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Partial Purification and Characterization of Rhinoceros Gonadotropins, Growth Hormone, and Prolactin: Comparison with the Horse and Sheep[1]

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ABSTRACT

The rhinoceros is an endangered species related to the horse family. Little is known of its reproductive endocrinology. The objectives of this study were to partially purify rhinoceros pituitary hormones, determine which assays could be used for their assessment, and to ascertain whether rhinoceros LH possesses the intrinsic FSH activity of equine LH.

A single pituitary each from a White (1.3 g) and a Black (1.2 g) Rhinoceros was homogenized and extracted (pH 9.5), then subjected to pH and salt fractionation, and ion-exchange chromatography (DEAE and Sephadex SP-C50) to yield partially purified fractions of LH, FSH, growth hormone (GH), and prolactin (PRL).

LH was readily measured by a rat Leydig cell assay (0.1–1% × equine LH) and an RIA using a monoclonal antibody to bovine LH (6–11% × equine LH). FSH activity detected in the LH by either an FSH RIA or a calf testis radioreceptor assay (RRA) was extremely low. No FSH activity could be detected in the White Rhinoceros pituitary "FSH" fraction, but was readily detected in the Black Rhinoceros fraction (RIA: 0.2% × equine FSH; RRA: 0.8% × equine FSH).

The presence of GH and PRL was determined by SDS-PAGE and Western blots. Results showed a single immunoreactive GH band and multiple immunoreactive PRL bands. Adsorption with Concanavalin A-Sepharose indicated that some of the PRL bands are glycosylated.

INTRODUCTION

All species of the family Rhinocerotidae are endangered and rapidly disappearing from their native habitat [1]; their survival depends on intensive management of native populations and maintenance of captive colonies. A survey of the recent literature reveals that little is known of the reproductive endocrinology in these species. Rhinoceros are members of the order Perissodactyla [2], which also comprises the horse family (Equidae: horse, donkey, zebra) and tapirs (Tapiridae).

This laboratory has had a major interest in the biochemical and biological properties of gonadotropins from the horse and related species. Particularly interesting is that both equine LH (eLH) and equine chorionic gonadotropin (eCG) appear to possess potent intrinsic FSH-like activity in a number of in vitro and in vivo assays [3, 4] and radioreceptor assays (RRAs) [3, 5]. So far, this characteristic has not been found in any other mammalian species. Thus, when we were offered pituitaries from a White (*Ceratotherium simum*) and a Black Rhinoceros (*Diceros bicornis*), we were interested in comparing the properties of several of the pituitary hormones with their equine counterparts, in determining which assays could be used for physiological detection studies, and whether or not the rhinoceros LH also possessed the FSH activity displayed by eLH.

To achieve these objectives, the rhinoceros pituitaries were subjected to purification techniques previously established for equine and other species. A variety of RIAs, RRAs, and a bioassay were used to characterize the various preparations. SDS-PAGE together with Western blotting were additionally used to study the growth hormone (GH) and prolactin (PRL) preparations obtained.

MATERIALS AND METHODS

Pituitaries and Hormones

A single pituitary each from a White Rhinoceros (1.3 g) and a Black Rhinoceros (1.2 g) was obtained post-mortem from female zoo animals. These pituitaries were obtained for us by Dr. J.H. Olsen, Busch Gardens, Tampa, FL, and Dr. S.B. Citino, Metro Zoo, Miami, FL. Each animal was 28 yr old at death. The White Rhinoceros was euthanized because of chronic severe health problems. Regrettably, its pituitary arrived in this laboratory in a thawed, ambient temperature state. It was immediately refrozen until extracted as described below. The Black Rhinoceros pituitary arrived frozen.

The hormones used in this study as standards or assay radioligands were purified in this laboratory and have been previously described as follows: equine LH and equine FSH (eLH, eFSH [3]), ovine LH (oLH, [6]), ovine FSH (oFSH, [7]), equine PRL (ePRL, [8]), and equine GH (eGH, obtained from C.H. Li, University of California, San Francisco, CA; deceased). Human FSH (hFSH) was obtained from the National Hormone and Pituitary Program of the NIDDK, NIH (Baltimore, MD).

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Purification

Each pituitary was extracted and purified separately. The extraction and purification techniques employed have previously been described in detail [3, 9, 10]. In brief, each pituitary was minced, homogenized, and extracted (pH 9.5) overnight at 4°C. After removal of insoluble residue, the GH-PRL fraction (White, 1P, 89 mg; Black, 13P, 74 mg) was precipitated with HPO_3 (pH 4.0), leaving a glycoprotein concentrate fraction (White 1S, 113 mg; Black, 13S, 57 mg) in the supernatant fluid. Separation and further purification of LH and FSH from the White Rhinoceros fraction (1S) proceeded as follows: fraction 1S was chromatographed on a column of DEAE-cellulose (BIO-RAD, Richmond, CA) with 0.03 M NH_4NCO_3, pH 9.0; the unabsorbed fraction (putative LH) was applied to a column of sulfopropyl-Sephadex (SP-C50; Pharmacia, Piscataway, NJ) with 0.03 M NH_4HCO_3; LH was adsorbed and eluted with 1 M NH_4HCO_3 (fraction 4B, 2.6 mg). On the DEAE column, FSH is normally adsorbed and eluted with 0.2 M NH_4HCO_3, pH 9.0. This fraction was chromatographed on SP-C50 with 0.03 M sodium acetate at pH 4.0; after serial elution with the pH 4.0 buffer and 0.03 M ammonium acetate, FSH is normally eluted with 0.03 M NH_4HCO_3 (fraction 5C, 0.1 mg). FSH activity was subsequently found to be absent in the White Rhinoceros fractions.

