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RAPID METHOD FOR QUANTITATION OF ANDROGEN BINDING PROTEIN IN SERTOLI CELL CULTURES AND ITS USE FOR MEASUREMENT OF BINDING KINETICS

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Summary—The accurate measurement of the kinetics of binding of 5α-dihydrotestosterone to the Sertoli cell specific protein, androgen binding protein (ABP), has been frustrated by the extremely rapid rate of dissociation of the ABP–dihydrotestosterone complex. We describe a rapid and highly sensitive assay suitable for ABP quantitation which utilizes DEAE Bio-Gel and [^{3}H]dihydrotestosterone. The assay has been used to accurately measure the rate of dissociation ($8.25 \times 10^{-4}\,s^{-1}$, $t_{\frac{1}{2}}$ 14 min) and the rate of association ($2.04 \times 10^5\,M\,s^{-1}$) of the binding of [^{3}H]dihydrotestosterone to rat ABP. The ratio of these rate constants is in perfect agreement with the equilibrium dissociation constant determined by Scatchard analysis (4.0 nM). This multipoint assay is extremely rapid such that binding can be measured at equilibrium, it has high precision (coefficient of variation 3%), and is particularly useful at low protein concentrations (50 ng/ml); furthermore, the assay background of nonspecific ^{3}H-binding is extremely low (0.2%). Since at such low protein concentrations a 10 point Scatchard analysis can be performed on 1 ml culture medium containing as little as 3 fmol ABP, the assay is suitable for monitoring changes in ABP secretion resulting from manipulations of cells in culture. The assay which utilizes DEAE Bio-Gel A is compared to five alternative methods: the standard method of steady state gel electrophoresis, Dextran-coated charcoal assay, hydroxylapatite assay, DEAE filter assay, and radioimmunoassay. The DEAE Bio-Gel assay has advantages over all of these alternative methods. In summary, this new assay is particularly useful for monitoring temporal changes in the secretion of ABP, and the method is equally effective in quantitating ABP in rat, rabbit and hamster Sertoli cell cultures.

INTRODUCTION

The main function of Sertoli cells appears to be maintenance and support of spermatogenesis [1]. This function appears to be regulated by follicle-stimulating hormone (FSH) and testosterone (T) [2]. Seven major polypeptides have been detected in Sertoli cell secretions under the influence of these two hormones [3]. These proteins are thought to be secreted into the lumen of the seminiferous tubules where they aid in the development of germinal cells. The significance and regulation of one of these proteins, androgen binding protein (ABP), has become the subject of intensive investigation [4–8].

We are interested in the effects of hormones and various agents on Sertoli cell function. To measure the direct effects of such agents involves their addition to monolayer cultures of Sertoli cells. The effects on the cells can then be measured by monitoring cell secretions. Since one of the best characterized secretory products is ABP, this protein has been selected as a monitor of Sertoli cell function.

The most commonly used method for quantitating ABP has been steady state polyacrylamide gel electrophoresis (SS–PAGE) which is most time-consuming and relatively insensitive. Furthermore, since each assay utilizes a single concentration of [^{3}H]dihydrotestosterone and the assay background is high, the method has poor accuracy and precision when low concentrations of ABP are assayed. An alternative method, the quantitation of rat ABP by radioimmunoassay, has limitations in that the ABP antibody does not measure the androgen binding capacity of ABP, and since it only recognizes specific antigenic determinants, it is unusable in heterologous systems. For these reasons, we sought a new method of ABP quantitation which would be free of these drawbacks. The new method would require the rapid removal of unbound steroid so that equilibrium binding would not be disrupted and should be of use at very low protein concentrations to allow monitoring of culture media for ABP secretion without incurring losses involved in concentrating the media prior to assay. This report describes the validation of a rapid, reproducible and sensitive method for the quantitation of ABP in Sertoli cell secretions which, because of its rapidity, has allowed measurement of the rate of formation and the rate of dissociation of the ABP–DHT complex.

