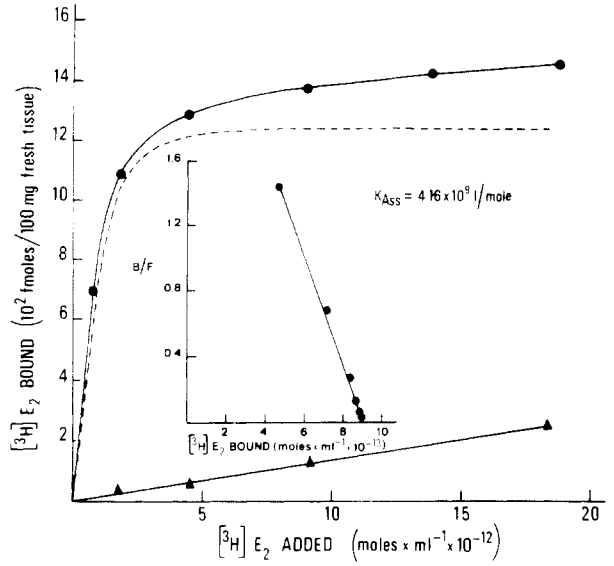

FIGURE 4: Determination of K_{assoc} with [^{3}H]estradiol of estrogen receptor in whole uterine homogenate. Homogenate aliquots of uteri from untreated ovariectomized rats were incubated with 0.5 M NaSCN and [^{3}H]estradiol according to the standard exchange method (see Materials and Methods) except that the concentration of radioactive hormone was varied. Experimental points refer to total binding (●), aspecific binding (▲), and specific binding (---), i.e., total minus aspecific binding. Insert: Results plotted according to Scatchard.

Table III: Accuracy and Precision of the NaSCN Exchange Method in Uterine Homogenates

assay	sp binding of [^{3}H]E_2 [a] (fmol/100 mg of fresh tissue)
b	1350 ± 11
b	1578 ± 15
c	1392 ± 26
c	1660 ± 23
c	1224 ± 48
c	1777 ± 35
c	1676 ± 35
c	1693 ± 29
c	1667 ± 49
c	1439 ± 27

[a] Specific binding of [^{3}H]E_2 by uterine homogenate incubated with [^{3}H]E_2 in the presence of NaSCN for 16 h at 4 °C. Values represent the mean ± SEM of two determinations performed in triplicate. [b] Intact animals. [c] Ovariectomized animals.

animals were not injected with cold hormone, the binding curve is not an isotope-dilution curve, as in the case of the experiment described in Figure 2, but it really represents the affinity of the estradiol–receptor interaction. Data plotted according to Scatchard are present (Figure 4, insert), and the association constant with estradiol has been estimated.

The value does not show any significant difference with previous estimates obtained in our laboratory (Puca & Bresciani, 1969; Puca et al., 1970, 1971) as well as in other laboratories by using a variety of tissue fractions and experimental techniques (Noteboom & Gorski, 1965; Toft & Gorski, 1966; Erdos et al., 1969; Shyamala & Gorski, 1969; Sanborn et al., 1971; Truong & Baulieu, 1971), thus indicating that we are really measuring estradiol receptor. The exchange assay in uterine homogenate is accurate and precise as shown by the low standard error (±48 ≥ SEM ≥ ±11) computed from assays performed in triplicate (Table III). It is worthwhile to note that no significant difference exists between total receptor concentration in ovariectomized compared to intact rats. In order to control if the receptor assay carried out in the homogenate is quantitative, i.e., if it really measures

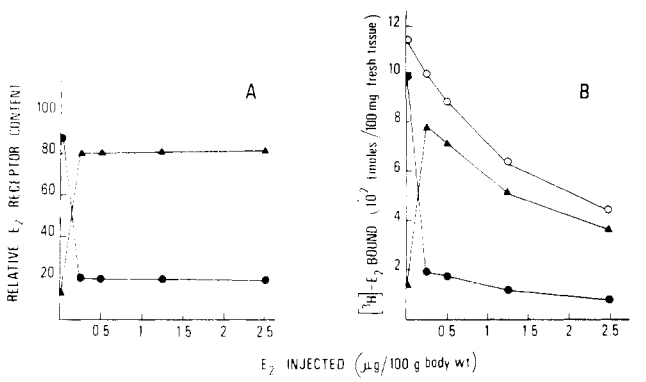

FIGURE 5: Total receptor content of uteri and of uterus nuclear and cytosol fractions 2 h after injection of increasing concentrations of 17β-estradiol into ovariectomized rats. Receptor content of tissue and tissue fractions was assessed by the standard exchange method (see Materials and Methods). (A) Results expressed as percent of total receptor in uterus. (B) Receptor content of whole uterus (○), nuclear fraction (▲), and cytosol (●).