The Black Rhinoceros glycoprotein concentrate was handled in a similar fashion except the order of the two chromatography steps was reversed. The procedure for the Black Rhinoceros yielded LH (fraction 14SB, 1.3 mg), and FSH (fraction 15D, 0.6 mg). For HPLC comparisons, an oLH was prepared in a similar fashion to fractions 4B and 14SB.

Analysis of fractions 1P and 13P by SDS-PAGE showed bands suggestive of GH and PRL in both (see Fig. 6). These hormones were further purified by subjecting the fractions to DEAE chromatography: GH and PRL were adsorbed at 0.03 M NH_4HCO_3, pH 9.0, and eluted with 0.2 M NH_4HCO_3, yielding fractions 11BC (White) and 16B (Black). The GH and PRL in 11BC was further purified by Concanavalin A-Sepharose (Con A; BIO-RAD) chromatography, yielding an unabsorbed fraction (12A) and an adsorbed fraction (12B). The Black Rhinoceros fraction 16B, which contained little PRL relative to GH, was put on a Sephadex G-100 (Pharmacia) column equilibrated in 0.05 M NH_4HCO_3. The GH was found in the peak eluting with a V_e/V_0 of 2.2, and yielded fraction 17B (5 mg).

Iodinations

Hormones to be used as radioligands in various assays were iodinated with Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycolril; Pierce, Rockford, IL). The iodinations were performed as follows: 500 ng of Iodo-Gen dissolved in 50 μl of chloroform was evaporated in a 12 × 75-mm glass tube. The tube was then rinsed with 0.05 M PBS, and 100 μl of PBS was added together with 5 μg of the protein to be iodinated in 50 μl of the same buffer and 500 μCi (5 μl) of ^{125}I (Amersham, Arlington Heights, IL). The reaction vessel was gently vortexed for 10 min; then the reaction mixture was transferred to 1 ml of PBS containing 0.1% BSA. This mixture was applied to a small desalting column (Econo-Pac 10DG, BIO-RAD), and 1-ml fractions were collected. The void volume peak was collected and used in subsequent assays.

RIAs and RRAs

The RIA employed for measuring LH utilized a monoclonal antibody against bovine LH and ^{125}I-eLH as radioligand and has been described in detail [11]. The antibody has cross-reacted with every mammalian species of LH thus far tested (>12). An RRA for LH as been described [12] that uses rat testis membranes as the receptor source and ^{125}I-eLH as radioligand. FSH was measured in an RIA with a rabbit polyclonal antibody raised against oFSH, with ^{125}I-hFSH as radioligand. This assay has been previously described [13] and the antibody also cross-reacts with FSH from many mammalian and nonmammalian species. An RRA for FSH was used as previously described [9, 12]. This assay employed calf testis membrane fractions as receptor source and ^{125}I-oFSH as radioligand. PRL was measured with a previously described RIA [9] using a rabbit polyclonal antibody raised against purified ePRL, with ^{125}I-ePRL as radioligand. The GH RIA used a monkey polyclonal antiserum raised against rat GH; this assay has been characterized elsewhere [14]. The antiserum was used at a final dilution of 1:75 000, with eGH as radioligand. The samples were assayed by the RIAs and the RRA in duplicate and on at least two separate occasions.

Bioassay

LH bioactivity was monitored in vitro in a rat Leydig cell assay as previously described [15], with minor modifications. The assay was carried out in 48-well tissue culture plates (Costar, Cambridge, MA). Approximately 100 000 cells were incubated in 500 μl of medium (M-199 with Earle's BSS and 0.1% BSA) to which was added the doses of standard or test material. The plates were incubated for 2 h at 37°C in the presence of CO_2 and O_2 mixture (5:95), after which testosterone production was determined by an RIA. The samples were assayed in triplicate.

HPLC

HPLC gel filtration was used to compare the elution characteristics of the rhinoceros LH preparations with that of a similarly prepared oLH. A Perkin-Elmer (series 410) HPLC with UV detector (LC-95) and integrator (LCI-100) (Perkin-Elmer Corp., Norwalk, CT) was used with a 300 × 7.5-mm TSK-250 (BIO-RAD) gel filtration column. The solvent was 0.1 M Na_2SO_4, 0.1 M NaH_2PO_4, pH 6.8, at a flow rate of 1 ml/min. UV absorption was measured at 225 nm,

and samples of 10–20 μg were injected onto the column. The column was calibrated using a mixture of five molecular mass standards (BIO-RAD). The molecular mass and V_e/V_0 values for each of the standards were as follows: thyroglobulin-67 kDa, 1.01; bovine gamma globulin-158 kDa, 1.50; ovalbumin-44 kDa, 1.72; horse myoglobin-17 kDa, 2.06; and vitamin B12-1.35 kDa, 2.45.

