Estrogen Receptor in the Rat Uterus. Physiological Forms and Artifacts†

Gary C. Chamness*,‡ and William L. McGuire

ABSTRACT: Estrogen receptors are always present in the cytoplasm of target tissues but can only be extracted from nuclei, their presumed site of action, after incubation with estrogens. In comparing cytoplasmic and nuclear estrogen receptors from rat uterus, we have shown that the cytoplasmic form sediments at 6 S at physiological ionic strength (0.15 M KCl), in contrast to 8 S and 4 S in low and high salt as usually studied. Receptor extracted from nuclei remains at 4.5 S in physiological as in high salt. We can, however, deliberately cause either form to assume any value between 4 S and 9 S by adjusting the concentration of the polyanion heparin, so that the previous emphasis on S value assignments appears less meaningful. An independent comparison of cytoplasmic and nuclear receptor is provided by DEAE-cellulose chromatography, which clearly separates the 4.5S nuclear receptor from the cytoplasmic 8S form.

Estrogen target tissues contain a high affinity estrogen binding protein which is thought to serve as a receptor, initiating responses to the hormone. Current knowledge of the estrogen receptor has been extensively reviewed (Jensen *et al.*, 1971, 1969; Gorski *et al.*, 1968). Briefly, it is found only in the cytoplasm in the absence of estrogen, but is translocated rapidly into the nucleus after binding the hormone; the transfer step is apparently temperature dependent. In the nucleus, receptor is bound to chromatin, where it presumably acts to stimulate the first phases in the complex sequence of estrogen responses (Hamilton, 1971).

Considerable effort has been devoted to determining the natural intracellular state or states of the receptor, with emphasis on its sedimentation through sucrose gradients; some of these results are assembled in Table I. In general, receptor obtained from the cytoplasm sediments at 8–9 S in the absence of salt and at 4–5 S in 0.3–0.4 M KCl, while receptor extracted from nuclei sediments at about 5 S in the latter condition and seems to form nonspecific aggregates in the former. The variation between different laboratories and different conditions has been considerable. Only two published studies (Reti and Erdos, 1971; Rochefort, 1972) have employed physiological salt concentrations for the sedimentation of cytoplasmic receptor; both reported values near 6 S. There are apparently no reports of the behavior of nuclear receptor under these conditions. Recently, Harris (1971) made the provocative discovery that, if polyanions are added, nuclear receptor sediments at 8 S in the absence of salt rather than aggregating. It was suggested that this results from complexing of the polyanions with basic proteins which would otherwise aggregate nonspecifically with the receptor, and that the true nuclear form of the receptor is therefore revealed. If so, it would be possible that this form is the same as that found in the cytoplasm.

Here we consider further the question of differences between nuclear and cytoplasmic receptor in rat uterus and the problem of determining the native state of each. We report first the sedimentation rates of both nuclear and cytoplasmic forms in sucrose gradients at physiological ionic strength. We then examine the polyanion effect on both types of receptor and show that a wide range of sedimentation values can be produced by varying polyanion concentration. Finally, we employ DEAE-cellulose chromatography to further compare nuclear and cytoplasmic forms of receptor by a method independent of sedimentation rates.

Methods

Preparation of Cytoplasmic Receptor. Uteri were removed from mature intact female rats, chilled and weighed, minced with scissors, and homogenized in TE 7.4 (0.01 M Tris-HCl (pH 7.4), containing 0.0015 M Na_2EDTA), 1 ml of buffer/400 mg wet weight of tissue, using a glass–glass homogenizer. The homogenate was centrifuged 40 min at 200,000*g* and the supernatant recovered after elimination of any floating fat. To the supernatant was added [^{3}H]estradiol (48 or 96 Ci per mmole) to 10^{-9} M. Radiochemical purity of the estradiol was checked by thin-layer chromatography (Bishara and Jakovljevic, 1969). After at least 30 min at 2° the labeled cytosol was ready for use, though for some applications free estradiol was removed by passage over Sephadex G-25 or by treatment with dextran-coated charcoal. The charcoal procedure, adapted from Korenman (1968), used a suspension of 2.5 g/l. of Norit A and 25 mg/l. of dextran in 0.01 M Tris-HCl (pH 8.0)–0.0015 M Na_2EDTA. A volume equal to that of the cytosol preparation to be treated was centrifuged 10 min at 2000*g* and the supernatant discarded; the pellet was resuspended directly in the cytosol and, after 15 min, centrifuged 10 min at 2000*g*, leaving the supernatant free of unbound estradiol. All operations were carried out at 2°, and all pH values were adjusted at this temperature.

Preparation of Nuclear Receptor. Mature intact rats were given intraperitoneal injections of 0.1 μg of [^{3}H]estradiol in 0.5 ml of saline. After 1 hr the uteri were removed, homogenized, and centrifuged as above. The pellet was rehomogenized in TE 7.4, about 1 ml/200 mg of original tissue, and the centrifugation was repeated. The resulting washed sediment was homogenized with TKE 8.5 (0.01 M Tris-HCl (pH 8.5)–0.4 M KCl–0.0015 M EDTA), about 0.5 ml/200 mg of original tissue, and allowed to remain 1 hr in the cold before

† From the Department of Physiology and Medicine, University of Texas Medical School at San Antonio, San Antonio, Texas 78229. *Received December 21, 1971.* Supported in part by U. S. Public Health Service Grant CA-11378 and American Cancer Society Grant BC-23A.

