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The Application of Rate Dialysis to the Determination of Free Steroids in Plasma

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Rate dialysis is used to obtain the free steroid fraction in undiluted plasma at 37°C. The free steroid fraction is determined from the rate at which a small amount of tritiated steroid diffuses from plasma on one side of a semipermeable membrane into an identical plasma sample on the other side which lacks radioactive steroid. The method may be generally applicable to steroids since the cell permeability constant, which is a function of the volume of the dialysis cell and the area and diffusion properties of the membrane, was similar for seven steroids tested. The method requires only 0.3 ml of plasma, is simple and economical to perform, and enables up to 120 determinations to be made in one day. The free fractions of cortisol, progesterone, and estradiol-17β were measured in plasma pooled from pregnant and non-pregnant women and pregnant and lactating sows. The results were compared with those obtained for the same plasma pools by centrifugal ultrafiltration.

It is generally assumed that the physiologically active component of a circulating steroid hormone is that fraction of it which circulates in a free or non-protein-bound form. Consequently, various attempts have been made to develop methods for measuring the free fraction of a steroid hormone because of its presumed biological role. Available methods usually require the separation of bound from free steroids after addition and equilibration of a known amount of the radioactive steroid to the plasma or serum sample. Such methods include zonal chromatography (1), steady-state gel filtration (2,3), equilibrium dialysis (4–7), flow dialysis (8), ultrafiltration through a semipermeable dialysis membrane (9–13), and modified forms of radioimmunoassay (14). Although similar results are obtained by several of these methods (4,7), they either require large sample volumes, correction for variable volume changes which occur when plasma is dialyzed against a hypotonic solution, or multiple determinations of radioactivity per sample. Furthermore, the changes in the concentration of low-molecular-weight solutes which occur with most of these methods, including ultrafiltration, may perturb steroid binding in an unpredictable way.

We report here the adaptation of a rate dialysis method (15) for the measurement of free cortisol, progesterone, and estradiol-17β in small volumes of undiluted plasma at 37°C. The method can be applied to a wide range of steroid hormones and large numbers of samples can be processed simultaneously.

MATERIALS AND METHODS

Materials. Crystalline human serum albumin was supplied by Behringwerke (Marburg, West Germany), Dextran C was purchased from British Drug Houses (Pool, England), and 6α-[1,2-^{3}H(N)]methyl-17α-hydroxyprogesterone acetate (60 Ci/mmol) was from New England Nuclear (Boston, Mass.). Amersham

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International (Sydney) supplied [1,2,6,7-^{3}H]progesterone (87 Ci/mmol), [1,2,6,7-^{3}H]cortisol (85 Ci/mmol), [2,4,6,7-^{3}H]estradiol-17β (95 Ci/mmol), 17α-hydroxy-[1,2,6,7-^{3}H]progesterone (70 Ci/mmol), and 20α-hydroxy[1,2,6,7-^{3}H]progesterone (65 Ci/mmol). Purity of the tritiated steroids was assessed as ≥98.5% by thin-layer chromatography in our laboratory using solvent systems recommended by the suppliers. Dialysis tubing (flat width 43 mm and mean dry thickness 0.020 mm) was purchased from Union Carbide (Chicago, Ill.). Perspex (grade A) was obtained as a clear acrylic sheet (Plastics Ltd., Western Australia). All other chemicals were of analytical grade.

Plasma. Individual frozen plasma samples from pregnant and non-pregnant women were thawed, pooled, and refrozen in aliquots. Heparinized blood was obtained from the ear veins of lactating sows and sows of various gestational ages. The plasma was separated and frozen as two pools. One week later, the two porcine plasma pools were thawed and frozen again in aliquots.

Apparatus. For rate dialysis, Perspex blocks were constructed to contain 12 half-cells (9 mm diameter and 3.1 mm depth). One layer of moist dialysis membrane was laid between two blocks, which were then bolted together (Fig. 1). Access to the assembled dialysis cells for addition and removal of samples was accomplished through threaded holes in the top of the blocks using an injection syringe (CR-700-200, Hamilton Co., Reno, Nev.) and the holes were sealed by 12-mm-long, 6BA nylon cheesehead screws.

For centrifugal ultrafiltration, a single YMT membrane was used in the MPS-1 device (Amicon Industries, Thebarton, South Australia), which is designed to separate free from bound ligands in serum or plasma.

