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Characterization and Partial Purification of an Estrogen Type II Binding Site in Chick Oviduct Cytosol†

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ABSTRACT: An estrogen binding site of moderate affinity (K_d ~10 nM) and high capacity (~25-70 pmol/g of tissue) was measured in DES-stimulated chick oviduct cytosol. Saturation analysis by [^{3}H]estradiol exchange demonstrated that this binding site displayed sigmoidal binding characteristics suggesting a cooperative binding mechanism. Competition analysis with a number of compounds demonstrated that the bioflavonoid luteolin was a better competitor for binding to type II sites in chick than either estradiol or DES. Steroid specificity was demonstrated by the inability of 17α-estradiol, progesterone, testosterone, corticosterone, and the triphenylethylene antiestrogen nafoxidine (U-1100A) to compete for [^{3}H]-17β-estradiol binding to chick oviduct cytosol preparations. In addition, the binding site appeared to be sensitive to sulfhydryl reducing reagents as evidenced by a 75% reduction in binding activity in the presence of dithiothreitol. Both prelabeling and postlabeling procedures used in conjunction with Sephacryl S-300 chromatography resulted in a single major peak of type II binding activity representing a molecular weight in the 40 000 range. Type II binding activity was recoverable after precipitation with ammonium sulfate, and this material was subjected to a variety of column chromatography procedures in order to achieve further purification of the type II site. Significant purification of the site was achieved with a bioflavonoid–Sepharose (quercetin–Sepharose) affinity matrix. The purified type II sites eluted from quercetin–Sepharose displayed the same sigmoidal binding curves characteristic of native cytosol.

Previous reports have described multiple classes of estrogen binding sites in rat uterus (Clark et al., 1978; Eriksson et al., 1978), mouse mammary tumors (Watson & Clark, 1980), human breast cancer (Syne et al., 1982a,b; Lopes et al., 1987), mullerian duct (MacLaughlin et al., 1983), ventral prostate (Pliner & Swaneck, 1985), and hepatocarcinoma cells (Tam et al., 1986). These components have been designated type I (estrogen receptor; K_d ~1 nM) and type II (K_d ~10-20 nM) (Markaverich & Clark, 1979). Although the estrogen (type I) receptor has long been the subject of extensive investigation by many laboratories and its detailed structure has recently

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been elucidated by recombinant DNA techniques (Green et al., 1986; Greene et al., 1986), the structure and precise function of the type II binding site is unknown. The presence of type II sites in rat uterus was first suggested by saturation binding analysis which used concentrations of [^{3}H]estradiol ranging from 0.05 to 80 nM (Eriksson et al., 1978). Cytosol displayed two binding sites according to Scatchard analysis. The lower affinity type II site displayed a sigmoidal binding pattern characteristic of a cooperative binding mechanism. Another distinguishing characteristic of type II binding is its sensitivity to the presence of sulfhydryl reducing reagents such as dithiothreitol (Markaverich et al., 1981b). In addition, a high-affinity endogenous inhibitor of estrogen binding to type II sites has been observed. Recent findings regarding the biochemical nature of this endogenous inhibitor of [^{3}H]estradiol binding to type II sites in rat uterus as well as the in vitro and in vivo effects of this inhibitor have provided new evidence for a functional role of the type II site in cell growth regulation (Markaverich & Clark, 1979; Markaverich et al., 1981a,b, 1984, 1988a,b). Finally , the induction and propagation of type II sites in human hepatocytes have recently been correlated quantitatively with an estrogen memory effect (Tam et al., 1986). While these findings provide the potential for a new approach to studying the type II site, little, however, is known about the physicochemical properties of this binding site, and no attempts to purify it have been published.

While the obvious importance of the biochemical characterization and purification of type II sites has never been in question, several factors have delayed the progress of this work until recently. First, the presence of a high-affinity endogenous inhibitor of estrogen binding to type II sites (Markaverich et al., 1987a) has complicated the accurate quantification of this binding site during or after a number of potential purification steps. The removal of this inhibitor is an important prerequisite for many of these procedures. However, the conditions which are optimal for the dissociation of this endogenous inhibitor (i.e., high temperature, activated charcoal treatment, etc.) and its subsequent removal from the preparation either are often not optimal with respect to the stability of the type II binding activity or cannot be conveniently maintained during various characterization procedures. Additional problems with characterizing the type II site relate to the relatively low affinity of estradiol for the site (K_d approximately 10 nM) which results in rapid dissociation of the radioligand. Ways to minimize these problems have recently been developed, allowing for a more in-depth analysis of this perplexing site.

Since chick and/or hen oviduct provides a significantly more plentiful source of tissue (approximately 1–2 orders of magnitude, respectively) when compared to the mature rat uterus, it was felt that the chick oviduct might provide an improved, if not ideal, model system for the scaled-up purification and the eventual cloning of the cDNA encoding the type II binding site. After preliminary investigations determined that a type II like binder was in fact present in chick oviduct and that this binding site behaved very much like that described by this laboratory and others for the rat uterus and other mammalian tissues, a more complete characterization and purification of this site from chick was attempted and is discussed below.

Materials and Methods

Animals. White Leghorn chicks aged 7–8 days were treated over a 21-day period with weekly implants of 20 mg of DES in Carbowax [poly(ethylene glycol)]. Birds were sacrificed 1–5 days after the final implant for most experiments although variable periods of withdrawal were examined to determine the effects on type II site concentration. For comparative purposes 45-day-old Holtzmann rats (Madison, WI) were ovariectomized 5–8 days before treatment. 17β-Estradiol was administered to rats by injection (10 μg) in a saline–ethanol vehicle or by beeswax implant (2 mg), as previously described (Markaverich & Clark, 1979). All animals were kept in a controlled environment, with 12 h of light each day starting at 0700. Food and water were provided ad libitum. All animals were sacrificed by cervical dislocation, and oviducts or uteri were removed, separated from extraneous tissue, and prepared as described below for binding analysis.

Cytosol Preparation. Stimulated chick oviducts (typically in the 1.5–3-g range) were quickly removed and stripped of connective tissue, blotted dry, and weighed, prior to being homogenized in TE buffer (10 mM Tris; 1.5 mM EDTA; pH 7.4 at 22 °C) either in a Kontes all-glass homogenizer with a motor-driven pestle or in a Sorvall Omni-Mixer (always on ice). Both methods of homogenization seemed to produce similar, if not identical, results, and preferences depended upon the quantity of tissue being homogenized for a particular procedure. The homogenate was centrifuged (30000g for 20 min) in a Beckman JS 7.5 rotor, and after the fat pad was aspirated from the top of the tube, the supernatant was recentrifuged at 105000g for 60 min.