‡ Special postdoctoral fellow of the National Cancer Institute.

LE I: Published Sedimentation Values for Estrogen Receptor.[a]

Reference	Sedimentation Value[b] Low Salt	0.3–0.4 M KCl	Notes
A. Cytoplasmic receptor			
Toft and Gorski (1966)	9.5		
Jensen *et al.* (1967)	9.5		
Erdos (1968)	8	5	Calf (also pig, sheep, rat)
Korenman and Rao (1968)	10	5	Rat (age not noted); also pregnant rabbit
Rochefort and Baulieu (1968)	8	4	Ovariectomized rat
Jensen *et al.* (1969)		4	
DeSombre *et al.* (1969)	4		Ca^{2+}-treated; calf
Steggles and King (1970)	8		Immature or ovariectomized rat
Steggles and King (1970)	8 and 4		Mature intact rat
Kyser (1970)	8		Mature intact rat
Brecher *et al.* (1970)		4	
Brecher *et al.* (1970)		5	Treated 28°, 30 min
Vonderhaar *et al.* (1970a)	4		Treated 37°, 15 min; intact mature rat
Giannopoulos and Gorski (1971)		4.2	
Giannopoulos and Gorski (1971)		5.5	Extracted 0.4 M KCl
Reti and Erdos (1971)	9.5		Press juice; calf
Reti and Erdos (1971)	6.4[c]		Press juice, gradient 0.15 M KCl; calf
Reti and Erdos (1971)	6.4[c]		Press juice, gradient press juice; calf
Puca *et al.* (1971)	8.6		Purified; calf
Puca *et al.* (1971)	4.5		Purified subunit; calf
B. Nuclear receptor			
DeSombre *et al.* (1967)		5	
Puca and Bresciani (1968)		5.1	Ovariectomized rat
Korenman and Rao (1968)	Aggregate	5	Same as cytoplasmic subunit
Rochefort and Baulieu (1968)	4	4	Same as cytoplasmic subunit; ovariectomized rat
Puca *et al.* (1969)		5.4	Ca^{2+}-treated; calf
Shymala and Gorski (1969)		5	From purified nuclei
Shymala and Gorski (1969)		5	From purified chromatin
Jensen *et al.* (1969)		5 and 4	Cell-free nuclear uptake
Jensen *et al.* (1969)		3–4	Cell-free; diaphragm nuclei
Musliner *et al.* (1970)		5 and 4	Cell-free
Musliner *et al.* (1970)		3–4	Cell-free; diaphragm nuclei
Puca *et al.* (1970)	4.5		Purified; calf
Giannopoulos and Gorski (1971)		4.8	
Harris (1971)	Aggregate	5	Mouse
Harris (1971)	8		Polyanion-treated; mouse

Except as noted, preparations were from immature rat uterus by procedures similar to those discussed under Methods. Where uterus was used, animals were usually described as prepuberal. [b] Sedimentation values in Svedberg units, determined according to Martin and Ames (1961). [c] Note that these gradients contained physiological rather than low salt concentrations.

entrifugation. The supernatant contained the extracted Jestradiol-labeled nuclear receptor.

Sucrose Gradients. All sucrose gradients were prepared in 7.4, and some also contained 0.15 M or 0.4 M KCl, as ed. Gradients were prepared by layering 0.5 ml of 20% rose, 1.2 ml each of 16.25%, 12.5%, and 8.75% sucrose, l 0.5 ml of 5% sucrose in cellulose nitrate tubes and allow- diffusion in the cold for 12 hr. Examination of fractions a refractometer showed that the resulting gradient was ar except for the top 300 μl, which were gently removed l replaced with a 200-μl sample just before centrifugation ually at 56,000 rpm in a Beckman SW 56 rotor). Each ple contained about 1000 cpm of ^{14}C-labeled bovine um albumin (Rice and Means, 1971) as an internal marker; the ^{14}C peak provided a check of the quality of each gradient as well as a more precise sedimentation standard than achieved with the usual separate albumin gradient. Neither the presence or absence of 0.4 M KCl in the gradient, nor any other variable used, appeared to affect the sedimentation of the ^{14}C-labeled albumin. After centrifugation, 4-drop fractions were collected and suspended in modified Bray's solution (125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, 0.38 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 1 l. of *p*-dioxane) for liquid scintillation counting in a Beckman LS 233 or a Packard Tri-Carb 3375; 80–90% of the applied counts were recovered on the gradients. Sedimentation values were determined according to Martin and Ames (1961).

