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Temperature-Dependent Binding of Estrogen Receptor to Chromatin†

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ABSTRACT: Estradiol enters a target cell and rapidly binds to a cytoplasmic protein receptor. The steroid–receptor complex translocates into the nucleus where it binds to chromatin at a presumed regulatory site. It has been reported that the temperature dependence of the nuclear accumulation of estradiol at least partially resides in the entry of estradiol into the cell. We now report that the binding of estradiol-charged receptor to chromatin in a cell-free system is markedly dependent upon the incubation temperature and may require re-examination of results obtained in low-temperature cell-free experiments.

We currently understand that estradiol (E) enters a target cell and rapidly binds to a cytoplasmic protein receptor (R). The R–E complex translocates into the nucleus where it binds to chromatin (C) at a presumed regulatory site (Jensen *et al.*, 1971). Investigators found the nuclear accumulation of E to be temperature dependent with most experiments pointing to the translocation of R–E from cytoplasm to nucleus as the critical temperature-dependent step (Jensen *et al.*, 1968; Shyamala and Gorski, 1969; Giannopoulos and Gorski, 1971). However, Williams and Gorski (1971) recently provided convincing evidence that entry of E into the cell was the initial and perhaps even exclusive temperature-dependent step. We studied R–E behavior in mammary carcinoma tissue (McGuire and Julian, 1971; McGuire *et al.*, 1971) and had preliminary evidence that in a cell-free system the binding of R–E to C was influenced by temperature (McGuire *et al.*, 1972). We now report that the rate of C, R–E interaction is proportional to temperature. Preincubation of the individual components (C, R–E) at 21° in an attempt to induce enzymatic activity or irreversible conformational changes in the components fails to reproduce the marked temperature-enhanced binding observed when all components are simultaneously warmed. Thus, nuclear accumulation of E is influenced by temperature-dependent binding of R–E to C as well as temperature-dependent entry of E into the target cell.

Materials and Methods

Cytosol Preparation. Cytosol was prepared by homogenizing minced uteri of mature recently ovariectomized Sprague Dawley rats in 0.01 M Tris-HCl–0.0015 M EDTA (pH 7.4) in a glass homogenizer (400 mg of uteri/ml of buffer). The homogenate was centrifuged at 105,000*g* for 45 min and the cytosol decanted out from between the floating fat layer and pelleted debris. All procedures were done at 4°. The protein concentration was determined by the method of Lowry *et al.* (1951). The cytosol was incubated with 17β-[^{3}H]estradiol (48 Ci/mmole), final concentration 3.3×10^{-9} M. After 1 hr at 4° the unbound 17β-[^{3}H]estradiol was removed with dextran-coated charcoal (Chamness and McGuire, 1972).

Chromatin Isolation. R3230AC mammary tumors were removed from Fisher rats after a 14- to 21-day posttransplantation growth period. The healthy tissue was minced and homogenized in 5–10 volumes of 0.5 M sucrose in 0.002 M $CaCl_2$–0.25 M KCl–0.05 M Tris-HCl (pH 7.5) (buffer 1) with a 5-sec burst of a Polytron PT-10 at a power setting of 5, followed by five strokes in a motor-driven glass–Teflon homogenizer. The

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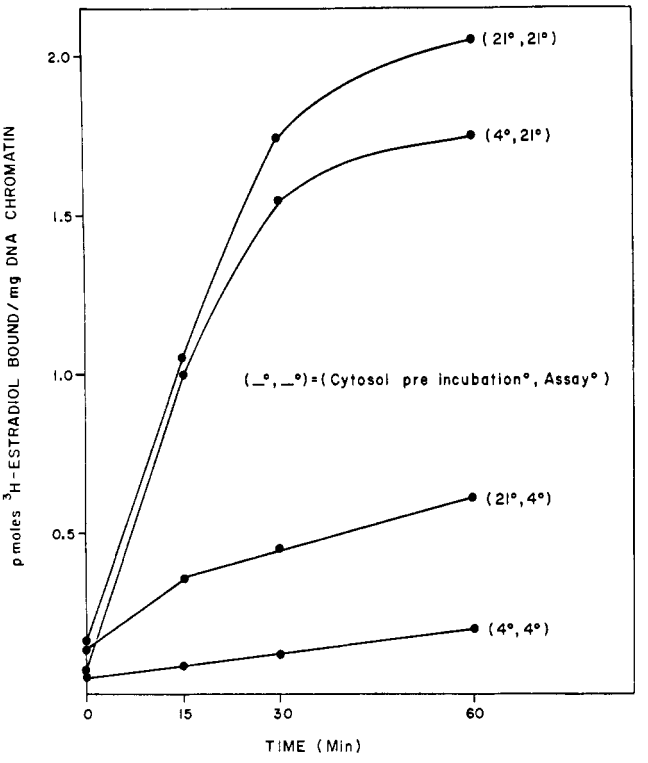