SDS-PAGE and Western Blotting

Purification of rhinoceros GH and PRL was monitored by SDS-PAGE as outlined by Laemmli [16]. A 4.5% stacking gel was used with a 12.5% gel. Samples were reduced with 5% β-mercaptoethanol (Sigma Chemical Company, St. Louis, MO) prior to electrophoresis. The following molecular mass standards (BIO-RAD) were used: phosphorylase-*b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Separated proteins were transferred to Hybond-N (Amersham) sheets, and then probed with antibodies in a manner similar to that described by Towbin et al. [17]. The antibody probes used were rabbit antiserum against ePRL [9] and bovine GH (obtained from C.H. Li) at a dilution of 1:500. A peroxidase-conjugated second antibody (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was applied and then developed with 4-chloro-1-napthol.

Statistical Analysis

Relative potencies of the rhinoceros preparations and the standard hormones were calculated from the ED_{50} of log-logit linear regression lines. Linear regression analysis was performed using SAS (SAS Institute Inc., Cary, NC) statistical software.

RESULTS

Gonadotropins

The rhinoceros LH preparations (4B and 14SB) were examined by HPLC and compared to a similarly prepared oLH. These results are shown in Figure 1. The major oLH peak eluted at about 11 min and comprised about 70% of the integrated area. A more highly purified oLH would also elute at 11 min and comprise closer to 90–95% of the total area (data not shown). The White Rhinoceros LH preparation (4B) and that of the Black Rhinoceros (14SB) each had a peak eluting at about 11 min. The percentage of this peak was only 27% in 4B and 39% in 14SB. Because far less of the FSH preparations were available and these preparations were not expected to be as purified as the LH preparations, HPLC analysis was not performed with them.

Three different assays were used to assess LH activity in the rhinoceros preparations. The results for the RIA are shown in Figure 2 and Table 1, and it can be seen that although the final LH preparations (4B, 14SB) were by no

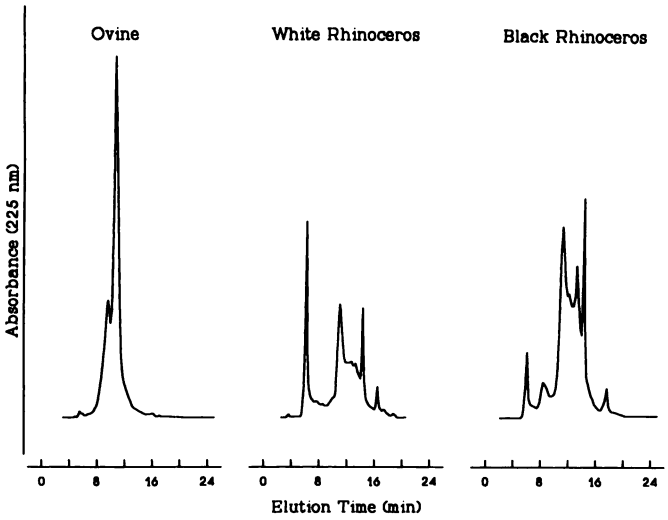

FIG. 1. Gel filtration HPLC chromatograms of oLH and the LH preparations from the White (4B) and the Black Rhinoceros (14SB) on a TSK-250 column (330 × 7.5-mm). The column was equilibrated and the sample eluted with 0.1 M Na_2SO_4, 0.1 M NaH_2PO_4(pH 6.8).

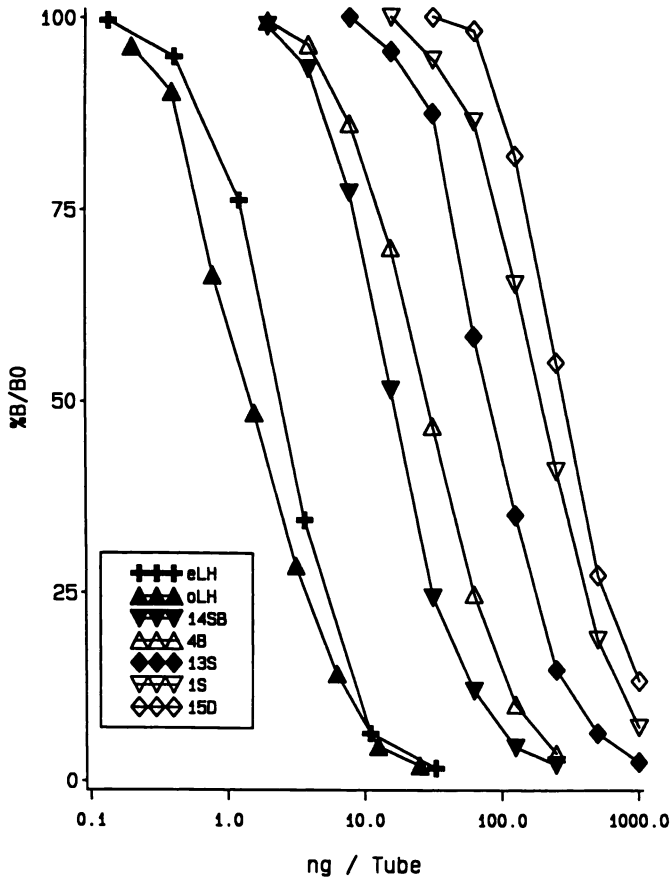

FIG. 2. Competitive binding curves of rhinoceros pituitary preparations compared with purified ovine and equine LH in an RIA using a monoclonal antibody against bovine LH and ^{125}I-eLH tracer.