the total receptor content of the tissue (cytoplasmic + nuclear), uterine homogenate of untreated mature rats was fractionated, and the NaSCN extraction–exchange assay method was performed in whole homogenate, cytosol, and nuclear fraction. The receptor concentration in the homogenate (1646 ± 55 fmol/100 mg of fresh tissue) actually represents the sum of the cytosol (1448 ± 45 fmol/100 mg of fresh tissue) and nuclear (224 ± 10 fmol/100 mg of fresh tissue) receptor contents.

Total Content of Estradiol Receptor after Intraperitoneal Injection of Increasing Doses of 17β-Estradiol into Ovariectomized Rats. It has been suggested that the translocation process of the cytoplasmic estrogen–receptor complex to nuclei is responsible for the characteristic retention of estrogenic hormones in target tissues (King et al., 1965; Jensen et al., 1968; Shyamala & Gorski, 1969).

The concentration of nuclear receptor is maximal at 1 h after estradiol administration (Anderson et al., 1972, 1974, 1975; Mester & Baulieu, 1975; Clark & Peck, 1976, Juliano & Stancel, 1976). The decline in nuclear receptor after the first hour after injection is dependent on the quantity of estradiol which was administered (Anderson et al., 1972). Using the NaSCN exchange method, we decided to determine the levels of estradiol receptor present in the whole homogenate as well as in the nuclear fraction and in the cytosol under a large range of hormone concentrations. Rats were injected intraperitoneally with different doses of nonradioactive hormone 2 h before sacrifice. The effects of estradiol dosage on the level of specific cytoplasmic, nuclear, and total homogenate binding sites are shown in Figure 5.

If the content of cytosol and nuclei is expressed as a fraction of total (Figure 5A), one can see that independently of the estradiol dose injected about 80% of the receptor is found in nuclei and 20% in cytosol. This finding is in accord with that of Williams & Gorski (1972), who found that at any dose of hormone administered a constant percentage of total receptor is transferred. However, when absolute values are considered (Figure 5B), one can see that the total tissue content of receptor in the homogenate, 2 h after estradiol treatment, decreases progressively as a function of estradiol injected. A similar finding has been described by Horwitz & McGuire (1978) in experiments with cell cultures.

Because of the way the experiment is constructed, the decrease observed as a function of dose may be the result of a dilution of specific activity due to the dose of injected unlabeled hormone.

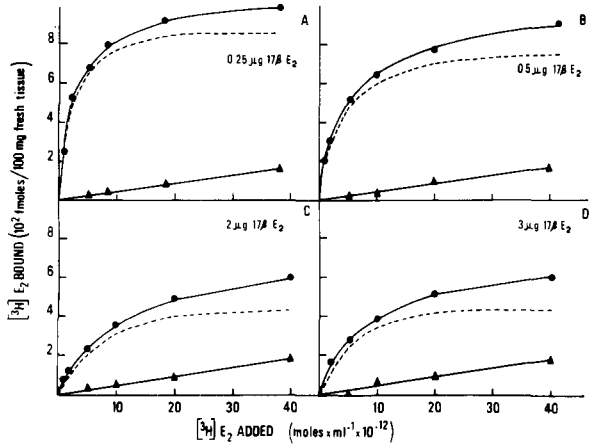

FIGURE 6: Exchange of [^{3}H]estradiol for 17β-estradiol at 4 °C in rat uterus homogenate as a function of [^{3}H]estradiol concentration after administration of varying concentrations of estradiol. Ovariectomized rats were injected with 0.25 (A), 0.5 (B), 2 (C), and 3 (D) μg of 17β-estradiol per 100 g of body weight. After 2 h, animals were sacrificed, and the NaSCN exchange assay was performed in the homogenate at increasing [^{3}H]estradiol concentrations. Experimental points refer to total binding (●), aspecific binding (▲), and specific binding (---), i.e., total minus aspecific binding.

