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Zinc Potentiation of Androgen Receptor Binding to Nuclei in Vitro†

Douglas S. Colvard and Elizabeth M. Wilson*

ABSTRACT: Zn^{2+} potentiates binding of the 4.5S [^{3}H]dihydrotestosterone-receptor complex to isolated rat prostate Dunning tumor nuclei in vitro when assayed in the presence of 300 μM $ZnCl_2$, 3 mM $MgCl_2$, 0.25 M sucrose, 5 mM mercaptoethanol, 0.15 M KCl, and 50 mM tris(hydroxymethyl)aminomethane, pH 7.5. In the presence of 5 mM mercaptoethanol, the concentration of 50 μM total Zn^{2+} required to promote half-maximal receptor binding to nuclei corresponds to a free Zn^{2+} concentration of 50 nM. The receptor–nuclear interaction appears to be selective for Zn^{2+}; other divalent cations when added at a concentration of 1 mM to a buffer containing 5 mM mercaptoethanol are less effective (Ni^{2+}) or have essentially no effect (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+}). Zn^{2+} does not alter the sedimentation rate of the 4.5S [^{3}H]dihydrotestosterone receptor in the presence of mercaptoethanol; however, in the absence of mercaptoethanol, Zn^{2+} causes the receptor to aggregate. Zn^{2+}-dependent nuclear binding of the 4.5S [^{3}H]dihydrotestosterone receptor is saturable at 1.4×10^{-13} mol of receptor sites/mg of DNA, corresponding to approximately 1150 sites/nucleus. In the presence of excess nuclei, up to 60% of added receptor is nuclear bound. An apparent binding constant for the receptor–nuclear interaction of 10^{13} M^{-1} was approximated. Pyridoxal 5′-phosphate ($\leq$10 mM), but not 0.4 M KCl, inhibits Zn^{2+}-dependent nuclear binding of the [^{3}H]dihydrotestosterone receptor. Up to 66% of nuclear-bound receptor can be extracted in buffer containing 3 mM ethylenediaminetetraacetic acid plus either 0.4 M KCl or 10 mM pyridoxal 5′-phosphate. Nuclear receptor extracted in buffer containing the protease inhibitor diisopropyl fluorophosphate (2 mM) sediments at 4.5 S on sucrose gradients, but the receptor sediments at 3 S when extracted without the inhibitor. Zn^{2+}-dependent nuclear binding of the [^{3}H]dihydrotestosterone receptor is temperature dependent, with association rate constants (k_a) at 0, 15, and 25 °C of 4.0×10^5, 2.8×10^6, and 9.7×10^6 M^{-1} min^{-1}, respectively. The activation energy of binding is 21 kcal/mol.

Androgens are believed to be concentrated in target cell nuclei through an unknown mechanism involving the androgen receptor (Rennie & Bruchovsky, 1973). Attempts to mimic this process of nuclear retention of steroid receptors in vitro have led to the realization that an alteration in the receptor occurs that is likely initiated by the binding of hormone. The terms "transformation" and "activation" have been adopted to encompass this poorly understood process whereby the steroid-receptor complex is converted to a nuclear or DNA binding form (Alger & Milgrom, 1976; Kalimi et al., 1975; Higgins et al., 1973; Buller et al., 1975a,b). In vitro transformation of certain steroid receptors can be induced by heat (Higgins et al., 1973; Buller et al., 1975a,b), dilution (Higgins et al., 1973), $(NH_4)_2SO_4$ (Buller et al., 1975a,b), or ATP (Moudgil et al., 1981).

Studies on androgen receptor binding to isolated nuclei have been complicated by the low concentration of receptor, the susceptibility of the receptor to proteolytic cleavage (Wilson

† From the Departments of Pediatrics and Biochemistry and the Laboratories for Reproductive Biology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514. *Received November 1, 1983.* This work was supported by U.S. Public Health Service Research Grants HD16910 and HD04466 and by American Cancer Society Institutional Grant IN-15-V.

* Address correspondence to this author at the Department of Pediatrics, University of North Carolina School of Medicine.

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& French, 1979), and the lability of the steroid binding site (Wilson & French, 1976). Consequently, little is known about the requirements for transformation of the androgen receptor. Early work with the androgen receptor demonstrated only slight effects of heat on receptor binding to prostate nuclei (Fang & Liao, 1971) or chromatin (Mainwaring & Peterken, 1971; Davies & Griffiths, 1974). In this report, we present evidence that low levels of Zn^{2+} potentiate androgen receptor binding to nuclei in vitro. Zn^{2+}-dependent receptor binding is temperature dependent, saturable, and inhibited by pyridoxal 5′-phosphate.

Experimental Procedures

Materials

[1,2,4,5,6,7-^{3}H]Dihydrotestosterone (120 Ci/mmol) was purchased from New England Nuclear; cellulose phosphate P11 was from Whatman; diisopropyl fluorophosphate was from Calbiochem; 2-mercaptoethanol was from Eastman; Trizma base [tris(hydroxymethyl)aminomethane (Tris)[1] buffer] and ovalbumin were from Sigma; bovine γ-globulin (fraction II) was from Miles; pyridoxal 5′-phosphate was from Aldrich Chemical Co.; $NaBH_4$, $ZnCl_2$, sodium molybdate, divalent ion salts, Scintiverse, and reagent-grade chemicals were from Fisher Scientific Co. The Ca^{2+}- and Zn^{2+}-selective ion electrode was purchased from HNU Systems Inc., Newton, MA.

Methods

Animals. Copenhagen-Fischer rats bearing the Dunning prostate tumor (R3327H) were obtained from the Papanicolaou Cancer Research Institute, Inc., Miami, FL. Rats were castrated through an abdominal incision under ether anesthesia when tumors reached 2–4 cm in diameter. After 18 h, rats were decapitated and tumors rapidly removed, rinsed in 0.9% NaCl at 0 °C, and immersed in liquid nitrogen. Tumors were stored at −70 °C until use for preparation of cytosol and nuclear fractions.

Preparation of Subcellular Fractions. All procedures were carried out at 4 °C unless otherwise indicated. Dunning prostate tumor was pulverized under liquid nitrogen and homogenized with an Ultraturrax homogenizer (three intermittent 10-s bursts) in buffer containing 0.25 M sucrose, 10% glycerol, 2 mM diisopropyl fluorophosphate, 2 mM 2-mercaptoethanol, and 50 mM Tris, pH 7.5. (Note: Diisopropyl fluorophosphate is highly toxic.) When nuclei were isolated, 3 mM $MgCl_2$ was included in the buffer. When cytosol was prepared without nuclei isolation, the homogenates were immediately centrifuged at 100000g for 75 min. Supernatants were stored at −70 °C before use.