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EXPERIMENTAL

Chemicals, reagents and animals

5α-Dihydro [1, 2, 4, 5, 6, 7-^{3}H] testosterone, 145 Ci/mmol, (DHT) was obtained from Amersham, Arlington Heights, IL. Radioinert DHT, testosterone, progesterone, estradiol and corticosterone were all obtained from Steraloids. Scintiverse, DE-81 and GF/A glass microfiber filters, EDTA, thioglycerol and hydrochloric acid were purchased from Fisher, Houston, TX. DEAE Bio-Gel A 100–200 mesh, hydroxylapatite, all polyacrylamide gel reagents and protein assay reagents were bought from Bio-Rad, Richmond, CA. Tris-base ultrapure was obtained from Schwartz–Mann, Spring Valley, NY. Hanks Balanced Salt Solution, trypsin, chick serum, and minimum essential medium (MEM) containing d-valine were all from GIBCO, Santa Clara, CA. Collagenase was purchased from Worthington Biochemical Corp., Freehold, NJ, and fetal calf serum from KC Biological, Lenexa, KS. Rabbits and Sprague–Dawley rats were obtained from Temco, Houston, TX.

Culturing of Sertoli cells

The testes were removed from 18-day old Sprague–Dawley rats, 200 g rabbits, or estrogen-treated, mature hamsters and the Sertoli cells isolated by a modification of the method of Welsh and Wiebe [9] as described by Smith *et al.* [10]. After 24 h, the cells had formed a monolayer and the medium was replaced with serum-free media containing FSH (NIH, FSH S-14, 0.2 μg/ml) and testosterone (200 ng/ml). Media was changed after 4 and 8 days in culture. The "spent" medium was centrifuged and stored at −25°C.

Steady-state polyacrylamide gel electrophoresis to measure ABP

For the ABP assay, 5 ml media was centrifuged at 1,000 ***g*** for 30 min to remove any detached cells, and 300 μl of this centrifuged media was preincubated with 5 nM [^{3}H]DHT for 2 h at 4°C and run at 4°C on 7% polyacrylamide gels containing 2 nM [^{3}H]DHT similar to that by Ritzen *et al.* [11]. The gels were then sliced into 2 mm pieces and counted for ^{3}H-incorporation.

Assay of ABP using DEAE Bio-Gel

The conditions and proportions of the reagents were optimized to maximize ABP quantitation (all procedures are performed in a cold room at a temperature of 2–4°C). One-hundred microliter aliquots of centrifuged media were added to assay tubes containing 300 μl of Buffer A (10 mM Tris–HCl, pH 7.5; 1 mM EDTA, 12 mM monothioglycerol; 10% v/v glycerol). A 300 μl aliquot of a 50% slurry of 100–200 mesh DEAE Bio-Gel in Buffer A, which had previously been thoroughly washed with Buffer A, was added. The suspension was then very gently shaken for 30 min, centrifuged at 1,000 ***g*** for 5 min and the supernatant discarded. Next, varying concentrations of [^{3}H]DHT were added to the DEAE-ABP pellets at a concentration range of 0.5–15 nM in a total volume of 300 μl. A duplicate set of assay tubes were prepared in the presence of a 100-fold excess of radioinert DHT. The suspensions were incubated for 2 h with brief, gentle agitation at 20-min intervals. After incubation, the tubes were centrifuged for 5 min at 1,000 ***g***, the supernatant was discarded and the tubes were blotted. To each tube 3 ml of Buffer A was added, using a repeating syringe, and the resulting suspension was immediately poured onto a Whatman GF/A 2.4 cm glass microfiber filter in a Millipore filter box. Vacuum was applied, so that each filter well drained in 5 s, and was then disconnected *to prevent drying* of the DEAE. Each well was then washed with 3 × 3 ml Buffer A in a similar manner and the filters were then dried with suction. The DEAE-coated filters were carefully removed with forceps, placed in scintillation vials and dried under an infrared lamp. Finally, 6 ml Scintiverse was added and the vials were counted for ^{3}H-incorporation.

Assay of ABP using hydroxylapatite

The procedure followed was identical to that described for DEAE Bio-Gel, except that 200 μl of a 50% hydroxylapatite suspension was used. These conditions were optimized to maximize ABP binding by varying incubation times and concentrations of hydroxylapatite.

Assay of ABP using DEAE filters (DE-81)

Aliquots (100 μl) of medium were incubated for 2 h with various concentrations of [^{3}H]DHT as described above, and then assayed using the procedure described by Santi *et al.*[12].

Assay of ABP using Dextran-coated charcoal

Aliquots (100 μl) of medium were incubated for 2 h in the presence of 0.5–15 nm [^{3}H]DHT as described above. The [^{3}H]DHT bound to ABP was assayed using a Dextran-coated charcoal assay (0.5% charcoal, 0.05% Dextran 80, in Buffer A with an incubation time of 5 min). An alternative method utilizing 0.1% gelatin, exactly as described by Musto *et al.* [7] was also used for comparison purposes.