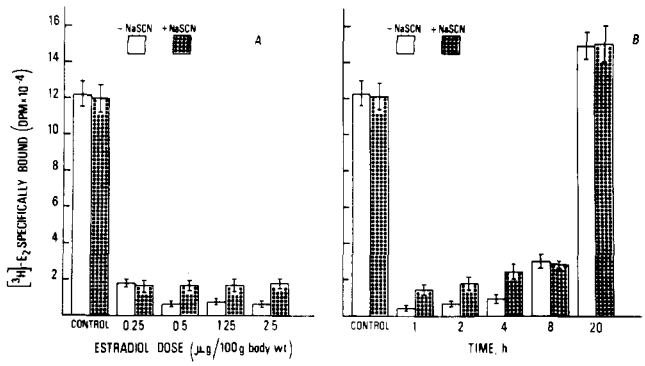

FIGURE 7: NaSCN exchange assay of cytoplasmic estradiol receptor compared with direct assay after administration of hyperphysiological doses of 17β-estradiol. (A) Ovariectomized rats were injected with the indicated doses of 17β-estradiol. Receptor was assayed by direct incubation of cytosol with [^{3}H]estradiol or by NaSCN standard exchange method. (B) Ovariectomized rats were injected with 17β-estradiol (1 μg/100 g of body weight). At the indicated time, animals were sacrificed, and the receptor was assayed by direct incubation of cytosol with [^{3}H]estradiol or by NaSCN standard exchange method (see Materials and Methods). The error bars represent the mean ± SEM of four determinations.

To clarify this point, the experiment was repeated, the estradiol receptor in the homogenate was assayed in the presence of increasing [^{3}H]estradiol concentrations, and isotope-dilution curves were constructed for each dose of estradiol administered (Figure 6). It can be seen that the total specific estradiol receptor really diminishes with increasing dosage of hormone.

From this experiment, it is also clear that at the hormone concentrations used throughout these studies (15–20 nM) the exchange is virtually complete even when hyperphysiological doses of estradiol have been administered.

Cytoplasmic Estradiol Receptor after Administration of Hyperphysiological Doses of Hormone. Ovariectomized rats were injected with increasing doses of 17β-estradiol, and after 2 h receptor was assayed in the cytosol by direct incubation with radioactive hormone (unfilled sites) and by NaSCN exchange assay (filled + unfilled sites). As shown in Figure 7A, a certain amount of hormone–receptor complex remains in the cytosol when the administered dose is higher than 0.25 μg/100 g of body weight. These filled sites can still be found

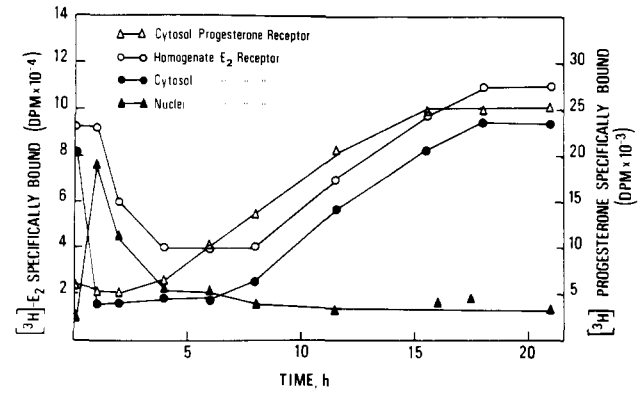

FIGURE 8: Effect of treatment time on estradiol receptor content and distribution in ovariectomized rats as a function of time after estradiol administration. Ovariectomized rats were injected with 17β-estradiol (1 μg/100 g of body weight) and at the indicated time were sacrificed. Estradiol receptor content of homogenate (○), cytosol (●), and nuclei (▲) was assayed by the standard NaSCN exchange method. Progesterone receptor in the cytosol (△) was assayed by direct incubation with [^{3}H]progesterone (see Materials and Methods).

in the cytoplasm for at least 4 h after a single injection of 1.25 μg/100 g of body weight of estradiol (Figure 7B).

Effect of 17β-Estradiol Administration on the Receptor Compartmentalization and Processing. Figure 8 shows the estradiol receptor levels in whole homogenate and cytoplasmic and nuclear fractions of rat uteri during 20 h after hormone treatment. Following estradiol (1 μg/100 g of body weight) administration, the receptor binds the hormone, and the receptor–hormone complex is rapidly translocated into the nucleus as shown by the reciprocal increase of nuclear receptor and decrease of cytoplasmic receptor by 1 h. Once the complex is translocated, a processing stage occurs in which the level of total detectable estradiol receptor is reduced by approximately 55–60% from the control level. The decline in the total receptor is practically complete between the first and fifth hours after injection.