DEAE-Cellulose Columns. Whatman DE-52 was degassed

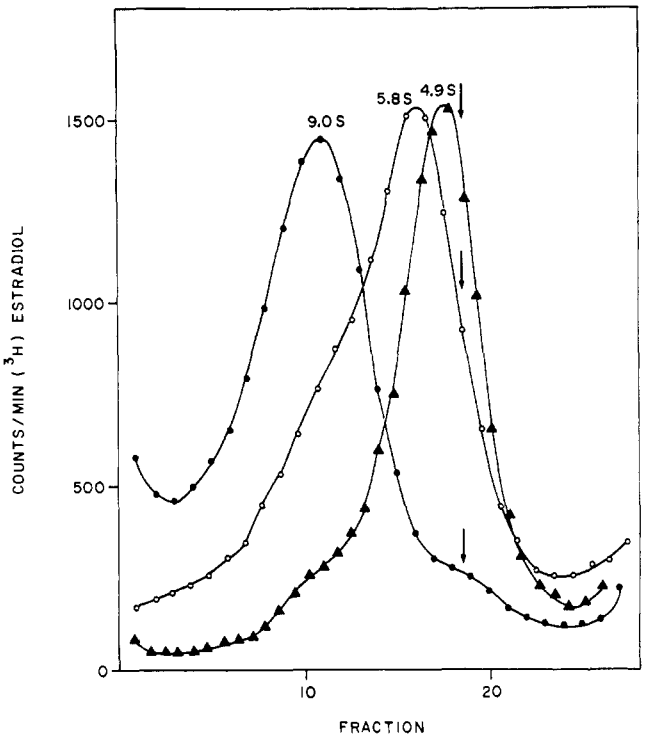

FIGURE 1: Sedimentation of cytoplasmic receptor on sucrose gradients containing 0.4 M KCl (▲), 0.15 M KCl (○), or no added salt (●). The sample applied to the 0.15 M KCl gradient was first brought to 0.4 M KCl for 15 min as disucssed in the text. Arrows mark the peak of ^{14}C-labeled bovine serum albumin included as an internal sedimentation standard in each gradient.

at pH 4.5, brought to pH 7.4, and equilibrated with TE 7.4 which had been boiled to remove carbon dioxide and then chilled before use. Columns were 0.8 × 10 cm. Samples were desalted if necessary by passage over Sephadex G-25 before application to the column. About 2000 cpm of ^{14}C-labeled bovine serum albumin were added as a marker. After application of sample, columns were washed with 40 ml of TE 7.4, required to elute any free estradiol, before application of a linear KCl gradient and collection of at least 40 2-ml fractions at about 20 ml/hr, all at 2°. Aliquots of each fraction were taken up in Aquasol (New England Nuclear) for radioactivity counting, and the remainder was used for determination of conductivity.

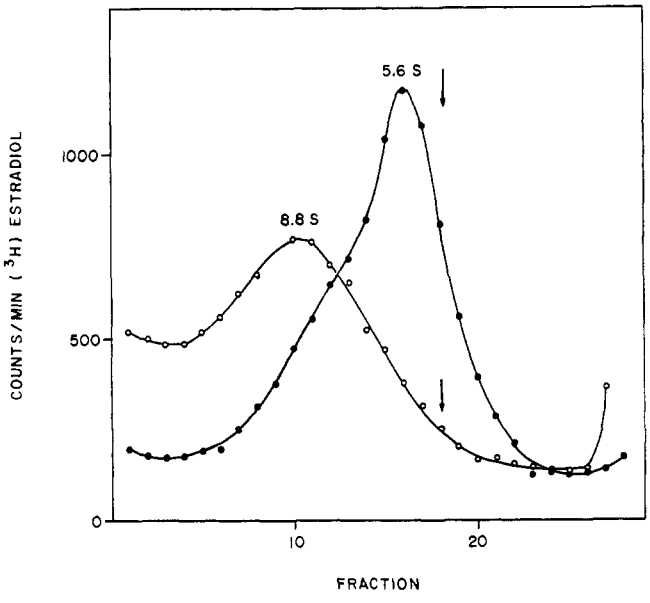

FIGURE 2: Effect of Ca^{2+} on sedimentation. Cytoplasmic receptor was treated with dextran-coated charcoal and brought to 0.4 M KCl with (●) or without (○) 0.004 M $CaCl_2$ 45 min before low salt sucrose gradient centrifugation. Pretreatment with KCl and $CaCl_2$ for 4.5 hr gave identical results. Arrows mark the peaks of ^{14}C-labeled albumin.

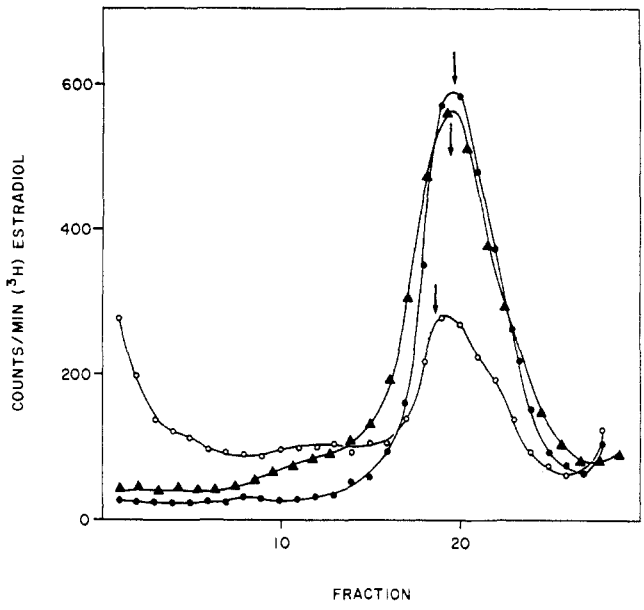

FIGURE 3: Sedimentation of nuclear receptor on sucrose gradients containing 0.4 M KCl (●), 0.15 M KCl (▲), or no added salt (○). Arrows mark the peaks of ^{14}C-labeled albumin.