Assay of ABP using radioimmunoassay

Rat ABP was measured by RIA from a kit provided by the National Hormone and Pituitary Program, supported by NIADDK and NICHD. The usable range of the assay is 3–200 fmol ABP/tube, the within assay variation is 5–6% and the between assay variation is 16%.

Measurement of the rate of association of ABP and DHT

Medium was harvested from Sertoli cell cultures after a 4-day accumulation of ABP secretions and

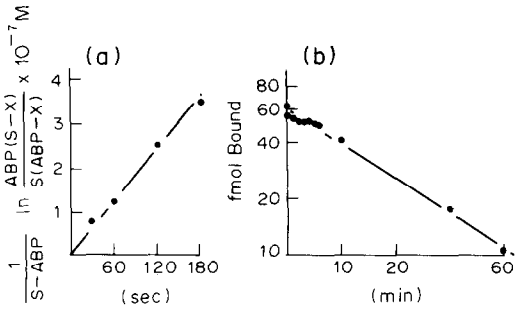

Fig. 1. Association and dissociation reactions of [^{3}H]DHT binding to ABP as determined using DEAE Bio-Gel. Panel A represents the association reaction for the first 180 s. The slope of the straight line equals the association rate constant, 2.04×10^5 M s^{-1}. Panel B represents the dissociation of the ABP-[^{3}H]DHT complex. The $t_{\frac{1}{2}}$ of dissociation was 14 ± 2 min ($N = 3$) which is equivalent to a dissociation rate of 8.25×10^{-4} s^{-1}.

centrifuged at 1,000 *g* to remove any cell debris. Aliquots of the media were then incubated at 4°C with [^{3}H]DHT (2.56 nM) for 0.5, 1, 2 and 3 min, and assayed for ABP binding using the DEAE Bio-Gel assay.

Measurement of the rate of dissociation of DHT from ABP

Medium was collected as above and labeled with [^{3}H]DHT (2.56 nM) for 2 h at 4°C. Radioinert DHT (460 nM) was added and the specific bound [^{3}H]DHT was assayed using DEAE Bio-Gel after 0.5, 1, 2, 3, 4, 5, 6, 10, 30 and 40 min.

RESULTS

Kinetic analysis of [^{3}H]DHT binding to ABP using DEAE Bio-Gel

The DEAE Bio-Gel assay is an extremely rapid assay which, therefore, allows measurement of fast association and dissociation reactions. The amount of DHT binding to ABP in media from cultured rat Sertoli cells was measured from 0.5 to 120 min using the DEAE Bio-Gel assay as described in Materials and Methods. No significant increase in binding could be demonstrated between 30–120 min of incubation at 4°C. At short incubation times relative to the rate of dissociation, and assuming a single class of DHT binding sites per ABP molecule, the association reaction approximates a second order reaction. This reaction is represented by the equation: $dX/dt = k(S - X)(ABP - X)$. Integration of this equation yields equation (i). By substitution of the values for the amount of DHT bound to ABP at times 0, 0.5, 1, 2 and 3 min in equation (i), the association rate constant (k) can be calculated:

$$\frac{1}{S - ABP} \cdot ln \frac{ABP(S - X)}{S(ABP - X)} = kt \qquad (i)$$

where S = DHT concentration, ABP = ABP concentration, and X = ABP–DHT concentration at times t. Figure 1A shows that substitution of binding data from the association of [^{3}H]DHT with ABP after 0, 0.5, 1, 2, 3 and 4 min into the above equation furnishes a linear plot consistent with a second order reaction. The association rate constant calculated from the slope of this line is 2.04×10^5 M s^{-1}.

The dissociation of [^{3}H]DHT from ABP in medium from cultured Sertoli cells was measured as described in Materials and Methods. In the presence of a large excess of radioinert DHT, the association or reverse reaction is nondetectable; thus, the observed reaction is first order. Figure 1B shows the log of the concentration of [^{3}H]DHT–ABP as a function of time. The half-life ($t_{\frac{1}{2}}$) of this complex was shown to be 14 ± 2 min ($N = 3$), since for a first order reaction, the rate of dissociation is equal to $ln\ 2/t_{\frac{1}{2}}$, the dissociation rate constant is 8.25×10^{-4} s^{-1}.