Measurement of Type II Sites. An aliquot of the cytosol preparation from above was poured over the pellet obtained by the centrifugation (1000g for 7 min) of an equal volume of hydroxylapatite (Bio-Gel HT) in TE buffer (60% v/v). The preparation was continually resuspended by gentle mechanical inversion for 15 min at 4 °C. Following this incubation, the hydroxylapatite–cytosol suspension was centrifuged (1000g for 7 min), and the resulting hydroxylapatite pellet (containing the type II sites) was washed three times by gentle resuspension in TE buffer and centrifugation (1000g for 7 min). The pellet was resuspended in a volume of TE equivalent to the volume of cytosol originally incubated with the hydroxylapatite suspension, and aliquots (250 μL) were pipetted directly into assay tubes containing [^{3}H]estradiol (1–40 nM) plus or minus a 300-fold molar excess of either DES or the bioflavonoid luteolin. This method of preincubating the cytosol with hydroxylapatite prior to incubation with radiolabeled steroid will be refered to as the hydroxylapatite adsorption assay (HAA). Following incubation for the appropriate time and temperature (typically either 16 h at 22 °C or 2 h at 30 °C), 1.5 mL of ice-cold TE buffer was added to the assay tubes, and the tubes were vortexed and centrifuged at 1000g for 7 min. The hydroxylapatite pellets were washed two additional times by resuspension in TE buffer and centrifugation to remove free hormone, the final washed pellet was either extracted with 1 mL of 100% ethanol (22 °C for 30 min) or simply resuspended in 4 mL of aqueous counting scintillant, and the bound counts were determined by liquid scintillation spectrometry.

Size Determination of the Type II Binding Site by Size Exclusion Chromatography. The apparent molecular weight of the cytosolic type II site was determined with both prelabeling and postlabeling procedures in conjunction with Sephacryl S-300 chromatography. The postlabeling procedure involved applying 1 mL of freshly prepared cytosol to a column (90 cm × 1.5 cm) and collecting approximately 2-mL (68-drop) fractions. The column was in a cold room at 2–4 °C, and fraction tubes were placed on ice upon completion of the run. Two aliquots (of variable volume depending upon the concentration of the sample applied to the column) of each fraction were incubated with 250 μL of hydroxylapatite suspension (60% in TE) and washed as described above for cytosol. This resulted in two complete sets of HAA incubation

tubes. After the final wash step, 250 μL of TE was added to each hydroxylapatite pellet, and [^{3}H]estradiol (40 nM unless otherwise stated) was added to one set of tubes (total binding) and [^{3}H]estradiol plus a 300-fold molar excess of DES was added to the other set of tubes (nonspecific binding). Specific binding was determined for each column fraction as previously described and then plotted to provide a complete column profile.

Chromatography of prelabeled sites first required the extraction of freshly prepared cytosol with dextran-coated charcoal in order to remove endogenous inhibitors of estrogen binding to the type II site. This extracted cytosol was then incubated directly with 40 nM [^{3}H]estradiol or 40 nM [^{3}H]estradiol plus a 300-fold molar excess of DES for 12 h at 2–4 °C. Although this procedure resulted in significantly less binding than the HAA procedure, the levels were high enough to allow for chromatography. Because dissociation of the steroid from the type II site during a lengthy column run would obviously reduce the resolution of the profile and possibly lead to erroneous interpretation of the results, prelabeled sites were run on a shorter Sephacryl S-300 column (45 cm × 1.5 cm). Fractions of approximately 2 mL (68 drops) were collected, and an aliquot of each fraction was counted directly.

The standard proteins including ribonuclease A (MW 13 700), chymotrypsinogen A (MW 25 000), ovalbumin (MW 45 000), and aldolase (MW 158 000) were run in addition to blue dextran 2000 (MW 2 000 000), KCl, and [^{3}H]water to calibrate each of the columns used. The blue dextran was detected visually; proteins were detected in the elution profile by absorbance at 280 nm; the KCl was detected by conductivity, and the [^{3}H]water was detected by directly counting an aliquot of each profile fraction.

Ion Exchange Chromatography of the Type II Site. Ion exchange chromatography of the type II site on DEAE-cellulose involved a postlabeling procedure. Freshly prepared cytosol was diluted to a concentration of 20 mg of tissue/mL of cytosol prior to being run through a 5-mL column of Whatman DE-52 DEAE-cellulose. The volume of cytosol run through the column varied with experiment. The flow-through volume was always collected and kept on ice for later type II binding and protein analysis. The column was then eluted with 10 mL of TE buffer, and this eluate was retained also on ice. The column was then eluted with a gradient of 0–1 M KCl or NaCl in TE, and fractions of approximately 2 mL (68 drops) were collected. An aliquot of each fraction was frozen for later protein analysis and ionic strength determination using a conductivity meter. The remaining volume of each fraction tube was then subjected to the HAA assay procedure previously described for the postlabeling of Sephacryl S-300 fractions.

Hydroxylapatite Chromatography of the Type II Site. As described for the Sephacryl S-300 chromatography, both postlabeling and prelabeling procedures were used for characterizing and partially purifying the type II site on hydroxylapatite chromatography. The hydroxylapatite was the same as was used for HAA assays, except that it was prepared in T buffer (10 mM Tris; pH 7.4 at 22 °C) instead of TE buffer since EDTA leads to increased packing of the hydroxylapatite in the column and results in a reduced flow rate. A column volume of 5 mL was used, though it was found that a shorter, wider column than was used for ion exchange was most appropriate due to the very slow flow rate through hydroxylapatite. Cytosol was prepared and applied to the column as described previously for ion exchange chromatography, except that cytosol was prepared in T instead of TE buffer. As before, all flow-through and wash eluates was then eluted with a gradient of 0–0.5 M sodium phosphate in 10 mM Tris, pH 7.4, and fractions of approximately 2 mL (68 drops) were collected. An aliquot of each fraction was frozen for later protein analysis and ionic strength determination. The remaining volume of each fraction tube was then subjected to HAA assay procedures as previously described for the postlabeling of Sephacryl S-300 fractions with one important exception: the aliquots incubated with hydroxylapatite were diluted 4-fold with T buffer to reduce the phosphate concentration and to allow for the rebinding of phosphate-eluted type II sites to hydroxylapatite.