Results

Sedimentation at Physiological Ionic Strength. Cytoplasmic receptor produced the expected peaks at 8–9 S and 4–5 S on sucrose gradients prepared in low salt or 0.4 M KCl, respectively (Figure 1). By contrast, on gradients prepared at approximately actual intracellular ionic strength (0.15 M KCl), cytoplasmic receptor sedimented neither at 8 S nor at 4 S but rather in a single sharp peak at about 6 S. Values for several experiments ranged from 5.8 to 6.0 S, referred to internal ^{14}C-labeled albumin standards at 4.6 S. A considerable amount of 8S material was present along with the 6S peak when the brief pretreatment with 0.4 M KCl was omitted.

An identical 6S sedimentation pattern appeared even in low-salt gradients when cytoplasmic receptor was treated for 45 min with 0.004 M $CaCl_2$ and 0.4 M KCl before centrifugation (Figure 2). The same result was obtained after a similar pretreatment lasting 4.5 hr. This finding will be discussed later.

Receptor extracted from cell nuclei in 0.4 M KCl has been said to form nonspecific aggregates in low-salt sucrose gradients (Korenman and Rao, 1968; Harris, 1971), but its sedimentation pattern at physiological ionic strength has not been previously reported. Figure 3 shows that a single nuclear receptor peak appeared at about 4.5 S in 0.15 M KCl, just as in 0.4 M KCl. The formation of a 6S form as seen in cytoplasm did not occur. Even in low salt a significant peak of protein-bound estradiol was found near 4.5 S, though considerable random aggregation also occurred. No other discrete peaks were present in low salt.

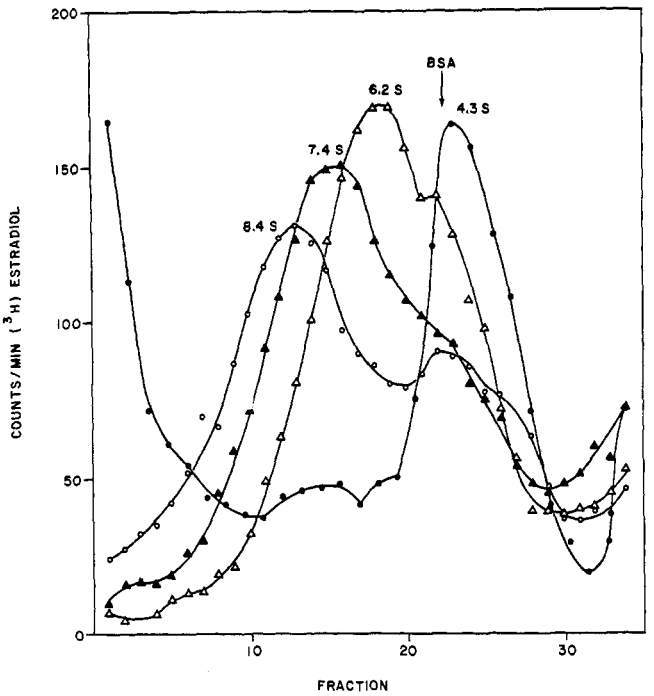

FIGURE 4: Effects of heparin on sedimentation of nuclear receptor. Immediately before application to low salt sucrose gradients, samples in TKE 8.5 were treated with sodium heparin at several final concentrations: 0 mg/ml (●), 0.3 mg/ml (○), 1.5 mg/ml (▲), 6.0 mg/ml (△).

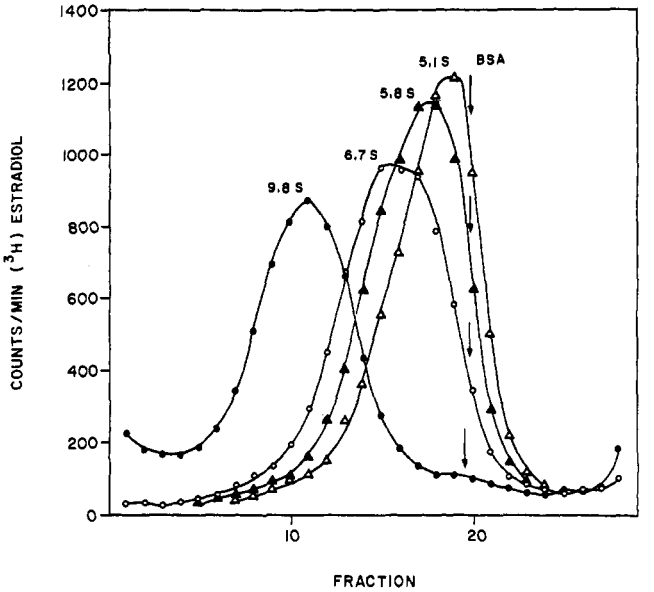

FIGURE 5: Effects of heparin on sedimentation of cytoplasmic receptor. Samples in TE 7.4 were treated as described in Figure 4.

