Studies on Estradiol Receptors of the Rat Uterus. Nuclear Uptake *in Vitro**

Thomas A. Musliner,† Gerald J. Chader,† and Claude A. Villee‡

ABSTRACT: In a study of the specificity of nuclear uptake of estradiol-6,7-*t*, a biphasic accumulation of radioactivity in uterine nuclear fractions was observed when intact uterine horns were incubated with steroid. Uptake, as a function of concentration, shifted from a rapid to a slower phase at hormone levels where the cytoplasmic 8S estrogen–protein complex was nearly depleted. Studies of the uptake phenomenon in a cell-free system showed that estradiol is transferred from the cytoplasmic 8S receptor to the nuclear fraction in two different processes. In the first, small amounts of 8S complex disappear from the supernatant fraction as 4S and 5S complexes become extractable from the nuclear pellet. In the second, large amounts of hormone are transferred from the 8S complex to the nucleus without the appearance of extractable 4S or 5S complexes. When the nuclear pellet was heated above 40° before incubation with the 8S complex, less of the 4S or 5S complex could be extracted. No 4S or 5S complex was observed in extracts of diaphragm nuclei after incubation with 8S complex. A complex sedimenting more slowly than the uterine 4S complex was observed, however, but did not appear to be heat sensitive.

The experiments suggest a role for a heat-labile intranuclear factor in the uptake and binding of estradiol by uterine nuclei.

An early event in the interaction of estradiol with target cells is believed to be the binding of the hormone to a specific cytoplasmic receptor, forming a complex with a sedimentation coefficient of approximately 8 S (Toft and Gorski, 1966; Toft *et al.*, 1967; Jensen *et al.*, 1969). This is followed both *in vivo* and *in vitro* by disappearance of the estradiol–receptor complex from the cytoplasm, and the simultaneous appearance of estradiol in the nuclear pellet fraction, part of which can be extracted as a complex sedimenting at 5 S (Jensen *et al.*, 1968; Shyamala and Gorski, 1969; Jensen *et al.*, 1969). The 5S complex has also been extracted from chromatin purified from the nuclear fraction (Shyamala and Gorski, 1969). In the presence of high salt concentration, the 8S cytoplasmic receptor releases an estradiol binding subunit reported to sediment at 5 S by Erdos (1968) and Korenman and Rao (1968) and at 4 S by Jensen *et al.* (1969). Furthermore, Brecher *et al.* (1970) have recently found that a 5S complex is formed from the 4S subunit in the soluble fraction of homogenized uteri incubated at 25°.

To study the factors involved in the nuclear uptake of hormone, several workers have utilized a cell-free system in which the nuclear pellet is incubated in buffered estradiol solution with or without the addition of the supernatant fraction of homogenized tissue (Brecher *et al.*, 1967). The uptake phenomenon in this system is presently not well defined, although the steroid specificity for estrogen has been investigated in detail (Brecher and Wotiz, 1968a,b, 1969). Jensen and his coworkers (1968, 1969) have reported that the 5S complex could be extracted from the nuclear pellet incubated with supernatant containing the 8S complex but not after incubation in buffered estradiol solution or from pellet incubated with supernatant in which the cytoplasmic receptor had been destroyed by heating. More recently, they have observed that a 4S complex also appears in KCl extracts of nuclei incubated with supernatant containing 8S complex and have reported that a complex, but no 5S material, was extractable from nonuterine tissue pellets (*i.e.*, diaphragm) incubated with uterine supernatant containing 8S complex. On the other hand, Clark and Gorski (1969) were unable to detect either the 4S or 5S species from uterine pellets under similar conditions, although the supernatant was found to transfer estradiol nonspecifically to nuclear pellets of the uterus and other tissues, as well as to finely ground glass.

The experiments presented here provide additional evidence concerning the specificity of interaction between cytoplasmic 8S complex and the cell nucleus in intact uterine horns and in cell-free preparations of the uterus and diaphragm.

