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Lassourd, V., V. Gayrard, V. Laroute, M. Alvinerie, P. Benard, D. Courtot, and P. L. Toutain. Cortisol disposition and production rate in horses during rest and exercise. *Am. J. Physiol.* 271 (*Regulatory Integrative Comp. Physiol.* 40): R25–R33, 1996.—The influence of a 56-km endurance exercise on cortisol kinetics and production rate was evaluated in six horses administered [^{3}H]cortisol. Exercise resulted in an immediate two- to threefold increase in plasma cortisol, with values returning very rapidly to preexercise levels. During exercise, clearance and steady-state volume of distribution of total cortisol were greatly increased (338 ± 95 vs. 137 ± 34 $ml \cdot kg^{-1} \cdot h^{-1}$ for clearance and 359 ± 82 vs. 229 ± 18 ml/kg for volume of distribution), whereas the terminal half-life decreased significantly (0.97 ± 0.16 vs. 1.55 ± 0.33 h). The estimated cortisol production rate was 4.41 ± 1.06 $\mu g \cdot kg^{-1} \cdot h^{-1}$ at rest and 26.75 ± 5.11 $\mu g \cdot kg^{-1} \cdot h^{-1}$ during exercise. We conclude that exercise triggers a large increase (×6) in the adrenal secretion rate, which is not accurately reflected by the more limited increase (×2–3) in plasma cortisol concentration, the actual measurement of plasma cortisol clearance being a prerequisite to assessment of adrenal gland function during exercise.

kinetics; deconvolution

THE PRODUCTION RATE of cortisol (hydrocortisone) has been extensively investigated in physiological and pathological states in humans (14, 19) and in different species, including the horse (30). However, conflicting results, due to methodological difficulties, have been obtained with both the urinary secretion methods (based on quantitation of endogenous metabolites excretion) and plasma production rate methods [based on evaluation of plasma cortisol clearance; see review by Esteban and Yergey (6)]. One of the most difficult issues with the plasma clearance method is the role played by the nonlinear binding of cortisol to the corticosteroid-binding globulin (transcortin), a glycoprotein that binds cortisol with high affinity, but low capacity. A maximal capacity of 90 ng/ml was reported in the horse (11), which is of the same order of magnitude as the maximal plasma physiological cortisol concentration (31). If it is assumed that only the free fraction of cortisol can be cleared from the plasma, it can be anticipated that plasma cortisol clearance is not an invariant parameter, but rather a permanently variable process reflecting the episodic secretory pattern of cortisol already described in different species, including the horse (12, 31). According to this view, the free fraction of cortisol (and consequently plasma cortisol clearance) should increase episodically and should display a circadian rhythm.

The existence of diurnal variations in plasma clearance of cortisol was reported 20 years ago in humans (14). More recently, the relationship between a burst-like hormonal secretion and the nonequilibrium kinetic behavior of a hormone, assuming the presence of a high-affinity binding protein, has been documented from a theoretical point of view (32).

Exercise is a physiological situation in which the preceding consideration must be taken into account. Cortisol is a key hormone involved in adaptation to exercise, and an increase in plasma cortisol concentration during exercise has been reported in different species, including humans (3, 7) and horses (5, 27). This has led to the generally accepted view that exercise increases adrenal secretion rate.

This increase in adrenal secretion has been generally assumed to be proportional to the corresponding increase in plasma cortisol concentration. During exercise, the plasma cortisol concentration may, in fact, exceed the maximal binding capacity of transcortin, leading to a possible relevant increase in the plasma cortisol clearance and, by consequence, to a less-than-proportional change in plasma concentration with respect to the actual increase of secretion rate of cortisol.

The purpose of the present experiment was to examine the disposition and production rate of cortisol in horses by considering the nonlinear binding of cortisol in relation to cortisol concentration and to document the influence of exercise, a physiological event in which plasma cortisol can greatly exceed the maximal binding capacity of transcortin.

MATERIAL AND METHODS

Animals

Six healthy saddlebred horses (~9 yr old), weighing 494 ± 51 kg, were used. All horses were regularly exercised for 1–2 h per day. Horses were kept in individual boxes and were fed an appropriate diet of two meals per day; straw and water were given ad libitum.

Experimental Design

Cortisol disposition was studied in all horses during rest and during sustained exercise.

Initially, horses were allocated to two groups of three to permit a crossover design with a washout period of at least 2 wk. Because of a technical difficulty unrelated to the experiment, one horse had to be replaced, and, finally, four horses received cortisol during rest first and then during exercise,

whereas the reverse was so for the two other horses. During the rest trial, horses were kept in their individual boxes and received labeled cortisol at 11:00 A.M. The exercise test consisted of an endurance ride of 56 km at ~200 m/min (corresponding to ~50% of maximal O_2 consumption ($\dot{V}O_{2max}$). The ride was carried out between 9:30 A.M. and 4:00 P.M. Horses were stopped for 18 min at ~11:00 A.M. for intravenous [^{3}H]cortisol administration and were allowed to rest for 60 min at 12:30 P.M. The horses were stopped every 27 min during the ride for 3 min for blood sampling. During stops, the horses were allowed access to water.

Test Article

[1,2,6,7-^{3}H]cortisol was purchased from Amersham-France in toluene:ethanol (9:1). The specific activity was 80 Ci/mmol, and the radiochemical purity was >97% as determined by radio thin-layer chromatography (dichloromethane/acetone 4:1). Each vial was rinsed three times with 1 ml of toluene. The solution was evaporated to dryness using nitrogen gas. Five milliliters of dimethyl sulfoxide were added to the residue, and the solution was kept at 37°C in a water bath for 15 min. Two aliquots of 10 µl were taken before the syringe was filled.

