with previously uninjected control groups.

Summary. 1) The effects of secondary injections of various soluble protein antigens, subsequent to disappearance from the circulating system of antibodies to a primary antigen, are described. 2) Secondary antigen injections do not elicit antibody responses to previously injected antigens, if the antigens are unrelated. 3) Quantitative levels of antibody response to secondary antigens are apparently unaffected by previous antigenic stimulations of the animal, if the antigens are unrelated.

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Nychthemeral Variations in Plasma Free Corticosteroid Levels of the Rat.* (24955)

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Measurements of "resting levels" of plasma corticosteroids in a series of experiments in the rat relating to CRF activity *in vivo*(1) suggested a diurnal rhythm in this index of adrenocortical function. If (diurnal) variations were as large as we suspected, it was important to obtain an accurate level/time relationship, and an exact estimate of extremes of these variations. No report on similar investigations has been found in the literature, owing probably to recent availability of methods to measure plasma corticosterone levels in the rat.

Materials and methods. A group of 100 animals (rats, males, Holtzman Farms) were housed in our animal quarters (illuminated with daylight only) 40 days prior to start of experiment. On day of experiment (Jan. 31 to Feb. 1), one animal at a time was taken from a different cage and was decapitated within 20 seconds. Sacrifice of animals (B.W. 200-230 g) was done outside animal quarters, at 12 noon, 4 p.m., 8 p.m., 12 midnight, 4 a.m., and 12 noon. Blood was collected in 50 ml beakers containing 0.01 ml of heparin solution 1/1000, and samples taken for hematological studies. Total white cell counts were done with routine laboratory methods. Air-dried blood specimens were stained with Wright-Giemsa solution, and 500 leukocytes identified for each differential count. The absolute number of eosinophils, neutrophils, lymphocytes, and monocytes/mm^3 of blood was calculated from total white cell and differential counts. Calculated eosinophil values determined in this manner are comparable to those obtained by direct counting methods (Higgins, G.M., personal communication).

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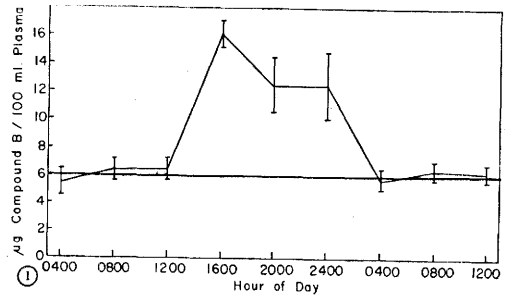

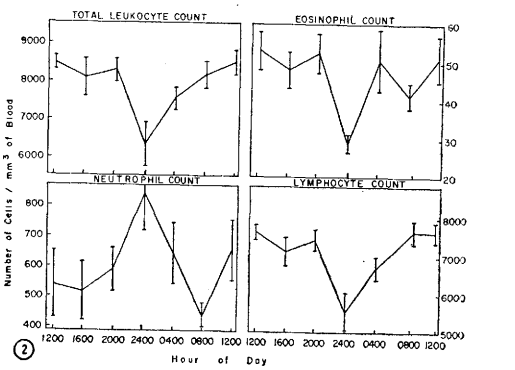

FIG. 1. Resting levels of plasma free corticosteroids at various times of day.

FIG. 2. White cell counts in peripheral plasma at various times of day.

Measurements of plasma-free corticosterone levels were done on 0.5 ml plasma samples as described by Guillemin *et al.*(2,3). Results are expressed in µg-corticosterone/100 ml plasma.

Results. Fig. 1 shows that striking variations occurred in concentrations of plasma free corticosteroids. With animals of this strain, body weight range, at this time of year and with schedule followed here for blood sampling, the highest concentration of plasma corticosteroids was observed at 4 p.m. with an elevated level persisting till midnight. In the rat, allegedly a "nocturnal" species, the nychthemeral rhythm appears to have an image inverted from the model known to exist in the human. Confirming other reports(4,5,6), variations were also noted in total white cell count (Fig. 2). Eosinopenia, lymphopenia, and neutrophilia at midnight are of interest in view of similar well documented responses of the peripheral blood elements following administration of ACTH or adrenal corticosteroids or exposure to stressful stimuli. The peak of leucopenia is not "in phase" with that of corticosteroid concentration, a fact best explained by the well known causative relationships between the 2 phenomena.

The literature on 24-hour periodicity of biological phenomena and particularly of pituitary-adrenal function is considerable (see review(7) by Halberg for references to 1953).† More recently evidence has been presented involving various structures of the limbic system in integration of cyclicity of the adrenocorticotrophic function of anterior pituitary (8). These studies were done on monkeys. The data reported here indicate that the rat may be used for similar studies since sensitive methods are available for measurement of plasma corticosteroids in this species. These data also add another reason to be considered in the care necessary(2) to obtain reliable "resting levels" of pituitary adrenal function in the rat.

Summary. Nychthemeral variations in levels of plasma free corticosteroids in the rat have been observed, with highest concentrations of steroid between 4 p.m. and midnight. The magnitude of these cyclic variations is such that they will have to be considered in measurement of "resting levels" of plasma corticosteroids in this species.

† Since this paper was submitted for publication, Halberg *et al.*(9) reported studies in mouse similar to those described here and with similar conclusions.

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