Ammonium Sulfate Precipitation of the Type II Site. Ammonium sulfate in solid form was added to aliquots of freshly prepared cytosol to result in final solutions of 30, 40, 50, 60, and 70% ammonium sulfate. The salt was added slowly while the cytosol was being continuously stirred and maintained at 0 °C on ice. After the complete dissolution of the ammonium sulfate, each of the cytosol tubes was allowed to sit on ice for an additional 15 min before being centrifuged in a Beckman J 20 rotor for 30 min at 14 000 rpm. After the supernatant was decanted and the inside of the tubes was carefully wiped free of any remaining liquid, the pellets were redissolved in enough cold TE buffer to bring the volumes back up to the starting level. Each of the preparations was then subjected to a complete [^{3}H]estradiol saturation binding analysis as previously described.

Chemicals and Reagents. Radiolabeled steroids were purchased from Amersham (Arlington Heights, IL). All other reagents were of the highest quality and were purchased from Sigma Chemical Co. (St. Louis, MO), Bio-Rad (Richmond, CA), or Pharmacia (Piscataway, NJ).

Purification of the Type II Site. An affinity column for type II binding sites was prepared with the bioflavonoid quercetin. This compound binds to cytosol and nuclear type II sites from the rat uterus with a high affinity (Markaverich et al., 1987). For these experiments, quercetin (approximately 5 μmol) was coupled to 5 mL of epoxy-activated Sepharose 6B (Pharmacia) according to the standard procedures recommended by the manufacturer. The residual active sites on the resin were blocked by treatment with an excess of ethanolamine, and the quercetin affinity resin was washed with TE buffer. In a typical experiment, oviduct cytosol (prepared as previously described) from DES-implanted chicks was stripped with 2% dextran-coated charcoal to remove any endogenous ligands for the type II sites as well as any free DES that might interfere with binding of the site to quercetin. After the charcoal-stripped cytosol was divided into two equal aliquots, one aliquot was loaded onto the quercetin affinity column at 4 °C, and the second aliquot was incubated under identical conditions for direct assay. Following sample loading, which depending upon the volume of cytosol loaded required from 1 to 2 h, the quercetin affinity column was washed extensively with TE buffer (200–400 mL) and eluted with a high concentration of 1 mM quercetin (10 mL for 30 min at 22 °C). The quercetin eluate from the column and the control cytosol for direct assay were cooled to 4 °C, stripped with 5% charcoal (4 °C for 15 min, to remove quercetin), and diluted to equivalent volumes, and aliquots (250 μL) were assayed for type II sites by the HAA assay procedure described earlier.

Results

Saturation Binding Analysis. Saturation analysis of chick cytosol over a wide range of [^{3}H]estradiol concentrations using the HAA assay resulted in a curve for specifically bound

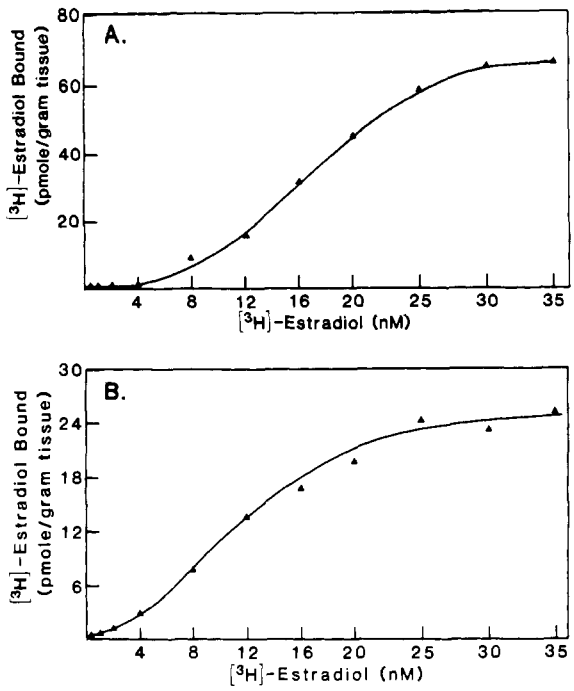

FIGURE 1: Saturation analysis of estrogen binding sites in chick oviduct and rat uterine cytosol. Saturation analysis for [^{3}H]estradiol binding was performed with the HAA assay and concentrations of the steroid ranging from 0.5 to 35 nM. Specific binding of [^{3}H]estradiol to the hydroxylapatite-bound estrogen binding sites was determined by subtracting nonspecific binding in the presence of a 300-fold molar excess of the bioflavonoid luteolin. Virtually identical results were obtained when nonspecific binding was determined in the presence of a 300-fold molar excess of DES. Nonspecific binding (not shown) was unsaturable and increased linearly. (A) Chick oviduct cytosol. (B) Rat uterine cytosol.

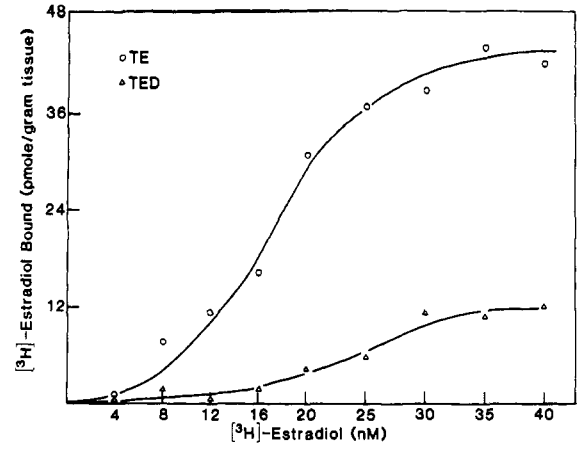

FIGURE 2: Effect of sulfhydryl reduction on estrogen binding to type II sites in chick oviduct cytosol. To determine what effect, if any, that sulfhydryl reduction has on type II binding activity, the hydroxylapatite-bound fraction of chick oviduct cytosol was incubated with the indicated concentrations of [^{3}H]estradiol in the presence or absence of a 10 mM concentration of the sulfhydryl-reducing reagent dithiothreitol (DTT). Binding indicated is specific binding. Nonspecific binding for each condition was determined in the presence of a 300-fold molar excess of the bioflavonoid luteolin. Nonspecific binding was approximately the same when determined in the presence of a 300-fold molar excess of DES.