TABLE 1. The potency of the rhinoceros gonadotropin preparations compared to highly purified equine (taken as 100%) and ovine preparations in various assays.[a]

Samples[b]	LH RIA	LH RRA	LH bioassay	FSH RIA	FSH RRA
eLH	100.0	100.0	100.0	0.01	15.5
oLH	115.0	29.1	10.2	<0.01	<0.01
4B	6.4	0.6	0.1	<0.01	<0.01
14SB	10.7	7.2	1.0	0.01	0.03
1S	0.9			<0.01	
13S	2.1			0.1	
eFSH				100.0	100.0
oFSH				53.0	94.7
15D	0.68			0.2	0.8

[a]Described in *Materials and Methods.*

[b]Numerically designated samples are described in detail in *Materials and Methods*: 1S and 13S, crude glycoprotein concentrates; 4B and 14SB, partially purified LH; 15D, partially purified FSH.

means pure (see Fig. 1), they were significantly more pure (5- to 7-fold) than the starting materials (1S, 13S). Their activities were about 6–11% that of eLH (Table 1). The FSH preparation (15D) was very low in activity (about 0.6% eLH). All the dose-response curves were relatively parallel, indicating a considerable immunological similarity between rhinoceros and eLH.

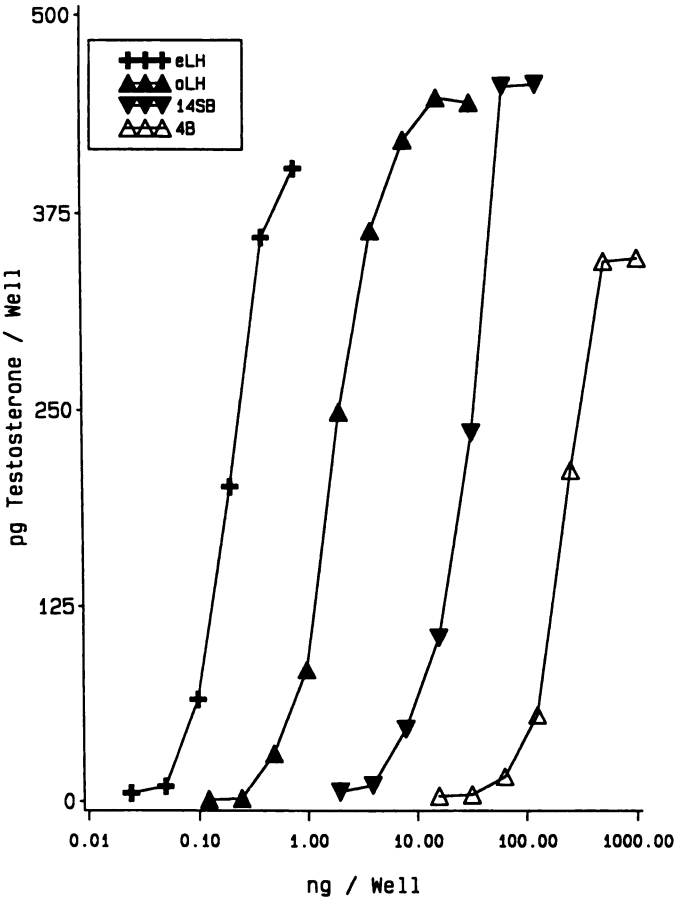

FIG. 3. Dose-response curves for rhinoceros LH preparations compared to those of purified ovine and equine LH in a rat Leydig cell assay in which testosterone production is measured.

The putative LH preparations (4B and 14SB) were also assayed for biological activity in an in vitro rat Leydig cell assay and compared with eLH and oLH. The results are shown in Figure 3 and Table 1. Maximum testosterone production was similar for all four preparations, but required different doses. The activity of eLH was the most potent, having 10 times the activity of oLH. The Black Rhinoceros LH preparation (14SB) was 105 times less potent than eLH, but was 12 times more potent than the White Rhinoceros LH material (4B), which was considerably different from that predicted by the LH RIA. The results from a rat testis membrane RRA (see Table 1) also indicated a 12-fold difference between the two rhinoceros LH preparations. Overall displacement activities of 4B and 14SB in the RRA were about 8 times higher than the results of the bioassay would predict.

When the rhinoceros LH preparations were tested in an FSH RIA and FSH RRA, the results shown in Figures 4 and 5 and Table 1 were obtained. FSH contamination, as measured by the RIA, was extremely low in both LH preparations (0.01% or less), comparable to that detected in the purified ovine and equine preparations of LH. The FSH activity in 4B and 14SB, as measured by an RRA, was also very low (<0.01% and 0.03%, respectively). In contrast, eLH, as has been reported previously, had a considerable degree of activity (15.5%), which cannot be accounted for by the small degree (0.01%) of contamination with FSH.

The Black Rhinoceros FSH preparation (15D) was active in the FSH RIA, as shown in Figure 4 and Table 1, but only a marginal improvement in purification over the starting material was indicated; thus, this preparation had a low potency compared to eFSH and oFSH. The slope of the dose-response curve was similar to that of eFSH, indicating a strong similarity to the standard FSH preparations. The oFSH was about half as potent as the eLH, as has been reported by Licht and Bona-Gallo [13]. No FSH activity could be found in any of the White Rhinoceros preparations.