[^{3}H]cortisol Administration and Blood Sampling

[^{3}H]cortisol (1 mCi) in 5 ml of dimethyl sulfoxide was injected in the right jugular vein within 10 s via an indwelling catheter. The precise dose administered to each horse was determined by weighing the syringe before and after injection and by measuring the activity of a weighed aliquot of [^{3}H]cortisol solution.

Blood samples (5 ml) were collected at 30-min intervals for 26.5 h (from 8:30 A.M. up until 11:00 A.M. the following day) and after cortisol administration at 1, 2, 4, 8, 15, and 30 min. The blood sample was obtained by direct venipuncture using a 22-gauge needle. Horses were well trained to venipuncture and were apparently not disturbed by the sampling procedure.

Blood samples were collected in heparinized tubes and centrifuged at 1,400 *g* for 10 min within 2 h of collection. Plasma was separated and stored at −20°C until it was processed for measurement of nonradioactive and radioactive cortisol.

Analytic Methods

Plasma cortisol concentration was determined using high-performance liquid chromatography (HPLC), after methylene chloride extraction as described previously (1). The quantification limit of the assay was 5 ng/ml, and the intra-assay coefficient of variation was less than 10%.

The cortisol-specific radioactivity was measured by coupling HPLC and scintillation liquid counting techniques. For each sample, the eluate (methylene chloride:methanol:acetic acid) was collected for 2 min (corresponding to the cortisol peak detection). This fraction was evaporated to dryness in a glass vial in a water bath at 50°C using nitrogen gas. The dry residue was dissolved in 100 µl methanol and was counted for 10 min in a Packard Tri-Carb 2200 CA Scintillation Counter after addition of 5 ml of liquid scintillation mixture (Instagel, Packard Instruments). The counts were corrected for background and quenching effects. The counts per minute were converted to disintegrations per minute (dpm) using the external standard ratio technique. The counting efficiency was 52 ± 1%.

Protein Binding

Equilibrium dialysis was performed to characterize the concentration-dependent binding of cortisol to plasma protein in all but one horse. Investigated plasma samples were obtained during a second ride similar to that described above; two samples (300 ml) were collected 2 days before the ride (at 11:00 A.M. and 12:30 P.M.) and after 16.2 and 32.4 km of a ride performed at 200 m/min in relatively cold conditions (4°C).

In vitro protein binding of cortisol was measured on plasmas collected at 11:00 A.M. and at 12:30 P.M. (rest or exercise) using a Dianorm system (CH8135; Langenau, Zurich, Switzerland) with Teflon half-cells (1 ml) under constant stirring (20 revolutions/min). All the experiments were carried out at 37°C. The two half-cells were separated by a semipermeable cellulose membrane that retained compounds with molecular weights greater than 10,000 (Diachema 16–10; Braun Scientetec, ZA Courtaboeuf, Les Ulis, France). One compartment contained cortisol (Hydrocortisone; Sigma, L'Isle d'Abeau Chesnes, La Verpillière, France) solution in 0.1 M phosphate buffer (0.9 ml, pH = 7.4), and the other contained plasma stripped of cortisol (0.9 ml). Charcoal-stripped plasma was prepared according to de Vries et al. (34) by mixing 5 g of charcoal with 100 ml of plasma for 1 h at room temperature; the charcoal was removed by centrifugation (4 times 10 min at 3,000 *g*). The plasma samples, free of steroid, were immediately frozen at −20°C and kept at this temperature until the cortisol binding study took place. Cortisol binding was studied over a wide range of concentrations from 0.0055 to 5.5 µM (2–2,000 ng/ml). Twenty microliters of a 1.5 nM tritium-labeled cortisol solution ([1,2,6,7-^{3}H]cortisol, 80 Ci/mmol; Amersham International, Buckinghamshire, UK) in toluene:ethanol (9:1, vol/vol) was added to each 1 ml cortisol solution as a tracer (10,000 dpm per cell). The radiochemical purity of the [^{3}H]cortisol solution kept at −20°C and controlled by HPLC before use was >98%. The time required to obtain the equilibrium of free cortisol concentrations between plasma and buffer compartments was 1 h.

After dialysis, 200- and 400-µl aliquots from plasma and buffer solution, respectively, removed from the cells were counted in a Liquid Scintillation Spectrometer (Kontron beta V, Montigny le Bretonneux, France) after addition of a liquid scintillation mixture (Ready Safe Beckman Instrument, Gagny, France; 4.5 ml). The counts per minute were converted to dpm using the external standard ratio technique.

The dpm counts were converted to equilibrium concentration of unbound (free) and bound cortisol using an appropriate computation (22). The plasma cortisol concentration after equilibration (P_E) was calculated from the known concentration of cortisol (P_T) added to the buffer solution and the radioactivity on each side of the membrane (*Eq. 1*)

$$P_E = P_T \times \frac{\text{dpm in dialyzed plasma}}{(\text{dpm in buffer}) + (\text{dpm in dialyzed plasma})} \quad (1)$$

Equations 2 and *3* were used to determine the molar concentrations of F (free cortisol) and B (bound cortisol).

$$F = P_E \times \frac{\text{dpm in buffer}}{\text{dpm in dialyzed plasma}} \quad (2)$$

$$B = P_E - F \quad (3)$$

Data Analysis

Protein binding. Protein-bound cortisol concentrations were plotted against unbound ones. The profiles indicated the presence of both saturable and nonsaturable protein binding,

transcortin, and albumin, respectively. These data were fitted according to *Eq. 4*

$$B = \frac{N_1 \times P_1 \times F \times K_{a1}}{1 + K_{a1} \times F} + \frac{N_2 \times P_2 \times F \times K_{a2}}{1 + K_{a2} \times F} \quad (4)$$

where N_1 and N_2 are the number of sites per molecule of protein; P_1 and P_2 (M) are the molar concentrations of transcortin and albumin, respectively; and K_{a1} and K_{a2} (M^{-1}) are the affinity constants of cortisol for transcortin and albumin, respectively. $N_1 \times P_1$ is the binding capacity of transcortin (B_{max}). N_1 was assumed to be 1 (24), which allowed calculation of P_1 (the transcortin concentration).