[^{3}H]estradiol as shown in Figure 1A. The curve exhibits the sigmoidal appearance known to be characteristic of the type II binding in rat uterine cytosolic and nuclear preparations (Eriksson et al., 1980). This sigmoidal pattern is suggestive of a cooperative binding mechanism. For comparison, rat uterine cytosol was prepared and assayed in an identical fashion as shown in Figure 1B. Clearly, these two preparations exhibit nearly identical [^{3}H]estradiol binding characteristics. The type II sites are present in much greater quantities than type I sites in both tissues. Nonspecific binding (not shown) was unsaturable and increased linearly. While the cytosolic type II binding capacity of chick oviduct was somewhat variable, depending upon age, hormone treatment status, and perhaps other conditions, it generally ranged from about 25 to 70 pmol/g of tissue. Because of problems encountered in determining binding capacity from Scatchard analysis, due to the apparent cooperative nature of the binding, binding capacity was estimated from the levels of binding acheived at near-saturating levels of [^{3}H]estradiol. On the basis of this fact and the possibility that some type II sites might still be occupied by the endogenous ligand, it is possible that the actual maximal binding capacity could be higher than we have estimated.

Effects of Reducing Reagents on [^{3}H]Estradiol Binding by Cytosolic Type II Sites. Reducing reagents, such as dithiothreitol and β-mercaptoethanol, are routinely used to stabilize most types of steroid hormone receptors and have been shown to affect the transformation and DNA binding properties of these receptors. It was discovered, however, that the addition of low concentrations of dithiothreitol (less than 1 mM) had marked effects on the rat uterine nuclear type II binding profile of [^{3}H]estradiol (Markaverich et al., 1981b). In contrast to the general stabilizing effect it has on most other steroid receptors, binding to these nuclear type II sites was shown to be completely eliminated upon addition of the dithiothreitol. To further confirm the type II nature of the binding seen in chick oviduct, we examined the effects of dithiothreitol on [^{3}H]estradiol binding to oviduct cytosol preparations. As shown in Figure 2, dithiothreitol had an inhibitory effect also on [^{3}H]estradiol binding in oviduct cytosol. A reduction in specific binding of approximately 75% was observed when a dithiothreitol concentration of 10 mM was used. Increasing the concentration of dithiothreitol to 20 mM did not further reduce the specific binding. While these results agree with those previously reported for rat (Markaverich et al., 1981b) in that reducing reagents are inhibitory with respect to type II binding, it is currently unknown why dithiothreitol was only partially effective in eliminating type II binding in chick while it was totally effective in eliminating type II binding in the rat at even lower doses (0.1 mM). Perhaps chick oviduct cytosol contains an additional reducing reagent insensitive site not present in rat, or the relevant sulfhydryls in the chick site are simply more resistant to reduction by dithiothreitol than those present in the rat uterine site. Aside from interspecies differences in the binding sites themselves, there is also the possibility that chick cytosol may have more or different dithiothreitol-reactive compounds than rat cytosol.

Interaction of Steroids and Bioflavonoids with Cytosolic Type II Sites. To determine the ligand specificity of the binding of [^{3}H]estradiol to the type II site in chick oviduct cytosol, competition analyses were performed with a variety of steroid and nonsteroid ligands that have been shown previously to interact with the type I and type II estrogen binding sites in other tissues. For these experiments, the hydroxylapatite-bound fraction of freshly prepared chick oviduct cytosol (HAA) was incubated with 30 nM [^{3}H]estradiol plus or minus the indicated concentrations of the compounds shown in Figure 3. The data demonstrate that luteolin was a better competitor for [^{3}H]estradiol binding to type II sites in chick than either estradiol or DES. One could therefore conclude that bioflavonoids interact with these sites with a reasonably high affinity (nanomolar). Ligand specificity was demonstrated by the inability of 17α-estradiol, progesterone, testosterone,

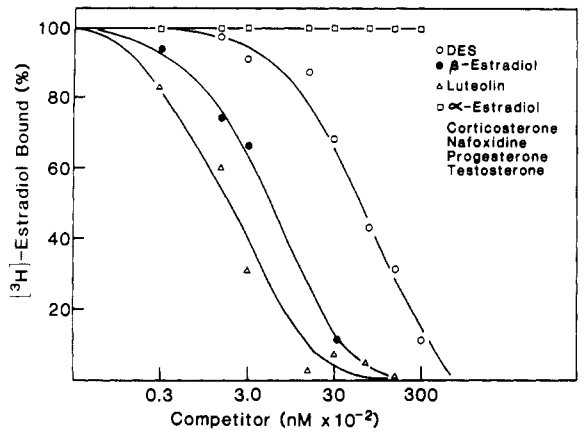

FIGURE 3: Steroid and bioflavonoid competition for estrogen binding sites in stimulated chick oviduct cytosol. The hydroxylapatite-bound fraction of chick oviduct cytosol was incubated with 30 nM [^{3}H]-estradiol plus or minus the indicated inhibitor at 30 °C for 3 h. Relative binding is expressed as a percentage of the specific [^{3}H]-estradiol binding in the absence of any competitor. All specific binding was determined by subtracting nonspecific binding from the total binding exhibited in the presence of a given concentration of competitor. Nonspecific binding was determined to be the binding resulting from the incubation of 30 mM [^{3}H]estradiol plus a 300-fold molar excess of luteolin.

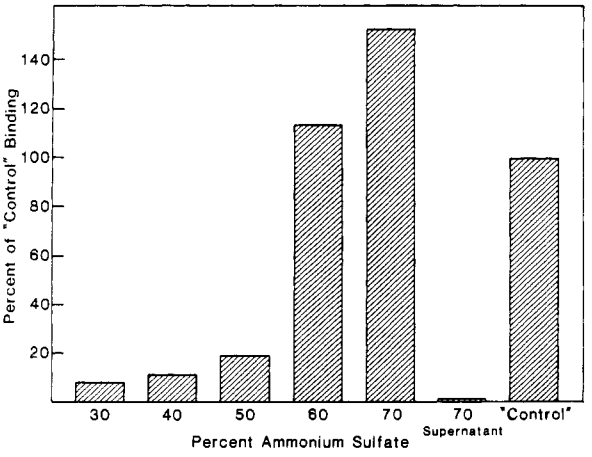

FIGURE 4: Ammonium sulfate precipitation of estrogen type II binding activity from chick oviduct cytosol. Binding activity of each fraction is expressed as percent of "control" binding (no ammonium sulfate). Binding was determined by incubating the hydroxylapatite-bound fraction of each redissolved ammonium sulfate precipitated pellet with 35 nM [^{3}H]estradiol plus or minus a 300-fold molar excess of the bioflavonoid luteolin.

corticosterone, and the triphenylethylene antiestrogen nafoxidine (U-1100A) to compete for [^{3}H]-17β-estradiol binding. These findings are very similar to those reported by this laboratory for the type II site in rat uterus (Markaverich et al., 1987, 1988c).

