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COMPARISON OF PROPRANOLOL-BINDING PLASMA PROTEINS IN SHEEP WITH THOSE IN HUMANS, DOGS AND RATS*

MARTIN D. HILL,† PAMELA R. BRISCOE and FRED P. ABRAMSON‡
Department of Pharmacology, The George Washington University Medical Center, Washington, DC 20037, U.S.A.

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Abstract—Alpha$_1$-acid glycoprotein (AGP), a plasma protein responsible for the binding of a variety of basic lipophilic drugs including propranolol, is different from other plasma proteins in being nonprecipitable after treatment with 1.2 M perchloric acid (PCA). To assess the contribution of AGP to drug disposition in sheep and three other species (rats, dogs, and humans), the binding of [^{3}H]propranolol was measured before and after PCA precipitation. PCA precipitation reduced propranolol binding 14-fold in sheep, compared to 2- to 3-fold in the other species. This implied either that sheep AGP binds less propranolol than other species, or that the AGP in sheep is more precipitable. It was not due to inherently poor propranolol binding, as whole sheep plasma bound a higher fraction than the other species. When samples of PCA-precipitated sheep plasma were analyzed using polyacrylamide gel electrophoresis, the concentration of AGP was 10–20% that of the other species. Phenobarbital induction was used as a tool to examine the changes in the plasma protein profile. Phenobarbital induced propranolol binding and AGP along with two other proteins in sheep. One of these proteins migrated similarly to AGP deglycosylated by peptide-*N*-glycosidase F. It is postulated that the greater precipitability of propranolol binding in sheep is due to a less glycosylated form of AGP which is not important in other species.

Alpha$_1$-acid glycoprotein (AGP§), an acute phase reactant, is the main plasma protein for the plasma binding of a wide variety of basic lipophilic drugs, including local anesthetics (e.g. lidocaine and bupivicaine), neuroleptics (e.g. chlorpromazine and thioridazine), and cardioactive drugs (e.g. propranolol and disopyramide) [1]. It is characterized in humans as being nonprecipitable after treatment with 1.2 M perchloric acid (PCA) [2] and this method has been used to isolate it for quantification [3, 4].

The plasma concentration of AGP is increased during acute phase reactions [5] and can be induced by agents that induce cytochrome P-450 [6, 7]. These higher AGP concentrations result in increased binding of propranolol [8, 9] which in turn lowers the free concentration of a drug, dramatically altering its pharmacokinetics and pharmacodynamics [10, 11].

The pregnant ewe is used as a pharmacokinetic model of transplacental drug concentrations [12]. We have been interested in plasma protein binding as a determinant of maternal–fetal drug concentrations [13], and so we examined the nature of the plasma protein binding of [^{3}H]propranolol in control and phenobarbital-treated pregnant sheep. Because the results were unusual, we compared them to results from several other mammalian species.

MATERIALS AND METHODS

Prior to surgery (at 124–138 days gestation), a total of fifteen pregnant ewes were anesthetized with halothane/N_2O/O_2. The maternal femoral artery and vein were cannulated, as were the fetal femoral artery and vein after hysterotomy. Maternal and fetal plasma samples were obtained the day of surgery to avoid acute phase reaction, and were stored frozen in heparin-containing glass tubes with Teflon-lined caps to avoid drug displacement by plasticizers associated with Vacutainers [8]. Although heparin has been shown to displace propranolol through induction of free fatty acids *in vivo*, its introduction *in vitro* does not affect binding to AGP [14]. Six of the pregnant sheep were pretreated with phenobarbital. The doses were 100–400 mg/day, p.o., phenobarbital in gel capsules, and were adjusted during a 3-week period based on how well the animals appeared to tolerate the drug. The last dose was given the day previous to surgery. The other nine sheep received no drug. Plasma samples from four nonpregnant adult female sheep were also analyzed.

Plasma samples from control and phenobarbital-treated male beagle dogs were used from previous AGP induction experiments [15]. Plasma from

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‡ Corresponding author: Dr Fred Abramson, Department of Pharmacology, George Washington University, 2300 Eye St., N.W., Washington, DC 20037.