For albumin, $K_{a2} \times F << 1$, and if N_2 is assumed to be unity, *Eq. 4* can then be simplified (*Eq. 5*)

$$B = \frac{(N_1 \times P_1)K_{a1} \times F}{1 + K_{a1} \times F} + NS \times F \quad (5)$$

in which $P_2 \times K_{a2}$ equal NS, a dimensionless proportionality constant for the nonspecific binding of cortisol to albumin. Initial binding parameters ($N_1 \times P_1$, K_{a1}, and NS) obtained using the method of Rosenthal (23) were optimized by a computerized nonlinear least-squares regression program Microparm (Vs. 1.6; Institut National de la Santé et de la Recherche Médicale, Paris, France). The dissociation constant (K_d) was then calculated as the inverse of the affinity constant (K_a). The parameter K_{a2} was obtained by dividing NS by P_2 (molar albumin concentration), which was determined chemically: proteins fractionated by electrophoresis on cellulose acetate in Veronal buffer (pH = 8.6, μ = 0.05; Sebia, Issy-les-Moulineaux, France) were analyzed by dry-chemistry procedures with a Kodak Ektachem XR700 (Kodak, Paris, France).

Using these estimates, the concentration of free cortisol was calculated from the quadratic equation derived by Tait and Burstein (28) for each measured total cortisol plasma level (*Eq. 6*)

$$K_a(1 + NS)F^2 + (1 + NS + K_a \times B_{max} - K_a \times TOT)F - TOT = 0 \quad (6)$$

where TOT is the physiological plasma cortisol concentration, and the other parameters are as previously defined.

Albumin-bound cortisol concentration (B_{alb}) was calculated from *Eq. 7*

$$B_{alb} = NS \times F \quad (7)$$

The concentration of transcortin-bound cortisol was determined from the difference between total cortisol and the sum of free and albumin-bound cortisol concentrations. The fractions of free, transcortin-bound, and albumin-bound cortisol were determined from the ratio between the respective concentrations and the corresponding total concentration.

Kinetic analysis: cortisol. The apparent kinetic parameters for total (free and bound) cortisol were calculated from plasma [^{3}H]cortisol concentration time profile using a program for nonlinear regression analysis adapted from Multi (36). The plasma concentrations of individual horses were fitted to the general polyexponential *Eq. 8*

$$C(t) = \sum_{i=1}^{n} Y_i \exp(-\lambda_i t) \quad (8)$$

In *Eq. 8*, $C(t)$ is the cortisol plasma activity concentration (dpm/ml) at time t, Y_i (dpm/ml) is the coefficient of the ith exponential term, and λ_i (h^{-1}) is the ith exponential term. Initial estimates were obtained using the method of residuals (9). These initial estimates were refined by a least-squares nonlinear regression. The inverse of the fitted values ($1/Y_i^2$) was used as weight.

Based on a F-test, a triexponential equation was selected for all horses (*Eq. 9*)

$$C(t) = Y_1 \exp(-\lambda_1 t) + Y_2 \exp(-\lambda_2 t) + Y_3 \exp(-\lambda_3 t) \quad (9)$$

where $C(t)$ is the plasma cortisol activity (dpm/ml) at time t; Y_1, Y_2, and Y_3 (dpm/ml) are preexponential coefficients; and λ_1, λ_2, and λ_3 (h^{-1}) are exponents of the equation. In consequence, the data could be described by a three-compartment open model with cortisol elimination from the central compartment.

The parameters (Y_1, Y_2, Y_3, λ_1, λ_2, λ_3) were used to solve for the first-order rate constants of transfer from central to peripheral compartments (K_{12}, K_{21}, K_{13}, K_{31}) according to the classical equations (9).

The volume of the central compartment (V_c) was obtained using *Eq. 10*

$$V_c = \text{dose}/(Y_1 + Y_2 + Y_3) \quad (10)$$

The steady-state volume of distribution (V_{ss}) was obtained using *Eq. 11*

$$V_{ss} = V_c(1 + K_{12}/K_{21} + K_{13}/K_{31}) \quad (11)$$

The apparent plasma clearance (Cl) was calculated using *Eq. 12*

$$Cl = \text{dose}/AUC \quad (12)$$

where AUC is the area under the curve obtained with *Eq. 13*

$$AUC = Y_1/\lambda_1 + Y_2/\lambda_2 + Y_3/\lambda_3 \quad (13)$$

The mean residence time (MRT) of the system, i.e., the mean time for a cortisol molecule to transit in the body, was calculated by a compartmental approach (*Eq. 14*)

$$MRT = \frac{Y_1/\lambda_1^2 + Y_2/\lambda_2^2 + Y_3/\lambda_3^2}{Y_1/\lambda_1 + Y_2/\lambda_2 + Y_3/\lambda_3} \quad (14)$$

with Y_1, Y_2, Y_3, λ_1, λ_2, λ_3 as previously defined.

Free and transcortin-free cortisol kinetics. In vivo, the administered [^{3}H]cortisol is distributed between free, transcortin-bound, and albumin-bound fractions. Assuming that the administered [^{3}H]cortisol has the same distribution as the adrenal-secreted cortisol, these fractions were determined from the actual physiological cortisol plasma concentration (ng/ml) and the individual binding parameters of each horse for each [^{3}H]cortisol plasma activity concentration profile (dpm/ml).

The free [^{3}H]cortisol plasma activity concentration time data were fitted as described for the total [^{3}H]cortisol plasma activity. Derived parameters (volume of distribution, plasma clearance. . .) were calculated from free [^{3}H]cortisol using the same equation as for total [^{3}H]cortisol.