Determination of equilibrium dissociation constant (K_d) and precise quantitation of ABP using Scatchard analyses

Having established the rate constants of association and dissociation, a suitable incubation period for the measurement of the equilibrium dissociation constant (K_d) of the ABP-DHT binding reaction was selected. Upon incubation of media containing ABP for 120 min with various concentrations of [^{3}H]DHT (0.5–15 nM), the DEAE Bio-Gel method was used to separate macromolecular bound DHT from free DHT as described in Experimental. When these resulting data were treated according to the method of Scatchard [13], the K_d and concentration of ABP were determined from plots typical to that depicted in Fig. 2. The concentration of ABP (N) depended on the particular treatment of the cultured Sertoli cells; however, the K_d was always approx 4.0 nM. This is in excellent agreement with the value obtained from

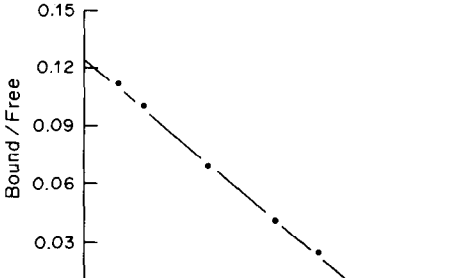

Fig. 2. Scatchard analysis using DEAE Bio-Gel of [^{3}H]DHT binding to ABP in tissue culture medium. The media was harvested after 4 days from rat Sertoli cell cultures. The [^{3}H]DHT binding in a concentration range of 0.5–15 nM was corrected for nonspecific binding by incubating a replicate assay tube in the presence of a 100-fold excess of radioinert DHT.

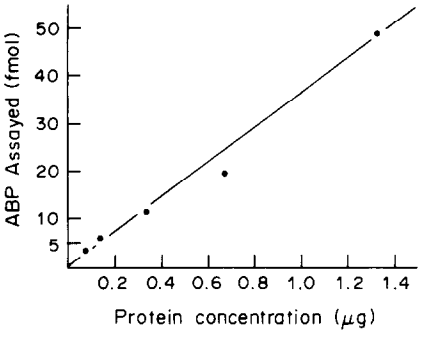

Fig. 3. Measurement of ABP in media at various dilutions using DEAE Bio-Gel. The ordinate represents the total amount of ABP assayed. The quantitation of ABP was performed using Scatchard analysis as described in Experimental.

the ratio of the rate constants which also gives a value of 4.0 nM.

The reproducibility of this assay was assessed by repeatedly determining the K_d and N values four times on the same batch of medium. The DEAE Bio-Gel method is remarkably reproducible with a range in K_d of 3.6–4.3 nM, and a range of N of 0.65–0.69 nM. The coefficient of variation of the assay was 3%.

Specificity of the DEAE Bio-Gel

To determine the specificity of this new assay, [^{3}H]DHT was incubated with medium in the absence or presence of 80 nM radioinert DHT, testosterone (T), estradiol (E_2), progesterone (P), corticosterone (C), or diethylstilbestrol (DES). The following displacement of DHT by the radioinert steroids was observed: 85% by DHT, 68% by T, 30% by E_2, <1% by P, C, and DES. Since both DHT and T displace the binding to a greater extent than E_2, P, C, and DES, the assay appears to be specifically measuring [^{3}H]DHT binding to ABP. The assay blank was 0.3% of the total [^{3}H]DHT added. It is possible that the specific DHT binding that we are observing could be due to androgen receptors which might be released from degenerating Sertoli cells. Scatchard analyses were performed on control medium and the same medium which was receptor depleted by heating at 50°C for 30 min [14]. The control medium bound 44 fmol of DHT/μg protein and heated medium bound 42 fmol of DHT/μg protein.

Utility of the DEAE Bio-Gel assay at low protein concentrations

The linearity of the assay was investigated by diluting the spent media from Sertoli cell cultures with MEM and then measuring ABP concentration by Scatchard analyses. Figure 3 illustrates such a quantitation at various dilutions. It can be seen from this figure that the assay is linear, even at a protein concentration as low as 50 ng/ml. The working capacity of the DEAE Bio-Gel assay was determined in 10-fold concentrated medium. The unconcentrated medium upon assay gave a value of 1.31 ± 0.13 ($\pm$standard deviation, $n = 3$) and the concentrated medium a value of 10.10 ± 0.70 pmol ABP ($n = 3$).