Materials and Methods

Incubation of Uterine Horns. Uterine horns from 24- to 26-day-old rats (Charles River Breeding Laboratory) were rapidly excised, chilled on ice, and washed at 2° with Tris-EDTA (0.01 M amino(hydroxymethyl)propanediol–0.0015 M disodium ethylenediaminetetraacetate, pH 7.4). Intact uterine horns were incubated at 37° in Tris-EDTA buffer (four uteri in 50 ml) containing appropriate concentrations of estradiol-6,7-*t* (New England Nuclear, specific activity 40–42 Ci/mmole). No differences in final results were observed when Krebs–Ringer–Henseleit–glucose buffer (Jensen *et al.*, 1968) was used instead of Tris-EDTA buffer for incubation of intact uteri. Uteri were homogenized with a ground-glass homogenizer at 2° in Tris-EDTA buffer (0.05 ml/horn). Supernatant and pellet fractions (referred to as "nuclear pellet" or

* From the Department of Biological Chemistry, Harvard Medical School, and the Laboratory of Reproductive Biology, Boston Hospital for Women, Boston, Massachusetts. *Received April 27, 1970.* This work was supported by Grant HD06 of the U. S. National Institute of Child Health and Human Development.

† Present address: Howe Laboratory, Massachusetts Eye and Ear Infirmary, Boston, Mass. 02114.

‡ To whom to address correspondence.

"nuclear fraction" in these studies) were prepared by centrifugation for 60 min at 105,000*g* in a Spinco Model L2 or Model L2-65B ultracentrifuge.

Incubations of Cell-Free Fractions. Uteri were homogenized and separated into nuclear and supernatant fractions prior to addition of estradiol-6,7-*t*. An individual incubation utilized material from seven uterine horns (3.5 uteri) homogenized in 0.5 ml of Tris-EDTA buffer. Pellet fractions were rehomogenized with supernatant to which 2.5 ng of estradiol-6,7-*t* had been added, and incubated for 6 min at 37°, after which they were again separated by ultracentrifugation at 2°. The 4S and 5S complexes were extracted by homogenizing the nuclei in 0.5 ml of Tris-EDTA buffer containing 0.4 M KCl (pH 8.5), freezing and thawing (Shyamala and Gorski, 1969), and finally centrifuging for 30 min at 2° at 105,000*g*. In some experiments, isolated nuclear pellets were homogenized in 0.5 ml of Tris-EDTA buffer, incubated for 10 min at appropriate temperatures. and recentrifuged before incubation with supernatant. Aliquots of 0.2 ml of the nuclear extracts and of cytoplasmic supernatant were analyzed by centrifugation in sucrose density gradients.

Experiments with the diaphragm were done with an amount of tissue equal in wet weight to the uterine tissue used. Diaphragm nuclei, prepared as above, were then incubated with uterine supernatant as indicated.

Gradient Density Ultracentrifugation. Linear sucrose density gradients (5–20%) were prepared in Tris-EDTA buffer (for supernatant fractions) or Tris-EDTA–0.4 M KCl buffer (for KCl pellet extracts) using RNase-free sucrose (Mann). Samples of 0.2 ml were layered on 4.5-ml cold gradients and centrifuged for 12–15 hr at 2° at 39,000 or 45,000 rpm in a Spino Model L2 or Model L2-65B ultracentrifuge, using an SW-39 or SW-50.1 rotor. Bovine serum albumin, 5 mg/ml in Tris-EDTA, was used as standard. Fractions of approximately 0.13 ml were collected in scintillation vials. When appropriate, the optical density of each fraction was measured with a Zeiss PMQ II spectrophotometer after addition of 0.5 ml of buffer. Samples were dried and a drop of water was added to each, followed by 0.5 ml of NCS solubilizer (Amersham–Searle). After KCl extraction, nuclear pellets were dissolved in 1 ml of NCS solubilizer. Samples were shaken gently for 1 hr, 10 ml of scintillation fluid containing 4 g of Omnifluor (New England Nuclear Corp.)/l. of toluene was added, and radioactivity was measured in a Packard spectrometer Model 3375 (40% efficiency). Results are expressed as counts per minute/fraction.

After a typical sucrose gradient ultracentrifugation of the supernatant fraction, steroid in the 8S peak and in regions sedimenting before and after it was extracted and shown by thin-layer chromatography to be unaltered estradiol-6,7-*t* (M. Fencl, E. Charreau, G. J. Chader, and C. A. Villee, 1969, unpublished data).