Tumor nuclei were isolated as previously described (Spelsberg et al., 1971) with some modifications by further dispersing the homogenate in a Thomas glass–Teflon homogenizer at 4 °C (four to six strokes). Samples were filtered through four layers of cheesecloth and centrifuged at 4000 rpm for 5 min in an SS34 Sorvall rotor. Supernatants were collected and centrifuged for 90 min at 100000g_{max} in a type 35 Beckman rotor. Crude nuclear pellets were resuspended in 1.9 M sucrose in TKM (0.025 M KCl, 3 mM $MgCl_2$, and 50 mM Tris, pH 7.5; 100 mL per 20 g of starting tissue), overlaid on the same buffer, and centrifuged at 15000 rpm for 10 min in an SS34 Sorvall rotor. Supernatants and surface debris were decanted and the pellets resuspended and centrifuged over a 1.9 M sucrose cushion in TKM as before. Nuclei were resuspended in 1 M sucrose in TKM, filtered through a nylon mesh cloth, and centrifuged at 9000 rpm for 10 min. Pellets were resuspended twice more in 0.25 M sucrose–TKM and centrifuged at 4000 rpm in the SS34 Sorvall rotor. Purified nuclei were resuspended in 0.25 M sucrose in TKM and stored at 0 °C for not more than 6 days. Protein/DNA weight ratios ranged from 2.5 to 4.5.

Cytosol fractions (100000g_{max} supernatants) were incubated with 15 nM [^{3}H]dihydrotestosterone and 10 mM 2-mercaptoethanol for 18 h at 0 °C as previously described (Wilson & French, 1979). Cytosol was centrifuged at 10000 rpm for 10 min in an SS34 Sorvall rotor to remove aggregated material and the supernatant applied to a phosphocellulose column (40 mL of cytosol/15 mL of packed column; column dimensions 2.5 × 3 cm) equilibrated in 1 mM EDTA, 10 mM mercaptoethanol, and 50 mM Tris, pH 7.5. The phosphocellulose column was washed with 0.15 M KCl, 10 mM mercaptoethanol, and 50 mM Tris, pH 7.5. The 4.5S androgen receptor was eluted with 0.3 M KCl, 15% glycerol, 10 mM mercaptoethanol, and 50 mM Tris, pH 7.5, as previously described (Colvard & Wilson, 1981), except EDTA was omitted. Sucrose gradient centrifugation showed that after phosphocellulose chromatography, the receptor remains in the 4.5S form in buffer containing 0.15 or 0.025 M KCl due to its separation from the 8S androgen receptor promoting factor (Colvard & Wilson, 1981). Glycerol was added to the receptor fraction to a final concentration of 30%. The receptor was stored in small aliquots at −70 °C prior to use.

Nuclear Binding Assay. The phosphocellulose-purified [^{3}H]dihydrotestosterone receptor was thawed rapidly and dialyzed for 2 h at 4 °C against a large volume of 0.025 M KCl, 10% glycerol, 0.25 M sucrose, 5 mM 2-mercaptoethanol, 3 mM $MgCl_2$, and 50 mM Tris, pH 7.5. Aliquots of isolated nuclei (20–750 μg of DNA) were incubated with the dialyzed 4.5S [^{3}H]dihydrotestosterone receptor (10–330 pM). Unless otherwise indicated, incubations were for 30 min at 25 °C in a total volume of 0.3 mL of 0.25 M sucrose, 0.15 M KCl, 5 mM 2-mercaptoethanol, 300 μM $ZnCl_2$, 3 mM $MgCl_2$, and 50 mM Tris, pH 7.5. In this buffer, a total Zn^{2+} concentration of 300 μM corresponds to 0.5 μM free Zn^{2+} due to complexation of Zn^{2+} by mercaptoethanol (see below). After incubation, nuclei were centrifuged at 4000 rpm in an HB-4 Sorvall rotor for 5 min. Supernatants were aspirated; nuclear pellets were resuspended in 0.3 mL of the same buffer, but without $ZnCl_2$ or mercaptoethanol, and centrifuged as before. The combined supernatant fractions were assayed for radioactivity in 6 mL of Scintiverse–toluene (1:1) as an indication of unbound radioactivity. Nuclear pellets were extracted with 0.25 mL of ethanol for 10 min at 0 °C, unless indicated otherwise, and the radioactivity of the entire suspension was determined. Receptor association with nuclei is expressed as the difference in radioactive counts sedimented in the presence and absence of nuclei. Under standard assay conditions, sedimentation of receptor alone ranged from 5 to 15%, where the lower limit primarily represents binding of ^{3}H-steroid to glass.

Determination of the Zn^{2+}–Mercaptoethanol Complexation Constant. The binding of Zn^{2+} and mercaptoethanol was estimated by monitoring proton release (Armanet & Merlin, 1961) with a pH meter at concentrations of mercaptoethanol from 0.1 to 5 mM and of $ZnCl_2$ from 0.01 to 0.3 mM in buffer containing 0.15 M KCl–0.1 mM HEPES, pH 7.5. Following each addition of 0.1 M $ZnCl_2$, pH 5, to buffer containing mercaptoethanol, the resulting drop in pH (not lower than pH

[1] Abbreviations: TKM, 0.025 M KCl, 3 mM $MgCl_2$, and 50 mM Tris, pH 7.5; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

Table I: Effect of Zn^{2+} Concentration on Androgen Receptor Binding in Prostate Tumor Nuclei[a]

$[ZnCl_2]_{total}$ (μM)	$[Zn^{2+}]_{free}$ (μM)	nuclear-bound androgen receptor	
		fmol/mg of DNA	% of total
0		3.8	9
25	0.024	6.8	17
50	0.051	7.9	21
100	0.11	10.5	28
200	0.26	13.5	35
300	0.47	14.6	38
400	0.98	15.4	41
500	2.0	15.8	41