Ammonium Sulfate Precipitation of Type II Binding Activity. Ammonium sulfate precipitation was investigated as a potential purification step for the type II binding site as well as to further distinguish it from the type I site and the other estrogen binding proteins. Soluble type II binding sites in chick oviduct cytosol are ammonium sulfate precipitable at high concentrations of the salt with no observable loss in binding activity. As shown in Figure 4, most type II binding appeared to precipitate at an ammonium sulfate concentration of between 60 and 70%. Essentially no binding activity was present in soluble form after precipitation with 70% ammonium sulfate. Precipitation of the binding site with concentrations of ammonium sulfate exceeding 60% appears to activate sites not

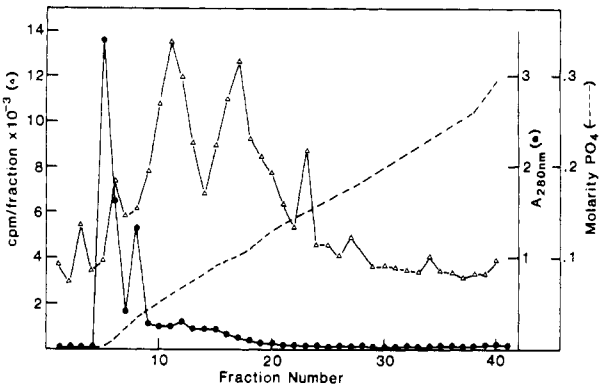

FIGURE 5: Hydroxylapatite chromatography of the type II site from chick oviduct cytosol. Chick oviduct cytosol was incubated with a 60% suspension of hydroxylapatite in Tris buffer (pH 7.4 at 22 °C) for 15 min at 0 °C. The hydroxylapatite was then washed by centrifugation with cold Tris buffer three times prior to being applied to a column. The resulting hydroxylapatite column was then eluted with a 0–500 mM phosphate gradient, and fractions were collected for subsequent analysis. An aliquot of each column fraction was reincubated with fresh hydroxylapatite after being diluted 3-fold with Tris buffer in order to allow rebinding of the phosphate-extracted type II sites to the matrix. These hydroxylapatite-bound sites were then incubated with 35 nM [^{3}H]estradiol plus or minus a 300-fold concentration of the bioflavonoid luteolin to assay type II binding. Additional aliquots of each of the original column fractions were used for measuring UV absorbance (280 nm) and ionic strength (to determine phosphate concentration).

measured in the nontreated control. This is perhaps due to a more efficient removal of the endogenous inhibitor of [^{3}H]estradiol binding to the site. Full saturation binding analysis of each group (data not shown) resulted in the same sigmoidal binding curves characteristic of nontreated cytosol, indicating that precipitation had no significant effect on the general binding characteristics of the site. In addition to its potential usefulness as a purification step, ammonium sulfate precipitation provides yet another distinction between the type II and the type I site (which precipitates at a much lower ammonium sulfate concentration).

Hydroxylapatite Chromatography of the Type II Site. Hydroxylapatite chromatography was investigated as another potential means of purification of the type II site as well as to shed some light on the chemical nature of this protein. The elution profile of type II binding determined by an HAA postlabeling procedure after dilution of the fractions revealed two major peaks of activity eluting at phosphate concentrations of around 70 and 110 mM and one minor peak eluting at around 140 mM (see Figure 5). Although some minor peaks of activity eluting at low phosphate concentrations were occasionally observed, their occurrence was not consistent. The 280-nm absorbance profile indicated that most protein present in oviduct cytosol eluted at very low concentrations of phosphate (less than 50 mM). In subsequent experiments, it was found that incubation of cytosol with hydroxylapatite in the presence of low concentrations of phosphate (50–75 mM) automatically eliminated a large proportion of the protein that would initially be retained by the matrix, thereby improving column capacity. Most cytosolic proteins are easily eluted with low levels of phosphate (Schrader et al., 1977), allowing for some degree of rapid purification using a step gradient approach to elution of the hydroxylapatite. Another advantage of hydroxylapatite is that the stability of the type II site appears to be significantly enhanced when it is bound to it, perhaps because proteases and other potentially degrading or destabilizing enzymes are rendered ineffective by being bound to the hydroxylapatite matrix. The step immediately prior to

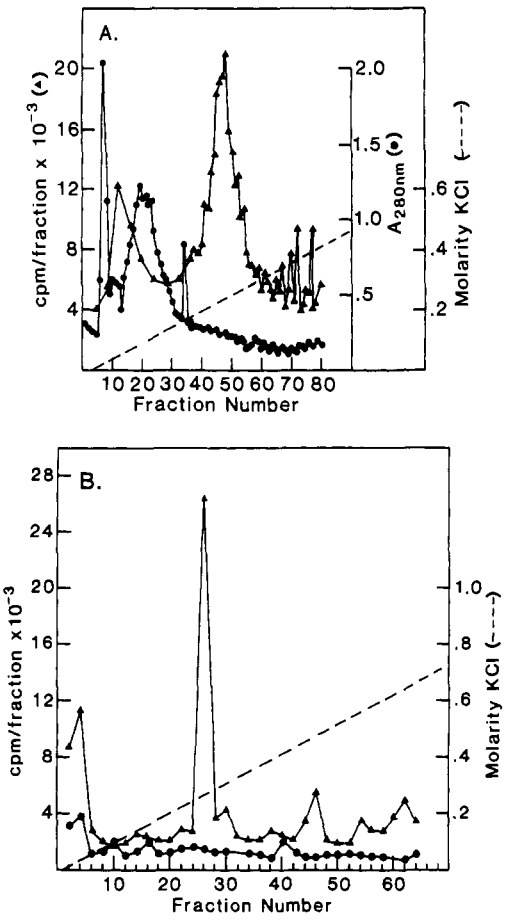

FIGURE 6: Ion exchange chromatography of the type II binding site from chick oviduct cytosol. (A) Chick oviduct cytosol was applied to a column of DEAE-cellulose and washed with TE buffer before being eluted with a 0–1 M KCl gradient. The hydroxylapatite-bound fraction of each KCl-eluted column fraction was then postlabeled with 35 nM [^{3}H]estradiol plus or minus a 300-fold concentration of the bioflavonoid luteolin and binding determined in the usual fashion. Aliquots of each column fraction were also subjected to protein and ionic strength analysis. (B) Ammonium sulfate precipitated (40–60% ammonium sulfate cut) type II sites were resolubilized in enough TE buffer so as to significantly reduce the ionic strength before being applied to a DEAE-cellulose column. The column was then washed with 10 mL of TE buffer before being eluted with a 0–1 M KCl gradient. Fractions (68 drops, approximately 2 mL) were collected and assayed for type II binding as previously described. Binding indicated for each column fraction is specific binding.

the phosphate elution of the site from the hydroxylapatite therefore makes a good "stopping point", during which the hydroxylapatite-bound type II pellet can sit on ice for many hours with only a minimal loss of binding activity.