Results

Kinetics of the 8S Complex Depletion and Nuclear Estradiol Uptake in Intact Uterine Horns. Intact uterine horns were incubated for 2 hr at 37° in solutions containing different concentrations of estradiol-6,7-*t*. The results of sucrose density centrifugation of the respective supernatant fractions are shown in Figure 1. Virtually all of the label in the supernatant fraction sedimented at 8 S (curve B, Figure 1) when the steroid concentration was 1×10^{-10} M or lower in the incubation medium. With increasing concentrations (curve A), there was a transient rise in the amount of 8S complex as more estradiol became available to the cytoplasmic receptor. Greater hormone concentrations, 1.5×10^{-9} M or higher, however, resulted in a smaller 8S peak and left increasing amounts of free radioactivity at the top of the gradient. The greater total radioactivity in the supernatant at 5×10^{-10} M estradiol than at 1×10^{-9} M estradiol suggests that the disappearance of the labeled 8S peak was not due simply to instability of the newly formed complex.

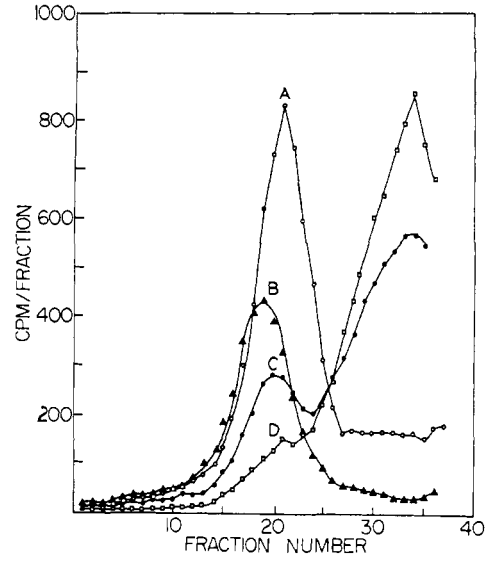

FIGURE 1: Sedimentation patterns of supernatant fractions from uterine horns incubated *in vitro* at 37° for 2 hr with varying concentrations of estradiol-6,7-*t*. (A) 5×10^{-10} M, (B) 1×10^{-10} M, (C) 1×10^{-9} M, and (D) 1.5×10^{-9} M. Supernatant was layered on a 5–20% linear sucrose gradient prepared in Tris-EDTA buffer and centrifuged 12 hr at 39,000 rpm in a Spinco Model L2 ultracentrifuge.

With increasing estradiol concentrations, the radioactivity bound in the nuclear fractions (Figure 2) increased in a biphasic pattern. At levels of hormone at which the disappearance of 8S peak neared completion, the rate of increase in nuclear binding diminished.

A similar transient appearance of the 8S peak was observed when uptake was studied at a fixed concentration of estradiol (2×10^{-9} M) and the time of incubation was varied (Figure 3). The large 8S peak present at 30 min was smaller at 60 min and had disappeared almost completely by 2 hr. Both the amount of steroid at the top of the gradient and the radioactivity in the pellet fraction increased during this period.

Interaction between 8S Complex and Nuclear Fraction in a Cell-Free System. Before studying the transfer of estradiol from the cytoplasmic complex to the nuclear fraction in a cell-free system, it was necessary to examine the stability of the cytoplasmic receptor under these conditions. Incubation of supernatant alone for 30 min at 37° reduced the amount of 8S complex detectable upon subsequent addition of estradiol-6,-7-*t* to approximately 5% of an initial value of 36,120 cpm. The presence of estradiol throughout the incubation stabilized the receptor, so that about 30% of the radioactivity remained in the 8S peak after 30 min with an additional 10% sedimenting more rapidly in heavier aggregates. In contrast, supernatant

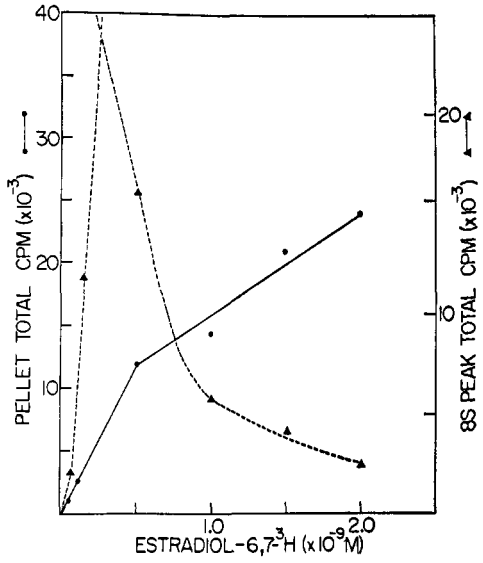

FIGURE 2: Radioactivities in nuclear pellet fractions and 8S peaks of supernatant fractions from uteri incubated *in vitro* at 37° for 2 hr with varying concentrations of estradiol-6,7-*t* in incubation medium. Concentrations were (1) 5×10^{-11} M, (2) 1×10^{-10} M, (3) 5×10^{-10} M, (4) 1×10^{-9} M, (5) 1.5×10^{-9} M, and (6) 2×10^{-9} M. The radioactivity in the pellets was measured by direct determination after incubation; the radioactivity in the 8S peaks was determined after the supernatant sample was subjected to density gradient analysis as described in the legend of Figure 1.