[a] Prostate tumor nuclei (400 μg) were incubated with the 4.5S [^{3}H]dihydrotestosterone receptor partially purified by phosphocellulose chromatography. Incubation was for 30 min at 25 °C in a total volume of 0.3 mL of 0.25 M sucrose, 0.15 M KCl, 3 mM $MgCl_2$, 5 mM mercaptoethanol, and 50 mM Tris, pH 7.5, as described under Methods. Receptor sedimentation in the absence of nuclei and adhesion of ^{3}H-steroid to the glass totaled 5–8% of total radioactivity and were subtracted from each point. The amount of receptor binding to nuclei is given in femtomoles per milligram of DNA and as the percent of total receptor added. Free Zn^{2+} concentration was estimated by using the three Zn^{2+}–mercaptoethanol binding constants, K_1 (1.4×10^3 M^{-1}), K_2 (6.0×10^3 M^{-1}), and K_3 (0.8×10^3 M^{-1}), determined from the modified Scatchard analysis shown in Figure 2. Free Zn^{2+} concentration was calculated by using the equation $[Zn]_{total} = [Zn]_{free}(1 + K_1[M]_{free} + K_1K_2[M]_{free}^2 + K_1K_2K_3[M]_{free}^3$ where M is mercaptoethanol and $[M]_{free} = [M]_{total} - [M]_{bound}$.

6.8) was back-titrated to pH 7.5 by the addition of 0.01 M NaOH. The small amount of NaOH required to neutralize $ZnCl_2$ additions in the absence of mercaptoethanol was subtracted from each determination. The molar amount of NaOH required to offset proton release was taken as a measure of bound mercaptoethanol. A modified Scatchard analysis[2] was carried out as shown in Figure 2 by plotting $[\text{mercaptoethanol}]_{bound}/([\text{mercaptoethanol}]_{free}[Zn^{2+}]_{total})$ vs. $[\text{mercaptoethanol}]_{bound}/[Zn^{2+}]_{total}$. The apparent K_1 is estimated from the y intercept, K_2 from the slope of the line extrapolated to the y intercept (slope = $2K_2$–K_1), n from the x intercept, and K_3 from the slope of the line extrapolated to the x intercept (slope = $-nK_3$).

Sucrose Gradient Centrifugation. Linear sucrose gradients (2–20% w/v) of 4.8 mL contained 10 mM 2-mercaptoethanol, 10% glycerol, 0.15 M KCl, and 50 mM Tris, pH 7.5, unless otherwise indicated. Receptor samples (0.15–0.3 mL) were layered together with the protein standards ovalbumin [3.6 S (40 μL of 40 mg/mL)] and bovine γ-globulin [7 S (40 μL of 35 mg/mL)]. Gradients were centrifuged in an SW 50.1 Beckman rotor for 20 h at 4 °C at 46 000 rpm. Gradients were fractionated (0.22 mL/fraction) and aliquots (25 μL) taken for protein determination (Lowry et al., 1951). Remaining fractions were combined with 5 mL of Scintiverse–toluene (1:1) and counted in mini vials. Counting efficiency was 47%.

Protein and DNA Assays. Protein concentration was estimated by the method of Lowry et al. (1951) using ovalbumin as the standard. DNA was assayed by the method of Burton (1956) using calf thymus DNA as the standard.

Results

Effects of Zn^{2+} and Mercaptoethanol on Androgen Receptor Binding in Nuclei. The androgen receptor in Dunning prostate tumor cytosol was labeled with [^{3}H]dihydrotestosterone in the absence of $ZnCl_2$ and partially purified by phosphocellulose chromatography as previously described (Colvard & Wilson, 1981). The resulting 4.5S [^{3}H]dihydrotestosterone receptor was incubated with Dunning prostate tumor nuclei for 30 min at 25 °C in buffer containing 0.25 M sucrose, 0.15 M KCl, 3 mM $MgCl_2$, 5 mM mercaptoethanol, and 50 mM Tris, pH 7.5. Under these conditions, only 9% (3.8 fmol/mg of DNA) of the total added receptor bound to nuclei (Table I). Addition of $ZnCl_2$ to the assay buffer prior to incubation at 25 °C resulted in an increase in receptor–nuclear binding (Table I). Maximal binding was reached at 200–400 μM total $ZnCl_2$, accounting for 41% of the total added receptor (15.6 fmol/mg of DNA).

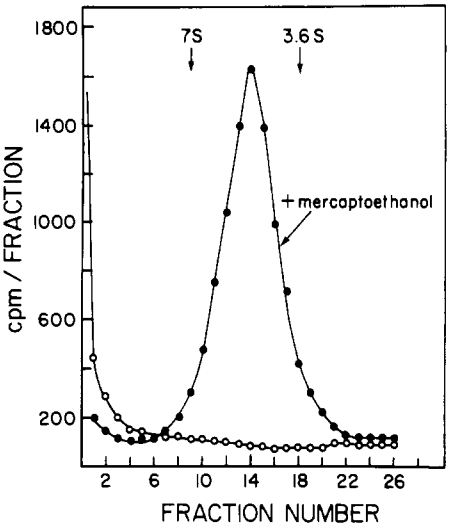

FIGURE 1: Effect of Zn^{2+} with or without mercaptoethanol on the sedimentation of the androgen receptor. Tumor cytosol was incubated with [^{3}H]dihydrotestosterone (15 nM) at 0 °C for 18 h and chromatographed on phosphocellulose as described previously (Colvard & Wilson, 1981). The partially purified 4.5S [^{3}H]dihydrotestosterone-receptor complex was dialyzed for 2 h at 4 °C against 0.025 M KCl, 10% glycerol, 0.3 mM $ZnCl_2$, and 50 mM Tris, pH 7.5, in the presence (●) or absence (○) of 10 mM mercaptoethanol. Aliquots (0.3 mL) were centrifuged through 2–20% sucrose gradients containing 0.15 M KCl, 10% glycerol, and 50 mM Tris, pH 7.5, in the presence or absence of 10 mM mercaptoethanol, respectively. Gradients were centrifuged in an SW 50.1 Beckman rotor at 46 000 rpm for 20 h at 4 °C. Internal molecular weight markers are indicated for bovine γ-globulin (7 S) and ovalbumin (3.6 S). In the absence of mercaptoethanol, essentially all of the radioactivity sedimented to the bottom as a pellet. Fraction 1 represents the lowest portion of the gradient above the pellet, and fraction 26, the top of the gradient.

