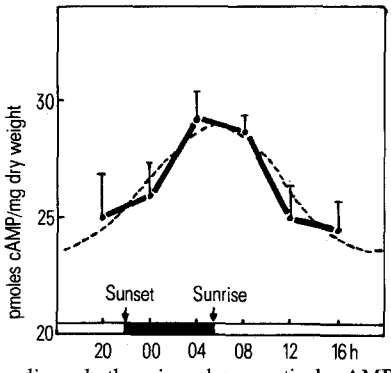

Fig. 1. Circadian rhythm in adrenocortical cAMP (fasciculata-reticularis zona) of mature male Wistar rats hypophysectomized 10 days before. Synchronization with natural day-light. Temperature 22±1 °C. Food and water available ad libitum. Sampling interval ≃4 h (8-10 animals). ·—· Raw data (chronogram) with time point mean±SEM in pmole cAMP/mg dry weight. --- Cosinor analysis: best sine function with period $\tau = 24$ h.

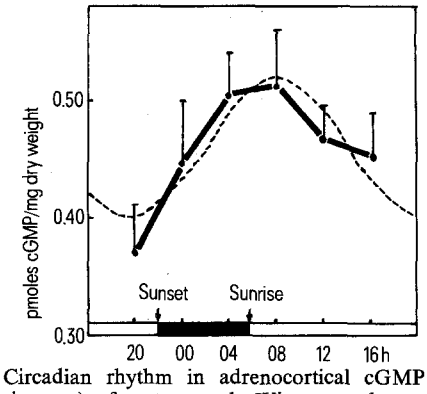

Fig. 2. Circadian rhythm in adrenocortical cGMP (fasciculata-reticularis zona) of mature male Wistar rats hypophysectomized 10 days before (cf. legend to figure 1). ·—· Raw data (chronogram) with time point mean±SEM in pmole cGMP/mg dry weight, --- Cosinor analysis: best fitting sine function with period $\tau = 24$ h.

Peak-trough differences are statistically significant with $p < 0.02$ for cAMP and $p < 0.05$ for cGMP. The results obtained from raw data were confirmed by Cosinor analysis (table and figures 1 and 2). Statistically significant circadian rhythms were validated for both variables (amplitude differing from zero with $p < 0.005$). The peaks are located at about 05.30 and 08.00 for cyclic AMP and cyclic GMP respectively, that is, at the end, or just after the end of the darkness period. The amplitudes expressed as a percentage of the mesor are comparable for both nucleotides, equalling 9.5% for cAMP and 13.7% for cGMP. Since the cyclic nucleotide concentrations have been expressed as a function of dry weights, these were verified to be constant throughout the experiment.

Discussion. As the adrenocortical dry weights remain constant throughout the nycthemere, it can be inferred that the circadian variations observed reflect a true modification of the cyclic nucleotide concentrations. Expressing the results as a function of adrenocortical protein concentrations (not shown here), gives comparable results. The somewhat surprising finding of a circadian variation of the adrenocortical cyclic nucleotides in hypophysectomized rats raises some unresolved questions about the origin and role of these cyclic nucleotides in the adrenal cortex. The persistence of rhythmic adrenal activity has been noted in hypophysectomized rats implanted with pellets of ACTH and thyroxine[6]. Although not much attention has generally been paid to the relationship between the nervous system and the adrenal cortex, some experiments indicate such a link. Denervation of the rat adrenal has been shown to diminish the elevation of cAMP induced by stress[7]. In rats with adrenal autotransplants, daily variations in plasma corticoids are no longer observed[8]. Following these observations, some authors have postulated the existence of a neural pathway to the adrenal which would regulate the circadian variation of adrenal steroidogenic activity[8]. On the other hand, persistence of circadian rhythms has been demonstrated in adrenal tissue cultures[9,10]. Whatever the mechanism involved in the present findings, the demonstration of a circadian variation of adrenocortical cyclic nucleotides in hypophysectomized rats is an additional proof of at least a partially extrapituitary regulation of the circadian rhythm in adrenocortical function.