Polyanion Effects on Receptor Sedimentation. Harris (1971) prepared nuclear receptor either by KCl extraction or by DNase treatment of nuclear sediment, and reported that both receptor preparations sedimented at 8 S in low-salt sucrose gradients if "Polytak-RNA," heparin, or dextran sulfate were first added at 1.25 mg/ml. Cytoplasmic receptor was not affected by the same treatment. This result naturally suggested that the nuclear receptor might actually be identical with the 8S cytoplasmic receptor, if basic proteins present in the nuclear extracts were prevented by polyanions from aggregating the subunits nonspecifically in low salt. But if this were true, it might also have been expected that nuclear receptor which was not randomly aggregated would appear at 8 S in low salt gradients or at 6 S in gradients with 0.15 M salt, rather than remaining at 4.5 S as actually occurred (Figure 3). We were therefore led to reexamine the polyanion effect.

Heparin was used as the polyanion. Nuclear receptor did indeed sediment faster in the presence of heparin, as reported by Harris (1971) (Figure 4). However, the effect was strongly dependent on the concentration of heparin; low levels converted most of the nuclear receptor to 8 S leaving a substantial fraction in a 4S peak, while higher concentrations progressively reduced the sedimentation rate of the major peak back toward 6 S.

Cytoplasmic receptor was affected by heparin in the same way as receptor from the nucleus, except that no residual 4 S appeared (Figure 5). Displacements were larger when heparin was added in the absence of KCl, as shown here. When cytoplasmic receptor was heparin treated in 0.4 M KCl as was the nuclear receptor, the peaks approximately matched the major peaks of the nuclear form while nuclear receptor desalted before addition of heparin produced peaks similar to those of Figure 5.

These results show that the same artifacts can be produced by heparin with either nuclear or cytoplasmic receptor. This is consistent with an identity between the two forms, but does not prove that the 8S nuclear form seen in the presence of heparin is identical with the cytoplasmic 8 S found in its absence. It is equally possible that polyanions dissociate the components of larger forms, if present, and operate on the presumed 4S subunit.

DEAE-Cellulose Chromatography. In a further comparison of nuclear and cytoplasmic receptor, each preparation was adsorbed to a DEAE-cellulose column and eluted with a linear KCl gradient. Nuclear receptor was first desalted on a small Sephadex G-25 column. Figure 6 shows that this form eluted early, at about 0.04 M KCl. It produced a single sharp peak, in spite of the fact that low-salt conditions seem to aggregate much of this form in sucrose gradients. This was designated peak I.

The cytosol receptor yielded a later peak, at about 0.2 M KCl, designated peak II (Figure 7). Peak II appeared whether or not the preparation was first treated with KCl and desalted on Sephadex G-25, showing that the nuclear peak I seen above was not an artifact produced by this treatment. A small peak in the position of peak I was always present in cytoplasmic preparations, however, and this faction was greater after treatments which tend to convert some 8S receptor to the 4S form (*e.g.*, warming or prior KCl exposure—Vonderhaar *et al.*, 1970a,b). This peak I could not be distinguished from that of the nuclear receptor.

^{14}C-Labeled albumin eluted conveniently between the two receptor peaks, at about 0.14 M KCl.

Somewhat similar results were obtained by DeSombre *et al.* (1969), who compared cytoplasmic 8 S and a calcium-stabilized cytoplasmic 4S receptor, both prepared from calf uterus, and found peaks at 0.22 and 0.10 M salt, respectively.

Discussion

The finding that cytoplasmic receptor sediments at about 6 S at physiological ionic strength, in contrast to 4 S or 8 S at high or very low salt concentrations, has also been re-

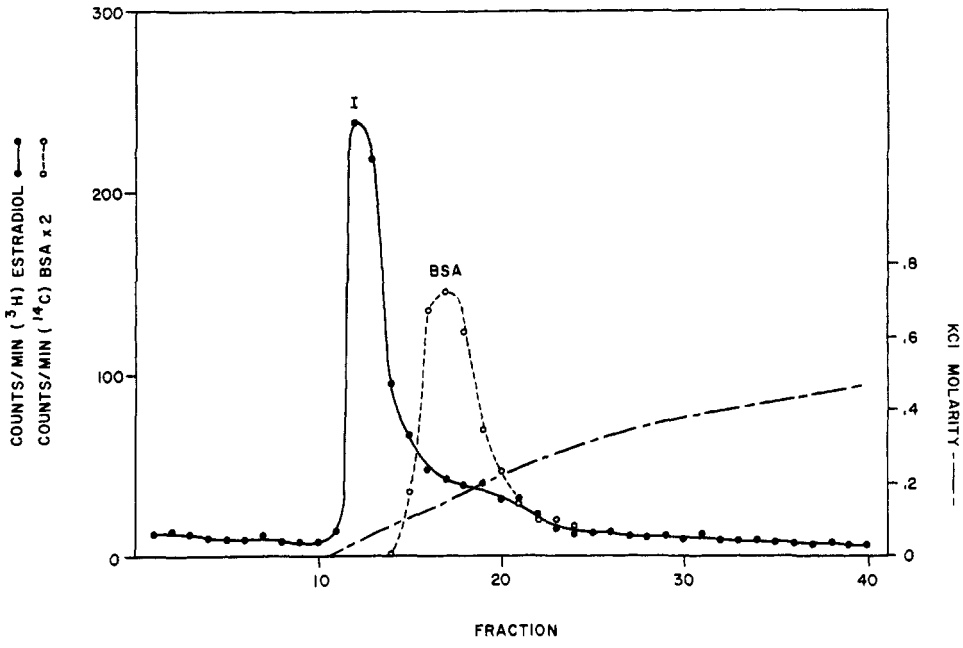

FIGURE 6: DEAE-cellulose chromatography of nuclear receptor, prepared as described in Methods except that a recently ovariectomized rat was used to achieve maximum possible uptake of label by nuclei. (As discussed in the text, we have never observed any qualitative differences in receptor from intact and ovariectomized animals.) The nuclear extract was desalted by passage over a small Sephadex G-25 column. After addition of ^{14}C-labeled albumin a 0.4-ml sample was applied in TE 7.4 to a DEAE-cellulose column 0.8 × 10 cm, eluted at 20 ml/hr by a linear KCl gradient in TE 7.4, and collected in 2-ml fractions. All procedures were carried out at 2°.