§ Abbreviations: AGP, α_1-acid glycoprotein; PCA, perchloric acid; *B/F*, ratio of bound to free concentrations of the drug; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; and PNGase, peptide-*N*-glycosidase F.

untreated 2-year-old Sprague–Dawley strain (Harlan–Sprague–Dawley, Indianapolis, IN) rats and plasma samples from healthy humans were also examined. Controls using human, rat, and dog AGP (Sigma) were made in double-distilled water.

Tritiated propranolol ([^{3}H-(G)]-DL-propranolol, 0.044 mM with 1 mCi/ml activity, Amersham) was purified by extraction into a heptane/isoamyl alcohol solution and then back-extracted into 0.01 N HCl. This was lyophilized overnight and reconstituted in ethanol for use at a 1:40 dilution.

Plasma protein binding was measured in both the supernatant fraction of perchloric acid precipitated plasma and untreated plasma using equilibrium dialysis. Plasma samples (1.2 ml) were divided in two: 0.7 ml in one set of tubes and the remaining 0.5 ml in the second set. To set the tubes with 0.7 ml plasma, 42 μl of 60% perchloric acid and then 658 μl of 0.5 M perchloric acid were added to induce plasma protein precipitation according to the method of Kattermann and Kreiger [16]. These samples were centrifuged at 1650 *g* for 10 min, and 1.0 ml of supernatant fluid was added to a cellophane dialysis bag. To readjust the pH, the bag was sealed and placed in a 4-liter flask filled with 0.111 M phosphate buffer at pH 7.38, and dialyzed overnight at 4°. The resulting pH was measured and found to be between 7.3 and 7.4. Samples were retrieved, and their volumes were measured and restored to 1 ml with the phosphate buffer solution. One-half milliliter of the phosphate buffer solution was added to the other set of tubes to provide for an equal dilution.

These samples were spiked with 10 μl of [^{3}H]propranolol for a final concentration of 11 ng/ml and then placed in one side of a dialysis chamber. The other side contained phosphate buffer (pH 7.38, 0.111 M). The two sides were separated by a cellophane membrane (Scienceware) retaining any materials with a molecular weight of 6000 and higher. Dialysis was allowed to proceed at 37° for at least 18 hr. Aliquots (50 μl) of each side of the dialysis chamber were counted in 10 ml of Safety-Solve scintillation fluid (Research Products International).

The amount of drug on the buffered side was considered equal to the free concentration and the amount of drug on the plasma sample side equal to the bound plus and free portions. The bound fraction was then determined by subtracting the amount of drug found on the buffered side from the amount found on the plasma sample side.

In the absence of saturation, the concentration of a binding protein (P) is directly proportional to the ratio of the bound to free concentrations (B/F) for a particular drug according to the formula: $B/F = nPK_a$, where K_a is the association constant for the drug to the protein, and n is the number of equivalent binding sites on that protein. If one assumes a single protein is responsible for binding, then the B/F ratio is the proper parameter to compare with the protein concentration. In this experiment, 2.2 ng/ml of [^{3}H]propranolol was used, well below what would saturate plasma protein binding [17]. For calculation purposes, the observed bound/free ratio was doubled to take into account the dilution of the samples with acid or buffer.

Phenobarbital concentrations were measured by gas chromatography with nitrogen selective detection, using a method described previously [15].

Protein patterns of untreated plasma and the supernatant fractions of PCA-precipitated plasma were examined using gradient polyacrylamide gel electrophoresis (PAGE). PCA-precipitated supernatant fractions were returned to physiological pH by dialysis against 0.111 M phosphate buffer, pH 7.4. Before electrophoresis, samples were dried using a Savant SVC-100 SpeedVac evaporator/concentrator and reconstituted in 50 μl of sample buffer and 10 μl of tracking dye, heated for 10 min at 80°, centrifuged, and then loaded on the gel. For molecular weight determinations, SDS–PAGE low molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were run simultaneously.