Ion Exchange Chromatography. The surface charge characteristics of the type II site from oviduct cytosol were investigated by DEAE-cellulose chromatography. Again, a postlabeling HAA procedure was employed to determine the type II binding activity profile. Unlike the phosphate-eluted fractions from hydroxylapatite chromatography, dilution or desalting of the ion exchange fractions was not a prerequisite since relatively high salt (NaCl or KCl) concentrations did not appear to significantly affect the binding of type II sites to hydroxylapatite. The effects of salt on [^{3}H]estradiol binding to hydroxylapatite-bound type II sites was not an issue since salt was washed from the hydroxylapatite pellets (containing the binding sites) prior to steroid incubation. The type II binding profile varied to some extent depending upon the amount of total protein bound to the DEAE-cellulose prior to salt elution. Although some heterogeneity was evident, the

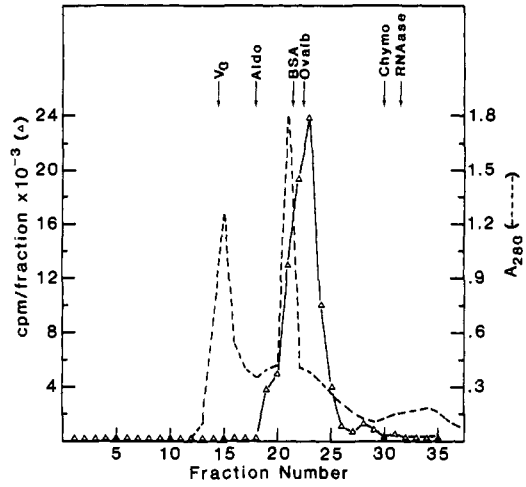

FIGURE 7: Molecular weight determination of the type II binding site from chick oviduct cytosol by Sephacryl S-300 chromatography. A 2-mL aliquot of chick oviduct cytosol was applied to the 50-cm column, and 68-drop (~2 mL) fractions were collected. The hydroxylapatite-bound fraction of each column fraction was postlabeled with 35 nM [^{3}H]estradiol plus (nonspecific) or minus (total) a 300-fold molar excess of the bioflavonoid luteolin. Binding indicated is specific binding.

bulk of the luteolin-competable estrogen binding eluted at a KCl concentration of 250–300 mM, although higher concentrations (about 400 mM) were required when a relatively high concentration of protein was bound to the DEAE column (see Figure 6A). Generally there was some binding activity eluting at a very low ionic strength (about 50 mM). As with hydroxylapatite chromatography, most cytosolic protein eluted at a lower salt concentration than did the major peak of type II binding activity as indicated by the 280-nm absorbance profile. When cytosol was first precipitated with ammonium sulfate (40–60% cut) prior to being run on DEAE-cellulose, a more highly resolved profile was obtained with the HAA postlabeling procedure (see Figure 6B). This is likely due to a significant decrease in the overall concentration of protein applied to the column.

Sephacryl S-300 Chromatography of the Type II Site. Chromatography of chick oviduct cytosol on a Sephacryl S-300 column followed by the HAA postlabeling procedure provided evidence that the type II site was a single major species with a molecular weight in the 40 000 range (see Figure 7). It should be noted that there was a significant degree of loss in the type II binding capacity during the long column run, so one cannot exclude the possibility that one or more less stable species with higher or lower molecular weights are present in cytosol. Although the MW 40 000 peak was consistently present in the S-300 profiles, a small peak of "specific" binding occurred occasionally at the void volume; this peak was thought to be an artifact of preparation. When prelabeled preparations were used, the results were less quantitatively consistent, perhaps due to steroid dissociation during the column run. Nevertheless, the major peak still appeared in the MW 40 000 range, further confirming the findings obtained with the HAA postlabeling procedure.

Affinity Purification of the Type II Site. Our preliminary experiments demonstrated that an affinity column for type II binding sites could be prepared with the bioflavonoid quercetin. This compound binds to cytosol and nuclear type II sites from the rat uterus with a high affinity (Markaverich et al., 1987). Results from estradiol saturation binding analyses have shown that the type II binding which eluted from a quercetin affinity purified preparation appeared identical with that obtained with native cytosol (see Figure 8). A KCl gradient elution profile

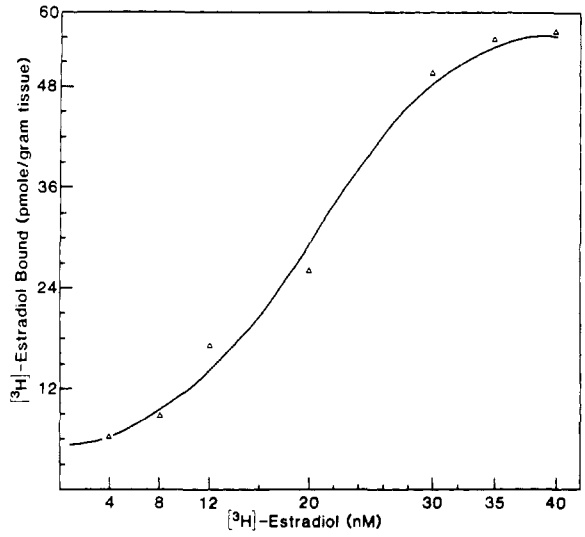

FIGURE 8: Saturation analysis of type II binding site purified from chick oviduct cytosol by bioflavonoid affinity chromatography. A bioflavonoid affinity matrix was made by linking the bioflavonoid quercetin to epoxy-activated Sepharose. This column was used to purify chick oviduct type II sites by loading the 40–60% ammonium sulfate precipitated fraction of cytosol onto the affinity column, incubating for an appropriate time at 4 °C, washing the column extensively with TE buffer, and eluting with 10 μM quercetin. The eluate was then charcoal stripped to remove free quercetin before being evaluated for type II estrogen binding activity with the hydroxylapatite absorption assay previously described.