The disposition parameters of the transcortin-free [^{3}H]cortisol, i.e., the sum of the free and albumin-bound fractions, were directly obtained from those calculated from the free [^{3}H]cortisol concentration profile. The mean residence time and the half-life for terminal phase were the same as for the free [^{3}H]cortisol. The V_c, the V_{ss}, and the clearance were those of the free [^{3}H]cortisol divided by (NS + 1) (see *Eq. 5*).

Cortisol production rate. Cortisol production rates (PR) were calculated from *Eq. 15*

$$PR = AUC\ (0 - t\ last) \times Cl/\Delta t \qquad (15)$$

where Cl is the free [^{3}H]cortisol clearance, and AUC ($0 - t$ *last*) is the area under the free cortisol plasma curve for the rest period and during the ride from *time 0* to the last time (*t last*) (Δt).

Instantaneous cortisol production rate was evaluated using a method of discrete deconvolution as previously described (30). Briefly, deconvolution analysis is based on modeling the disposition kinetics of the hormone as a linear and time invariant (stationary) system. Cortisol secretion rate was modeled by the following convolution integral (*Eq. 16*)

$$C(t) = \int_0^t F(t - \tau) \times S(\tau) \times d\tau \qquad (16)$$

where $C(t)$ is the measured plasma concentration of free cortisol in time t, $S(\tau)$ is the unknown function (i.e., input rate of hormone to be calculated), $F(t)$ is the response function to a unit impulse, and τ is the integration variable. $F(t)$ corresponds to the intravenous kinetics of free cortisol. *Equation 16* was solved for $S(\tau)$, given $C(t)$ and $F(t)$. Raw data were smoothed with an unweighted least-square Spline function (subroutine EO 2 BAF from the NAG library, document no. 135470).

Statistical analysis. Statistical analysis was carried out using Statgraphics (STSC, Rockville, MD). Values are reported as means ± SD or means ± SE.

The effect of physiological condition (rest or exercise) was assessed using an analysis of variance (ANOVA) including two factors (horse and physiological condition).

RESULTS

Protein Binding

The mean protein binding parameters of cortisol are presented in Table 1.

The ANOVA indicated an absence of effect between rest and exercise ($P > 0.05$) with respect to transcortin and albumin binding parameters. The overall binding capacity of transcortin was 0.28 ± 0.07 × 10^{-6} M; the transcortin K_a was 53.8 ± 18.6 × 10^6 M^{-1}, corresponding to a K_d of 0.019 × 10^{-6} M (6.75 ng/ml); the albumin concentration was 508 ± 40 × 10^{-6} M; and the albumin affinity constant (K_{a2}) was 2.37 ± 0.23 × 10^3 M^{-1}, corresponding to a K_d of 422 M (153 μg/ml).

Table 1. *Plasma protein binding parameters of cortisol during rest and exercise in five horses*

Parameters	Experiment	
	Rest	Exercise
P_2, ×10^{-6} M	514 ± 23	503 ± 13
NS	1.19 ± 0.04	1.21 ± 0.07
K_{a2}, ×10^6 M^{-1}	0.0023 ± 0.0001	0.0024 ± 0.0001
N_1P_1, ×10^{-6} M	0.27 ± 0.03	0.28 ± 0.03
K_{a1}, ×10^6 M^{-1}	55 ± 8	53 ± 10

Values are means ± SE. P_2, molar concentration of albumin; NS, dimensionless proportionality constant of the nonspecific binding of cortisol to albumin; K_{a1} and K_{a2}, affinity constants of cortisol for transcortin and albumin, respectively (K_{a2} is calculated by the ratio between estimated NS and measured albumin plasma levels, assuming one site of cortisol binding per molecule of albumin); $N_1 \times P_1$, binding capacity of transcortin.

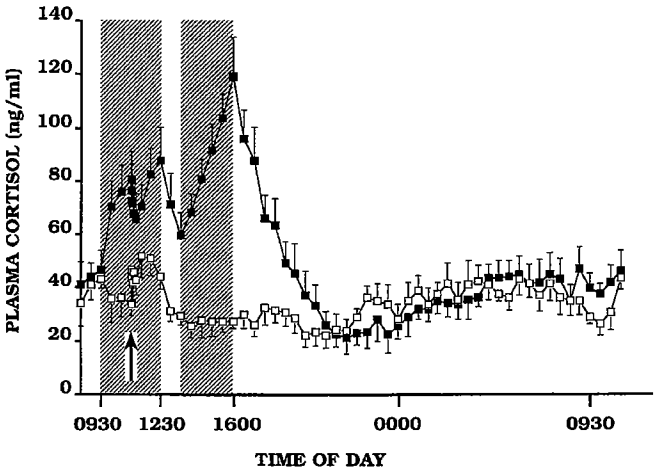

Fig. 1. A 26.5-h plasma cortisol concentration profile in 6 horses (means ± SE) during a rest day (□) and during a day (■) that included 2 bouts of an endurance ride (56 km, hatched areas). [^{3}H]cortisol was injected at 1100 (arrow).

Total and Free Plasma Cortisol Concentration Profile

Figure 1 shows the mean plasma concentration time profile of total cortisol during the control period and the test ride of six horses. Visual inspection clearly indicates that plasma cortisol was immediately increased at the start of the ride and attained an initial maximum at the end of the first part of the test ride. The plasma cortisol concentration then decreased immediately during the 1-h stop and increased again at the second start to attain a second maximum value at the final end of the test ride. The overall mean plasma cortisol concentration calculated by the trapezoidal rule was 36.2 ± 9.9 ng/ml during the 6.5-h control period (from 9:30 A.M. to 4:00 P.M.), 73.7 ± 22.5 ng/ml during the first part of the test ride (from 9:30 A.M. to 12:30 P.M.), and 86.9 ± 20.1 ng/ml during the second part of the test ride; the difference between the two parts of the test ride and the rest was significant ($P < 0.05$, Bonferroni test).