The 5–15% gradient gels were prepared using the method described by Irwin *et al.* [18] with the following modifications: Stock solution A was made using 30% acrylamide and 1.5% bisacrylamide in double-distilled water. Stock solution B was made at pH 9.6 using HCl. Electrode buffer was made using 11.25 g Tris base, 54.4 g glycine, and 7.5 g SDS in 5 liters of double-distilled water. The dimensions of the gel were 12.5 cm × 14 cm × 0.15 cm using a total of 30 ml running gel solution.

These gels were run at 20 mA/gel until the sample entered the stacker, 25 mA/gel through the stacker, and 30 mA/gel through the gel for approximately 6 hr at 4°. Gels were stained in Coomassie Blue R 250, destained, and then photographs were scanned for density using a Joyce Loebl Chromoscan 200/201 system at 585 nm. Known amounts of human α_1-acid glycoprotein (Sigma) were used as standards.

Human AGP samples were treated by peptide-*N*-glycosidase F (PNGase, EC 3.5.1.52, Genzyme, Boston, MA) before electrophoresis to remove carbohydrate moieties from the glycoproteins according to the method provided [19].

RESULTS

The results from the binding experiments are given in Table 1. Maternal sheep bound propranolol to a greater degree than dogs, humans, or rats. The difference between pregnant sheep and any of the other species was significant (ANOVA, $P < 0.05$).

The binding of propranolol in maternal sheep plasma was reduced 14-fold by perchloric acid precipitation, far more than in the three other species (ANOVA, $P < 0.005$). Similarly, for nonpregnant sheep, PCA treatment reduced binding by approximately 13-fold. For rat, human, and canine plasma samples, PCA treatment reduced binding by only 2.5- to 3.1-fold. The binding for human AGP standards remained virtually the same after PCA precipitation.

In maternal sheep, the concentration of phenobarbital was 9.7 μg/ml (±6.2 SE). The binding of propranolol was induced by phenobarbital by 46% for nonprecipitated plasma and 212% for PCA-precipitated supernatant fraction although only the latter was statistically significant (unpaired t-test $P < 0.05$). The precipitability of binding in these

Table 1. Binding of [^{3}H]propranolol in the precipitated and nonprecipitated plasma of various species

Species/treatment	Nonprecipitated %B, B/F (SE)		Precipitated %B, B/F (SE)		Nonppt/Ppt ratio
Maternal sheep (control, N = 9)	90.9%,	10.02 (±1.84)	41.5%,	0.71 (±0.16)	14.11
Adult nonpregnant sheep (control, N = 4)	90.6%,	9.63 (±3.53)	48.4%,	0.94 (±0.45)	12.68
Adult beagle (control, N = 5)	81.9%,	4.52 (±0.80)	64.0%,	1.78 (±0.08)	2.54
Adult human (control, N = 5)	87.9%,	7.24 (±1.20)	70.0%,	2.33 (±0.75)	3.11
Adult rat (control, N = 4)	79.6%,	3.90 (±0.62)	60.2%,	1.51 (±0.16)	2.58
Maternal sheep (phenobarbital, N = 6)	93.6%,	14.64 (±2.81)	68.9%,	2.22 (±0.78)	6.59
Adult beagle (phenobarbital, N = 4)	96.1%,	22.37 (±2.40)	95.7%,	22.16 (±1.81)	0.99
Human AGP (Sigma) (1 mg/ml)	71.2%,	2.47	69.1%,	2.24	0.91

samples was only 7-fold. For canine plasma the binding of propranolol was induced 5-fold by phenobarbital in nonprecipitated samples (unpaired t-test, $P < 0.005$) and was even more pronounced in the supernatant fraction of PCA-precipitated canine plasma, increasing by 13-fold (unpaired t-test, $P < 0.0001$). For phenobarbital-treated dogs, the binding of propranolol was similar in PCA-precipitated and untreated samples.