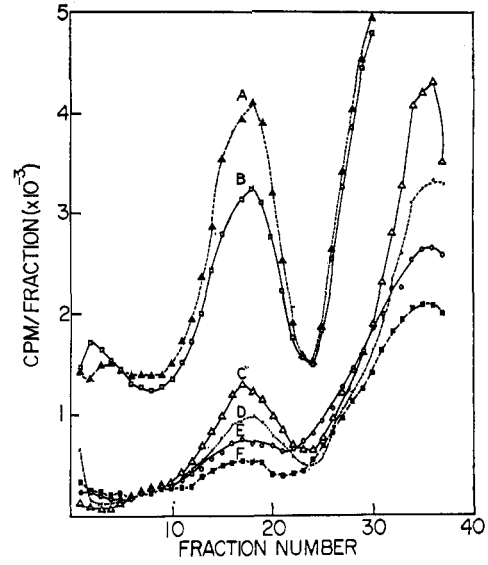

FIGURE 4: Sedimentation patterns of uterine supernatant fractions showing the effect of heating the nuclear pellet before incubation with supernatant fraction. (A) Supernatant incubated for 6 min at 0°; (B) supernatant incubated for 6 min at 37°; (C) supernatant incubated with pellet for 6 min at 37°; pellet previously heated at 60° for 10 min; (D) supernatant incubated with pellet for 6 min at 37°; pellet previously heated at 80° for 10 min; (E) supernatant incubated with pellet for 6 min at 37°; pellet previously heated at 40° for 10 min; and (F) supernatant incubated with pellet for 6 min at 37°; pellet kept at 0° for 10 min before the incubation. Other conditions as in Figure 1.

containing 8S complex, incubated with nuclear pellet for 30 min at 37°, contained less than 5% of the complex in the 8S or heavier forms. Thus, incubation with the nuclear pellet depleted the supernatant 8S complex to a far greater extent than incubation of supernatant fraction alone. The contrast was still more marked with shorter incubation times. After 6 min at 37°, 85–90% of the total radioactivity was found in the 8S complex in the absence of the nuclear pellet, but only approximately 15% in its presence. A typical experiment is shown in Figure 4 (curves A, B, and F).

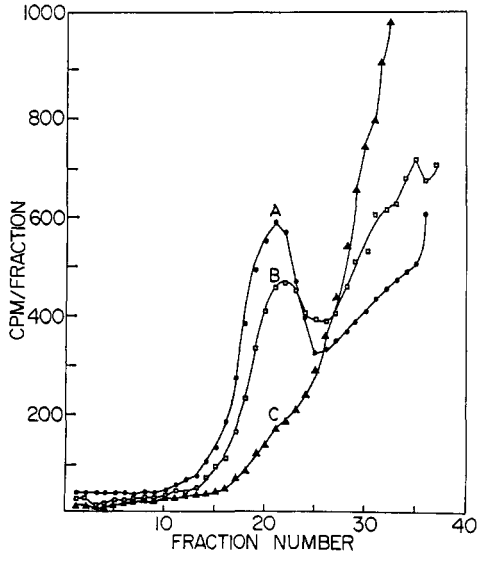

FIGURE 3: Sedimentation patterns of supernatant fractions from uterine horns incubated *in vitro* with 2×10^{-9} M estradiol-6,7-*t* for varying lengths of time. (A) 30 min, (B) 60 min, and (C) 120 min. The experimental conditions were as given in the legend to Figure 1.