from a DEAE-cellulose column exhibited the same major peaks present in profiles of unpurified preparations (not shown). SDS–polyacrylamide gel electrophoresis of affinity-purified preparations have proven impressively devoid of contaminating proteins. One band is consistently present at around MW 40 000–45 000, the approximate size of the type II site as determined by Sephacryl S-300 chromatography. However, since there are no antibodies to the type II site and since electrophoresis appears to destroy type II binding activity, it is uncertain as to whether this band represents the type II site or a copurifying contaminant of the same approximate molecular weight. It should be noted that ovalbumin also falls into this molecular weight range and is present in very high concentrations in the estrogen-stimulated chick oviduct.

High-capacity, low-affinity (nonspecific) binding of cytosolic proteins to the matrix-bound quercetin appears to occur and reduce the capacity of the affinity matrix for high-affinity binding by type II sites. We felt it was logical to remove as many of these contaminants as possible prior to the affinity chromatography step. This was done by first submitting the native cytosol to a combination of ion exchange chromatography, hydroxylapatite chromatography, or ammonium sulfate precipitation prior to application of the type II binding sites to the affinity column. The use of one of these steps or a series of steps prior to the affinity column step acted to further remove endogenous ligands to the type II sites and allowed a better interaction with the affinity matrix.

Overall, the best results were obtained with the following steps: (1) prepare fresh cytosol in the usual manner and bring the cytosol to a phosphate concentration of 50 mM; (2) incubate this preparation with hydroxylapatite; (3) wash the hydroxylapatite three times with cold TE buffer plus 50 mM phosphate; wash the hydroxylapatite with cold TE buffer plus 300 mM phosphate to remove the type II sites and retain the supernatant; dilute the supernatant 10-fold with cold TE buffer to decrease the phosphate concentration; (4) incubate the diluted preparation with DEAE-cellulose batchwise with

Table I: Purification of Chick Oviduct Type II Binding Site[a]

fraction	sp act. (pmol/mg of protein)	purification (x-fold)	yield (%)
cytosol	0.8	1	100
HAP	6.4–7.9	8–10	35–70
DEAE	35–80	44–100	12–45
affinity chromatography	550–1100	685–1375	1–5

[a] *Values represent the range over several experiments.*

periodic gentle mechanical inversion; wash the DEAE-cellulose pellet three times with cold TE buffer; apply the washed DEAE-cellulose to a column and elute with a 0–500 mM NaCl gradient in TE buffer containing 10% glycerol; (5) assay a small aliquot of each collected fraction with the HAA procedure in order to find the type II peak; (6) apply the peak fractions to a quercetin–agarose matrix and follow the affinity purification procedure; (7) subject the aliquots of the resulting preparation to estradiol saturation analysis, SDS–polyacrylamide gel electrophoresis, and protein analysis. Unfortunately, the degree of purification is impossible to determine precisely because of problems in accurately determining maximal binding capacity. In addition, there is evidence that while some purification steps lead to the destabilization of the type II site, other steps actually appear to result in further dissociation of endogenous ligand, uncovering previously unseen sites. The estimates for the degree of purification as well as yield are approximate but appear to be in the range of 1000-fold or greater with a yield of only 1–5% (see Table I).

In an effort to overcome the problem of apparent instability during many of the purification or characterization procedures, a variety of agents were tested for their ability to enhance binding site stability. Glycerol appeared to have only minimal effect on binding site stability, and a variety of protease inhibitors were shown to be without effect as well. Molybdate, which is known to stabilize a variety of steroid receptors, particularly in their unoccupied state, was also without significant effect.

Another approach to purifying the type II site involved the use of an estradiol affinity column, which has been used to purify the estrogen (type I) receptor (Greene et al., 1980). One advantage of using such a column would be that the binding properties of the type II site for estradiol are relatively well-known, whereas interactions of the site with bioflavonoids are understood only indirectly by virtue of their inhibitory effects on [^{3}H]estradiol binding to the type II sites. Unfortunately, our attempts to purify the type II site using a commercially available estradiol agarose affinity resin were unsuccessful.

Discussion

This study has provided evidence for an estrogen type II binding site in a nonmammalian species which exhibits binding characteristics remarkably similar to those described previously for rat uterine preparations (Markaverich & Clark, 1979). The existence of such a site in two widely different species provides some evidence of a physiological function important enough to be conserved among at least higher vertebrates. From a more practical standpoint, the existence of this binding site in relatively high concentrations in the chick provides for a new, and perhaps better, model for the study of the type II binding site and its function. The estrogen-stimulated chick and hen oviducts are generally 1–2 orders of magnitude larger, respectively, than the rat uterus, thereby providing a more plentiful source of tissue for the purification of the site.

Although the actual function of the type II site is still in

question, recent findings by the laboratories of Markaverich and Clark regarding the biochemical nature of the endogenous inhibitor of [^{3}H]estradiol binding to type II sites in rat uterus as well as in vitro and in vivo effects of this inhibitor have provided new evidence for a functional role of the type II site in cell growth regulation. These workers found that the endogenous inhibitor in rat uterus was a bioflavonoid metabolite (Markaverich et al., 1988c) and subsequently demonstrated that several known bioflavonoids (luteolin, quercetin, pelargonin) compete for [^{3}H]estradiol binding to cytosol and nuclear type II sites in rat uterine preparations. The inhibition of binding of [^{3}H]estradiol to type II sites was specific, and these bioflavonoids did not interact with the uterine estrogen (type I) receptor. Since estradiol stimulation of nuclear type II sites in the rat uterus is highly correlated with cellular hypertrophy and hyperplasia (Markaverich & Clark, 1979; Markaverich et al., 1981a,b), this laboratory assessed the effects of these compounds on the growth of MCF-7 human breast cancer cells in culture and on estradiol stimulation of uterine growth in the immature rat. These findings demonstrated that addition of bioflavonoids to MCF-7 cell cultures resulted in a dose-dependent inhibition of cell growth. It was found also that injection of these compounds blocked estradiol stimulation of type II sites in the immature rat uterus, and this correlated with an inhibition of uterine growth. These findings are particularly interesting in light of the previous observation by this laboratory that while normal mouse mammary gland cytosol contained high levels of endogenous inhibitor activity, cytosol from mouse mammary tumors and human breast cancer contained very low quantities of this inhibitor (Markaverich et al., 1984). Most recently, the endogenous inhibitor(s), presumably the endogenous ligand(s) for the type II site, was (were) isolated from bovine serum and identified as (*p*-hydroxyphenyl)lactic acid (HPLA) and methyl (*p*-hydroxyphenyl)lactate (MeHPLA), bioflavonoid metabolites (Markaverich et al., 1988a). The presence of MeHPLA in rat liver has recently been confirmed (Markaerich et al., 1988b). The ability of commercially available bioflavonoids such as luteolin and quercetin to compete with and apparently bind to the chick oviduct [^{3}H]estradiol type II binding site with high affinity provides even more evidence for a potentially common physiological role for these sites in rat and chick.