Rate dialysis theory. The theoretical basis of rate dialysis has been described previously in detail (15). Briefly, a dialysis cell of two identical compartments of volume V, separated by a semipermeable membrane of area A, is filled with the same sample (in this case undiluted plasma). Tracer hormone is added to one compartment initially so that after dialysis for time t, the isotope distribution H is $(h_1 - h_2)/(h_1 + h_2)$ where h_1 and h_2 are the amounts (or radioactivities) of hormone in compartments 1 and 2.

The free hormone fraction (f) is calculated from $-ln\ (h_1 - h_2)/(h_1 + h_2) = 2ADft/V$, where D is the "membrane diffusion coefficient," and comprises the diffusion coefficient of the hormone in plasma, membrane thickness, and a factor relating the diffusion area of the membrane to its total area.

In practice the "cell permeability constant," $2AD/V$, is determined for a particular membrane and size of cell from preliminary experiments using hormone solutions of known free fraction. Thereafter, the free fraction of unknown solutions of hormone can be obtained from their isotope distribution after dialysis for a known time.

The technique assumes that diffusion of tracer hormone across the membrane is a first-order rate process according to Fick's law. It requires also that equilibration between the hormone and its binding macromolecules in plasma is much more rapid than the rate of diffusion of tracer hormone across the membrane so that equilibrium is maintained in spite of the movement of tracer hormone. This condition is satisfied in plasma since added steroid hormone exchanges with endogenous hormone, either bound or unbound, within seconds (16) whereas the rate of dialysis is measured in terms of hours. Finally, it is necessary that bound and free forms of endogenous hormone exchange equally well with the tracer hormone.

Procedure for rate dialysis. Radioactive steroid (10,000–25,000 dpm, 0.05–0.1 pmol) dissolved in ethanol was added to a small glass test tube. The solvent was evaporated and 0.2 ml of plasma was added. After equilibration at 37°C for 1–3 h, 0.15 ml of the plasma was loaded into one side of a dialysis cell. The other side of the dialysis cell received 0.15 ml of the same plasma sample which had been maintained at 37°C in the absence of tritiated

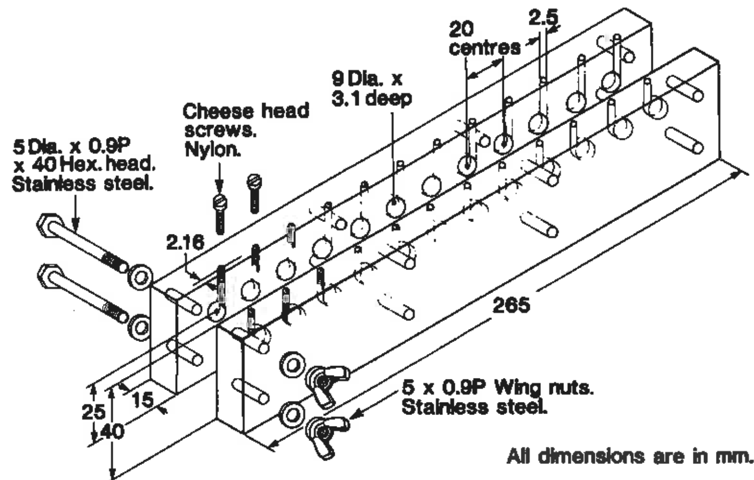

FIG. 1. Rate dialysis apparatus. Two Perspex blocks are clamped together with a sheet of semipermeable membrane lying between them. Plasma is loaded via the threaded holes into the wells on one side and plasma plus tritiated steroid is loaded similarly into the wells on the opposite side. The wells are sealed by nylon screws.

steroid. Dialysis was carried out at 37°C accompanied by horizontal shaking (40 oscillations/min) for 3–18 h depending on the particular steroid. A sample (0.075 ml) from each side of the cell, together with a sample of the original plasma containing tracer, was taken for determination of radioactivity by liquid scintillation counting.

Procedure for centrifugal ultrafiltration. Plasma (0.6 ml) was equilibrated with 50,000–80,000 dpm (0.2–0.45 pmol) tritiated steroid at 37°C for 1–3 h, and 0.5 ml was centrifuged in the MPS-1 device at 37–38°C (Sorvall RC-5B) for 10 min at 220*g* to yield 0.1–0.2 ml of ultrafiltrate. The percentage free steroid in the plasma was calculated as (dpm/ml ultrafiltrate/dpm/ml plasma) × 100. Background radioactivity in our counter was ≤20 dpm.