The results of the FSH RRA are shown in Figure 5 and Table 1. The rhinoceros FSH (15D) dose-response curve had a similar slope to that of eFSH and was about 1% as

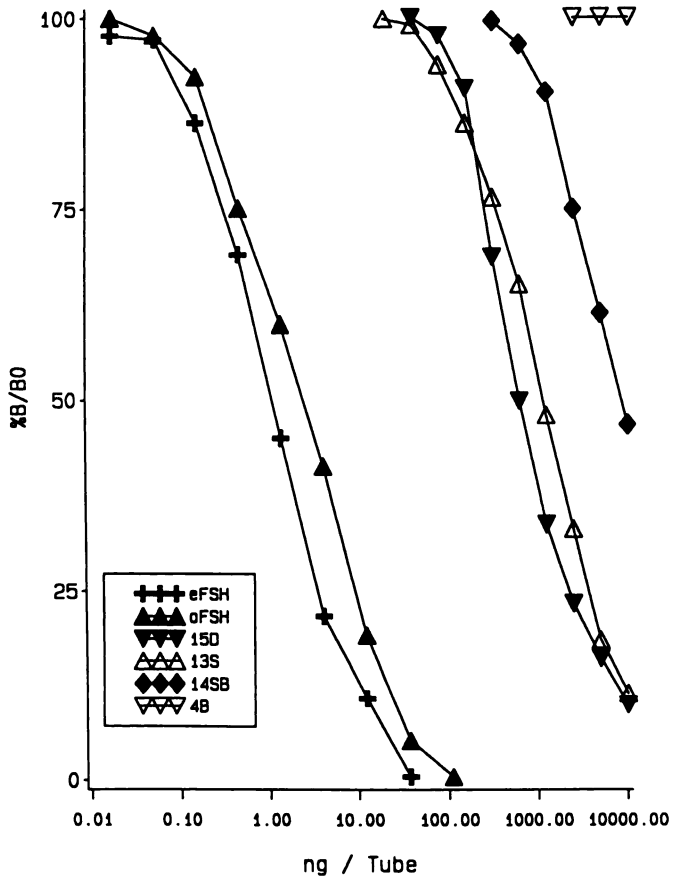

FIG. 4. Competitive binding curves of various rhinoceros pituitary preparations compared with purified ovine and equine FSH in an FSH RIA using ^{125}I-hFSH tracer and antiserum against oFSH.

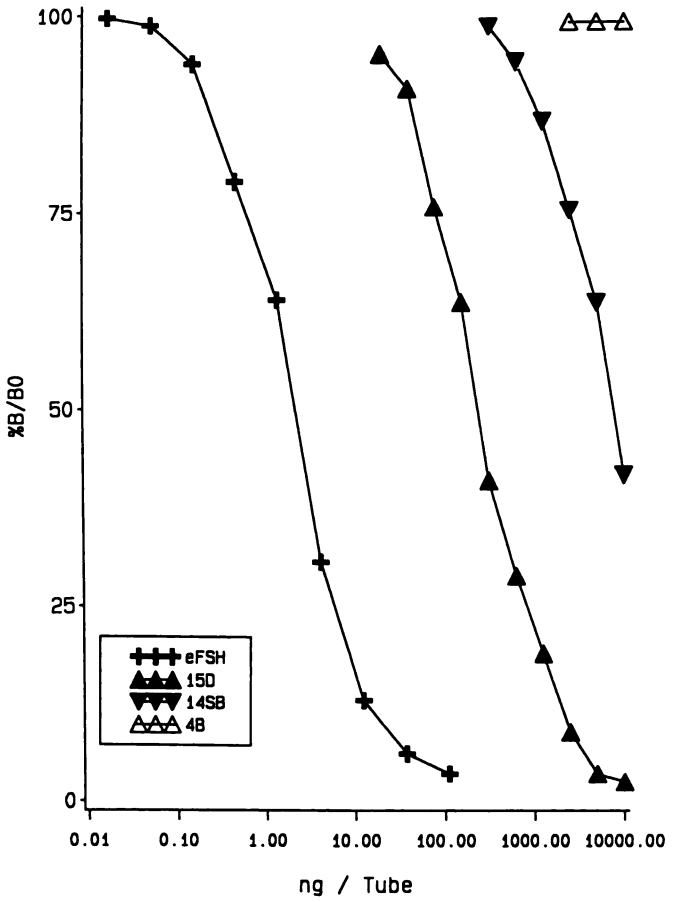

FIG. 5. Competitive displacement curves of rhinoceros LH and FSH preparations compared with ovine and equine FSH in an FSH RRA using ^{125}I-oFSH tracer and calf testis membrane as receptor source.

potent. Although this potency was low, it was considerably higher than in the RIA, indicating that if the FSH could have been purified to the same degree as the oFSH and eFSH, it would probably have a similar biological potency (i.e. specific biological activity). The higher potency of the rhinoceros LH (14SB) in the FSH RRA (0.03%) most likely reflects the higher activity of the rhinoceros FSH in this assay (0.8%) over the RIA (0.2%), rather than intrinsic FSH receptor binding by the LH. Ovine FSH has a potency and displacement curve similar to that of eLH, and was not included in the figure.