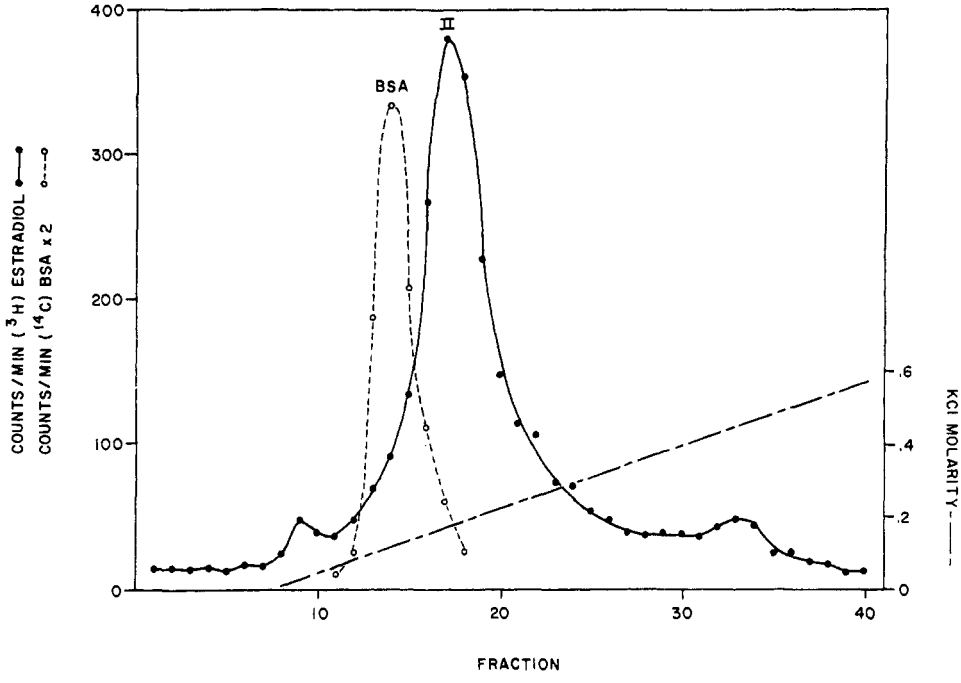

FIGURE 7: DEAE-cellulose chromatography of cytoplasmic receptor, prepared as given in Methods, treated with dextran-coated charcoal, and applied and eluted as described in Figure 6.

ported by Rochefort (1972) and Reti and Erdos (1971). The latter also found only 6S receptor in juice pressed directly from uteri with no added buffer, when sedimented in sucrose gradients prepared in the same press juice deproteinized. It seems likely that the 6S form is the form which predominates in the living cytoplasm, and that the 8S and 4S forms are artifacts of preparation in very low or very high salt. In our experiments, with cytoplasmic receptor extracted in low salt rather than at physiological salt concentrations, a variable but large amount of 8S material always appeared along with the 6S major peak in 0.15 M KCl gradients unless the sample had been brought to 0.4 M KCl for a few minutes before layering on the sucrose gradients. It seems most likely that the 6S form associates to yield an 8S molecule in low salt, and that the dissociation of 8 S back to 6 S is slow in 0.15 M KCl at 2°, leaving a significant amount of 8S material on the gradient. Brief treat-

ment with 0.4 M KCl would then speed the dissociation to 6 S (or to 4 S, with relatively rapid return to 6 S in 0.15 M salt).

If the cytoplasmic receptor is treated briefly with 0.004 M $CaCl_2$ in the presence of 0.4 M KCl before centrifugation, we find even in low salt gradients a 6S peak identical with that seen above in 0.15 M KCl. Nuclear receptor from calf uteri yields a similar peak in the presence of calcium ion (Puca *et al.*, 1969). DeSombre *et al.* (1969) reported that calcium added to preparations of calf uterine cytosol in high salt stabilized the receptor in its 4S form, while Puca *et al.* (1971) added the calcium ion 2 hr before addition of salt to yield an equally stable 8S receptor which they considered to be a tetramer involving the 4S units. If so, it is possible that the 6S form, as produced here by a slightly different calcium treatment or found at physiological ionic strength, is a dimer. Giannopoulos and Gorski (1971) have seen a similar form in high-salt gradients after extraction of receptor from whole tissue with 0.4 M KCl, and they also noted as we have that small amounts of this 6S form occur even in high-salt gradients with cytoplasmic receptor prepared in low salt. Puca *et al.* (1971) have hypothesized the existence of a receptor transforming factor, activated by calcium and inactive in low salt, to explain some of these results.