Radioimmunoassay. Progesterone, cortisol, and estradiol-17β were measured by specific radioimmunoassays (34).

Statistical analysis. For each combination of steroid and plasma, statistics were computed from a one-way analysis of variance for differences between assays. The SE of the overall mean was computed as the square-root of the mean square error associated with estimates between assays divided by the total number of estimations (17). Unless stated otherwise, values are given as the mean ± SE (n) where n is the number of observations.

RESULTS

Linearity of rate of dialysis of steroids. The mass transfer of tracer hormone across the dialysis membrane was a first-order process conforming to Fick's law during the first 24 h since log plots of the tracer distribution (H) were linear with time (Fig. 2). In this system, equilibrium was not reached until 36–40 h of dialysis at 37°C. For the routine estimation of percentage free steroids, dialysis times were adjusted for each steroid so that approximately one-half of the tracer was on the other side of the membrane ($-ln\,H = 0.7$). This provided the optimal distribution for accurate counting of the radioactivity in both compartments. Thus, cortisol, progesterone, and estradiol-17β in human plasmas were dialyzed for about 9, 16, and 18 h, respectively, whereas in porcine plasmas the steroids were dialyzed for 3.5, 8, and 11 h, respectively.

Cell permeability constant for steroids. This was determined as 0.030 min^{-1} for six different steroids over a range of free concentrations using a sample volume of 0.15 ml on each side of the membrane (Table 1). The steroids were totally free when dialyzed in phosphate buffer (25 and 50 mM) at pH 7.4. However, in order to more closely mimic the distribution of steroid between bound and free forms occurring in undiluted plasma, tritiated steroid

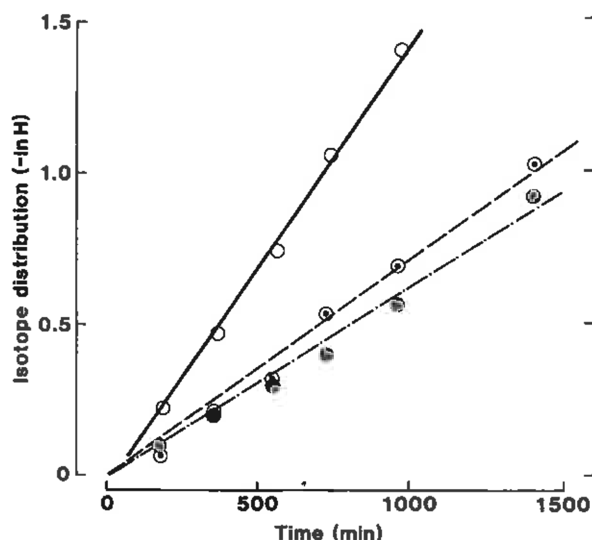

FIG. 2. The distribution of tritiated steroid with increasing time of dialysis. Radioactive steroid is added with plasma to one compartment initially and dialyzed for time t across the semipermeable membrane into the other compartment. The isotope distribution H is $(dpm_1 - dpm_2)/(dpm_1 + dpm_2)$ where dpm_1 and dpm_2 are the amounts of radioactivity in the two compartments. The lines were obtained by least-squares analysis. ($r \geq 0.994$ and $P < 0.001$ in each case). Estradiol-17B, (⊛); progesterone, (⊙); and cortisol, (○).

dissolved in buffer containing 1 or 15 mg human serum albumin was dialyzed against buffer alone. When 15 mg albumin was used, 18 mg/ml of Dextran C was added to the other side of the membrane to minimize volume changes due to osmotic effects. The volume change after dialysis was ≤5%, as judged by changes in the concentration of added I^{125} transferrin. At the same time, parallel dialyses were taken to equilibrium (40 h at 37°C) in order to determine the proportion of free steroid in the original solution. Dialysis of steroids in solutions containing albumin provided a 16-fold range in the amount of free steroid (Table 1). The addition of 0.1% w/v Tween 80 detergent to solutions of tritiated steroid and albumin, or of 100- and 1000-fold excesses of radioinert steroid (which increased the amount of free tritiated steroid) did not alter the cell permeability constant.