The PAGE analysis of the plasma protein profiles for the various species is shown in Fig. 1. One protein in the precipitated plasma supernatant fraction of sheep (lanes 4 and 5) corresponded in migration to AGP standards of other species (lanes 6–8), equivalent to a molecular weight of approximately 44 kD. Although 50 μl of precipitated plasma supernatant fraction was sufficient to quantify AGP in the other species (lanes 1–3), AGP in maternal sheep was barely detectable at this amount (lane 4), and 125 μl (lane 5) or more was used to quantify it. The concentration of sheep AGP correlated well to the propranolol B/F values in PCA-treated samples (r = 0.79, $P < 0.01$, N = 15). Using human AGP as a standard, the concentration of this protein averaged 0.066 mg/ml (±0.012) in control maternal sheep.

AGP concentration increased to 0.174 mg/ml (±0.013) in phenobarbital-induced sheep (lanes 16 and 17). Using the formula, $nK_a = (B/F)/P$, and a molecular weight of 44 kD for the above protein, we calculated an nK_a product of $4.73 \times 10^5\ M^{-1}$ for the PCA-treated plasma in control maternal sheep, and $5.61 \times 10^5\ M^{-1}$ for phenobarbital-treated maternal sheep. In the phenobarbital-treated dog sample (lane 14), only 10 μl was necessary to see a large amount of AGP due to the high susceptibility of this species to induction [15]. Although there was a high concentration of AGP in the nonpregnant female sheep sample in this gel (lane 20), generally this was not true.

Untreated samples for each of the species were also analyzed (lanes 9–12, and 20 for control samples, lanes 15 and 18 for PB-treated animals). Two proteins in the unprecipitated plasma samples were initially more concentrated in sheep than in other species and were induced significantly in sheep by phenobarbital (compare lane 12 with lane 18). The first of these proteins had a molecular weight of 28.5 kD and was induced by 103%; the second has a molecular weight of 48.5 kD and was induced by 64%. Because their identity was unknown, absolute concentrations could not be calculated. In optical density units they were approximately 40 and 230 times as concentrated, respectively, as control sheep AGP. There was too little variation in the binding of propranolol in whole plasma to allow for a correlation between either of these proteins and the binding of propranolol. The relative increase in the binding between control and phenobarbital-treated sheep that was not accounted for by the increase in AGP was 33% (from Table 1, whole plasma binding minus AGP-related binding in PCA-treated samples in control sheep compared to binding in phenobarbital-treated sheep).

Incubating human AGP with PNGase produced a new band which migrated similarly to the 28.5 kD protein from ovine samples (Fig. 2, lanes 5–13 compared to lane 20).

Although not presented in Table 1, binding was low for fetal sheep. There was no transplacental induction of unprecipitated protein binding (control B/F 1.57 ± 0.44, N = 10; phenobarbital-treated B/F 1.53 ± 0.26, N = 6). Phenobarbital concentrations in fetal sheep were 6.0 μg/ml (±2.4).

DISCUSSION

Several studies have attempted to examine the contribution of AGP to the binding of basic lipophilic drugs in humans through its preferential elimination by immunoprecipitation [20] or by column liquid chromatography [21]. These studies demonstrated that AGP is the most significant binding protein for these drugs.

Perchloric acid has been shown to eliminate 98% of the proteins in human plasma but not AGP [22]. Using a method similar to our own, Suzuki *et al.* [21] also looked at the binding of drugs after precipitation of most other proteins by sulphosalicylic acid and