Individual analysis of cortisol binding permitted calculation of the free and transcortin-free cortisol plasma concentration profiles. Because there was no difference between rest and exercise for the binding parameters, we used the individual overall value of the binding parameters for each horse to calculate the different fractions of cortisol. Figure 2 shows the relative variation of total and free plasma cortisol concentration profiles for the six horses during 26.5 h that included rest and exercise. Visual inspection indicates that, under rest conditions, free cortisol increased almost proportionally to total cortisol, whereas during exercise, the free cortisol increased more than proportionally to total cortisol. The plasma profiles of transcortin-free cortisol are parallel to those of free cortisol, because there is only a proportionality constant (NS + 1) between the two profiles. When plasma cortisol was maximal (121 ng/ml), free cortisol (23 ng/ml) was

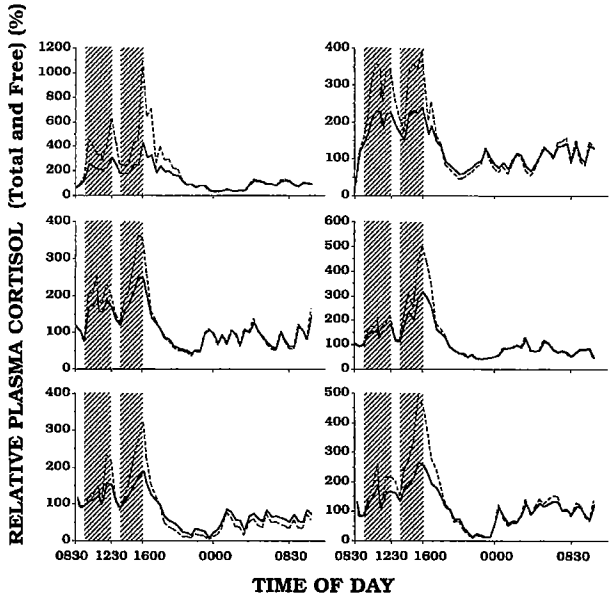

Fig. 2. Time course of the relative total (solid line) and free (dashed line) plasma cortisol concentrations in 6 horses during a day that included an endurance ride (56 km, hatched areas). Total and free cortisol concentrations are expressed as a percentage of the mean of the control concentrations for each horse.

increased by a factor of 7.7, whereas total cortisol was only increased by a factor of 3.3.

Total [^{3}H]cortisol Disposition

The total plasma [^{3}H]cortisol activity concentration after intravenous administration of [^{3}H]cortisol during rest and exercise is shown for the six horses in a semilogarithmic plot in Fig. 3.

Visual inspection of Fig. 3 indicates that cortisol disappears from the plasma much more rapidly when horses are exercising than when they are at rest.

The total cortisol clearance was 137 ± 34 ml·kg^{-1}·h^{-1} during rest and 338 ± 95 ml·kg^{-1}·h^{-1} during exercise. The difference was highly significant (ANOVA, $P < 0.01$). The V_{ss} was 229 ± 18 ml/kg during rest and 359 ± 82 ml/kg during exercise (ANOVA, $P < 0.01$). The terminal half-life averaged 1.55 ± 0.33 h during rest and 0.97 ± 0.16 h during exercise. The differences were significant (ANOVA, $P < 0.01$). Mean disposition parameters are given in Table 2.

Free and Transcortin-Free [^{3}H]cortisol Disposition

Free and transcortin-free [^{3}H]cortisol, similar to total [^{3}H]cortisol, disappeared faster during exercise than during rest.

The free cortisol clearance was 1,600 ± 624 ml·kg^{-1}·h^{-1} during rest and 2,863 ± 1,374 ml·kg^{-1}·h^{-1} during exercise. The difference was significant (ANOVA, $P < 0.05$). The V_{ss} was 2,471 ± 659 ml/kg during rest and 3,267 ± 1,472 ml/kg during exercise. The difference was significant for $P = 0.07$ (ANOVA). The terminal half-life was 1.77 ± 0.37 h during rest and 1.27 ± 0.09 h during exercise. The difference was significant (ANOVA, $P < 0.05$).

The corresponding disposition parameters of the transcortin-free (i.e., free and bound to albumin) cortisol were 731 ± 285 ml·kg^{-1}·h^{-1} for the clearance during rest and 1,295 ± 622 ml·kg^{-1}·h^{-1} during exercise; the V_{ss} averaged 1,128 ± 300 ml/kg during rest and 1,478 ± 666 ml/kg during exercise. The terminal half-lives were 1.77 ± 0.37 h during rest and 1.27 ± 0.09 h during exercise. The statistical significance of the differences of the parameters between rest and exercise was the same as for the disposition parameters of free cortisol.

Mean kinetic parameters based on free and transcortin-free cortisol are given in Table 2.

Cortisol Production Rates

Cortisol production rates were estimated using free cortisol data. The cortisol production rate was 4.41 ± 1.06 µg·kg^{-1}·h^{-1} during the rest day, 26.75 ± 5.11 µg·kg^{-1}·h^{-1} during the ride and up until the return to cortisol rest values (6:30 P.M.), and 4.60 ± 1.46 µg·kg^{-1}·h^{-1} after the return to cortisol rest values. The cortisol production rate was significantly higher during the last hour of exercise than during the first hour (43.5 ± 6.0 µg·kg^{-1}·h^{-1} vs. 19.2 ± 4.8 µg·kg^{-1}·h^{-1}, $P < 0.01$).

During the rest day, the total (24 h) cortisol production was 106 ± 25 µg/kg; during the day including the ride, the production was 311 ± 64 µg/kg.