The effects of Zn^{2+} and mercaptoethanol on the sedimentation properties of the partially purified 4.5S [^{3}H]dihydrotestosterone receptor were analyzed by sucrose gradient centrifugation in the presence of 300 μM $ZnCl_2$ with and without 10 mM mercaptoethanol. Receptor was preserved in the 4.5S form when mercaptoethanol was included in sample and gradient buffers containing 0.025 M KCl (not shown) or 0.15 M KCl (Figure 1). In the absence of mercaptoethanol, the receptor sedimented to the bottom of the gradient, indicating receptor aggregation. The extent of receptor aggregation in the absence of mercaptoethanol was dependent on Zn^{2+} concentration, increasing from 15% in the absence of $ZnCl_2$ to essentially 100% in 300 μM total $ZnCl_2$. In the presence of 10 mM mercaptoethanol, less than 10% of the receptor pelleted at $ZnCl_2$ concentrations ranging from 0 to 300 μM. These results suggested that the addition of mercaptoethanol to a Zn^{2+}-containing buffer prevents aggregation of the 4.5S receptor.

The effectiveness of mercaptoethanol in lowering receptor aggregation suggested the possibility that mercaptoethanol binds Zn^{2+}. In a preliminary experiment, the free Zn^{2+} concentration in a medium containing 300 μM Zn^{2+} and 5 mM mercaptoethanol was determined to be less than 10 μM, which

[2] L. Pedersen, unpublished results.

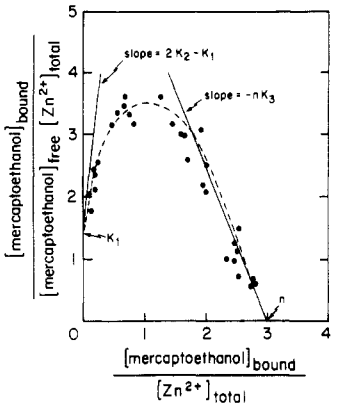

FIGURE 2: Modified Scatchard analysis of the binding of Zn^{2+} and mercaptoethanol. To 20 mL of buffer containing 0.15 M KCl, 0.1 mM HEPES, pH 7.5, and mercaptoethanol up to 5 mM were added sequential aliquots of 0.1 M $ZnCl_2$, pH 5, resulting in a drop in pH proportional to the amount of mercaptoethanol bound to Zn^{2+}. After each addition of $ZnCl_2$ to make a total Zn^{2+} concentration of 0.01, 0.025, or 0.05 μM, a measured amount of a fresh solution of NaOH (0.01 M) was added to back-titrate to pH 7.5. At mercaptoethanol concentrations in excess of 1 mM, total Zn^{2+} concentrations of 0.1, 0.2, and 0.3 mM Zn^{2+} were used as well. The molar amount of NaOH required to neutralize released H^+ indicates the extent of binding of mercaptoethanol to Zn^{2+}. Addition of $ZnCl_2$ to buffer without mercaptoethanol lowered the pH by up to 0.05 pH unit and was subtracted from values measured in the presence of mercaptoethanol. Treatment of the data was based on unpublished studies of L. Pederson[2] and those of Klotz & Hunston (1971, 1975, 1979), where the apparent K_1 is estimated from the y intercept, K_2 from the slope of the tangent extrapolated to the y intercept (slope = $2K_2 - K_1$), n from the number of mercaptoethanol molecules bound per Zn^{2+} atom at saturation, and K_3 from the slope of the line extrapolated to the x intercept (slope = $-nK_3$). The points are experimentally derived. The dashed curve represents the function described by the following equation using the three experimentally determined binding constants ($K_1 = 1.4 \times 10^3$ M^{-1}, $K_2 = 6.0 \times 10^3$ M^{-1}, and $K_3 = 0.8 \times 10^3$ M^{-1}): $[M]_b = [Zn]_T[(K_1[M]_{free} + 2K_1K_2[M]_{free}^2 + 3K_1K_2K_3[M]_{free}^3)/(1 + K_1[M]_{free} + K_1K_2[M]_{free}^2 + K_1K_2K_3[M]_{free}^3)]$ where $[M]_b$ is the concentration of bound mercaptoethanol, $[Zn]_T$ is the total $ZnCl_2$ concentration, $[M]_{free}$ is the free mercaptoethanol concentration (i.e., the difference between total and bound mercaptoethanol concentrations), and K_1, K_2, and K_3 are the three binding constants.

was the limit of detection using a Ca^{2+}- and Zn^{2+}-sensitive ion electrode. The extent of Zn^{2+} binding to mercaptoethanol was therefore further monitored by measuring proton release (Armanet & Merlin, 1961; Sillen & Martel, 1971) with a pH electrode at varying concentrations of Zn^{2+} and mercaptoethanol as described under Methods. Analysis of the data in the form of a modified Scatchard plot[2] as shown in Figure 2 allowed estimation of the three apparent binding constants: $K_1 = 1.4 \times 10^3$ M^{-1}, $K_2 = 6.0 \times 10^3$ M^{-1}, and $K_3 = 0.8 \times 10^3$ M^{-1}. The constants indicate cooperative binding of the second molecule of mercaptoethanol to Zn^{2+}. The computed curve (dashed curve) derived from the equation in the legend of Figure 2 is in excellent agreement with the experimental data (solid circles). The product of the three binding constants (6.7×10^9 M^{-3}) differs substantially from that previously reported (Sillen & Martell, 1971).

The free Zn^{2+} concentration in the presence of 5 mM mercaptoethanol was estimated from experimentally determined concentrations of free mercaptoethanol ($[M]_{free} = [M]_{total} - [M]_{bound}$), the total Zn^{2+} concentration, and the three apparent binding constants derived from the modified Scatchard analysis (Figure 2) by using the following equation:

$$[Zn]_{total} = [Zn]_{free}(1 + K_1[M]_{free} + K_1K_2[M]_{free}^2 + K_1K_2K_3[M]_{free}^3)$$

The estimated free Zn^{2+} concentrations are indicated in Table I. Half-maximal binding of receptor to nuclei observed at 50 μM total Zn^{2+} corresponds to 50 nM free Zn^{2+}. Although direct measurements of intracellular free Zn^{2+} concentration are not available, this amount of free Zn^{2+} could be well within the physiological range in the prostate. The optimum concentration of $ZnCl_2$ (200–300 μM total) for receptor binding to nuclei corresponds to 0.3–0.5 μM free Zn^{2+}.