Cytoplasmic receptor replenishment begins 4 h after hormone administration and is virtually complete after 20 h, when the total receptor level reaches a higher value, as compared to the control. Similar results have been obtained by several other groups by using different exchange assays in experiments with prepuberal animals or cell cultures (Anderson et al., 1972, 1974, 1975; Mester & Baulieu, 1975; Clark & Peck, 1976; Ruh & Baudendistel, 1977; Clark et al., 1977; Horwitz & McGuire, 1978a–c).

Nuclear processing of estrogen receptor seems to be an important intermediate step between nuclear receptor translocation and the initiation of hormonal response, such as synthesis of estrogen-regulated progesterone receptor (Horwitz & McGuire, 1978a). Even in in vitro experiments it seems that the turnover of estradiol receptor precedes the increasing of progesterone receptor level in the cytosol (Figure 8).

Effect of Actinomycin D and Cycloheximide on Nuclear Turnover of Estradiol Receptor. It has been described that actinomycin D inhibits the nuclear processing of estrogen receptor in the human breast cancer cell line MCF-7 whereas other intercalators and inhibitors of translation or of other cell functions fail to prevent the nuclear processing step (Horwitz & McGuire, 1978b).

In ovariectomized rats, when high doses of actinomycin D (400 μg/50 g of body weight) and cycloheximide (200 μg/50 g of body weight) are administered 1 h before 17β-estradiol injection (1 μg/100 g of body weight), the turnover of receptor is completely inhibited, and all the translocated estradiol–receptor complex can be found in the nuclei (Figure 9).

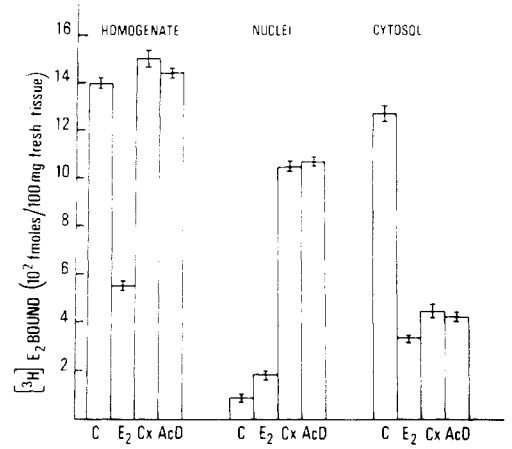

FIGURE 9: Effect of actinomycin D (AcD) and cycloheximide (Cx) on nuclear processing of estradiol–receptor complex. Ovariectomized rats were injected intraperitoneally with cycloheximide (200 μg/50 g of body weight) or actinomycin D (400 μg/50 g of body weight). They received 17β-estradiol (1 μg/100 g of body weight) 1 h after inhibitor administration. After 4 h, animals were sacrificed, and the estradiol receptor was assayed in the whole homogenate, in the nuclei, and in the cytosol by the NaSCN standard exchange method (see Materials and Methods). C, control, i.e., animals treated with saline; E_2, animals injected with 17β-estradiol alone; AcD and Cx, animals pretreated with inhibitors. The error bars represent the mean ± SEM of four determinations.

Discussion

We have shown that NaSCN, a chaotropic salt previously used in our laboratory to prevent the age-dependent aggregation of receptor (Sica et al., 1976), can be used to quantitate nuclear binding sites by a *single-step* method involving extraction of estradiol–receptor complex from nuclei and contemporary exchange of exogenous hormone with labeled estradiol. The same salt has proved very helpful in an exchange assay of estradiol–receptor complex in calf uterus cytosol (Sica et al., 1980) and has been used to elute estradiol receptor from affinity columns (Sica & Bresciani, 1979).

Previous reports in the literature suggested the feasibility of assaying estrogen-filled binding sites (Anderson et al., 1972; Katzenellenbogen et al., 1973; Chamness et al., 1975; Zava et al., 1976).

Consideration of the above methods and results points out very clearly that a delicate balance must be struck between the noxious temperature–time factor (Peck et al., 1973) and achievement of a sufficiently rapid rate of exchange for practical purposes.

An optimal method would be one in which exhange is performed at low temperature and which, in the case of nuclear receptor, could be carried out by direct incubation with the nuclear fraction.

The NaSCN procedure is performed at 4 °C, a condition compatible with a stable estradiol–receptor complex for more than 20 h even in the presence of high concentrations of thiocyanate, provided that the receptor contains bound hormone (Sica et al., 1980). Moreover, at 4 °C, the proteolytic activities should be reduced to a minimum.