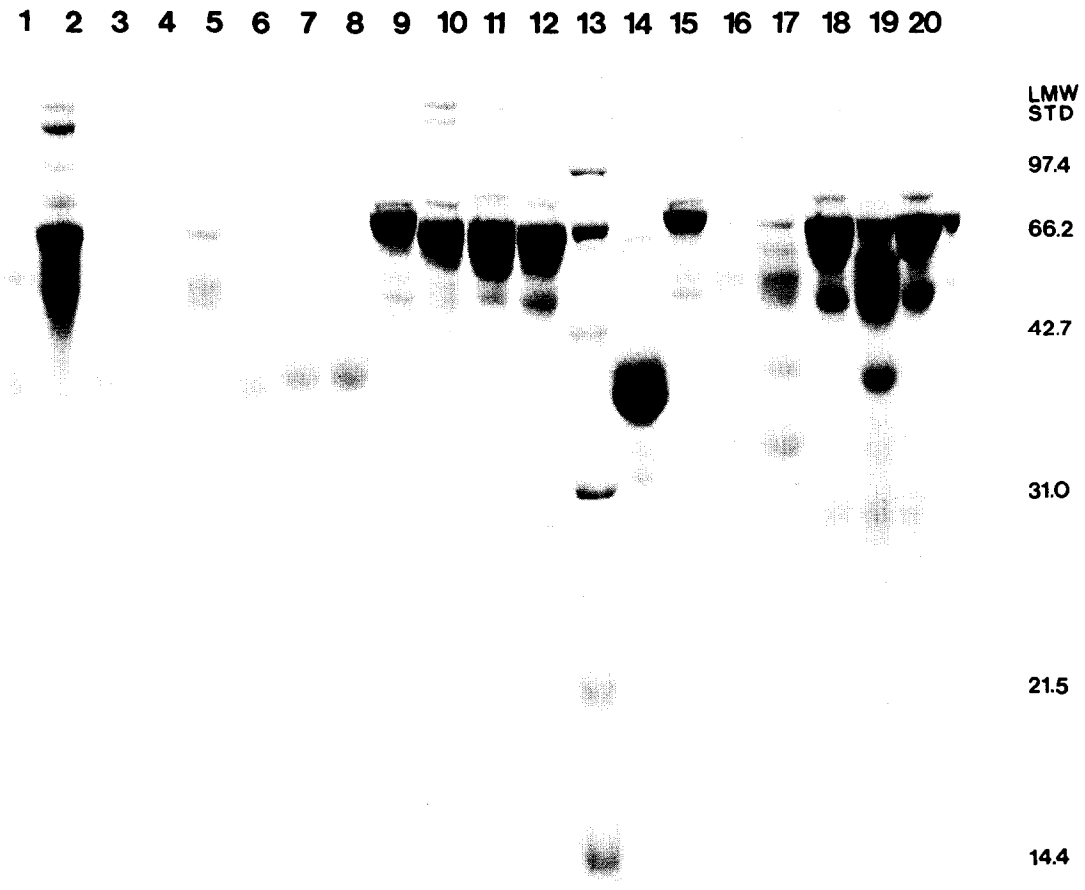

Fig. 1. Electrophoresis of plasma from dogs, rats, humans, and sheep. Total amounts of sample used, species, and treatment of sample: Lanes 1–4, 50 μl ppt. (the supernatant fraction following 1.2 M PCA precipitation) of, respectively, dog, rat, human, and sheep; lane 5, 125 μl ppt. sheep; lanes 6–8, 10 μg of dog, rat, and human AGP standards, respectively; lanes 9–12, 0.3 μl of, respectively, dog, rat, human, and sheep plasma; lane 13, low molecular weight standard (LMW); lane 14, 10 μl ppt. plasma from phenobarbital (PB)-treated dog; lane 15, 0.3 μl of plasma from PB-treated dog; lane 16, 50 μl ppt. plasma from PB-treated sheep; lane 17, 125 μl ppt. plasma from PB-treated sheep; lane 18, 0.3 μl of plasma from PB-treated sheep; lane 19, 50 μl ppt. of nonpregnant sheep; and, lane 20, 0.3 μl of nonpregnant sheep plasma. The additional protein of approximately 34 kD present in ppt. PB-treated sheep plasma (lanes 16 and 17) was not induced consistently.

found the binding of propranolol reduced by 3.7-fold, a figure similar to our findings in humans, dogs, and rats.