When the nuclei were incubated with supernatant containing 8S complex and then extracted with Tris-EDTA-KCl buffer, subsequent density gradient analysis of the extract showed radioactivity sedimenting in the 4–5S region as well as at the top of the gradient (presumably free estradiol). As seen in Figure 5, the macromolecular-bound estradiol-6,7-*t* sedimented as two peaks on either side of a bovine serum albumin marker (4.6 S). Only 20–30% of the radioactivity in the nuclear pellet could be extracted with KCl and about 60% of this sedimented in the 4–5S region (see Table I). Neither 4S nor 5S complex could be detected in pellets incubated with estradiol-6,7-*t* in the absence of supernatant complex. No 5S material was found in supernatant fractions incubated with or without pellet fraction in these experiments, presumably because the conversion of 4S to 5S complex described by Brecher *et al.* (1970) is inhibited by EDTA. It is not clear at present why 4S and 5S nuclear complexes were obtained in the present study and in that of Jensen *et al.* (1969) but not by Clark and Gorski (1969).

The total radioactivity recoverable in the pellet fraction, KCl fraction, and residual supernatant fraction generally amounted to 60% of the radioactivity present in the supernatant fraction prior to incubation. We assume this difference results from nonspecific binding of estradiol to tube surfaces, as described by Clark and Gorski (1969).

Studies on the Heat Sensitivity of Nuclear Uptake of Steroid. To clarify the role of the nuclear pellet in its interaction with the 8S complex, the cell-free experiment was repeated using

TABLE I: Distribution of Radioactivity in Nuclear Pellet after Incubation with Supernatant 8S Complex.[a]

Pellet Incubated at (°C)	Total Pellet (cpm)	KCl Extractable (cpm)	4–5S Complex (cpm)	Residual (cpm)
0	81,420	18,650	11,100	62,770
30	81,050	17,710	10,500	63,340
40	75,135	14,305	6,760	60,830
50	83,665	11,880	3,995	71,785
60	101,720	9,510	2,580	92,210

[a] Supernatant 8S-estradiol-6,7-^3H complex was incubated for 6 min at 37° with pellet fractions that had been previously kept for 10 min at 0, 30, 40, 50, or 60°. Pellets were separated and extracted with buffer containing 0.4 M KCl. Aliquots (0.2 ml) were subjected to density gradient ultracentrifugation. The radioactivity of the KCl extracts and of the residual nuclear pellets were measured and summed to obtain the radioactivity of the total pellet.

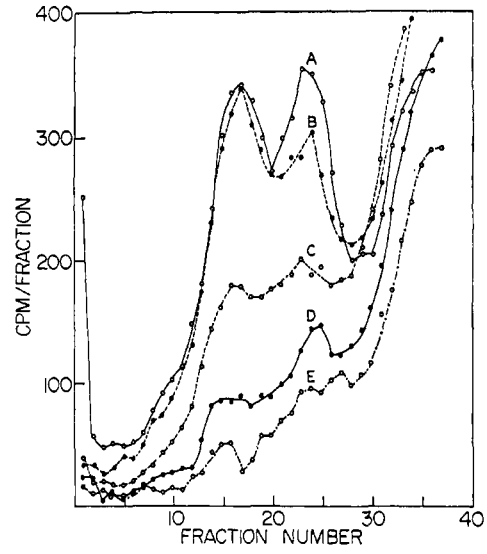

FIGURE 5: Sedimentation patterns of KCl extracts of nuclear pellets after incubation with supernatant 8S complex for 6 min at 37°. Pellets were previously treated for 10 min at (A) 0°, (B) 30°, (C) 40°, (D) 50°, and (E) 60°. Extraction was with Tris-EDTA buffer containing 0.4 M KCl. Density gradient ultracentrifugation was performed at 45,000 rpm for 15 hr in a Spinco Model L2-65B ultracentrifuge.

nuclear fractions which had been heated before incubation with supernatant 8S complex. Representative density gradients of the resultant supernatant fractions and KCl extracts of the nuclear pellet fractions are shown in Figures 4 and 5. The depletion of the 8S complex that results from the incubation of supernatant with the nuclear pellet was slightly inhibited when the pellets were preheated at 40, 60, or 80° (Figure 4, curves E, C, and D). The maximal inhibition, however, was never greater than 15% of the total depletion observed with unheated pellet with these or other (30, 50, and 65°) temperatures.