Table II: Effect of Mercaptoethanol Concentration on Receptor Sedimentation in the Presence and Absence of a Control Membrane Fraction or Nuclei

$[Zn]_{total}$ (μM)	[M] (mM)	% receptor sedimentation[a] −sarcoplasmic reticulum[b]	% receptor sedimentation[a] +sarcoplasmic reticulum
300	0	55	75
	1	45	55
	5	19	23
	10	16	14

$[Zn]_{total}$ (μM)	[M] (mM)	% receptor sedimentation[a] −nuclei[c]	% receptor sedimentation[a] +nuclei
300	0	21	53
	0.25	16	53
	1	9	52
	5	7	45
	10	6	37

[a] Percent of total added 4.5S [^{3}H]dihydrotestosterone receptor that sedimented following centrifugation. [b] The 4.5S [^{3}H]dihydrotestosterone receptor (22 fmol) was incubated for 30 min at 25 °C in the presence or absence of rabbit skeletal sarcoplasmic reticulum vesicles (0.6 mg of protein) in 0.3 mL of 0.25 M sucrose, 300 μM $ZnCl_2$, 0.15 M KCl, 3 mM $MgCl_2$, and 50 mM Tris, pH 7.5, with increasing concentrations of mercaptoethanol. Following incubation, samples were centrifuged at 30000 rpm in a Beckman type 40 rotor ($100000g_{max}$) for 10 min at 4 °C. Pellets were extracted with ethanol, and the radioactivity was determined as described under Methods. [c] The 4.5S [^{3}H]dihydrotestosterone receptor (22 fmol) was incubated as described above except in the presence or absence of prostate tumor nuclei (860 μg of DNA). Following incubation, samples were centrifuged at 2600g for 5 min at 4 °C. Radioactivity in supernatants and ethanol extracts of pellets was determined.

Various control experiments were carried out in order to rule out receptor aggregation with nuclei in the presence of $ZnCl_2$ and mercaptoethanol. The 4.5S [^{3}H]dihydrotestosterone receptor was incubated in nuclear binding buffer in the presence of 300 μM total $ZnCl_2$ with increasing concentrations of mercaptoethanol either without or with a control membrane fraction, sarcoplasmic reticulum, isolated from rabbit skeletal muscle as previously described (Meissner, 1974). After incubation at 25 °C for 30 min, the membranes were sedimented by centrifugation at 81000g for 10 min at 4 °C. The amount of receptor sedimented in the presence or absence of sarcoplasmic membranes was inversely proportional to the concentration of mercaptoethanol, ranging from ≥55% in the absence of mercaptoethanol to ~15% in 10 mM mercaptoethanol (Table II). In the presence of 5 and 10 mM mercaptoethanol, no receptor binding to sarcoplasmic reticulum membranes was observed.

Only small amounts of the partially purified receptor sedimented at the low centrifugal force required to pellet nuclei, decreasing from 21% in the absence of mercaptoethanol to 6% in 10 mM mercaptoethanol (Table II). Addition of nuclei to the incubation buffer resulted in sedimentation of up to 53% of the receptor in the absence or presence of low amounts of mercaptoethanol and 37% at 10 mM mercaptoethanol. Optimal mercaptoethanol concentrations at 300 μM total Zn^{2+} were between 1 and 5 mM. At higher concentrations of mercaptoethanol, receptor binding to nuclei decreased some-

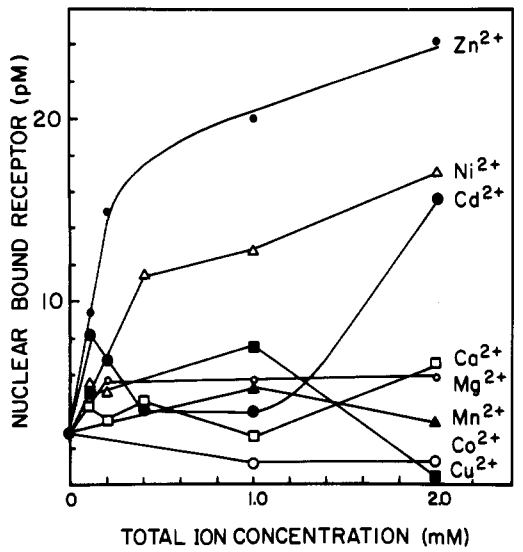

FIGURE 3: Effects of divalent cations on androgen receptor binding to nuclei in vitro. The 4.5S [^{3}H]dihydrotestosterone receptor (22 fmol) partially purified from prostate tumor cytosol by phosphocellulose chromatography was incubated with prostate tumor nuclei (100 μg of DNA) at 25 °C for 30 min as described under Methods. The incubation buffer contained 0.25 M sucrose, 0.15 M KCl, 5 mM mercaptoethanol, and 50 mM Tris, pH 7.6, plus the indicated amounts of the following divalent cations: $ZnCl_2$ (•); $NiCl_2$ (△); $CdCl_2$ (●); $CaCl_2$ (□); $MgCl_2$ (o); $MnCl_2$ (▲); $CoCl_2$ (○); $CuCl_2$ (■). Parallel incubations were carried out in the absence of nuclei to indicate nonspecific precipitation of the receptor. These values were subtracted from binding observed in the presence of nuclei, yielding specific nuclear binding. Nonspecific aggregation of the receptor in the absence of nuclei following centrifugation at 6000*g* was less than 10% for all ions, except at concentrations ≥2 mM and at both concentrations of $CoCl_2$.

what, probably due to the lowering of the free zinc concentration as a result of Zn^{2+} binding to mercaptoethanol. Therefore, receptor binding to nuclei is most likely not due to simple aggregation.

Effect of Zn^{2+} on Androgen Binding Activity of the Receptor. We investigated the effect of Zn^{2+} on androgen binding activity of the steroid-free receptor by adding $ZnCl_2$ (300 μM total concentration) to prostate tumor cytosol and incubating for 90 min at 0 °C in the presence of 5 mM mercaptoethanol with or without 0.15 M KCl. After incubation, aliquots of cytosol were labeled with 15 nM [^{3}H]dihydrotestosterone in the presence or absence of a 100-fold excess of unlabeled dihydrotestosterone. Specific receptor binding was assayed by using a charcoal adsorption method as previously described (Wilson & French, 1976). Approximately 40% of androgen binding activity in the absence of KCl and ~77% in the presence of 0.15 M KCl were lost as a result of exposure of the unbound receptor to Zn^{2+}. This loss in binding activity was not observed when receptor was bound with [^{3}H]dihydrotestosterone prior to exposure to Zn^{2+}. Thus, the androgen binding site of the receptor is sensitive to inhibition by Zn^{2+} in its free form but not in its bound form. Inhibition of androgen binding activity of the mouse prostate receptor by Zn^{2+} has been reported (Thomas et al., 1981).