In our study, plasma proteins bound propranolol to a greater degree in sheep than in the other species tested. Combining this fact with the significantly greater precipitability of binding in sheep compared with other species led us to conclude that sheep plasma propranolol binding protein(s) is anomalous; binding of this relatively selective ligand was high, yet unusually precipitable. This observation was not due merely to the status of pregnancy since it was also true for nonpregnant sheep.

Like propranolol binding in dogs [8, 15], binding in sheep was inducible by phenobarbital. Three proteins were induced by phenobarbital in sheep, two of which were precipitable and one which was not. The concentration of the two proteins induced in whole plasma could not be correlated with whole plasma propranolol binding, in part due to the fact that the binding in whole plasma had a much smaller range of induction than the binding in PCA-treated plasma. The one induced protein band from precipitated plasma supernatant fractions migrated similarly to AGP standards of the other species, and its concentration was significantly correlated to propranolol binding. This indicates that this protein is AGP. Its concentration in control maternal sheep plasma (0.066 mg/ml) was well below the concentrations found in the plasma of adult humans, 0.66 to 0.81 mg/ml [23]; dogs, 0.37 mg/ml [11]; or rats, 0.32 mg/ml [7]. This concentration is only 6–9% of what would be required to produce the observed control B/F of 10 using an affinity constant appropriate to either humans or dogs [8, 24]. If one were to include the contribution of albumin, 562 μM in