FIGURE 1: Effect of preincubating the 17β-[^{3}H]estradiol–receptor complex at 4 and 21° on subsequent binding to R3230AC chromatin. 17β-[^{3}H]estradiol (3.3×10^{-9} M) was incubated with uterine cytosol at 4° for 60 min or 21° for 20 min. Aliquots of each cytosol (0.4 mg of protein) were then incubated with chromatin (21 μg of DNA) at 4 or 21°. The first in each pair of temperatures is the 17β-[^{3}H]estradiol–cytosol preincubation temperature, the latter is the chromatin binding temperature.

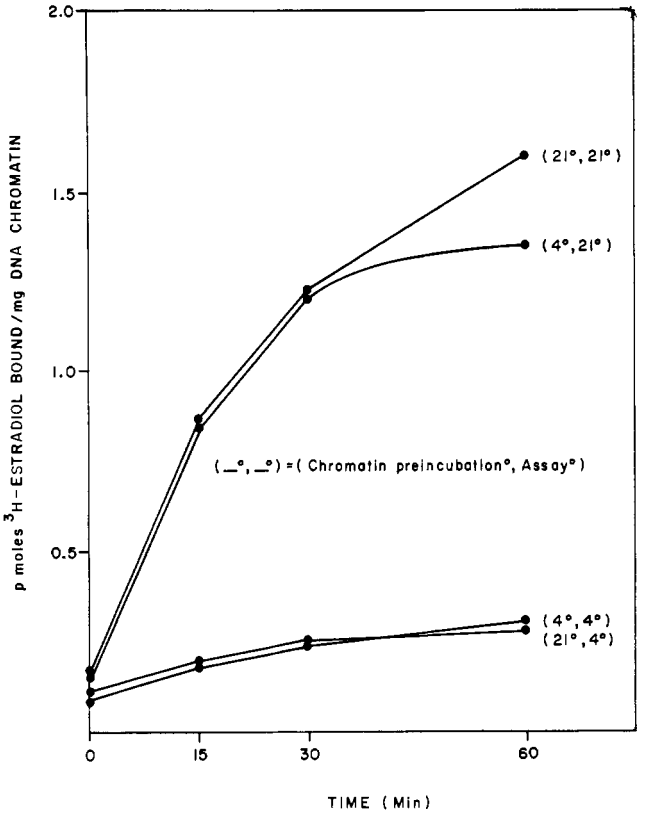

FIGURE 2: Effect of preincubating R323OAC chromatin at 4 and 21° on subsequent binding with the 17β-[^{3}H]estradiol–receptor complex. Each assay tube containing chromatin (25 μg of DNA) was incubated at 4 or 21° for one hour before adding the 17β-[^{3}H]estradiol–receptor complex (0.4 mg of cytosol protein). The receptor complex was incubated with the chromatin at either 4 or 21° for periods up to 1 hr. The first of each pair of temperatures is the chromatin preincubation temperature. The second number is the temperature of estradiol–receptor binding to chromatin.

homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 4000g. The pellet was rehomogenized in 2.0 M sucrose in buffer 1 with a final adjustment to 1.75 M sucrose. This homogenate (5 g of starting material/30-ml volume) was centrifuged at 26,000 rpm in a SW 27 rotor for 60 min. The supernatant and pellet were separated and the supernatant with floating fat layer was rehomogenized and recentrifuged for 1 hr. The pellets of nuclei were combined and resuspended gently by hand in 0.2% Triton-X100 with 0.5 M sucrose and buffer 1. The nuclear suspension was filtered through white organza cloth (100 mesh) and centrifuged at 10,000g for 10 min. The purified nuclei were stored frozen in a few milliliters of 0.08 M NaCl + 0.02 M EDTA (pH 6.3) at −76° in a Revco freezer.