Instantaneous production rate. The actual instantaneous secretion rate of free cortisol was reconstituted by deconvolution. Figure 4 shows the free plasma cortisol concentration vs. time profile and the corresponding instantaneous secretion rate vs. time profile for a representative horse. Cortisol secretion was clearly increased at each onset of the ride: first start and starts

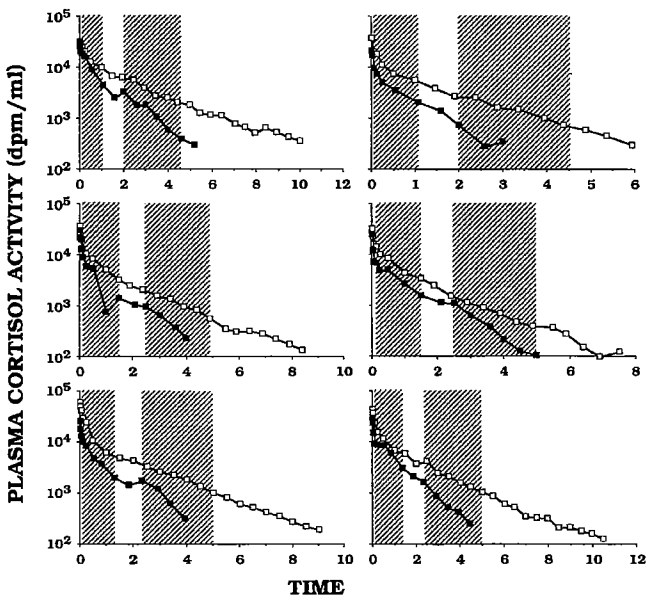

Fig. 3. Semilogarithmic plot of tritiated cortisol activity concentration vs. time in 6 horses after an intravenous administration of tritiated cortisol (1 mCi) during a rest day (□) and a day (■) that included 2 bouts of an endurance ride (56 km, hatched areas). dpm, disintegrations per minute.

Table 2. *Pharmacokinetic parameters describing the disposition kinetics of total, free, and transcortin-free cortisol after an intravenous bolus of tritiated cortisol (1 mCi in toto) in six horses at rest and during exercise*

Parameters	Total		Free		Transcortin-Free	
	Rest	Exercise	Rest	Exercise	Rest	Exercise
$t_{1/2\lambda 3}$, h	1.55 ± 0.33	0.97 ± 0.16	1.77 ± 0.37	1.27 ± 0.09	1.77 ± 0.37	1.27 ± 0.09
V_c, ml/kg	73.2 ± 28.4	82.8 ± 26.0	949 ± 226	738 ± 221	433 ± 103	334 ± 100
V_{ss}, ml/kg	229 ± 18	359 ± 82	2,471 ± 659	3,267 ± 1,472	1,128 ± 300	1,478 ± 666
Cl, ml·kg^{-1}·h^{-1}	137 ± 34	338 ± 95	1,600 ± 624	2,863 ± 1,374	731 ± 285	1,295 ± 622
MRT, h	1.73 ± 0.36	1.09 ± 0.16	1.63 ± 0.35	1.17 ± 0.15	1.63 ± 0.35	1.17 ± 0.15

Values are means ± SD. $t_{1/2\lambda 3}$, Plasma half-time for terminal phase; V_c, volume of the central compartment; V_{ss}, steady-state volume of distribution; Cl, body clearance calculated by dividing the dose of [^{3}H]cortisol by the area under the curve obtained by integrating the equation describing the fate of [^{3}H]cortisol plasma activity concentration; MRT, mean residence time. V_c, V_{ss}, and MRT were obtained using a compartmental approach.

of the two intermediary phases. The cortisol secretion continued to rise progressively during the exercise and then fell very abruptly and immediately at each of the three stops. A similar profile was observed in all six horses. The cortisol productions evaluated by deconvolution were very similar to those calculated using *Eq. 15*: 106 ± 25 vs. 111.3 ± 24.7 μg/kg ($P > 0.05$) during the rest day (24 h) and 240.7 ± 46.0 vs. 243.4 ± 42.4 μg/kg during the exercise and postexercise periods.

DISCUSSION

The main result of the present experiment is that the plasma cortisol concentration profile cannot be used directly to assess the actual influence of exercise on the adrenal secretion rate. Indeed, the actual production rate was increased by a factor of six, whereas the cortisol concentrations were only increased by a factor of ~2.4. The discrepancy is due to the large increase (~2.5-fold) in plasma clearance during the exercise. The mechanism responsible for the clearance increase during exercise merits special attention.

Cortisol is mainly eliminated by the liver, and it is generally considered that only the unbound compound can translocate into the hepatocyte. Assuming that the free hormone hypothesis is valid for cortisol, the subsequent rate of compound elimination is a function of the concentration of this moiety (15, 35).

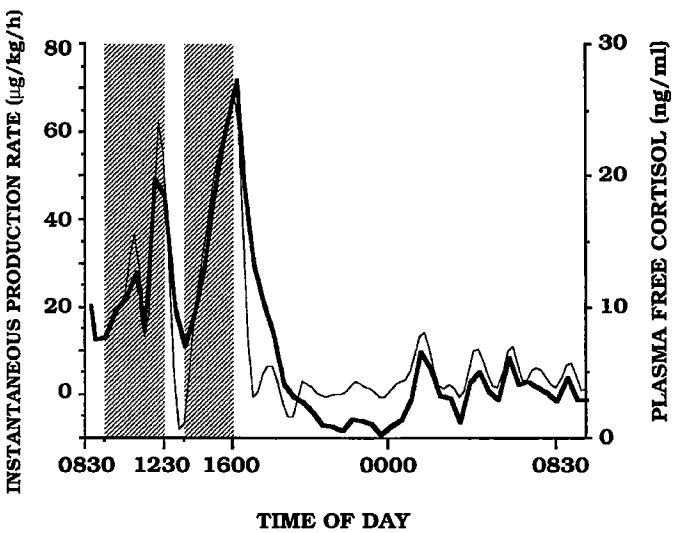

Fig. 4. Time course of free plasma cortisol concentration (thick line) and corresponding instantaneous production rate (fine line) in a representative horse during a day that included an endurance ride (hatched areas).

