Effects of Diurnal Variation in Plasma Corticosterone Levels on Adrenocortical Response to Stress.* (32172)

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Many species demonstrate circadian rhythmicity in pituitary-adrenal function in the absence of applied stress(1). In the female rat, daily fluctuations in peripheral plasma concentrations of corticosterone are especially large(2). Although negative feedback is implicated in the control of pituitary-adrenal function, the effect of these conspicuous physiological excursions in "background" levels of circulating corticosterone on the acute response to stress has received little attention. The purpose of the experiments reported here was to compare increments in plasma corticosterone concentrations produced by stress applied at times of day when, under the conditions used, plasma levels of corticosterone differ markedly(2).

Materials and methods. All experiments were performed using adult Sprague-Dawley female rats (Charles River) weighing 200-300 g. Each study was limited to animals of the same shipment that had been grouped 3/cage for at least 3 weeks under conditions of controlled lighting (fluorescent illumination from 04:00-18:00) and temperature (26 ± 1C). Purina Laboratory Chow and tap water were available *ad libitum*. Three days before each experiment, animals were transferred to individual cages. Vaginal smears were taken each morning for 3 days prior to the day of an experiment. To standardize conditions for studying pituitary-adrenal activity in undisturbed rats, the animal quarters were not entered during an 18-24-hour period preceding each experiment. In all studies, rats were taken individually to an adjoining room where treatment and collection procedures were begun approximately 15 seconds following onset of stress. Onset of stress is defined as the time of cage opening.

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Latency of acute rise in corticosterone levels following exposure to ether. To test the feasibility of studying stress-induced increments in plasma corticosterone levels in individual rats by means of a double bleeding procedure, 6 groups of 6 rats were decapitated at 15 seconds or 2, 4, 8, 15, and 30 minutes following onset of an ether stress procedure. A single sample of blood was collected in a heparinized beaker from each rat decapitated 15 seconds or 2 minutes following onset of stress. Each rat killed 4 minutes or longer after onset of stress was bled twice as follows: subsequent to complete anesthesia (approximately 1 minute after exposure to ether vapor), a skin incision was made to expose an external jugular vein and 1.5 ml of blood was drawn into a heparinized syringe. This initial blood sample was collected within 3 minutes from time of cage opening. At 3 minutes, exposure to ether was terminated and each rat was placed in a separate cage situated in the same room. Although this procedure is designated as ether stress, it included such additional stress stimuli as handing, placement in a novel environment, skin incision, superficial neck dissection, acute loss of 1.5 ml of blood, and hypoxia due to 3 minutes exposure to ether. Decapitation was performed at the times indicated and a sample of trunk blood was collected from each rat. All blood was immediately centrifuged and plasma saved. Both adrenals were promptly removed, cleaned, weighed to the nearest 0.2 mg, and ground in cold alcohol-saline. Corticosterone concentrations in plasma and adrenals were determined by the fluorometric method of Guillemin *et al*(3). No correction was made for residual fluorescence. However, the fluorescence obtained in this laboratory in plasma from adrenalectomized female rats is equivalent to 6.6 ± 0.6 μg corticosterone/100 ml plasma (mean ± S.E., 15 determinations). The experiment began at 08:30 and lasted 2 1/2 hr.

Morning and afternoon responses to ether stress. To compare increments in plasma corticosterone levels induced by stress applied at different stages in the 24-hour light-dark cycle, 10 rats were subjected to 3-minute ether stress at 08:30 and 10 others received the same treatment at 16:30 on the same day. One hour was needed to complete all procedures at each of these times. Each rat was taken from its cage, exposed to ether vapor, and 1.5 ml of blood was obtained from an exposed external jugular vein in less than 3 minutes from time of cage opening. Following 3-minute exposure to ether, each rat was transferred to a separate cage and decapitated 12 minutes later. Following decapitation, a second sample and adrenals were collected and processed as described above.