When the nuclei from approximately 100 g of R3230AC tumor tissue had accumulated the nuclei were thawed at 4°, and this temperature was maintained through the following procedures. The nuclei were washed three times in 0.08 M NaCl–0.02 M EDTA (pH 6.3) and once in 0.35 M NaCl in a Teflon–glass homogenizer. Each washing was followed by a 10-minute centrifugation at 10,300g. The partially purified chromatin was allowed to swell in 0.001 M Tris-HCl–0.001 M EDTA (pH 7.5) for 30 min, then centrifuged for 10 min at 18,000g. The chromatin became increasingly more gelatinous as it was washed twice more in 0.001 M Tris-HCl–0.001 M EDTA (pH 7.5), filtered through organza cloth (100 mesh), and allowed to swell overnight. The chromatin was recentrifuged at 10,300g for 10 min, then diluted with the above Tris buffer to give a DNA concentration of 0.5–1.0 mg/ml. The chromatin was stored frozen at −76° in 2- to 5-ml aliquots and analyzed for histone, non-histone, and DNA content as described previously (Spelsberg *et al.*, 1971). The ratios of histone protein to DNA and non-histone protein to DNA were 0.94–1.36 and 0.79–1.09, respectively.

Chromatin Binding Assay. Generally, for these experiments a quantity of cytosol-17β-[^{3}H]estradiol was allowed to react with a constant amount of R3230AC tumor chromatin at different temperatures for varying lengths of time. The preincubation temperature of the cytosol–receptor 17β-[^{3}H]-estradiol complex and the chromatin was 4° unless otherwise stated. The chromatin (representing 20–35 μg of DNA) was combined with cytosol receptor 17β-[^{3}H]estradiol complex in a medium of 0.15 M NaCl–0.5% bovine serum albumin–0.01 M Tris-HCl–0.0015 M EDTA (pH 7.4) in a total volume of 0.5 ml and incubated at the desired temperature. All assays were done in polypropylene tubes to eliminate the problem of chromatin sticking to glass. During the incubation the tubes were shaken every 10–15 min to resuspend the chromatin. At the end of the appropriate incubation time, the reaction was stopped with the addition of 1 ml of cold 0.15 M NaCl–0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.5), followed by centrifugation for 10 min at 2000g. The sedimented chromatin was washed and recentrifuged twice more, resuspended in 0.5 ml of the same solution, and transferred to a Millipore filter (0.45 μ pore size, 24 mm) under vacuum. The assay tubes were washed with two 1-ml washes and each filter was washed through with 15 ml

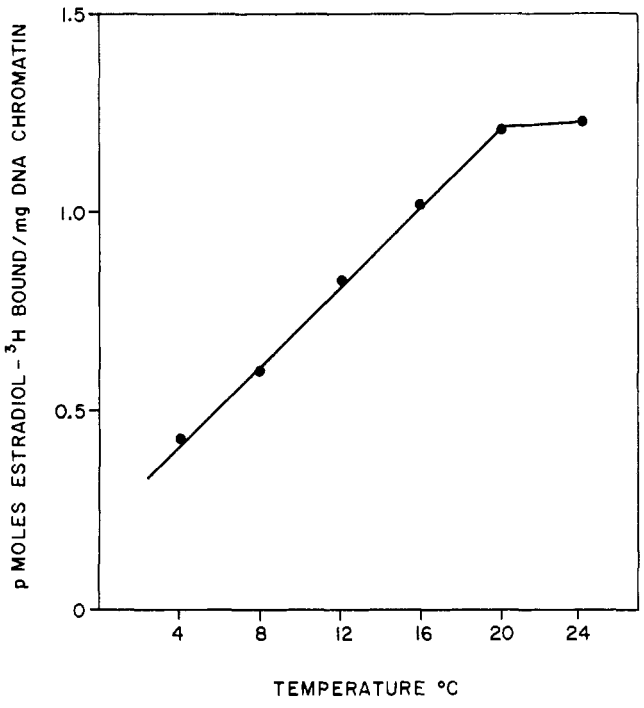

FIGURE 3: Estrogen-receptor binding to chromatin as a function of temperature. 1.0 mg of uterine cytosol charged with 17β-[^{3}H]estradiol was incubated with 29 μg of DNA-chromatin at various temperatures for 1 hr as described in Materials and Methods.