In the Zava et al. (1976) protamine exchange method, estrogen receptor is first extracted with KCl and then precipitated with protamine sulfate and the precipitate subjected to ligand exchange with radioactive estradiol for 2.5 h at 37 °C. Although KCl extraction has been used by other groups to extract calf and rat uterine nuclear pellets (Puca & Bresciani, 1968; Giannopoulos & Gorski, 1971; Zava et al., 1976), the recovery is variable and fails to remove part of the receptor. Other authors have reported the presence of KCl-resistant nuclear estradiol binding sites. Clark & Peck (1976) found two types of estrogen receptors in rat uterine nuclei. When measured at saturating concentrations of estradiol, one of these types, accounting for 10% of the total nuclear receptor, was not removed by a single KCl extraction, was capable of long-term nuclear retention, and was thought to be required for long-term stimulation of uterine growth. The other type, amounting to 80–90% of nuclear-bound receptor, was salt extractable, exhibited only short-term nuclear retention, and was not thought to be required for uterine growth. However, contradictory results have been reported by Juliano & Stancel (1976), who have questioned the concept of two distinct classes of nuclear binding sites. Muller et al. (1977) suggest that nuclear salt-resistant estrogen binding sites might simply be an experimental artifact and represented a small amount of estradiol-receptor complex sticking to the viscous gelatine-like nuclear pellet. Barrack et al. (1977) postulated that this nuclear component may represent high-affinity sites associated with the uterine nuclear matrix, a resistant structure of the nucleus. When NaSCN instead of KCl is used to extract the nuclear estrogen receptor, no residual radioactivity remains tightly bound to the nuclear pellet (Table I). It appears then that NaSCN can extract even the KCl-resistant receptor sites and can give a more complete quantitation of nuclear binding sites.

The ability of thiocyanate to extract nuclear receptor and to exchange radioactive vs. unlabeled hormone has been used to measure the total estrogen receptor in the whole tissue homogenate. This *single-step* extraction–exchange assay provides a very simple and helpful method when both unfilled and filled receptor sites must be assessed. The values obtained with the exchange assay in the homogenates are highly reproducible (Table III) and can be performed even in the presence of hyperphysiological concentrations of estradiol (Figure 6). Moreover, the amount measured in the homogenate accounts for the sum of cytoplasmic plus nuclear receptors.

The current mode of estrogen action is based on the assumption that once receptor binds estrogen it translocates into the nucleus. The possibility exists that when hyperphysiological doses of hormone are administered a certain amount of receptor complexed with the estradiol remains for some time in the cytoplasm, as a consequence of a "saturation" of nuclear components interacting with estrogen receptor.

Indeed, after injection of high amounts of estradiol, some receptor–estradiol complex can be found in the cytosol for at least 4 h. The presence of receptor-filled sites in the cytoplasmic fraction may be the result of "saturation" of chromatin binding sites of estradiol–receptor complex or may represent bound nuclear receptors which appear in the cytoplasmic compartment on homogenization as suggested by Williams & Gorski (1972) and, more recently, by Sheridan et al. (1979).

The possibility to assay total tissue receptor in whole homogenate furnishes a simple tool to study the turnover of receptor in vivo as a function of time and hormone dose. According to previous observations (Anderson et al., 1972, 1974), we have found that between the first and fourth hour after estradiol treatment approximately 60% of the receptor has been degraded or inactivated.

This phenomenon has recently drawn more attention following the findings of Horwitz & McGuire (1978a–c) demonstrating that nuclear turnover or processing may be related to the biological activity of estradiol and specifically to progesterone receptor induction. Our results agree with those in vitro findings, and the nuclear turnover of estradiol receptor

in vivo precedes the appearance of progesterone receptor in the cytoplasm.

The replenishment of cytoplasmic receptor beginning about 6 h after estradiol injection is completed at 20 h, when uterine cytoplasmic binding capacity has increased to a level which is greater than the value obtained from nontreated rats (Sarff & Gorski, 1971).

Horwitz & McGuire (1978b) have reported that actinomycin D, but not inhibitors of translation (cycloheximide), prevents nuclear processing in the human breast cancer cell line MCF-7. In experiments in vivo in ovariectomized rat uterus, both compounds inhibit the nuclear turnover. We have no explanation for this apparent discrepancy at present, and further experiments are required to clarify this point.

In conclusion, we describe a simple and rapid method to quantitate estradiol receptor content in whole tissue homogenate and tissue fractions, including nuclei. This method may become of considerable importance to assess receptor content in normal or neoplastic tissues.

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