Cation Specificity. Nuclear binding of the 4.5S [^{3}H]dihydrotestosterone-labeled androgen receptor was assayed for 30 min at 25 °C in the presence of 5 mM mercaptoethanol plus various divalent cations in order to test ion specificity of the receptor–nuclear interaction. As shown in Figure 3, potentiation of receptor–nuclear binding was greatest with Zn^{2+}, assayed here in the absence of $MgCl_2$. Binding increased nearly linearly up to 200 μM total $ZnCl_2$ and then gradually increased up to a concentration of 2 mM. $NiCl_2$ enhanced

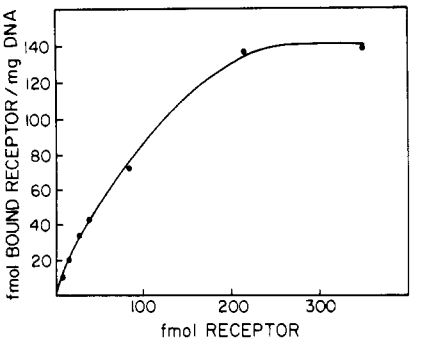

FIGURE 4: Saturation of androgen receptor binding sites in nuclei. Increasing amounts of the phosphocellulose-purified [^{3}H]dihydrotestosterone-receptor complex were incubated with prostate tumor nuclei (430 μg of DNA) as described under Methods, except in a final volume of 0.9 mL.

receptor–nuclear binding to a lower extent. Except for $CdCl_2$ which at high concentrations caused marked protein aggregation, other divalent cations were ineffective in potentiating receptor binding to nuclei, including Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, and Cu^{2+}. Furthermore, addition of 1–3 mM $MgCl_2$ in the presence of 300 μM total Zn^{2+} and 5 mM mercaptoethanol did not alter potentiation of receptor binding to nuclei by $ZnCl_2$.

The degree of pH change upon addition of metal to a mercaptoethanol-containing buffer was used as an indication of the extent of mercaptoethanol–metal binding. No interaction occurs between mercaptoethanol and Ca^{2+}, Mg^{2+}, or Mn^{2+}. The divalent cations that interacted with mercaptoethanol did so with similar affinity in the following order: $Cd^{2+} > Cu^{2+} > Ni^{2+} \sim Zn^{2+}$. Binding of $CoCl_2$ could not be determined due to its tendency to precipitate in the presence of mercaptoethanol. Thus, while some divalent cations do not potentiate receptor binding to nuclei (Ca^{2+}, Mg^{2+}, Mn^{2+}), binding is enhanced by Zn^{2+} and Ni^{2+}. Possible effects by Cd^{2+}, Co^{2+}, or Cu^{2+} were not ruled out due to their binding to mercaptoethanol.

Number of Androgen Receptor Binding Sites in Nuclei. Incubation of a constant amount of the phosphocellulose-enriched 4.5S [^{3}H]dihydrotestosterone receptor (13 fmol/0.3 mL of assay) with increasing amounts of nuclei resulted in increased binding of receptor (not shown). Under the conditions of the incubation (25 °C for 30 min), the amount of receptor bound reached a maximal level of ~42% of added receptor at a nuclear concentration of 1 mg of DNA/mL. If the time of incubation was increased to 90 min or 3 h, receptor binding increased to 51% and 56%, respectively. As discussed below, incubations for periods longer than 3 h at 25 °C were avoided due to appreciable inactivation of the androgen receptor.

Incubation of increasing amounts of 4.5S [^{3}H]dihydrotestosterone receptor with a constant amount of nuclei (450 μg of DNA) resulted in a saturation of binding at a receptor concentration of 200 pM (Figure 4). In order to minimize loss of receptor steroid binding activity (see below), incubations were carried out for 30 min at 25 °C. From these data, it can be estimated that there are approximately 1.4×10^{-13} mol of nuclear receptor binding sites per mg of DNA. If it is assumed that there are 12 pg of DNA/rat cell nucleus (Bruchovsky et al., 1975), then there would be approximately 1150 sites/nucleus. The apparent binding constant of 10^{13} M^{-1} is higher than that previously reported for in vitro nuclear binding of other steroid receptors (Kon & Spelsberg, 1981; Kuhn et al., 1977; Clark et al., 1976).

Effects of Ionic Strength and Pyridoxal 5'-Phosphate. The effect of ionic strength on receptor–nuclear binding was in-

Table III: Extraction of Zn^{2+}-Dependent Nuclear-Bound Androgen Receptor[a]

extraction medium	extracted radioactivity (% of total bound)
ethanol	100
buffer (no addition)	3
3 mM EDTA	8
0.4 M KCl, 0.3 mM Zn^{2+}	27
0.4 M KCl	42
0.4 M KCl, 3 mM EDTA	66
10 mM pyridoxal 5′-phosphate, 0.3 mM Zn^{2+}	15
10 mM pyridoxal 5′-phosphate	40
10 mM pyridoxal 5′-phosphate, 3 mM EDTA	61

[a] Prostate tumor nuclei (250 μg of DNA) were incubated with the 4.5S [^{3}H]dihydrotestosterone receptor (23 fmol) as described under Methods. After sedimentation of nuclei at 2600*g*, pellets were dispersed in 0.3 mL of ethanol or buffer containing 0.025 M KCl, 5 mM mercaptoethanol, 50 mM Tris, pH 7.5, and the compounds indicated in the table. Extractions were for 90 min at 0 °C followed by sedimentation at 2600*g*. Radioactivity in the supernatant was measured as described under Methods. The amount of extracted receptor is expressed as the percent released relative to that extracted with ethanol. Nonspecific sedimentation of radioactivity in the absence of nuclei of 3–8% for buffered samples and 12% for the ethanol extract was subtracted from the amount sedimented in the presence of nuclei. Note: The presence of 0.15 M KCl did not alter receptor extractability by 10 mM pyridoxal 5′-phosphate in the presence of 0.3 mM Zn^{2+} and 5 mM mercaptoethanol.

vestigated under standard assay conditions of Zn^{2+} and mercaptoethanol at concentrations of KCl ranging from 0.025 to 0.5 M. It was found that receptor binding in nuclei is unaltered by salt concentrations from 0.15 to 0.5 M KCl (not shown). The use of concentrations of salt lower than 0.15 M KCl was complicated by aggregation of the receptor.