Estimation of free steroid in plasma by rate dialysis. The percentage of free cortisol, progesterone, and estradiol-17β, and their free concentrations, is shown for four plasma pools in Table 2. The fractional free steroid contents of each plasma pool were measured 12 times in five different experiments, in order to assess within-assay and between-assay variability. The four plasma pools provided ranges of percentage free of three- to fivefold for each steroid hormone, and an overall range of 14-fold, extending from 1.17% free estradiol-17β

TABLE 1

CELL PERMEABILITY CONSTANT FOR RATE DIALYSIS OF STEROIDS[a]

Hormone	Albumin (mg/ml)	Percentage free steroid	Cell permeability constant $(min^{-1}) \times 10^2$
Cortisol	0	100	2.97 ± 0.27 (6)
Cortisol	15	63.9	2.67
Progesterone	0	100	2.88
Progesterone	1	57.0	2.76
Progesterone	15	8.8–10.1	3.37 ± 0.40 (4)
Estradiol-17β	0	100	3.37
Estradiol-17β	15	11.0–12.3	3.48, 2.73
17α-Hydroxyprogesterone	10	22.6	2.88
17α-Hydroxyprogesterone	15	50.4	2.82
20α-Hydroxyprogesterone	15	3.9	3.36
Medroxyprogesterone acetate	15	15.9, 18.4	2.75, 2.32

[a] The cell permeability constant (2AD/V) was determined from the slope of the line drawn through the plot of *ln* (hormone distribution) vs. dialysis time. The mean ± SE for 21 experiments was $3.06 \times 10^{-2} \pm 0.09 \times 10^{-2}$.

TABLE 2

FREE STEROID DETERMINATION BY RATE DIALYSIS

Steroid	Plasma pool			
	Human pregnant	Human non-pregnant	Porcine pregnant	Porcine lactating
Cortisol				
Percentage free[a]	4.92 ± 0.16	6.68 ± 0.27	14.24 ± 0.41	16.50 ± 0.31
$-S_T^2$ [b]	0.3289	0.5622	1.2086	0.8837
$-S_W^2$ (%)	66	37	34	48
$-S_B^2$ (%)	34	63	66	52
Total, ng/ml	286.14	189.73	13.34	8.90
Free, ng/ml[c]	14.08	12.67	1.90	1.47
Progesterone				
Percentage free	2.95 ± 0.01	3.28 ± 0.10	7.53 ± 0.16	7.58 ± 0.16
$-S_T^2$	0.0404	0.0765	0.3054	0.3893
$-S_W^2$ (%)	100	61	63	74
$-S_B^2$ (%)	0	39	37	26
Total, ng/ml	187.75	4.16	14.88	1.34
Free, ng/ml	5.54	0.14	1.12	0.10
Estradiol-17β				
Percentage free	1.17 ± 0.03	1.37 ± 0.08	5.78 ± 0.26	5.96 ± 0.18
$-S_T^2$	0.0189	0.0487	0.7809	0.4008
$-S_W^2$ (%)	84	36	61	66
$-S_B^2$ (%)	16	64	39	34
Total, pg/ml	1182	355	85	25
Free, pg/ml	13.8	4.9	4.9	1.5

[a] The mean ± SE of 60 measurements, determined as 12 replicates in 5 experiments.

[b] The variance of a single measurement (S_T^2) is subdivided into the percentage due to within-assay variance (S_W^2) and that due to between-assay variance (S_B^2).

[c] Free hormone concentrations were determined from the product of their total concentration (measured by radioimmunoassay) and free fraction.

in human plasma to 16.5% free cortisol in porcine plasma.

In any technique where small amounts of added tracer hormone are partitioned, it is important to be able to account for all of the added radioactivity, and rate dialysis is no exception. Recovery of tritiated steroid was quantitative for the three hormones added to human and porcine plasmas; it ranged between 99.2% ± 0.3% and 102.0 ± 0.3%. The mass of added tritiated steroid (0.1 pmol) was ≤0.15% of the total cortisol and ≤1% of the total progesterone in the different plasmas. The added tritiated estradiol-17β amounted to 15 and 50% of the endogenous steroid in pregnant and non-pregnant sow plasma, respectively. In separate experiments, the amount of added radioactive estradiol-17β relative to endogenous hormone in the plasma pooled from lactating sows, pregnant sows, and non-pregnant women was varied in five steps, from 1.8 to 24×, 0.15 to 2×, and 0.1 to 1.7×, respectively. For each plasma the free estradiol-17β fraction was unchanged from the value shown in Table 2.