Heating the nuclear pellet before incubation with supernatant had a much more marked effect on the formation of 4S and 5S complex (Figure 5 and Table I). An incubation at 40° for 10 min reduced the amount of 4–5S material that could be subsequently extracted to approximately 60% of the control value. Heating at 60 or 80° virtually eliminated the two peaks (Figure 5, curve E). It may be significant that the portion of 8S complex depletion that was heat sensitive (12,870 cpm when curves C and F are compared) was comparable to the amount of 4S and 5S complex extracted from unheated pellet (11,100 cpm). The total uptake of radioactivity by the pellet fraction was not decreased by heating; in fact, heating pellets to 60 or 80° consistently increased the total uptake of radioactivity by 10–20%. In view of the slightly increased levels of radioactivity remaining in the supernatant fractions at these temperatures, it seems most likely that pellet heating enhances total nuclear uptake at the expense of nonspecific binding of hormone to tube surfaces (Clark and Gorski, 1969).

Interaction between Uterine 8S Complex and Nuclei from Diaphragm. Uterine supernatant fraction containing 8S complex was incubated with the nuclear fraction from homogenized rat diaphragm. This resulted in a 65–70% depletion of the 8S complex. Total uptake of radioactivity by the diaphragm nuclear pellets was comparable to that observed with nuclei of the uterus, but only 15% of this was solubilized by Tris-EDTA–KCl buffer. When the extracted material was analyzed by density gradient centrifugation, approximately one-half of the radioactivity was found at the top of the gradient while the remainder sedimented in a peak in the 3–4S region, close to but slightly slower than the 4S complex of uterine nuclei. No 5S peak was observed (Figure 6).

Heating of diaphragm pellet for 10 min at 60° before incubation with uterine supernatant had little effect on the depletion of the 8S complex initially present. In contrast to the heat stability of nuclear complex formation in the uterus, approximately equal amounts of 3–4S material were extracted from heated and unheated diaphragm pellets incubated with uterine supernatant (Figure 6). As with unheated diaphragm nuclei, no 5S material was detected.

Discussion

As first demonstrated in the laboratories of Jensen and Gorski, uterine horns from immature rats accumulate estradiol in a process characterized by the appearance of an 8S hormone–receptor complex in the cytoplasm and its subsequent depletion with simultaneous concentration of steroid in the nuclear pellet. In the present study, the same sequence of appearance and disappearance of 8S complex was observed both as a function of length of incubation and of hormone concentration. Uptake by the pellet followed a biphasic course with increasing estradiol concentrations. The first phase of uptake corresponded to low levels of hormone where amounts of 8S complex still remained in the supernatant after the 2-hr incubation. The second phase, in which the rate of increase in nuclear uptake was not as great, corresponded to higher concentrations where 8S complex was largely depleted. It seems likely, therefore, that estradiol accumulates in the nuclear pellet in two processes—one requiring the presence of 8S

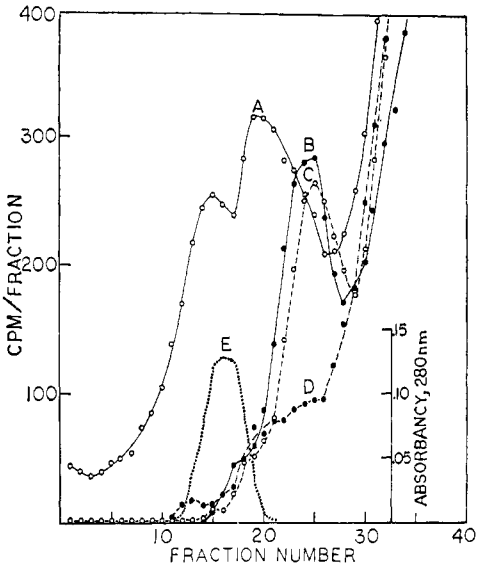

FIGURE 6: Sedimentation patterns of 0.4 M KCl extracts of nuclear pellets after incubation with uterine supernatant complex for 6 min at 37°. (A) Uterine nuclear pellet previously kept at 0° for 10 min; (B) diaphragm nuclear pellet previously heated at 60° for 10 min; (C) diaphragm nuclear pellet previously kept at 0° for 10 min; (D) uterine nuclear pellet previously heated at 60° for 10 min; and (E) bovine serum albumin control. Density gradient ultracentrifugation was performed at 45,000 rpm for 17 hr in a Spinco Model L2-65B ultracentrifuge.

complex and resulting in its depletion from the cytoplasm, and the other occurring with free, unbound hormone, independent of the cytoplasmic receptor.