Since it is well-known that a major portion of nuclear-bound steroid receptors can be extracted in high-salt buffer, we examined the possibility that Zn^{2+} might diminish salt extractability of the nuclear-bound receptor. Nuclear binding of [^{3}H]dihydrotestosterone receptor was carried out as described under Methods in the presence of mercaptoethanol and Zn^{2+}. Nuclear pellets were extracted as outlined in Table III. Only 27% of receptor was released in 0.4 M KCl containing 0.5 μM free Zn^{2+} (300 μM total $ZnCl_2$ + 5 mM mercaptoethanol). This result is in agreement with the above observations that receptor binds to nuclei in high-salt concentrations when Zn^{2+} is present. Inhibition was also observed with Ni^{2+} (300 μM), but not by Cd^{2+} (300 μM) or Mn^{2+} (300 μM) (not shown). Omission of Zn^{2+} from the high-salt extraction buffer resulted in extraction of 42% of nuclear-bound receptor. Extraction in 0.4 M KCl increased to 66% when the buffer contained 3 mM EDTA, an effective chelating agent of Zn^{2+}. Treatment with a KCl-free buffer with or without EDTA was ineffective in extracting receptor (Table III).

The salt-extractable receptor sedimented at 4.5 S on sucrose gradients containing 0.15 M KCl when diisopropyl fluorophosphate (2 mM) was included in the extraction buffer (not shown). Omission of diisopropyl fluorophosphate from the extraction buffer often resulted in a 3S receptor which we have shown previously to result from proteolytic degradation during extraction from nuclei (Wilson & French, 1979).

Since pyridoxal 5′-phosphate blocks progesterone receptor binding in nuclei (Nishigori & Toft, 1979) and can extract nuclear-bound glucocorticoid (Dolan et al., 1980) and androgen (Hiipakka & Liao, 1980) receptors, we tested its effect on the Zn^{2+}-potentiated androgen receptor–nuclear interaction. The presence of pyridoxal 5′-phosphate in the nuclear binding buffer (carried out in the dark to prevent decomposition)

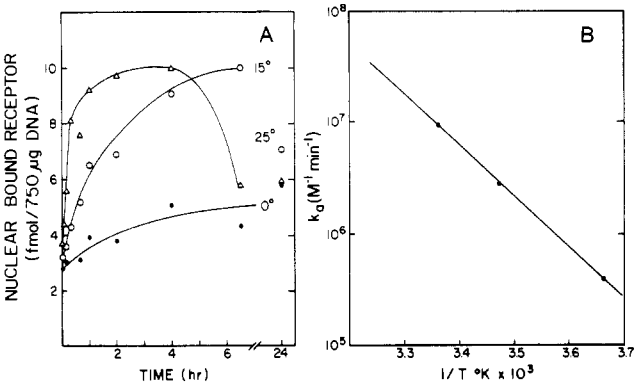

FIGURE 5: Temperature dependence of androgen receptor binding in nuclei. (A) The phosphocellulose-purified 4.5S [^{3}H]dihydrotestosterone-receptor complex (18 fmol in 0.3 mL) was incubated with prostate tumor nuclei (750 μg of DNA) as described under Methods for varying periods of time from 10 min to 24 h at 0 (●), 15 (○), and 25 °C (△). Control incubations were carried out in parallel in the absence of nuclei to establish nonspecific receptor sedimentation. Data points represent the difference in the amount of receptor in 2600*g* pellets of incubations in the presence and absence of nuclei. (B) Arrhenius plot of temperature-dependent receptor binding in nuclei. Association rate constants (k_a) for receptor binding to a nuclear fraction at 0, 15, and 25 °C were calculated by using the equation $[tk_a(b - a)]/2.303 = \log (a/b) + \log [(b - x)/(a - x)]$ where a is the maximal number of nuclear receptor binding sites (400 fmol/mL), b is the total [^{3}H]dihydrotestosterone-labeled receptor added to the binding reaction (68 fmol/mL), and x is the nuclear-bound receptor (femtomoles per milliliter) at a given time t (in minutes). Activation energy was calculated from the slope of the Arrhenius plot: $\Delta H = (\log k_1/\log k_2)[2.303R/(T_1 - T_2)]$ where $R = 1.987$ cal mol^{-1} deg^{-1}.

resulted in a concentration-dependent inhibition of receptor binding in nuclei (not shown). Pyridoxal 5′-phosphate did not reduce the concentration of Zn^{2+} as measured with an ion-selective electrode, suggesting that its ability to inhibit nuclear binding was not due to binding of Zn^{2+}.

Despite its effectiveness in blocking nuclear binding in the presence of Zn^{2+}, pyridoxal 5′-phosphate could release only 15% of nuclear-bound receptor in the presence of 0.5 μM free Zn^{2+} (300 μM total $ZnCl_2$ + 5 mM mercaptoethanol). When Zn^{2+} was omitted from the extraction buffer, however, receptor release by pyridoxal phosphate increased to 40%; in the presence of EDTA, up to 61% of nuclear-bound receptor was extracted by pyridoxal 5′-phosphate. Thus, EDTA, perhaps by chelating Zn^{2+}, enhanced the extractability of receptor in high salt or pyridoxal 5′-phosphate, suggesting that Zn^{2+} potentiation of in vitro receptor–nuclear interaction is a reversible process. The nature of the nonextractable radioactivity is not clear at this time.

Effect of Temperature. The rate of binding of the 4.5S [^{3}H]dihydrotestosterone receptor to nuclei in vitro was dependent on temperature (Figure 5). Receptor binding in nuclei was slow at 0 °C, approaching in 24 h half the maximal binding observed at 15 °C (Figure 5A). Receptor binding was of an intermediate rate at 15 °C, approaching a maximum by 6 h and then decreasing at 24 h. Nuclear binding reached a peak between 2 and 4 h at 25 °C but decreased rapidly between 4 and 6 h. The decrease in nuclear binding noted after prolonged incubation at 15 and 25 °C appeared to be due to loss of receptor steroid binding activity. Analysis of the supernatant fractions on sucrose gradients after prolonged incubation with nuclei at 15 or 25 °C revealed the loss of the 4.5S androgen receptor and the appearance of free [^{3}H]dihydrotestosterone at the top of the gradient (not shown). Thus, some component of nuclei appears to enhance receptor degradation since receptor steroid binding activity was stable when receptor was incubated in the absence of nuclei under these

conditions. Neither diisopropyl fluorophosphate (5 mM) nor sodium molybdate (10 mM) at pH 7 or 8 slowed degradation of the receptor in the presence of nuclei.