In most studies where the concentration of free steroid is measured in serial samples, the samples are collected over a period of time and frozen until analysis can be performed on groups of them simultaneously. Therefore, the effect of freezing and thawing of plasma, and prolonged storage, on the determination of percentage free steroid was investigated (Table 3). A pool of human plasma from preg-

nant women was analyzed before and after freezing and thawing (0 weeks), and following additional cycles of freezing and thawing after storage for 5 and 20 weeks. Percentage free progesterone and estradiol-17β remained constant after repeated freezing and thawing and prolonged storage whereas the proportion of free cortisol increased.

Estimation of free steroid in plasma by centrifugal ultrafiltration. Percentage free cortisol, progesterone, and estradiol-17β were determined by centrifugal ultrafiltration at 37°C in the same four plasma pools which had been analyzed by rate dialysis (Table 4). Ultrafiltration provided higher estimates of percentage free cortisol and estradiol-17β but lower estimates of percentage free progesterone than rate dialysis. For the plasma pool from pregnant women, the free fraction of each of the three steroids was measured by ultrafiltration in quadruplicate on separate days. Free progesterone was 1.61 ± 0.18% (4), and 1.69 ± 0.19% (4), respectively, and free estradiol-17β was 3.97 ± 0.14% (4) and 3.88 ± 0.16% (4), respectively. However, ultrafiltration provided an estimate of free cortisol of 7.48 ± 0.16% (4) on the first day and 9.22 ± 0.22% (4) on the second day. The differences were not due to different centrifugation conditions as the ultrafiltration cells were centrifuged randomly in batches of six cells. Furthermore, differential recovery of the added tracer hormone did not account for this within-day variability in the estimation of percentage free cortisol, since recovery of [^{3}H]cortisol, [^{3}H]progesterone, and [^{3}H]estradiol-17β after ultrafiltration was 81.3 ± 1.0 (8), 79.6 ± 1.3 (8), and 79.7 ± 1.5% (8), respectively.

The ultrafiltrates contained protein in proportion to their volumes. For example, when 0.5 ml of plasma was ultrafiltered, each 0.1 ml of filtrate contained 1.49 ± 0.02% (34) of the protein in the plasma, irrespective of the source of plasma or of the presence of added tritiated steroid.

TABLE 3

FREE STEROID CONCENTRATION AFTER REPEATED FREEZING AND THAWING OF PLASMA POOL

Steroid	Cycles of freezing and thawing			
	0	1	2	3
Free cortisol, ng/ml	4.91	5.25	7.46	7.39
Free progesterone, ng/ml	0.81	0.82	0.83	0.85
Free estradiol-17β, pg/ml	20.9	19.5	18.7	19.7

Note. The percentage free steroids were determined in duplicate by rate dialysis in plasma pooled from pregnant women directly and after three cycles of freezing and thawing. The total concentrations of cortisol, progesterone, and estradiol-17β, measured by radioimmunoassay, were 169.95, 37.70, and 1.82 ng/ml, respectively, from which the concentration of free steroid was calculated.

DISCUSSION

Available methods for determining free ligand fractions measure either directly or indirectly the amount of free hormone in an equilibrium system *in vitro,* which only approximates the fluctuating steady-state system characteristic of flowing blood. Also, all current methods assume implicitly that the free fraction of each steroid in plasma remains constant during the measuring process and that added tracer steroid equilibrates with both free and protein-bound steroid. In rate dialysis, the rate of dialysis of tritiated steroid depends on the free steroid fraction. The basic assumption of the method is that tracer steroid added to the plasma on one side of the cell diffuses through the membrane by a first-order rate process. This was shown experimentally for cortisol, progesterone, and estradiol-17β in human plasma (Fig. 2) and has been confirmed for 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, corticosterone, and medroxyprogesterone acetate in plasma from humans, pigs, and rats examined so far in our laboratory.