In a cell-free system, incubating the nuclear pellet with the supernatant fraction resulted in depletion of the cytoplasmic 8S complex accompanied by the appearance of both 4S and 5S nuclear complexes. The nuclear complexes accounted for only a small fraction of the total steroid taken up by the nuclei or depleted from the 8S complex. A similar extraction procedure has been reported to solubilize almost 100% of the radioactivity from the nuclear fraction of uterine horns exposed to estradiol *in vivo* (Puca and Bresciani, 1968). This suggests that part of the hormone accumulated by the nuclear fraction during cell-free incubations may be bound in a fashion different from that observed in the nuclei of uteri exposed to estrogens *in vivo*. After heating the nuclear pellet, depletion of 8S complex from the supernatant was slightly inhibited, while the formation of nuclear 4S and 5S complexes was drastically reduced, even though the total uptake of estradiol into the nuclei remained unchanged or was slightly increased at the higher temperatures.

These results can be explained if one assumes that the nuclear uptake of hormone observed in cell-free incubations involves at least two concurrent processes. The first would be relatively nonspecific and account for the larger fraction of depletion of 8S complex observed. This process would be slightly increased in pellets that had been heated to high temperatures and would not result in the appearance of extractable 4S and 5S complex from the nuclear pellet. The second uptake process would be sensitive to heat treatment of the nuclei and result in the specific formation of small amounts of 4S and 5S nuclear complexes with concomitant utilization of 8S complex from the supernatant. Alternatively, one may postulate that depletion of the 8S complex involves a single process which is only loosely coupled to the formation of 4S and 5S complexes in a subsequent second step. If the first step were heat stable and the second heat labile, heating the pellet would eliminate the appearance of 4S and 5S complexes, but not the depletion of the 8S complex. Since even drastic treatment of the pellet fraction (*i.e.*, heating at 60 or 80°) only slightly decreases the depletion of the 8S complex, it seems more likely that most of this depletion results from a separate, nonspecific process. The increased nuclear uptake of estrogen after pellet heating suggests that new nuclear binding sites become available after exposure to higher temperatures. It is interesting to note that this temperature range roughly corresponds to thermomelting curves of isolated chromatin and that DNA has been shown to interact directly with 17β-estradiol (Ts'o and Lu, 1963; Goldberg and Atchley, 1966; Cohen and Kidson, 1969; Speichinger and Barker, 1969).

To examine the specificity of nuclear complex formation, pellet from diaphragm muscle was incubated with uterine supernatant containing 8S complex. Accumulation of estradiol by diaphragm pellet quantitatively resembled that observed with uterine nuclei. Depletion of 8S complex was little affected by prior heating of the pellet to 60°. Extraction of diaphragm nuclei produced small amounts of a complex sedimenting more slowly than the 4S complex of uterine nuclei, but no material in the 5S region. It seems reasonable to postulate that the 8S complex depletion observed with diaphragm pellets might be similar to the nonspecific depletion process seen with uterine pellets, although the nature of the heat-stable formation of 3–4S complex in diaphragm remains to be clarified.

The formation of extractable 4S and 5S complexes appears to be dependent, in these experiments, not only on the presence of 8S complex, but also on a heat-labile nuclear factor present in the uterus but not the diaphragm. The sensitivity to heat may reside in the 4S and 5S nuclear complexes themselves, assuming they are entirely or in part of nuclear origin. This seems improbable, however, since the 8S cytoplasmic complex contains a 4S subunit which can be converted to a 5S complex in a particle-free system (Brecher *et al.*, 1970). More likely, the heat-sensitive component may be a separate nuclear factor required for the breakdown or processing of the 8S supernatant complex or the intranuclear binding of 4S and 5S complexes.

References

Brecher, P. I., Numata, M., DeSombre, E. R., and Jensen, E. V. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 249.

Brecher, P. I., Vigersky, R., Wotiz, H. S., and Wotiz, H. H. (1967), *Steroids 10*, 635.

Brecher, P. I., and Wotiz, H. H. (1968a), *Proc. Soc. Exp. Biol. Med. 28*, 470.

Brecher, P. I., and Wotiz, H. H. (1968b), *Steroids 9*, 431.

Brecher, P. I., and Wotiz, H. H. (1969), *Endocrinology 84*, 718.

Clark, J. H., and Gorski, J. (1969), *Biochim. Biophys. Acta 192*, 508.

Cohen, P., and Kidson, C. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 458.