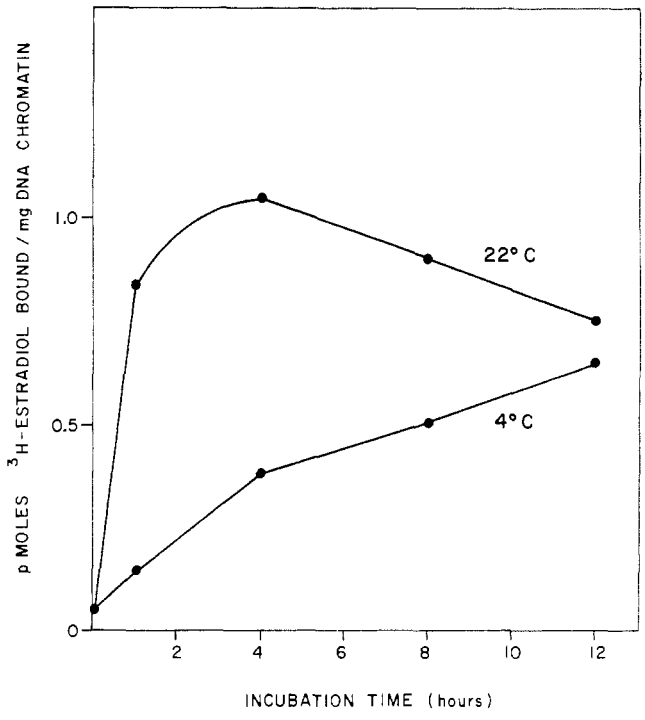

FIGURE 4: Prolonged low-temperature binding of estradiol-charged receptor to chromatin. Cytosol protein (0.85 mg) was incubated with DNA chromatin (39 μg) as described in the text.

of the NaCl–$MgCl_2$ solution. The filters were air-dried, then counted at room temperature in a Beckman LS-230 scintillation spectrometer in 5 ml of toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene solution.

After counting, the filters were removed from the vials and allowed to air-dry. The filters were cut into small pieces, then the DNA was extracted out by heating in 0.5 N $HClO_4$ for 30 min at 90°. The filter extracts were quantitated for DNA content by the diphenylamine reaction.

With each experiment assay tubes containing the cytosol and 17β-[^{3}H]estradiol but no chromatin were included. The radioactivity and diphenylamine reaction color of these blank filters are subtracted as background from the filters containing chromatin. The results are expressed as picomoles of 17β-[^{3}H]-estradiol–receptor complex bound per milligram of DNA–chromatin.

Results

In order to investigate temperature enhancement of C–R–E interaction we first examined whether preincubating the cytosol (R–E) alone at 21° and then interacting with C at 4° would show the enhancement, since it would be reasonable to postulate that the temperature effect is to induce a conformational change of R–E such that the complex now recognizes certain C acceptor sites (Jensen *et al.*, 1971). In Figure 1 we see the effect of preincubating cytosol (R–E) at 4 or 21° and then binding to C at either 4 or 21°. The 21° cytosol preincubation does slightly enhance binding to C and is explained by the fact that more E is complexed with R in the 21° preincubated group compared to the 4° preincubated group, but clearly this effect is small compared to amount of binding seen if all components (C–R–E) are present together at 21°.

This experiment did not exclude the alternate possibility that the 21° effect was to induce a conformational change in C which then permitted R–E binding to the specific acceptor sites. In Figure 2 we see the effect of preincubating C at either 4 or 21°. We see that preincubation at either 4 or 21° yielded identical results at a binding assay temperature of either 4 or 21°. Again, the 21° enhancement required the simultaneous presence of all components (C, R–E).