The distribution of cortisol between protein-bound and free fractions depends on both plasma protein binding parameters and cortisol secretion. We have shown that plasma protein binding parameters, namely maximal binding capacity of transcortin, K_d, and NS, were not modified in exercising horses, whereas cortisol plasma concentrations were increased by a factor of 2.4. Consequently, changes in the percentage of free cortisol can only be attributable to the increase of cortisol plasma secretion elicited by exercise.

In the present experiment, the estimated free plasma cortisol clearance was 1,600 ml·kg^{-1}·h^{-1} in the rest condition. Such a value is inconsistent with the hypothesis that only the free fraction can be cleared: the hepatic plasma clearance cannot be higher than the hepatic plasma flow, which is ~30% of the cardiac output, i.e., 600 ml·kg^{-1}·h^{-1} in horses (10). It was shown in sheep that the hepatic extraction of cortisol corresponds to ~80% of the unbound and albumin-bound cortisol entering the liver (20). Assuming that the same condition holds in the horse, the transcortin-free cortisol plasma clearance (i.e., calculated from free plus albumin-bound cortisol) is the physiologically relevant cortisol plasma clearance. In the present experiment, we found a value of 731 ml·kg^{-1}·h^{-1}, which is slightly higher than hepatic plasma flow, suggesting that a fraction of cortisol is metabolized in a site other than the liver. If we assume a hepatic extraction ratio for transcortin-free cortisol of 0.8 for horses, similar to sheep, the hepatic clearance must be equal to the product of plasma flow (600 ml·kg^{-1}·h^{-1}) and the extraction ratio (0.8), i.e., 480 ml·kg^{-1}·h^{-1} or 65% of the transcortin-free cortisol plasma clearance, which was actually measured. This figure is in agreement with that found in sheep, a species for which it was experimentally demonstrated that 61% of the cortisol produced in the control condition was cleared by the splanchnic viscera (16).

The cortisol concentration-dependent binding to transcortin and the fact that the transcortin-free cortisol is the driving concentration for hepatic clearance suggest that, whereas the total cortisol concentration kinetics are nonlinear, both the free and transcortin-

free cortisol concentration kinetics are linear. This was verified by injection of a 1 mg/kg dose of cortisol in the same horses by intravenous route, the clearance being increased by a factor of four when total cortisol was considered, but only by a factor of 1.2 (not statistically different from 1.0) for the free and transcortin-free fractions (results not shown).

The total cortisol (338 ± 95 $ml \cdot kg^{-1} \cdot h^{-1}$) and transcortin-free cortisol (1,295 ± 622 $ml \cdot kg^{-1} \cdot h^{-1}$) plasma clearances were greatly increased (2.5- and 1.8-fold, respectively) during exercise. One explanation for the increase should be the rise in transcortin-free fraction during exercise due to the limited binding capacity of transcortin in horse (mean maximal binding capacity of transcortin of 100 ng/ml). The influence of protein binding on hepatic clearance can be assessed using the general relationship that has been proposed for the well-stirred model of hepatic clearance (26, 35), i.e.

$$Cl_H = \dot{Q}\left[\frac{(F_u + F_{alb}) \times Cl_{int}^{free}}{\dot{Q} + (F_u + F_{alb}) \times Cl_{int}^{free}}\right] \quad (17)$$

where Cl_H is the hepatic clearance estimated to be ~480 $ml \cdot kg^{-1} \cdot h^{-1}$ (see above); $\dot{Q}$ is the hepatic plasma flow (600 $ml \cdot kg^{-1} \cdot h^{-1}$); Cl_{int}^{free} is the free intrinsic clearance, which indicates the maximal eliminating ability in the absence of flow limitation; and $F_u + F_{alb}$ is the relevant free fraction (i.e., free plus albumin-bound cortisol). From the Cl_H calculated in rest condition and taking into account a $F_u + F_{alb}$ equal to 0.18 (corresponding to the free plus albumin-bound fraction, which is calculated for a mean total cortisol concentration of 40 ng/ml), the estimated Cl_{int}^{free} is ~13,500 $ml \cdot kg^{-1} \cdot h^{-1}$. Using this intrinsic clearance and assuming that hepatic plasma flow was not decreased during exercise while $F_u + F_{alb}$ was increased to 27% (corresponding to the transcortin-free fraction when the mean cortisol was elevated to ~80 ng/ml during the ride), the well-stirred model predicts a hepatic clearance of 515 $ml \cdot kg^{-1} \cdot h^{-1}$ (with free plus albumin-bound concentration as the driving concentration); this value is only 7% higher than the hepatic clearance calculated in the rest condition (480 $ml \cdot kg^{-1} \cdot h^{-1}$), whereas a 77% increase in the transcortin-free plasma clearance has actually been observed. It is also generally admitted that the hepatic plasma flow is not maintained during a test exercise corresponding to 50% of $\dot{V}O_{2max}$ (25). Thus the large increase in plasma cortisol clearance during exercise cannot be of hepatic origin.

This nonhepatic origin of the increase in plasma cortisol clearance during exercise remains difficult to explain in terms of classical organ (i.e., kidney, lungs. . .) metabolism. In a study on men by Few (7), it was shown that the half-life for [^{3}H]cortisol was decreased from 74.5 to 31.5 min during heavy exercise. Because there was no corresponding change in the rate of appearance of ^{3}H in the conjugated steroid fraction of plasma or in the urine, the author speculated that the skeletal muscle was responsible for a specific uptake of cortisol during exercise. It was shown in man that the extraction ratio of cortisol by leg muscle (0.2) before and during exercise at a work load of 60% of $\dot{V}O_{2max}$ was unmodified (21), whereas the mean muscle blood flow was greatly increased. If muscle clearance is also of relevance in horse, it can be assumed that the very large increase in blood flow to muscle during our exercise condition (18) would be the origin of the twofold increase in cortisol clearance.