Each increase in temperature increased the association rate constant (k_a) of the receptor–nuclear interaction as follows: 4.0×10^{-5} M^{-1} min^{-1} at 0 °C, 2.8×10^{-6} M^{-1} min^{-1} at 15 °C, and 9.7×10^{-6} M^{-1} min^{-1} at 25 °C. Arrangement of these data in the form of an Arrhenius plot (Figure 5B) yielded an activation energy of 21 kcal/mol. These results indicate that Zn^{2+}-mediated in vitro androgen receptor binding to nuclei is temperature dependent in a manner similar to the temperature-dependent transformation reported for other steroid receptors (Higgins et al., 1973; Buller et al., 1975a,b).

Discussion

We have demonstrated that low levels of Zn^{2+} potentiate in vitro nuclear binding of the 4.5S [^{3}H]dihydrotestosterone-labeled androgen receptor in a concentration- and temperature-dependent manner. The importance of Zn^{2+} in steroid receptor–nuclear interactions is supported by previous indirect evidence suggesting that steroid receptors are metalloproteins, e.g., the mouse mammary gland estrogen receptor (Shyamala & Yeh, 1975) and the chick oviduct progesterone receptor (Lohmar & Toft, 1975). A high concentration of Zn^{2+} (3 mM) that slightly altered the sedimentation of the androgen receptor (Liao et al., 1975) was presumed to reflect an altered conformation of the receptor. In other studies suggesting that receptors require divalent cations, the metal chelator, 1,10-phenanthroline caused inhibition of binding of the activated glucocorticoid receptor to DNA–cellulose (Schmidt et al., 1981) and progesterone receptor binding to nuclei, ATP–Sepharose, and DNA–cellulose (Lohmar & Toft, 1975; Toft et al., 1979). It was speculated that inhibition results from chelation of metal ions associated directly with the glucocorticoid receptor (Schmidt et al., 1981).

Recent evidence indicates that steroid hormone receptors bind to specific nucleotide sequences (Mulvihill et al., 1982; Payvar et al., 1980; Govindan et al., 1982). Certain proteins known to interact with specific DNA sequences require Zn^{2+} either in tight association or as a cofactor. Some examples are DNA (Springgate et al., 1973) and RNA (Halling et al., 1977) polymerases, reverse transcriptase (Poiesz et al., 1974), the restriction enzyme *Eco*RI (Barton et al., 1982), and S1 nuclease (Vogt, 1973). Our experiments indicating that Zn^{2+} might play a role in nuclear binding of androgen receptor support the notion suggested previously (Barton et al., 1982) that DNA binding proteins in general have a requirement for Zn^{2+}.

Although other steroid receptors may be metalloproteins, potentiation of in vitro steroid receptor binding in nuclei has been reported in other systems without the addition of Zn^{2+}. The glucocorticoid receptor was shown to be heat transformed with the result that 50–60% of the receptor population could bind nuclei (Atger & Milgrom, 1976; Bailly et al., 1977) with high affinity ($K_d \sim 10^{-10}$ M) (Higgins et al., 1973). Saturable binding of up to 29% of added progesterone receptor was noted when nuclei from target or nontarget tissues were used (Buller et al., 1975a). Transformation of the progesterone receptor can be caused by 5–10 mM ATP (Moudgil et al., 1975), increasing in vitro nuclear uptake from 4 to 45% of added receptor. Interestingly, transformation of the progesterone receptor by ATP was unaffected by the presence of pyridoxal 5′-phosphate or 1,10-phenanthroline. In our experience, neither heat nor ATP exposure in the absence of Zn^{2+} and mercaptoethanol has resulted in a significant increase in in vitro nuclear binding of the androgen receptor.

Pyridoxal 5′-phosphate inhibited androgen receptor binding in a concentration-dependent manner and promoted release of receptor in the presence of EDTA. Pyridoxal 5′-phosphate is a well-known coenzyme that interacts at active sites through a Schiff base with lysine residues (Whitman & Tabita, 1978). More recently, it has been shown to interact through a similar mechanism (Nishigori et al., 1978) to block progesterone receptor binding to nuclei, ATP–Sepharose, and DNA–cellulose (Nishigori & Toft, 1979). Pyridoxal phosphate is a useful agent in extracting nuclear-bound glucocorticoid (Dolan et al., 1980) and androgen (Hiipakka & Liao, 1980) receptors and in preventing transformation of the estrogen receptor (Traish et al., 1980).

Zn^{2+} is thought to be important in male sexual development and fertility (Sandstead et al., 1967). Radiotracer techniques in the rat have shown that the testis and male accessory sex organs, such as the prostate, accumulate $^{65}Zn^{2+}$ from blood (Wetterdal, 1958). Zn^{2+} accumulates in the prostate to high levels (Mann, 1964). It has been suggested that 2 mM total Zn^{2+} may correspond to a free Zn^{2+} concentration of 20 μM in the prostate (Thomas et al., 1981). Spermatozoa within the testis avidly bind Zn^{2+} and are maintained in a high-Zn^{2+} environment within the seminal fluid (Wetterdal, 1958). Dietary Zn^{2+} deficiency leads to degeneration of the testis, reduction in spermatozoa, and decrease in the weight of the male accessory sex organs (Sandstead et al., 1967). Uptake of Zn^{2+} by the rat prostate is androgen dependent; castration causes a decrease in Zn^{2+} levels that can be restored by administration of testosterone (Gunn et al., 1961). Rats with the syndrome of testicular feminization, a genetic defect in the production of the androgen receptor that renders them insensitive to androgens, have reduced levels of zinc in the testis (Chan et al., 1981). Whether the above-cited physiological distribution and effects of zinc in the male reproductive system are related to the in vitro effects of zinc in androgen receptor binding to nuclei remains to be established.

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Registry No. Zinc, 7440-66-6; nickel, 7440-02-0.

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