Morning and afternoon responses to immobilization stress. To compare morning and afternoon increments in corticosterone levels following another type of stress, an immobilization procedure was employed using the time considerations described above with ether stress. Ten rats were removed from individual cages and rapidly immobilized in the supine position at either 08:30 or 16:30 on the same day. Procaine (1%, 0.5 ml) was injected subcutaneously over an external jugular vein. Approximately 30 seconds later, the vein was exposed. An initial 1.5 ml blood sample was obtained in less than 3 minutes following cage opening. After 3-minutes immobilization, each rat was placed in a separate cage. Twelve minutes later, *i.e.,* 15 minutes after onset of stress, decapitation was performed, trunk blood was collected, and adrenals were removed. Plasma samples and adrenals were processed as described above. Each sampling period lasted approximately 1 hour. As with the ether stress described above, this procedure probably involves many stress stimuli.

The difference between corticosterone levels in first and second plasma samples from each rat was calculated and used as an index of the pituitary-adrenal response to stress. Statistical probabilities were determined by analysis of variance(4) performed by the Common Research Computer Facility.§

Results. Latency of acute rise in corticosterone levels following exposure to ether. As shown in Fig. 1, plasma and adrenal concentrations of corticosterone 2 minutes after initiation of ether stress did not differ from those of rats decapitated before exposure to ether. Four minutes after cage opening, a significant increase ($P<0.01$) was evident in adrenal but not in plasma levels. Both plasma and adrenal corticosterone concentrations were elevated ($P<0.01$) at 8 minutes. While plasma levels were highest at 15 and 30 minutes, concentrations in adrenals showed no increase above those present 8 minutes following onset of stress. Corticosterone levels in initial plasma samples obtained during 3-minute exposure to ether in groups killed 4, 8, 15, and 30 minutes after stress did not differ significantly from those obtained by decapitation before exposure to ether.

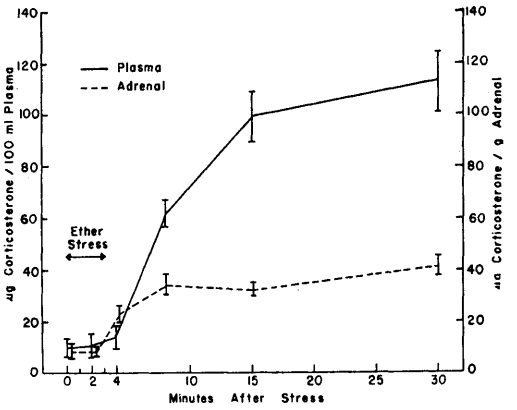

FIG. 1. Time-course of acute plasma and adrenal corticosterone responses associated with 3-min ether exposure and rapid sampling of jugular blood. Each point represents mean values for 6 rats; vertical lines indicate ± standard error.

Morning and afternoon responses to ether stress. Concentrations of corticosterone in peripheral plasma collected during 3-minute exposure to ether were higher ($P<0.05$) at 16:30 than at 08:30 (Fig. 2). Likewise, corticosterone concentrations in plasma obtained 15 minutes following onset of stress were higher ($P<0.05$) in the afternoon than in the morning. However, as shown in the summary of the analysis of variance (Table I), increments in corticosterone levels did not differ significantly at the two times of day. Of the

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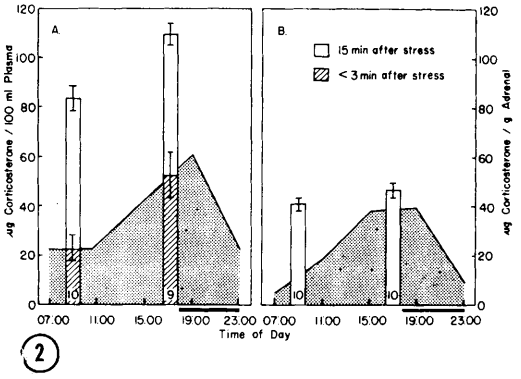