Finally, this study has also provided physicochemical information regarding the type II binding site for the first time. This information provides additional means for comparison of type II binding activity to other better understood steroid binding sites such as the high-affinity "classic" intracellular receptors for the various endogenous steroids, the moderate- to high-affinity plasma carrier proteins, and the various proteins which bind steroids intracellularly or in plasma with low affinity. Clearly, the information gained thus far indicates that the type II site is not likely to be a modification of the type I site, better known as the estrogen receptor. This is based on several pieces of evidence. First, the relatively low molecular weight (about 40000) is smaller than that reported for the estrogen receptor in either its multimeric or monomeric forms [about 300000 and 60000–70000 respectively (Lubahn et al., 1985; Parmar et al., 1988)]. The sensitivity of the type II site to sulfhydryl reducing reagents is contrary to the findings for essentially all of the intracellular high-affinity steroid receptors, including those for estrogen (Jensen et al., 1967), progesterone (Coty et al., 1983), glucocorticoids (Rees & Bell, 1975), mineralocorticoids (Emadian et al., 1986), and androgens (Wilson et al., 1986), which have generally been shown to be stabilized by the presence of sulfhydryl reagents. In some of these cases, binding capacity lost in the absence of reducing reagents, but under otherwise stabilizing conditions, could be reactivated upon addition of dithiothreitol.

Despite the relatively low yields of type II binding sites achieved with the purification protocol described here, the relatively high concentrations of the site in some tissues may eventually allow for purification to a degree suitable for the production of antibodies as well as other analytical procedures requiring highly purified preparations. Further studies regarding the stabilization of the site during purification, the kinetics of association and dissociation of bioflavonoids and other ligands (used in affinity chromatography), conditions favorable for the complete elimination of the endogenous ligand(s) [inhibitor(s)] prior to purification, and more precise methods of determining binding site concentration may lead to improvements in the overall purification process. For the time being, even a partially purified preparation should allow for a variety of analyses which may enhance our understanding of this potentially important molecule.

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Substrate Reduction Properties of Dinitrogenase Activated in Vitro Are Dependent upon the Presence of Homocitrate or Its Analogues during Iron-Molybdenum Cofactor Synthesis†

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ABSTRACT: (*R*)-2-Hydroxy-1,2,4-butanetricarboxylic acid [(*R*)-homocitrate] has been recently reported to be an integral constituent of the otherwise thought to be inorganic iron-molybdenum cofactor of dinitrogenase [Hoover, T. R., Imperial, J., Ludden, P. W., & Shah, V. K. (1989) *Biochemistry 28*, 2768-2771]. Different organic acids can substitute for homocitrate in an in vitro system for iron-molybdenum cofactor synthesis and incorporation into dinitrogenase [Hoover, T. R., Imperial, J., Ludden, P. W., & Shah, V. K. (1988) *Biochemistry 27*, 3647-3652]. Dinitrogenase activated with homocitrate-FeMo-co was able to reduce dinitrogen, acetylene, and protons efficiently. Homoisocitrate and isocitrate dinitrogenases did not reduce dinitrogen or acetylene, but showed very high proton reduction activities. Citrate and citramalate dinitrogenases had very low dinitrogen reduction activities and intermediate acetylene and proton reduction activities. CO inhibited proton reduction in both these cases but not in the case of dinitrogenases activated with other homocitrate analogues. By use of these and other commercially available homocitrate analogues in the in vitro system, the structural features of the homocitrate molecule absolutely required for the synthesis of a catalytically competent iron-molybdenum cofactor were determined to be the hydroxyl group, the 1- and 2-carboxyl groups, and the *R* configuration of the chiral center. The stringency of the structural requirements was dependent on the nitrogenase substrate used for the assay, with dinitrogen having the most stringent requirements followed by acetylene and protons.

Nitrogenase catalyzes the ATP-[1] and reductant-dependent reduction of N_2 to ammonia (Bulen & LeComte, 1966), in addition to the reduction of other triple-bonded molecules. In the absence of any other substance, nitrogenase catalyzes the reduction of protons to H_2. Nitrogenase consists of two proteins: dinitrogenase (or MoFe protein) and dinitrogenase reductase (or Fe protein) (Bulen & LeComte, 1966; Hageman & Burris, 1978). Substrate reduction occurs on dinitrogenase. A unique prosthetic group, the iron-molybdenum cofactor (FeMo-co) containing Mo, Fe, S (Shah & Brill, 1977), and homocitrate (Hoover et al., 1989), has been proposed as the site for substrate reduction (Shah & Brill, 1977; Rawlings et al., 1978; Hawkes et al., 1984; Hoover et al., 1988a, 1989). The structure and biosynthetic pathway of FeMo-co remain undefined. A system for in vitro synthesis of FeMo-co that requires molybdate, ATP, at least the *nifB*, *nifN*, and *nifE* gene products (Shah et al., 1986), the *nifH* gene product (Robinson et al., 1987; Shah et al., 1988), and (*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid (homocitrate) (Hoover et al., 1987) has been described. Unmodified homocitrate can be recovered from FeMo-co in a stoichiometric ratio of 1 homocitrate to 1 Mo (Hoover et al., 1989).

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[1] Abbreviations: FeMo-co, iron-molybdenum cofactor of dinitrogenase; ATP, adenosine 5'-triphosphate; homocitrate acid, (*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid; homoisocitric acid, 1-hydroxy-1,2,4-butanetricarboxylic acid; citramalic acid, 2-hydroxy-2-methylbutanedioic acid; tricarballylic acid, 1,2,3-propanetricarboxylic acid.

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