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High affinity binding of labeled androgens in the androgen-target tissues of the male rhesus monkey[1]

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Summary. High affinity testosterone (T)-specific and dihydrotestosterone (DHT)-specific binding sites exist in a 1:2 ratio in the cytosol fractions of the caput epididymidis, prostate, seminal vesicles and the ductus deferens of the rhesus monkey. The number of androgen-binding sites in the caput epididymidis is 3 times greater than that of the other 3 tissues.

The interaction of a steroid hormone with a cytoplasmic macromolecular receptor in the target cell is one of the earliest events recognized in the mechanisms of steroid hormone action. In an earlier study on the androgen metabolism in the monkey[3,4], we had reported that after in vivo treatment with ^{3}H-testosterone, the tissue-bound an-

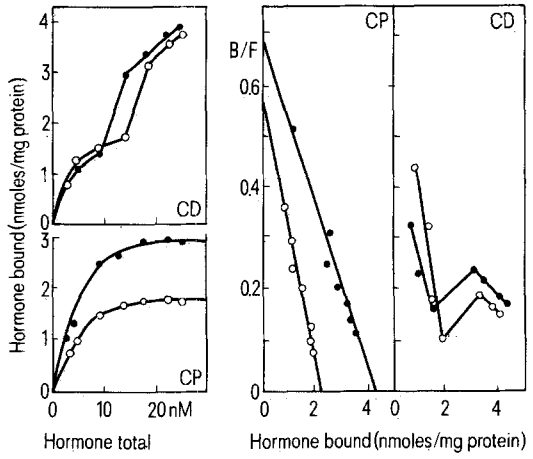

Fig. 1. Left. Equilibrium binding (specific) of testosterone (−○−) and DHT (−●−) to cytosol macromolecules of the caput (CP) and cauda (CD) epididymides. Right. Scatchard plot of the same data.

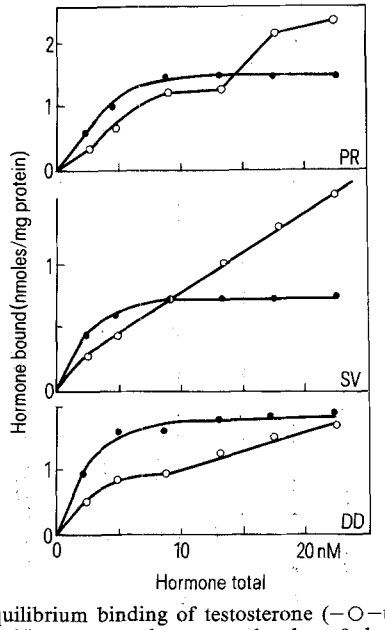

Fig. 2. Equilibrium binding of testosterone (−○−total) and DHT (−●−specific) to cytosol macromolecules of the prostate (PR), seminal vesicle (SV) and the ductus deferens (DD).

drogen metabolite pattern of the epididymis was found different from that of the accessory glands. 1 h after the labeled hormone treatment, the epididymis contained T and DHT in a 1:2 ratio while in the prostate, seminal vesicles and the ductus deferens more than 85% of the radioactivity was found associated with DHT. This pattern persisted even after the total decline in the levels of circulating labeled androgen. This observation raised the possibility that specific testosterone and DHT-receptors might be present in the epididymis while the androphilic proteins of the other three tissues remained mainly DHT-specific. The present study was carried out in order to examine this hypothesis.