A summary of the various assay results for the rhinoceros preparations and the equine and ovine standards are shown in Table 1.

GH and PRL

The results of SDS-PAGE of the rhinoceros PRL and GH preparations are shown in Figure 6. Results with the HPO_3-precipitated starting material from each rhinoceros (Lanes 3 and 5) indicated the presence of both PRL and GH in the rhinoceros pituitaries as bands of similar molecular mass to those of the equine standards of GH and PRL. The use of antibody probes for GH and PRL after Western blotting confirmed these bands to be GH and PRL. This analysis also indicated that an additional band of higher molecular mass was also PRL, suggesting the possibility of a glycosylated form. This was confirmed by Con A chromatography; the nonglycosylated form was unadsorbed (12A, Lane 8), and the glycosylated form was adsorbed (12B, Lane 9). The Black Rhinoceros preparation (13P), which contained much less PRL than the White Rhinoceros material (1P), was further purified to produce a GH-rich fraction (17B, Lane 6).

PRL RIA results for the HPO_3 precipitates (1P, 13P) and the Black Rhinoceros GH-rich preparation (17B), compared to ePRL and eGH, are shown in Figure 7. This figure shows that 1P and 13P contained immunoreactive PRL, although the slope of the competition curve for 13P was slightly nonparallel to ePRL. The low degree of cross-reactivity (0.3–1% of ePRL) exhibited by eGH and the purified rhinoceros GH (17B) probably represents residual contamination with PRL.

Figure 8 shows the results of a PRL RIA in which a concentrated GH-PRL fraction (DEAE-purified; 11BC) from the White Rhinoceros was tested as well as fractions obtained by submitting 11BC to Con A chromatography (unadsorbed

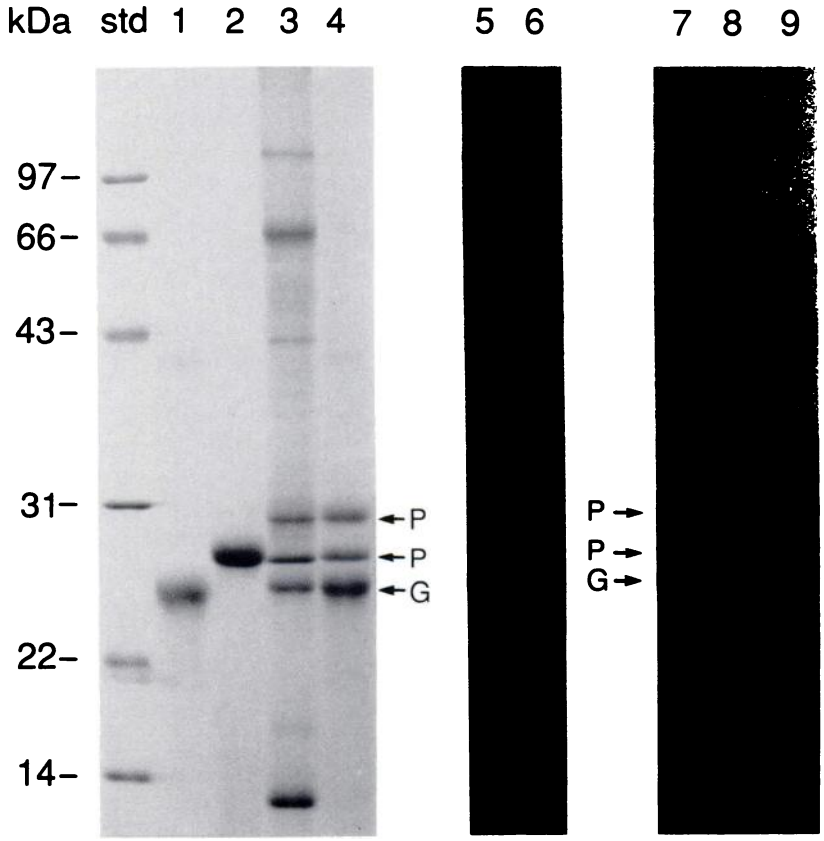

FIG. 6. SDS-PAGE of the rhinoceros pituitary extracts and the immunoreactive bands identified after Western blotting with GH and PRL antisera. 1) eGH, 2) ePRL, 3) 1P (White Rhinoceros HPO_3 precipitate), 4) 11BC, 5) 13P (Black Rhinoceros HPO_3 precipitate), 6) 17B, 7) 11BC, 8) 12A (Con A unadsorbed), 9) 12B (Con A adsorbed). P, PRL immunoactive; G, GH immunoactive.

12A, adsorbed 12B). It is evident that both the unadsorbed (nonglycosylated) and adsorbed (glycosylated) fractions were immunoreactive. Whether one form is more or less reactive cannot be ascertained until highly purified preparations of each are prepared. The ePRL antiserum, however, was raised against nonglycosylated ePRL [9].

The results from the GH RIA are shown in Figure 9. The slopes of the rhinoceros preparations were slightly different from those of the equine samples, but it was evident that the fractions contained GH. The Black Rhinoceros pituitary fraction (13P) had about twice the content of GH as the White Rhinoceros pituitary fraction (1P), which allowed its preparation in purified form. The rhinoceros GH preparation (17B) was extremely potent in this assay, being approximately of the same potency as the ePRL; however, it had a slightly more shallow slope.