Comparison of other methods of assaying ABP

Figure 4 represents a scatter-gram which clearly shows that there is a good positive correlation between ABP measured by DEAE Bio-Gel and the standard SS-PAGE method ($r = 0.97$, $P < 0.001$). For comparison with alternative methods, the same specimen of culture medium from Sertoli cell cultures was assayed by Dextran-coated charcoal adsorption (DCC), hydroxylapatite filter assay (HAP), and DEAE filters (DE-81). Scatchard analyses were used to quantitate the amount of ABP present. The values obtained were: 382 pmol by DEAE Bio-Gel, 314 by DCC, 110 by HAP, and 108 by DE-81. It is of interest that at this particular protein concentration (5 μg protein/assay tube), the DCC method gave a value in reasonable agreement with that obtained using DEAE Bio-Gel. This result is misleading. The apparent "agreement" is not good at lower protein concentrations, and the sensitivity at these lower levels is poor because of the relatively high background of radioactivity retained by the supernatant following charcoal adsorption. These high assay backgrounds are more pronounced if bovine serum albumin is added to the media.

In Table 1, a comparison has been made using a modified Dextran-coated charcoal assay in the presence of 0.1% gelatin. This particular method has been shown to be usable at low protein concentrations [7]. Although this procedure does appear to be an improvement over the more conventional charcoal adsorption assay, it does not give reproducible results below a protein concentration of 2.5 μg/ml. Each of the results shown in Table 1 were obtained by conducting Scatchard analyses at five different [^{3}H]DHT concentrations on the same sample of

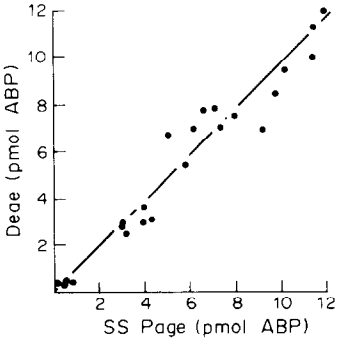

Fig. 4. Comparison of DEAE Bio-Gel with SS-PAGE for the quantitation of ABP. The DEAE Bio-Gel determinations were performed by Scatchard analysis (0.5–15 nM [^{3}H]DHT). SS-PAGE was conducted at a concentration of 5 nM [^{3}H]DHT as described in Materials and Methods, and the binding at saturation was computed according to reference 11.

Table 1. Quantitation of ABP at various protein concentrations using DEAE Bio-Gel or Dextran-coated charcoal.

Protein concentration (μg/ml)	[ABP] DEAE Bio-Gel (fmol)	[ABP] DEAE Bio-Gel (fmol/μg protein)	[ABP] Dextran-coated charcoal (fmol)	[ABP] Dextran-coated charcoal (fmol/μg protein)
2.5	134 ± 6	54 ± 2	119 ± 16	48 ± 7
1.25	74.2 ± 0.7	59 ± 0.5	a	a
0.25	13.4 ± 0.7	54 ± 3	a	a

The errors denote the standard deviation of the assay, where $n = 4$.
a—All 4 replicate assays gave a standard deviation of $> 50\%$.
The DEAE Bio-Gel assay was performed as described in Experimental. The Dextran-coated charcoal was performed according to Mustro *et al.* [7].

media; the assays were repeated four times to provide an estimate of the between assay standard deviation. Only the DEAE Bio-Gel procedure gave reproducible results at protein concentrations lower than 2.5 mg/ml.

The remaining two methods for comparison—hydroxylapatite filter assay and DE-81 filters—demonstrate good Scatchard plots and increased sensitivity, but the ABP concentration was underestimated by 65%. Similar results were obtained when the ABP-containing media from rabbit and hamster Sertoli cells were used, where DEAE Bio-Gel again proved to be the method of choice. The K_d of [^{3}H]DHT binding to hamster and rabbit ABP determined in Sertoli cell culture media was 8 and 10 nM respectively. When quantitation of rabbit ABP was compared using SS-PAGE and DEAE Bio-Gel was made, values of 9.5 and 8.3 fmol/μg protein were obtained respectively.

In another series of experiments, rat ABP was also measured by RIA and compared to the DEAE Bio-Gel method (Table 2). There is clearly a very good correlation between the two methods. A maximal concentration of FSH produced approx a 7-fold stimulation of ABP secretion from rat Sertoli cells when quantitated by either method.