The second basic kinetic parameter to take into account to explain cortisol disposition is the volume of distribution. The V_{ss} of total cortisol was increased by a factor of ~50% during exercise. The simplest quantitative expression relating the volume of distribution to plasma and tissue binding is given by the equation of Gibaldi and McNamara (8) (*Eq. 18*)

$$V_{ss} = V_p + V_t \frac{F_u}{F_t} \quad (18)$$

where V_p is plasma volume, V_t is tissue volume, and F_u and F_t are fractions of unbound cortisol in plasma and tissues, respectively.

Equation 18 indicates that an elevated unbound fraction (F_u) in plasma will result in an increase in the value of the distribution volume. Assuming a V_p of 54 ml/kg in horses (2) and knowing F_u during the rest condition (0.08 for a mean plasma cortisol concentration of 40 ng/ml) and V_{ss} (229 ml/kg), the estimated ratio V_t/F_t will be 2,188 ml/kg. During exercise, the free fraction increased to 0.12 for a mean cortisol concentration of 80 ng/ml. Assuming that the V_p and V_t/F_t were not modified, the preceding equation predicts a new V_{ss} of 317 ml/kg, which is very close to that actually found (359 ml/kg). In other words, the increase in the volume of distribution of cortisol during exercise can only be due to an increase in the free cortisol fraction. Cortisol concentration-dependent changes in binding of cortisol were also advocated to be the reason for the apparent increase in V_{ss} in humans when increasing doses of cortisol are injected (29). The increase in cortisol distribution should lead to larger hormone exposure receptors and hormone effectors. Because free cortisol is considered to be the biologically active form, one might postulate that a relative increase in free cortisol is a potentiating factor for the feedback modulation of adenocorticotropic hormone (ACTH) secretion.

The cortisol production rate calculated in the rest condition was ~0.1 $mg \cdot kg^{-1} \cdot 24\ h^{-1}$, a value lower than that previously reported (0.46 $mg \cdot kg^{-1} \cdot 24\ h^{-1}$; see Ref. 30). The origin of this discrepancy is due to the overestimation of plasma clearance measured during a 24-h perfusion of unlabeled cortisol. The advantages of a single injection of labeled cortisol as a tracer are evident both from theoretical and practical points of view, but the method requires an appropriate analytic technique (i.e., the coupling of HPLC and a radioactivity detector) because a large fraction of the plasma radioactivity does not correspond to cortisol but to its metabolites (results not shown).

The cortisol production rate obtained in rest condition was similar to that found in sheep (0.3 $mg \cdot kg^{-1} \cdot 24$

h^{-1}) (17) and man (2.7–9.9 mg/24 h, i.e., ~0.04–0.14 $mg \cdot kg^{-1} \cdot 24\ h^{-1}$ for a man of 70 kg body wt) (6).

Exercise brought about a sixfold increase in cortisol production rate, from 4.41 $\mu g \cdot kg^{-1} \cdot h^{-1}$ to 26.75 $\mu g \cdot kg^{-1} \cdot h^{-1}$. The increase in cortisol production has been hypothesized by numerous authors to explain the rise in plasma cortisol concentrations observed during exercise in men (3), but to our knowledge, the present experiment is the first to measure the actual cortisol production rate during exercise. The present experiment shows that secretion rate was instantaneously increased and dramatically decreased at the onset and end, respectively, of each bout of exercise. Mechanisms responsible for the plasma cortisol production increase during exercise remain largely unknown. If we accept that only ACTH can trigger an adrenal response, the present experiment shows that exercise directly and instantaneously influences pituitary ACTH activity as an on/off system and that adrenal response develops with minimal hysteresis.

During a standardized exercising test in the horse, a lag time of ~30 min between the ACTH and peak plasma cortisol concentration was demonstrated (4). This result is not in disagreement with our results because such a delay only reflects the time required for cortisol to reach its steady-state concentration. Indeed, for a constant cortisol production rate, the time required to reach cortisol steady-state concentrations is about five half-lives, which is significantly longer for cortisol (1.5 h) than for ACTH (~10–20 min in humans). The apparent on/off response of the adrenal gland supports the concept of neural rather than humoral control of the hypothalamus-hypophysis-adrenal axis. The exact mechanism has yet to be studied, but the absence of anticipatory response suggests that factors such as a stress-induced rise in corticotropin-releasing factor are unlikely to occur. To explain an on/off pattern of adrenal secretion, the existence of a direct relationship between locomotion and adrenal secretion must be suggested. The hypothesis that the stimulation of afferent nerves from working muscles elicits neuroendocrine and metabolic response was tested in humans (13) by using lumbar epidural blockade at rest and during submaximal exercise and in cats by stimulating muscular nerves (33). The authors thereby showed that, during exercise, afferent neuron activity from working muscles stimulates secretion of ACTH and β-endorphin. If this hypothesis is correct, it is interesting to note that the muscle is probably at the origin of both the increase in cortisol secretion rate and the rise in clearance during exercise.

In conclusion, exercise triggers a large increase in adrenal secretion rate in horses according to an on/off process. Because of a large increase in cortisol clearance during exercise, the extent of the adrenal response is higher than that provided by the plasma cortisol concentration profile. Finally, it is hypothesized that the large increases in plasma cortisol clearance and production rate are both related to the increase in muscle activity during exercise via an increase in muscle blood flow and stimulation of the muscle afferent nerves.

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