We have not succeeded in finding a clear distinction between nuclear and cytoplasmic receptors. The fact that nuclear receptor does not form a 6S species in physiological salt or 8S in low salt, but rather remains at about 4.5 S throughout except for a certain amount of nonspecific aggregation, does provide an apparent difference. It might well be argued, however, that differences in preparation or in the other proteins present in the two extracts, rather than differences in the receptors themselves, are responsible for these observations. Polyanions such as heparin affect both forms alike. DEAE-cellulose chromatography separates nuclear receptor from the primary cytoplasmic form found in low salt, presumably 8 S, but not from the peak associated with cytoplasmic 4 S. Partially purified preparations of nuclear receptor and cytoplasmic 4 S are also not distinguished by several criteria applied by Puca *et al.* (1971). It may well be that these forms are actually identical, with apparent differences such as a slight difference in sedimentation rate in crude extracts caused by different conditions or different nonreceptor proteins.

The nature of the effect of polyanions on receptor sedimentation unfortunately remains unclear. It is difficult to reconcile the results presented here with a model in which heparin simply permits recovery of a single "native" form of the receptor from among the nuclear proteins. It seems more likely that a more complex interaction would be required to produce this wide range of artifacts. Yet it is improbable that heparin is binding directly to the receptor: in addition to the fact that all forms of the receptor examined so far are rather acidic proteins (Puca *et al.*, 1971, DeSombre *et al.*, 1969) and hence unlikely candidates for nonspecific associations with polyanions, the density of heparin is considerably greater than that of proteins (Laurent, 1961), so that increased binding with higher heparin concentrations should raise rather than lower the sedimentation rate of the resulting complex. It may also be necessary to account for preliminary results which seem to indicate that receptor extracted from purified nuclei or from chromatin rather than from crude nuclear pellets does not yield 8 S in the presence of polyanions, but rather sediments primarily at about 4 S.

Steggles and King (1970) have reported the presence of a specific distinct 4S estrogen receptor in mature rats in addition to the 8S form. This 4S receptor would not reassociate to 8 S. It disappeared after ovariectomy and was not found in immature animals. Our experiments, however, have never shown any specific 4S cytoplasmic receptor in low-salt sucrose gradients, either from mature intact (Figure 1) or from ovariectomized rats. No other qualitative differences in either nuclear or cytoplasmic receptor between intact and ovariectomized rats have been noted. The absence of specific 4S receptor even in uterus from intact rats has also been reported by Kyser (1970), while both Kyser and Vonderhaar *et al.* (1970a) note the similarity of receptor from mature and immature rats in several different experiments.

It is clear that slight variations in conditions can affect estrogen receptor forms considerably. The polyanion effect in particular allows production of almost any desired sedimentation artifact, and several types of polyanion are undoubtedly present along with undefined concentrations of small ions such as calcium in the crude extracts in which receptor has usually been studied. These results warn against overinterpretation of sedimentation data as well as providing further information about the complex behavior of estrogen receptors.

References

Bishara, R. H., and Jakovljevic, I. M. (1969), *J. Chromatogr. 41*, 136.

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

DeSombre, E., Hurst, D., Kawashima, T., Jungblut, P., and Jensen, E. V. (1967), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 26*, 536.

DeSombre, E. R., Puca, G. A., and Jensen, E. V. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 148.

Erdos, T. (1968), *Biochem. Biophys. Res. Commun. 32*, 338.

Giannopoulos, G., and Gorski, J. (1971), *J. Biol. Chem. 246*, 2530.

Gorski, J., Toft, D., Shyamala, G., Smith, D., and Notides, A. (1968), *Recent. Progr. Horm. Res. 24*, 45.

Hamilton, T. H. (1971), *in* The Biochemistry of Steroid Hormone Action, Smellie, R. M. S., Ed., London, Academic Press, p 49.

Harris, G. (1971), *Nature (London), New Biol. 231*, 246.

Jensen, E. V., Hurst, D. J., DeSombre, E. R., and Jungblut, P. W. (1967), *Science 158*, 385.

Jensen, E. V., Numata, M., Brecher, P., and DeSombre, E. (1971), *in* The Biochemistry of Steroid Hormone Action, Smellie, R. M. S., Ed., London, Academic Press, p 133.

Jensen, E. V., Suzuki, T., Numata, M., Smith, S., and DeSombre, E. R. (1969), *Steroids 13*, 417.

Korenman, S. G. (1968), *J. Clin. Endocrinol. Metab. 28*, 127.

Korenman, S. G., and Rao, B. R. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1028.

Kyser, K. A. (1970), Ph.D. Dissertation, Department of Physiology, University of Chicago.

Laurent, T. C. (1961), *Arch. Biochem. Biophys. 92*, 224.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.

Musliner, T. A., Chader, G. J., and Villee, C. A. (1970), *Biochemistry 9*, 4448.

Puca, G. A., and Bresciani, F. (1968), *Nature (London) 218*, 967.

Puca, G. A., Nola, E., and Bresciani, F. (1969), *Atti Accad. Naz. Lincei, Cl. Sci. Fis., Mat. Natur., Rend. 46*, 286.