DISCUSSION

The difficulty in studying the kinetics of the ABP–DHT interaction is inherent in the rapid rate of dissociation of the ABP–DHT complex under non-equilibrium conditions. Any method for the measurement of the rate of formation and dissociation of the complex must, therefore, be a rapid one. The DEAE Bio-Gel method described herein required only 20 s per assay point; thus, it is a most appropriate method for measuring the association and dissociation rate constants. The rate of association at 4°C was $2.04 \times 10^5\ M^{-1}$, and the rate of dissociation was $8.25 \times 10^4\ s^{-1}$ ($t_{\frac{1}{2}}$, 14 min). The measured half-life of dissociation of DHT is somewhat slower than the 4.5 min reported previously [15]. This can be explained by the fact that the incubation time required to separate the complexed DHT from free DHT was always in excess of 12.5 min, whereas the DEAE assay requires only 20 s. To support the accuracy of the $t_{\frac{1}{2}}$ of dissociation obtained by DEAE Bio-Gel assay, the ratio of the rate of dissociation and the rate of association was in perfect agreement with the equilibrium dissociation constant (4.0 nM) obtained by Scatchard analysis. Since the $t_{\frac{1}{2}}$ of dissociation is 14 min and the removal of free steroid takes 20 s, any changes in the equilibrium are infinitesimal.

Table 2. Measurement of ABP in culture media from rat Sertoli cells by DEAE Bio-Gel and radioimmunoassay

		DEAE Bio-Gel fmol/100 μl media	RIA
Control	1	2.5	2.4
	2	2.8	2.4
	3	3.8	2.5
FSH (0.2 μg/ml)	4	18.7	15.2
	5	19.5	18.5
	6	17.9	14.0

The specificity of the binding was confirmed by competition binding assays which showed that only radioinert DHT or T were efficient in displacing macromolecular bound [^{3}H]DHT. Furthermore, since this specific binding could not be reduced by heating at 50°C for 30 min, the preparation was not contaminated by androgen receptors.

To quantitate ABP under equilibrium conditions, in general, 1 ml of tissue culture medium from Sertoli cells cultured for 4 days was used to set up a Scatchard analysis consisting of five different concentrations of [^{3}H]DHT in the absence or presence of a 100-fold excess of radioinert DHT. When this assay was repeated four times on the same batch of media, the precision was excellent (coefficient of variation of 3%). Thus, the method is highly reproducible and suitable for monitoring media from cells in culture, since only a small amount of media is required per assay.

To further determine the utility of this assay for monitoring cell cultures which secrete relatively small amounts of protein, the media obtained as described above was diluted to determine the linearity and assay sensitivity as a function of protein concentration. The assay was found to be useful at protein concentrations as low as 50 ng/ml and was linear at least in the range of 0.05–5 μg/ml.

We carefully evaluated possible alternative methods for the rapid quantitation of ABP. In particular, we considered that a hydroxylapatite filter assay might be appropriate because it could be performed in 20 s in a similar manner to the DEAE Bio-Gel

method. Another more obvious method was the DE-81 filter method previously described by Santi *et al.* [12]. In addition to these we also compared a Dextran-coated charcoal assay and SS-PAGE. Of all these methods which were compared using the same ABP-containing Sertoli cell culture medium, the DEAE Bio-Gel method was superior. The additional method for ABP quantitation we compared was radioimmunoassay [16]. The concentration of immunoassayable ABP was in very good agreement with the ABP quantitated by DEAE Bio-Gel. However, the DEAE Bio-Gel offers a number of advantages: 4-h assay compared to 4 days for the RIA; measures androgen binding capacity and affinity; and most importantly, DEAE Bio-Gel can be used to measure ABP secretions from Sertoli cells other than the rat.

The non-species specificity of the DEAE Bio-Gel ABP assay has been demonstrated by utilizing media from immature rat, estrogen-treated mature rat, immature rabbit and Sertoli cells from estrogen-treated adult hamsters. In all cases, the assay was effective in quantitating ABP.

The data we have presented demonstrates that the DEAE Bio-Gel assay method for quantitating ABP is highly sensitive and specific, offering considerable advantages over the commonly used SS-PAGE. The new method is more sensitive, and is much less laborious and less costly in both time and materials. It is more reproducible than SS-PAGE, particularly at low concentrations of ABP, since it may be conveniently utilized as a multipoint rather than a single point assay; moreover nonspecific backgrounds are very low. The method is infinitely more practical for large numbers of samples and does not require concentration of the media when low concentrations of ABP are present. Finally, since the DEAE Bio-Gel method is highly sensitive and requires only small samples of tissue culture media for a multipoint Scatchard plot, it is exceptionally well-suited for the temporal monitoring of ABP secretion by cells in culture.

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