The above data seemed to eliminate the possibility that the 21° effect was to induce a permanent conformational change in any of the individual components; rather, the effect required all components to be present together. This suggested either that some reversible enzymatic or conformational event was occurring at the actual time of binding or that the 21° effect was strictly affecting the rate of binding independent of other effects. To explore these possibilities we examined the binding process at a series of temperatures between 4 and 21° to see if the enhancement would be proportional to the temperature increase or would increase abruptly as a critical temperature was exceeded. Figure 3 demonstrates that the binding of R–E to C was directly proportional to the increase in temperature between 4 and 21° suggesting that the temperature was primarily affecting the rate of interaction. If this were true an incubation of C and R–E at 4° should result in maximal binding identical with that of a 21° incubation if the reaction were carried out for a sufficient length of time. In Figure 4 we see that at 22°, binding of R–E to C occurs rapidly but begins to decline after several hours due to the heat lability of R. The incubation at 4° does not demonstrate this lability of R and the C–R–E interaction at this temperature shows a progressive increase up to levels approaching those found at 22°.

Discussion

The data in this report indicate that the binding of R–E to C is temperature dependent. This temperature enhancement ap-

pears primarily to increase the rate of binding since prolonged incubations in the cold will achieve binding levels comparable to short incubations at elevated temperatures. Williams and Gorski (1971) have suggested that the marked temperature dependency of the accumulation of E in uterine nuclei might be totally concerned with the entry of E into the cell. Our data in a cell-free system indicate that equally dramatic effects of temperature can be observed with R–E and C interactions.

It is noteworthy that other investigators studying C–receptor–steroid hormone interactions have not reported on the influence of temperature in these systems and have incubated the components for 1 hr at 4° (Spelsberg *et al.*, 1971; Steggles *et al.*, 1971; Mainwaring and Peterken, 1971). Our data would suggest that 4° for 1 hr would not permit sufficient interaction and would lead to an underestimation of the number of C acceptor sites. This may be important since experiments designed to demonstrate the specificity of receptor–steroid–chromatin binding have largely depended on a comparison of the number of chromatin acceptor sites in various tissues.

Added in Proof

It has been brought to our attention that the EDTA present during the incubation depicted in Figure 1 might inhibit temperature-dependent transformation of receptor in the cytosol which is thought to be required for nuclear binding (Jensen *et al.*, 1971). We have therefore repeated the experiment in the complete absence of EDTA and still obtain the same result.

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Solubilization of a Specific Tetrodotoxin-Binding Component from Garfish Olfactory Nerve Membrane†

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ABSTRACT: Extraction of the membranes from garfish olfactory nerve cells with detergent has yielded a soluble tetrodotoxin-binding component with an apparent molecular weight of about 500,000. The binding of tetrodotoxin, which is reversible, occurs with a dissociation constant of 6 nM and a dissociation rate of 0.95 min^{-1}. Saxitoxin competes for the same site with an almost identical affinity. The binding of tetrodotoxin is independent both of pH, in the region 6.5–8.5, and of ionic strength, above 0.01 M. It is concluded that there exists in garfish nerve membrane a specific tetrodotoxin binding component, which probably forms part of the sodium ion channel involved in nerve impulse propagation.

Tetrodotoxin and saxitoxin are both potent inhibitors of the regenerative sodium ion conductance change which causes excitability in nerve and muscle membranes. Their action, which is normally reversible, can be explained well on the basis of a one-to-one combination with a specific membrane constituent whose contribution to the sodium ion conductivity is then eliminated (Hille, 1968, 1970; Cuervo and Adelman, 1970). Dissociation constants for binding of toxin ranging between 10^{-8} and 10^{-9} M have been obtained with several nerve preparations, both on the basis of electrical measurements (Hille, 1968; Cuervo and Adelman, 1970; Colquhoun and Ritchie, 1972a,b) and from measurements of the binding of radioactive [^{3}H]tetrodotoxin to intact cells (Colquhoun *et al.*, 1972; Hafemann, 1972). This paper is a report of some of the properties of a soluble tetrodotoxin-binding component obtained from garfish olfactory nerve membrane by extraction with detergents.

The ultimate aim of these experiments is to isolate the sites of toxin binding. Hopefully these will turn out to form at least

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