Materials and methods. 1,2-^{3}H-T (sp. act. 40 Ci/mM) and 1,2-^{3}H-DHT (sp. act. 40 Ci/mM) were obtained from New England Nuclear. Sub-adult male rhesus monkeys were castrated 2 months before they were used in experiments. The tissues, at autopsy, were cleared of fat and other extraneous matter and homogenized in 10 vol. of TEM buffer (0.05 M Tris-HCl, pH 7.6, 0.0015 M disodium EDTA

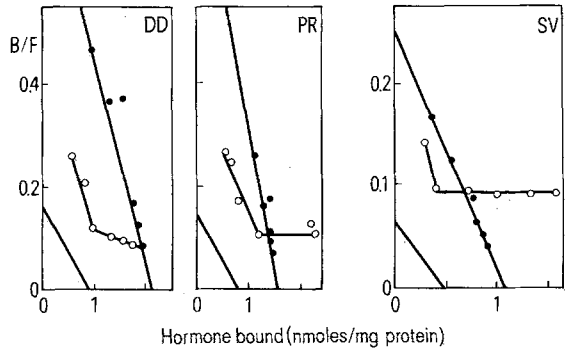

Fig. 3. Scatchard plots of the data given in figure 2. The solid line towards the bottom left indicates specific binding of testosterone after deducting values of non-specific binding from the original data.

and 0.0015 M β-mercaptoethanol). After an initial centrifugation of the homogenate at 10,000 rpm for 10 min, the supernatant was re-centrifuged at 105,000 × g for 90 min. Solid $(NH_4)_2SO_4$ was added to the cytosol to give 40% saturation. The precipitate was redissolved in the original volume of TEM buffer and dialysed against the same overnight. For in vitro binding studies, the Sephadex gel equilibration method of Pearlman and Crepy[5] was followed. The following modifications were introduced: TEM buffer, pH 7.6 was the medium employed and incubations were carried out at 4 °C for 2 h. Duplicate samples were incubated in the presence of a 100-fold excess of the respective unlabeled androgen, in order to calibrate the non-specific androgen binding levels. Protein was estimated according to Lowry et al.[6]. Radioactivity in the aqueous samples was analyzed in a mixture of 1 ml ethanol and 5 ml scintillation fluid (50 mg POPOP and 5 g PPO in 1 l distilled toluene). More than 95% of the bound radioactivity was recovered in the form of the respective unmetabolized androgen on TLC analysis and recrystallization to constant specific activity.

Results and discussion. High affinity binding of both T and DHT is demonstrated in the caput and cauda epididymides (figure 1). The Kd-values obtained for T and DHT binding in the caput cytosol were 0.262 nM and 0.157 nM, respectively. The numbers of binding sites specific for T and DHT appear in a 1:2 ratio (2100 and 4200 pmoles/mg protein, respectively). The saturation curve for androgen binding in the cauda epididymidis was biphasic. The binding constants were not calculated from the non-linear Scatchard plots[7]. However, it may reasonably be assumed that this cytosol fraction contains at least 2 classes of binding sites with varying affinities for T and DHT. The Kd-values calculated for DHT binding in the prostate, seminal vesicles and ductus deferens were 0.544, 0.235 and 0.414 nM, respectively (figures 2 and 3). The respective n-values (number of binding site in pmoles/mg protein) were 1600, 1100 and 2100. The high affinity T-binding in these 3 tissues (Kd: 0.188 nM, 0.156 nM and 0.194 nM, respectively) involved a much reduced number of binding sites (800, 450 and 850 pmoles/mg protein, respectively).

We have taken care to prevent androgen binding macromolecules of testicular origin from appearing in epididymal homogenates even though the presence of an androgen binding protein (ABP) in the rhesus monkey has not so far been reported. The prolonged period of castration did not eliminate the capacity of the tissues to metabolize and bind androgens, possibly indicating that the amount of circulating androgen present in the monkey during the postcastration period[8] is enough to maintain the receptors in a viable condition. An alternative hypothesis suggested by Sullivan

and Strott[9] indicates that an androgen-independent mechanism is responsible for the post-castrational restoration of androgen-receptors. Preliminary observations (unpublished) show that about 30% reduction in T-binding is effected by a 100-fold excess of DHT and vice versa, in both epididymis and the accessory glands. This may be construed as evidence for an apparent overlap existing between T-specific and DHT-specific binding sites. 5α-androstanediol inhibits labeled DHT-binding, probably due to its conversion to DHT by 3α-reductase. These results indicate that the androgen response created in a tissue is a net result of the combined action of 2 or more androgen metabolites.