DISCUSSION

The objectives of this study were to gain information on the biochemical behavior of the gonadotropins GH and PRL in the rhinoceros pituitary, to determine assay methods that could detect GH and PRL, and to ascertain if rhinoceros LH, like eLH, possessed intrinsic FSH-like activity. The results fulfilled these objectives and, for the first time, provide information on the pituitary hormones of this endangered species.

It should be noted that the data reported here are based on two pituitaries, each from a different species (White and Black) of rhinoceros. None of the hormone preparations obtained from either pituitary can be considered highly purified. This is evident in the HPLC chromatograms obtained with the LH preparations 4B and 14SB (Fig. 1). To obtain highly purified materials, we would have required greater numbers of pituitaries, which was unreasonable, considering the endangered status of these animals. Nonetheless, it is evident that during fractionation the rhinoceros hormones behave in the same way as the numerous mammalian and nonmammalian species that we have worked with in the past [18, 19].

Rhinoceros LH from both pituitaries were readily detected by the RIA, RRA, and the rat Leyding Cell assay. There were, however, significant differences in the potency of each

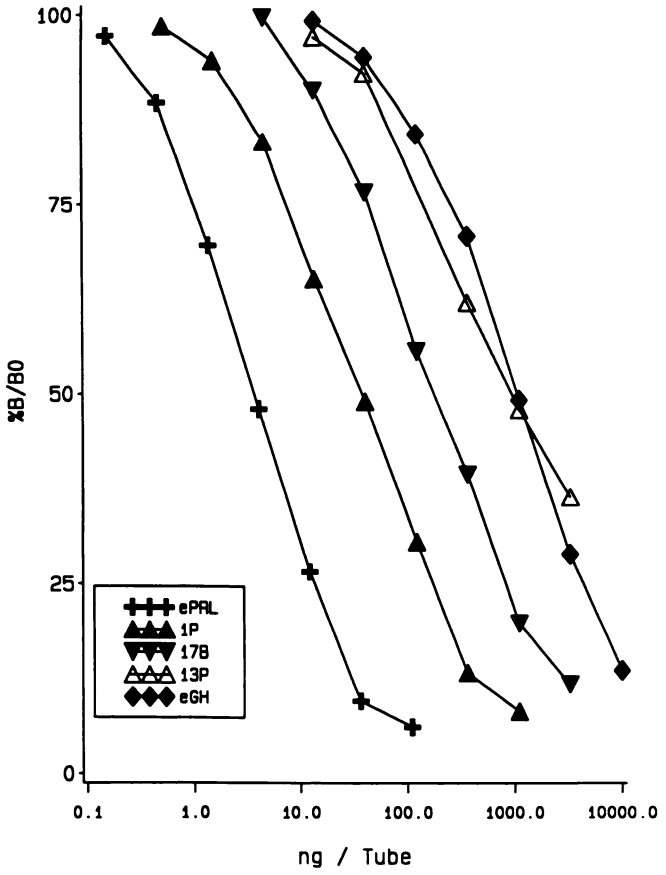

FIG. 7. Competitive binding curves of the HPO_3-precipitated starting materials and purified GH from the rhinoceros compared with purified equine PRL and GH in an equine PRL RIA.

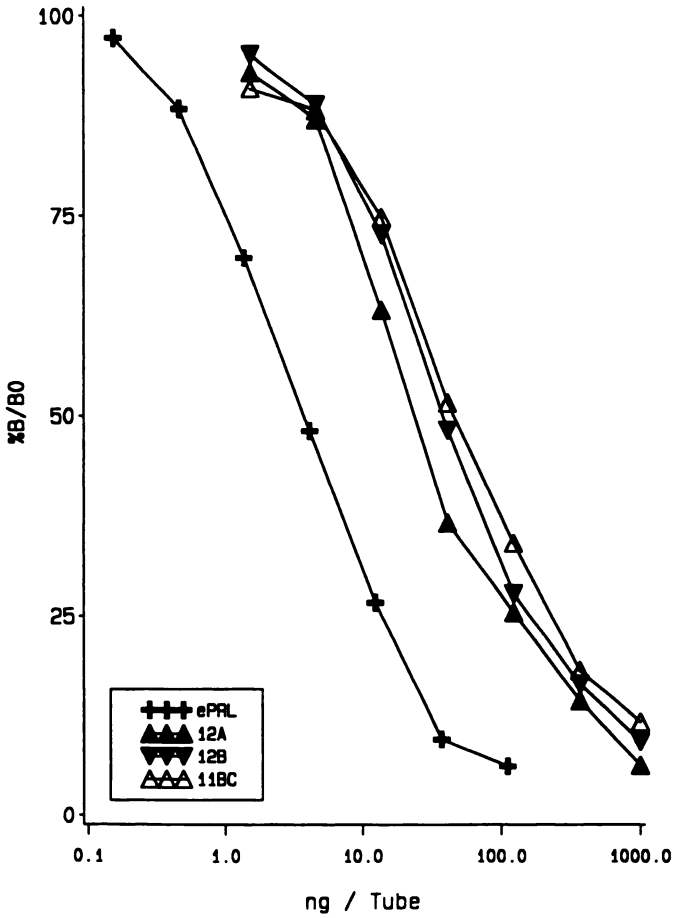

FIG. 8. Competitive binding curves of rhinoceros PRL preparations compared to purified equine PRL in an equine PRL RIA.

in the different assays and with respect to each other. Thus while the potency of Black Rhinoceros LH (14SB) was comparable in both RIA and RRA (11% and 7% of eLH), the bioassay were much lower (1% of eLH). The White Rhinoceros LH (4B) showed large variations in all 3 assays (ranging from 0.1% for bioassay to 6% for RIA).