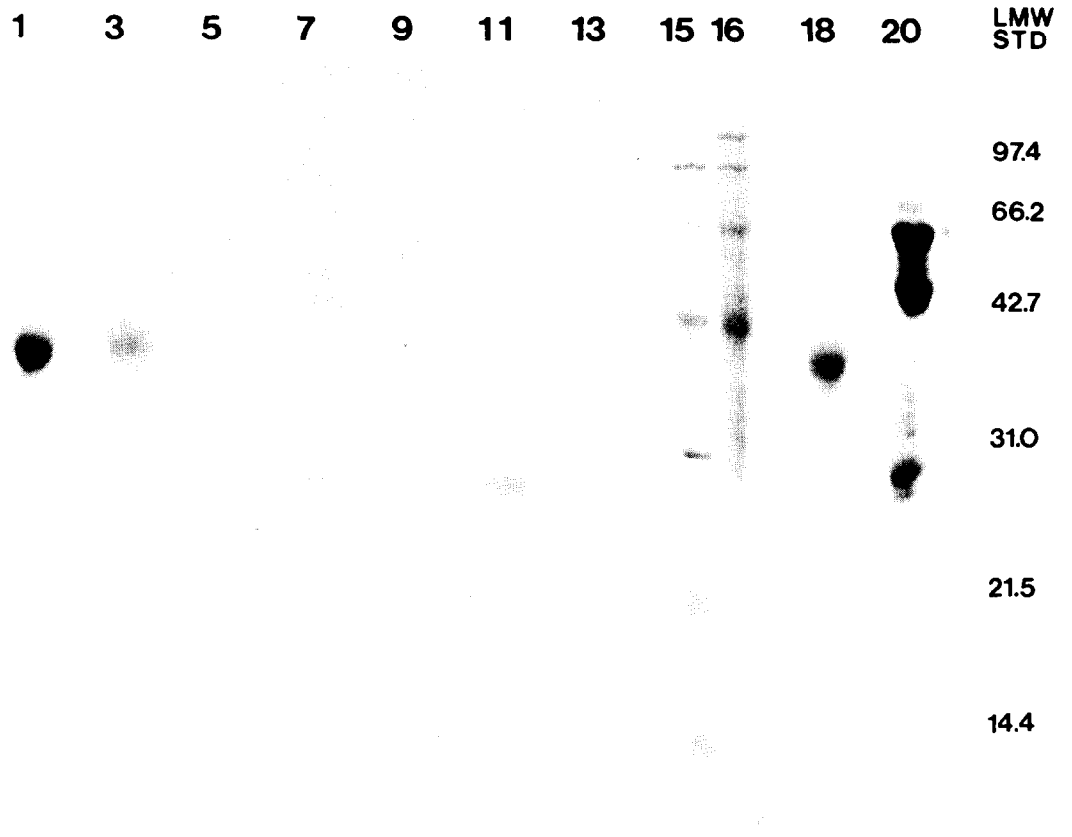

Fig. 2. PAGE analysis of plasma proteins comparing the migration of normal and deglycosylated human AGP to the bands in maternal sheep plasma. Lanes 1 and 3 are 20 μg human AGP standards. Lanes 5, 7, 9, 11, and 13 are separate samples of PNGase-treated 20 μg human AGP. Lanes 15 and 16 are low molecular weight standards and high molecular weight standards respectively. Lane 18 is 20 μg human AGP. Lane 20 represents 0.3 μl plasma from a phenobarbital-treated pregnant sheep.

near-term pregnant sheep [25], and assume that it bound propranolol with an affinity constant similar to humans ($2.1 \times 10^3\,M^{-1}$, [9]), the combination of AGP and albumin would still account for only one-fifth of the total binding.

Chauvelot-Moachon *et al.* [26] found that the presence of albumin potentiates the binding of drugs to AGP by as much as 2-fold. If this phenomenon were occurring here, it could contribute to some of the decreased binding after the PCA precipitation of albumin. Nevertheless, it would still not account for the great difference seen in sheep as compared to other species.

The fetal binding of propranolol was only a fraction of the maternal and was not inducible. It should be noted that the plasma phenobarbital levels were lower than what has been shown to be necessary to induce significant binding in our canine studies [15].

In contrast to whole plasma, the low concentration of AGP found in precipitated plasma supernatant fractions does correspond well to the low binding exhibited in these samples. The approximately $5 \times 10^5\,M^{-1}$ affinity constant calculated for sheep AGP is in close agreement with those for human AGP. This comparable affinity constant supports the notion that nearly all of the binding in the precipitated plasma supernatant fraction is the result of AGP.

Only carbohydrate-rich glycoproteins are non-precipitable after treatment with 1.2 M perchloric acid [27]. After using PNGase to remove glycan units from the human AGP standards, we found that the band corresponding to fully glycosylated AGP disappeared and a band corresponding to the 28.5 kD protein from sheep appeared (Fig. 2). The molecular weight of nonglycosylated AGP is 24.5 kD for humans [28], so our observed protein weighed somewhat more than perfectly deglycosylated human AGP. While this evidence is circumstantial, it indicates that this unknown protein is either a non-glycosylated form of AGP with a higher molecular weight than human, or else a less glycosylated form of AGP. Thus, a large portion of AGP in sheep is precipitable due to it being less glycosylated. Further, if the glycan units do not interfere with the protein region of AGP, then their presence or absence would not grossly affect the binding.

The identity of the 48.5 kD protein remains obscure. Several studies have shown that a variety of proteins beside AGP are inducible by enzyme-

inducing drugs, including sex hormone binding globulin (moi. wt 115 kD) [29], serum amyloid A (100+ kD), fibrinogen (340 kD), and hemopexin (57 kD) [30–32].

Our study demonstrates that the proteins that bind propranolol in sheep plasma were qualitatively dissimilar to its binding proteins in several other species. Therefore, the greater precipitability of binding in sheep plasma means that AGP, defined as a non-precipitable highly glycosylated protein with a molecular weight of approximately 44 kD, is present in a lower concentration in sheep than in other species. Therefore, the large fraction of binding that is precipitable must be due to at least one other protein. To account for the remainder of the binding, it is necessary to postulate a precipitable protein with affinity for propranolol that is not important in other species. Based on the presence of a 28.5 kD protein which was inducible in sheep but not dogs (Fig. 1, lanes 9 and 15) or rats (unpublished data) and which corresponds to a deglycosylated form of AGP, we speculate that this form of AGP is responsible for the unaccounted portion of propanolol binding in sheep. Definitive characterization, by immunological means or through protein sequencing, remains to be done.

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