Wilson[10] demonstrated an interesting correlation that exists between embryonic differentiation and metabolism of testosterone by different organs of the reproductive system. The Wolffian duct which differentiates to form the epididymis, ductus deferens and seminal vesicles develops the capacity to metabolize testosterone very late during the process of differentiation while the urogenital sinus which later undergoes differentiation to produce the prostate has the T:DHT equilibrium shifted towards the latter. The role of T as an essential factor for Wolffian duct differentiation is thereby indicated while more of DHT than of T is involved in the development of urogenital sinus. It is interesting to note that the fully differentiated epididymis retains a greater amount of testosterone than that done by the other 3 tissues. The ductus deferens and the seminal vesicles at the same time show a metabolite pattern similar to that of the prostate, a urogenital sinus derivative. The significance of this phenomenon is yet to be ascertained.

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PRO EXPERIMENTIS

A method for the determination of free neuraminic acid split from red blood cell receptors by attached Newcastle disease virus during simultaneous elution and hemolysis

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Summary. Determination of free neuraminic acid in chicken red blood cell (RBC) hemolysate becomes possible after deproteinization of the hemolysate by ethanol-chloroform followed by removal of the solvents by evaporation. This procedure permits the determination of in situ neuraminidase activity of virions preadsorbed on RBC receptors when the virus elution and hemolysis proceed simultaneously.

The interaction of myxoviruses with RBC includes attachment of virions to cell receptors at 4 °C and their elution at a temperature optimum of 37 °C[1,2]. The latter process is due to enzymatic action of viral neuraminidase which splits the N-acetyl-neuraminic acid (NANA) moiety from cell receptor glycoproteins[3-8]. However, the question arises whether there is a quantitative correlation between virus neuraminidase (Nase)activity in vitro (using appropriate substrate) and in situ Nase activity of the virus preadsorbed on RBC at 4 °C and eluting at 37 °C. The experimental approach to the problem is based on direct determination of free NANA accumulated in eluate as a result of in situ Nase action of the preadsorbed virus splitting NANA from RBC receptors[8]. However, this approach meets with an obstacle when paramyxoviruses, inducing hemolysis (Newcastle disease virus, in particular), are used. Preliminary data have suggested that in this case hemoglobin accumulated in eluate as a result of hemolysis interferes with the thiobarbituric method of determination of NANA accumulated in the same eluate as a result of Nase activity of eluting virus. This was demonstrated by mixing hemoglobin obtained from osmotic hemolysis of chicken RBC with pure NANA (figure 1). In the present paper, a method for determining free NANA accumulated in the NDV-RBC system as a result of elution which proceeds simultaneously with hemolysis is described.

Materials and methods. The avirulent strain 'Queensland' of NDV, grown in embryonated eggs was used. The virus was partially purified by differential centrifugation at 5000 × g and at 50,000 × g. The pellet was resuspended in 1/20 of the original volume, then treated with fluorocarbon 113 (1:1 v/v) for 1 min and centrifuged at 7500 × g for 10 min. The virus in the upper phase was used. The virus was adsorbed on fowl RBC (15% final concentration) for 30 min at 4 °C. The cells were centrifuged at 4 °C, resuspended in cold saline and immediately transferred to 37 °C. Samples were taken at intervals and centrifuged immediately in an Eppendorf centrifuge at 12,000 rpm for 1 min at 4 °C. Eluted virus, free NANA and hemoglobin were determined in the supernatants. Eluted virus was determined by hemaggluti-

Recovery of NANA from mixtures with different concentrations of hemoglobin

Red blood cell concentration (%)	Hemoglobin (A_{540})	N-acetyl-neuraminic acid recovered (A_{549}*)
0**	-	0.63
1	1.45	0.61
3	4.35	0.63
5	7.9	0.66
8	12.6	0.64
12.5	20.7	0.63

NANA (25 μg) was added to each hemolysate preparation and treated with ethanol-chloroform as described above. * Values after subtracting A_{549} values of hemolysate preparations without NANA. ** Untreated control-NANA in water.