Noteworthy was the virtual absence of FSH activity in the LH as measured either by RIA or RRA. In contrast, eLH displayed a high degree of activity in the FSH RRA. We concluded, therefore, that the rhinoceros LH does not possess a similar intrinsic FSH activity. With respect to the variation of results between assays, three points are noteworthy. Firstly, it has been well documented that the same hormone preparation will have different specific activities in different assays [20]. Secondly, different species of equally purified hormone will have different specific activities in the same assay [21, 22]. Equine LH has a remarkably high specific activity in the rat Leyding cell assay compared to oLH [3]. Thirdly, we would note that for several decades the purified human pituitary hormones distributed in this country have been derived from pituitaries obtained at autopsy when the postmotem period is usually 12–24 h. Further, most of these pituitaries have been from elderly, often chronically ill, individuals. In a sense, the White Rhinoceros pituitary was of a similar nature in having arrived in the laboratory in a thawed condition.

FSH was detected in the Black Rhinoceros pituitary by both RIA and calf testis RRA. The potencies were much lower (0.2% and 0.8% of eFSH) than those of LH (with respect to eLH), which probably reflects the lesser purity of the FSH preparation (15D) and the fact that the concentration of FSH in pituitaries is intrinsically very much lower than LH. The inability to detect FSH in the White Rhinoceros pituitary fractions may well relate to the thawing of the pituitary during shipment or to the fact this animal had been chronically ill for a long period of time and had been subjected to many medications. In addition, an unsuccessful attempt had been made prior to euthanasia to superovulate the animal [23], and the hormonal treatments may have affected pituitary FSH content.

Monitoring the various rhinoceros fractions with SDS-PAGE and immunoblotting techniques clearly showed the

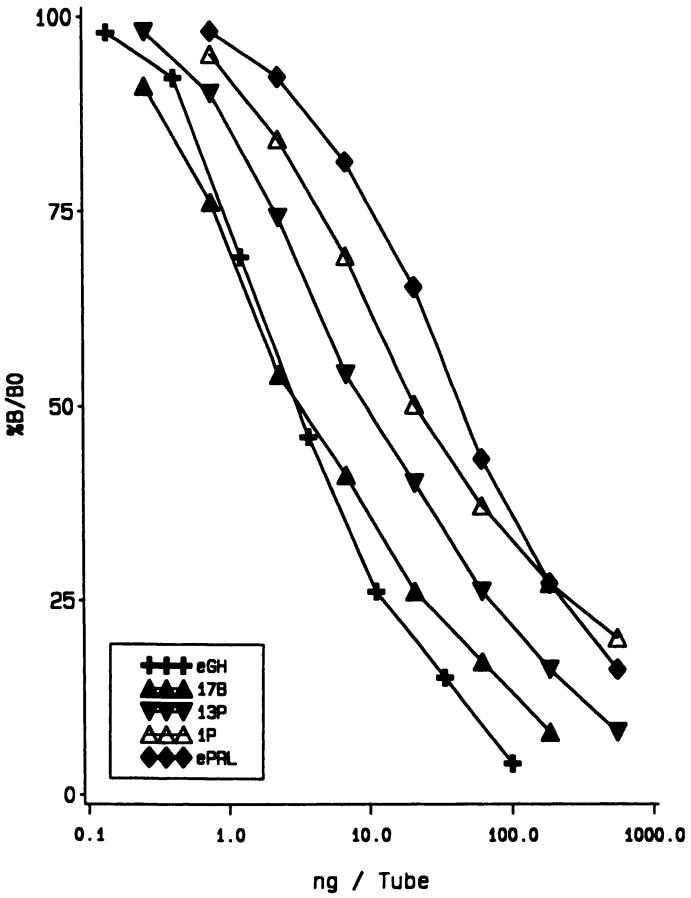

FIG. 9. Competitive binding curves of various rhinoceros GH and PRL preparations compared to purified equine GH and PRL in a rat GH RIA.

presence of GH and PRL in the expected HPO_3 precipitates of the initial extracts. Further purification could be achieved by DEAE chromatography, and, for GH, by an additional gel filtration step. Each of these hormones could be readily quantified by the respective RIAs we employed (rat GH RIA and ePRL RIA). The presence of multiple forms of PRL was observed in the SDS-PAGE gels. The presence of at least one form of glycosylated PRL was strongly suggested by the fact that it could be adsorbed with Con A, leaving the non-glycosylated form unadsorbed.

In conclusion, we have provided data on several of the pituitary hormones of the rhinoceros. Although the rhinoceros is phylogenetically related to the horse, rhinoceros LH, unlike eLH, does not possess intrinsic FSH-like activity. It appears also that several of the assays we have employed for the rhinoceros pituitary fractions will be efficacious in making physiological measurements in this endangered species. Such studies will be useful for efforts in reproductive management.

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