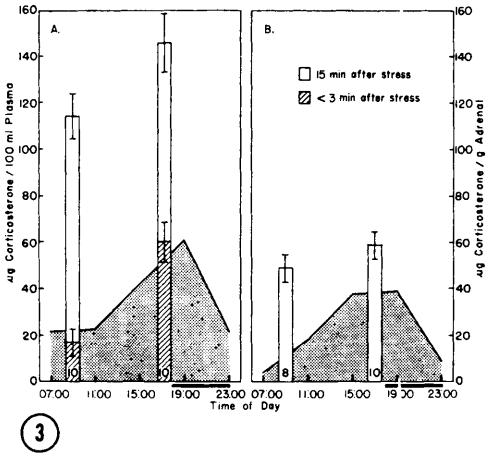

FIG. 2. Effect of diurnal variation in corticosterone levels on plasma (A) and adrenal (B) corticosterone responses to 3-min ether stress in female rats. Stippled areas indicate previously reported circadian patterns of corticosterone levels under conditions used in present sudies (2). The horizontal black bar indicates the first portion of the daily dark period; figures in columns denote number of rats and vertical lines represent ± standard error.

FIG. 3. Effect of diurnal variation in corticosterone levels on plasma (A) and adrenal (B) corticosterone responses to 3-min immobilization stress in female rats. See Fig. 2 for key to notations and symbols.

total plasma concentration of corticosterone 15 minutes after application of stress in the afternoon (115 μg/100 ml), approximately 50% was attributable to the effect of ether stress.

Morning and afternoon responses to immobilization stress. As summarized in Fig. 3 and Table II, levels of corticosterone in plasma obtained in less than 3 minutes after onset of stress were higher ($P<0.01$) at

TABLE I. Analysis of Variance for Morning *vs* Afternoon Adrenocortical Responses to Ether Stress.

	Source of variation	df	MS	F	P
Plasma corticosterone at <3 min	Time of day	1	4362.573	7.10	<.05
	Error	17	614.697		
Plasma corticosterone at 15 min	Time of day	1	3145.434	16.78	<.01
	Error	17	187.426		
Increment in plasma corticosterone	Time of day	1	99.313	.20	N.S.
	Error	17	493.149		
Adrenal corticosterone at 15 min	Time of day	1	196.565	2.95	N.S.
	Error	18	66.572		

TABLE II. Analysis of Variance for Morning *vs* Afternoon Adrenocortical Responses to Immobilization Stress.

	Source of variation	df	MS	F	P
Plasma corticosterone at <3 min	Time of day	1	9370.120	16.50	<.01
	Error	18	567.821		
Plasma corticosterone at 15 min	Time of day	1	5037.141	3.78	N.S.
	Error	18	1332.865		
Increment in plasma corticosterone	Time of day	1	667.012	.76	N.S.
	Error	18	875.346		
Adrenal corticosterone at 15 min	Time of day	1	437.208	1.31	N.S.
	Error	16	334.109		

16:30 than at 08:30. Levels following stress did not differ significantly at the two times of day. Nevertheless, as observed with ether stress, increments in corticosterone levels resulting from immobilization at 08:30 did not differ from those following this procedure at 16:30. Again, the plasma concentration of corticosterone present prior to stress in the afternoon represents a major portion (approximately 45%) of the total concentration existing 15 minutes following initiation of stress.

Similar to the results obtained using ether stress, adrenal concentrations of corticosterone 15 minutes after onset of immobilization at 08:30 did not differ significantly from those collected at 16:30 (Fig. 3 and Table II).