Erdos, T. (1968), *Biochem. Biophys. Res. Commun. 32*, 338.
Goldberg, M. L., and Atchley, W. A. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 989.
Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W., and DeSombre, E. R. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 632.
Jensen, E. V., Suzuki, T., Numata, M., Smith, S., and DeSombre, E. R. (1969), *Steroids 13*, 417.
Korenman, S. G., and Rao, B. R. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1028.
Puca, G. A., and Bresciani, F. (1968), *Nature (London) 218*, 967.
Shyamala, B., and Gorski, J. (1969), *J. Biol. Chem. 244*, 1097.
Speichinger, J. P., and Barker, K. L. (1969), *Steroids 14*, 132.
Toft, D., and Gorski, J. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1574.
Toft, D., Shyamala, B., and Gorski, J. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1740.
Ts'o, P. O. P., and Lu, P. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 17.

An Extraordinary Temperature Dependence of the Reovirus Transcriptase*

A. M. Kapuler†

ABSTRACT: The RNA-dependent RNA polymerase (transcriptase) found in the subviral core of reovirus has an unusual temperature dependence for polyribonucleotide synthesis. The rate of RNA synthesis is 13–15-fold higher at 44° than at 34°. The temperature optimum is between 47 and 52° and Arrhenius plots are biphasic. There is a profound activation between 30 and 40° and a more normal temperature coefficient between 40 and 50°. There are no major differences in the species of RNA being synthesized at 37.5, 45, and 52°. Replacement of normal substrates by the analogs 5-bromocytidine 5′-triphosphate or 5-bromouridine 5′-triphosphate does not qualitatively alter the temperature dependence and nucleoside 5′-triphosphates (NTPases) bound with the transcriptase in the subviral core do not show the abnormal temperature effect. The kinetics of synthesis of a reaction preincubated for 2 min at 51° and shifted to 34° are no different from those of a reaction maintained exclusively at 34°. A cooperative conformational change in the template RNA which directly affects polymerase activity is considered as a potential explanation for the phenomenon.

Analysis of the life cycles of animal viruses has revealed some novel phenomena. One such unexpected event was the discovery that several diverse viruses contain template-bound polymerases as a structural component of the mature virion. Kates and McAuslan (1967) and Munyon *et al.* (1967) found a transcriptase in the core of vaccinia, a DNA virus, while Shatkin and Sipe (1968) and Borsa and Graham (1968) uncovered a RNA-dependent RNA polymerase in reovirions and Baltimore *et al.* (1970) a comparable enzyme in vesicular stomatitis virions.[1] We succeeded in isolating the reovirus transcriptase directly from virus infected L cells in subviral particles (Levin *et al.*, 1970). This enzyme, as well as other preparations from mature virions (Skehel and Joklik, 1969) or infected cells (Gomatos, 1968), synthesizes ten mRNA molecules which correspond to the ten double-stranded RNA molecules or chromosomes found within the virus particle. While characterizing the transcriptase, we found that a NTPase[2] activity capable of hydrolyzing either ribo- or 2′-deoxyribo 5′-triphosphates to their corresponding 5′-diphosphates was intimately associated with the polymerase in the subviral particle (Kapuler *et al.*, 1970). In this paper, we examine the effect of temperature on the synthesis of reovirus mRNA by the subviral particle polymerase. RNA synthesis is measurable at 30–32°, but not below, and has an optimum rate at 50–52°. Between 34 and 44°, a 13–15-fold stimulation of RNA synthesis takes place without a qualitative alteration in the species of RNA being made. A potential explanation of the phenomenon is that at physiological temperatures a conformational change in the template RNA occurs leading to a profound effect on the transcriptase activity.

Experimental Section

The Dearing strain, type 3, reovirus RNA polymerase (or transcriptase or mRNA synthetase) was isolated in the

* From the Institute of Cancer Research, New York, New York. *Received July 14, 1970.* The work was supported in part by the Muscular Dystrophy Association of America.

† Present address: Life Sciences, Microbiology Section, University of Connecticut, Storrs, Conn.

[1] This line of investigation has recently culminated with the demonstration that the virions of RNA tumor viruses contain RNA-dependent DNA polymerases (Temin and Mizutani, 1970; Baltimore, 1970), thus substantiating Temin's heretical DNA provirus hypothesis (Temin, 1964).

[2] The following unusual abbreviations are used: SVP, subviral particle; NTPase, nucleoside 5′-triphosphatase; E_a, activation energy; 5BrCTP, 5-bromocytidine 5′-triphosphate; 5BrUTP, 5-bromouridine 5′-triphosphate.