Between-assay variability was not generally greater than within-assay variability (Table 2). Thus, it may be acceptable in routine studies using serial plasma samples to run replicate determinations in the same experiment. This is easier in practice and slightly reduces the amount of plasma and time required for each estimate of percentage free steroid. The percentages of free cortisol, progesterone, and estradiol-17β varied more between species than

TABLE 4

FREE STEROID DETERMINATION BY CENTRIFUGAL ULTRAFILTRATION

Steroid	Plasma pool: Human pregnant	Human non-pregnant	Porcine pregnant	Porcine lactating
Percentage free cortisol[a]	8.34 ± 0.95	10.75 ± 0.11	17.35 ± 0.39	17.87 ± 0.67
Free cortisol, ng/ml	23.86	20.40	2.31	1.59
Percentage free progesterone	1.65 ± 0.18	2.36 ± 0.40	4.49 ± 0.35	4.14 ± 0.36
Free progesterone, ng/ml	3.10	0.10	0.67	0.06
Percentage free estradiol	3.92 ± 0.15	4.12 ± 0.11	7.31 ± 0.35	7.74 ± 0.15
Free estradiol, pg/ml	46.3	14.6	6.2	1.9

[a] The percentage free data is expressed as the mean ± SE of four determinations for each steroid except for the pool of human pregnant plasma, in which eight determinations were made. The sample plasma pools were the same as used for rate dialysis in Table 2.

they did between pregnancy and non-pregnancy within each species (Table 2). When the endogenous hormone concentration was very low, eg., estradiol-17β in the plasma of lactating sows, the addition of radioactive hormone as part of the rate dialysis procedure increased the total hormone concentration by up to 50%. However, the ratio of free:bound estradiol-17β remained constant at this and higher total concentrations, so that the determination of the free hormone fraction was unaffected.

If the free fraction of a steroid is not measured in freshly obtained plasma, then it should be established that freezing and thawing, and/or storage, does not alter the binding equilibria and hence the estimates of percentage free steroid. Steroids are bound in plasma principally to albumin and to high-affinity proteins such as transcortin (18). Differential stability of these proteins to freezing and thawing may affect the free fraction of a steroid. For example, the concentrations of free progesterone and estradiol-17β were unaltered by three cycles of freezing and thawing, whereas the concentration of free cortisol increased progressively (Table 3). This increase in the free fraction of cortisol with repeated freezing and thawing of plasma has been observed by others (25).

We compared the measurement of percentage free steroid by rate dialysis to that of centrifugal ultrafiltration. The latter is theoretically equivalent to equilibrium dialysis (23,26,27) and, therefore, equivalent to rate dialysis. The two methods yielded similar results for the same four plasma pools (Tables 2 and 4). By ultrafiltration, percentage free cortisol and estradiol-17β were greater ($P < 0.05$ for the four plasmas) and percentage free progesterone was less ($P < 0.05$ for the four plasmas) than the corresponding values determined by rate dialysis. The free fraction would be overestimated by ultrafiltration if the tracers contained freely diffusible radioactive impurities. However, the same batches of tritiated steroids were used in both procedures and these were determined to be $\geq$98.5% isotopically pure. Therefore, the reason for the differences between the techniques is unknown. It may be attributable partly to leakage of 1.5% of the plasma protein into the ultrafiltrate. If the membrane passed all plasma proteins equally, such leakage would lead to an overestimate of 13% in the fractional free steroid if 90% of the steroids were protein-bound. Progesterone is a relatively hydrophilic steroid (18), so that it may have been adsorbed to the ultrafiltration membrane. This would cause an underestimate in the fraction of free progesterone when measured by ultrafiltration. However, other factors must be operating since the recovery of added tritiated steroid after ultrafiltration was uniformly only 80% for all

three steroids. This compares with quantitative recovery of the added radioactivity after rate dialysis.

The free fractions of steroid hormones in human plasma have been measured previously by a variety of techniques. The values reported for percentage free cortisol, progesterone, and estradiol-17β in plasma from pregnant and non-pregnant women range from 6.3 to 8.5% for cortisol (5,7,20,25,28,29), 1.9 to 9.1% for progesterone (3,5,11,30,31), and 0.96 to 1.57% for estradiol-17β (6,11,32). The values determined by rate dialysis in human plasma fall within these ranges for all three steroids (Table 2). However, free steroid fractions in porcine plasma have not been measured before. The value of 16.5% free cortisol in the plasma pool from lactating sows was double the highest value reported for percentage free cortisol in human plasma, although 25% of the cortisol in the plasma of squirrel monkeys is present in the free form (33).

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