Puca, G. A., Nola, E., and Bresciani, F. (1970), *in* Research on Steroids, Vol. IV, Finkelstein, M., Klopper, A., Conti, C., and Cassano, C., Ed., New York, N. Y., Pergamon Press, p 319.
Puca, G. A., Nola, E., Sica, V., and Bresciani, F. (1971), *Biochemistry 10*, 3769.
Reti, I., and Erdos, T. (1971), *Biochimie 53*, 435.
Rice, R. H., and Means, G. E. (1971), *J. Biol. Chem. 246*, 831.
Rochefort, H. (1972), *Int. Congr. Hormonal Steroids, 3rd, 1970* (in press).
Rochefort, H., and Baulieu, E. E. (1968), *C. R. Acad. Sci. Ser. D. 267*, 662.
Shyamala, G., and Gorski, J. (1969), *J. Biol. Chem. 244*, 1097.
Steggles, A. W., and King, R. J. B. (1970), *Biochem. J. 118*, 695.
Toft, D., and Gorski, J. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1574.
Vonderhaar, B. K., Kim, U. H., and Mueller, G. C. (1970a), *Biochim. Biophys. Acta 208*, 517.
Vonderhaar, B. K., Kim, U. H., and Mueller, G. C. (1970b), *Biochim. Biophys. Acta 215*, 125.

Thermodynamic Studies of Transfer Ribonucleic Acids. I. Magnesium Binding to Yeast Phenylalanine Transfer Ribonucleic Acid†

Giovanni Rialdi,‡ J. Levy,§ and R. Biltonen*

ABSTRACT: The thermodynamics of magnesium ion (Mg^{2+}) binding to yeast phenylalanine transfer ribonucleic acid ($tRNA^{Phe}$) have been determined calorimetrically. At low temperature, where $tRNA^{Phe}$ exists in its folded state, the enthalpy of Mg^{2+} binding was found to be 0 ± 100 cal/mole of ligand. This result is taken as evidence for the absence of a thermodynamically significant conformational change upon Mg^{2+} binding. Using the large heat of reaction between Mg^{2+} and EDTA, the extent of Mg^{2+} binding to $tRNA^{Phe}$ in both its folded state and unfolded state has been measured over a free Mg^{2+} concentration range of 0–2.5 mM. Mg^{2+} binding to folded $tRNA^{Phe}$ can best be represented in terms of two sets of independent binding sites characterized by occupancy numbers $N_{1A} = 4$ and $N_{2A} = 20$ with association constants $K_{1A} = 10^6\ M^{-1}$ and $K_{2A} = 1.1 \times 10^4\ M^{-1}$. Analysis of the combination of the present results and data previously obtained for mixed tRNA at higher Mg^{2+} concentration suggest the existence of a third set of independent binding sites. Mg^{2+} binding to unfolded $tRNA^{Phe}$ can be interpreted in terms of a single set of independent binding sites with $K_B = 7 \times 10^3\ M^{-1}$. These results show that Mg^{2+} binding to tRNA is thermodynamically characterized by a large, positive entropy change, presumably due to release of water from solvated Mg^{2+} upon binding. In addition it now appears that Mg^{2+} stabilizes the folded conformation of $tRNA^{Phe}$ simply because Mg^{2+} binds better to the folded form than to the unfolded form of the macromolecule.

The relationship between the structure and function of transfer ribonucleic acid has been extensively studied (Fresco *et al.*, 1966; Gantt *et al.*, 1969; Dudock *et al.*, 1970). An important result of such studies was the recognition that a unique three-dimensional conformation is necessary for the macromolecule to perform its biological function (Fresco *et al.*, 1966). The significance of the role of Mg^{2+} in the structure–function relationship has also been examined (Henley *et al.*, 1966; Lindahl *et al.*, 1966; Adams *et al.*, 1967; Reeves *et al.*, 1970; Ishida and Sueoka, 1968a,b; Ishida *et al.*, 1971; Robison and Zimmerman, 1971b). For example, Mg^{2+} has been found to influence the thermodynamic stability of the folded conformation of tRNA (Dudock *et al.*, 1970). Some investigators have also suggested that Mg^{2+} is an absolute requirement for the biologically active conformation (Lindahl *et al.*, 1966; Adams *et al.*, 1967; Reeves *et al.*, 1970). Since the interaction of Mg^{2+} with tRNA appears to be a rapid and reversible phenomenon (Ishida and Sueoka, 1968a), Mg^{2+} must exert thermodynamic, rather than kinetic, control on the structure of tRNA. For this reason a complete thermodynamic description of the interaction of Mg^{2+} with tRNA is necessary to understand this problem. Sander and Ts'o (1971) have studied the binding of Mg^{2+} to tRNA using a divalent cation-specific electrode, but were unable to obtain reliable data below free Mg^{2+} concentrations of about 0.1 mM. Others have investigated manganese binding to tRNA using proton magnetic relaxation (Cohn *et al.*, 1969) and electron spin resonance (Danchin and Gueron, 1970a) techniques. However, the characterization of the interaction has been limited because complete thermodynamic information has not been obtained.

† From the Department of Physiological Chemistry, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205. *Received February 8, 1972.* This investigation was supported by grants from the National Science Foundation (GB 7243) and National Institutes of Health (CA-11693).

‡ Senior Postdoctoral Fellow supported by the CNR, Italy, 1970–1971.

§ Predoctoral Trainee supported by National Institutes of Health Grant GM-00181, 1967–1971.

* To whom correspondence should be sent. Present address: Department of Pharmacology, University of Virginia, Charlottesville, Va. 22903.