Discussion. The results of the first experiment indicate a latency of approximately 4 minutes between exposure to ether and the associated increase in concentration of corticosterone in peripheral plasma. Cann *et al*(5) reported similar results following ether stress and, in unpublished studies, we observed a comparable latency following immobilization stress. Because of this latency and under the conditions used in these experiments, corticosterone concentrations in plasma obtained in less than 3 minutes following onset of stress are designated as "non-stress" and are regarded in the discussion below as an index of the level of pituitary-adrenal function existing before application of stress. If concentration of corticosterone in plasma 15 minutes after onset of stress is considered as the "stress" level, the difference between "non-stress" and "stress" levels provides a useful measure of the acute pituitary-adrenal response to stress.

Physiological variations in "non-stress" levels of plasma corticosterone at different periods in the 24-hour light-dark cycle appear to exert little or no effect on acute adrenocortical responses to the two stress procedures used. In spite of marked differences (approximately 40 μg/100 ml) between morning and afternoon "non-stress" levels, similar stress-induced increments in concentration of plasma corticosterone were observed whether stress was applied in the morning when "non-stress" levels were low or in the late afternoon when such levels were relatively high.

These results suggest that acute plasma corticosterone responses to 3-minute ether and immobilization stresses are independent of endogenous variation in "non-stress" levels of this steroid in the female rat. Ader *et al*(6), using male rats, obtained similar results with open field but not with handling stress. Although different stresses were used, the results of the present studies are compatible with those of Hodges and Jones(7) and Smelik(8). These investigators demonstrated that administration of corticosterone in amounts which produced high but physiological levels in peripheral plasma did not suppress corticosterone responses to histamine and laparotomy stresses. The present findings, however, are not consistent with the report of Haus(9) which described periodicity in mouse serum and adrenal corticosterone responses to injection of saline or ACTH.

Although "non-stress" levels of corticosterone in adrenals were not determined in the present morning-afternoon stress studies, adrenal levels in female rats, under conditions used in these experiments, show a considerable difference between morning and afternoon levels. In the morning, "non-stress" levels are approximately 10 μg/g; concentrations in the afternoon are normally 3-4 times higher(2). Because glandular concentrations 15 minutes following initiation of ether or immobilization stress did not differ significantly between morning and afternoon, it appears, unlike the situation in plasma, that stress-induced increments are smaller during the diurnal peak in pituitary-adrenal function than during the trough. Despite the apparent failure of stress applied at 16:30 to produce an increment in adrenal levels of corticosterone equivalent to that produced at 08:30, and assuming that peripheral clearance mechanisms play a minor role under the acute conditions of these studies, the unimpaired stress-induced rise in plasma corticosterone levels in the afternoon suggests that stress results in similar increases in rate of corticosterone secretion at these two times of day. It seems that 50-60 μg/g represents maximal adrenal concentration of corticosterone in the intact female rat. Apparently, stress applied at a time of day

when glandular concentrations normally approach these levels in undisturbed rats produces an increase in rate of corticosterone secretion without concomitant elevation in adrenal concentration of this steroid. These findings suggest that at high but physiological levels of adrenocortical activation, adrenal concentration of corticosterone cannot be used unconditionally as an index of degree of stimulation by ACTH or of adrenal secretory rate.

In so far as increments in peripheral plasma corticosterone levels following stress reflect acute increases in pituitary secretion of ACTH, the present studies suggest that the amount of ACTH secreted by the female rat in response to 3-minute ether or immobilization stress is quantitatively similar at the two times of day tested, even though marked differences exist in "non-stress" levels of corticosterone. However, in view of possible 24-hour variations in adrenal responsivity to ACTH(9), this possibility requires verification with assays of plasma ACTH.

Superimposition of similar stress-induced increments in plasma levels of corticosterone upon varying "non-stress" levels suggests that in the female rat there is little or no interaction between mechanisms regulating acute pituitary-adrenal response to stress and those responsible for circadian rhythmicity in "non-stress" pituitary-adrenal function. Because considerable evidence indicates that the nervous system is primarily responsible for both the circadian rhythm and acute responses to stress stimuli, these findings point to a possible functional dissociation of neural mechanisms underlying these two modes of pituitary-adrenal activation. Such dissociation was reported previously by Slusher(10) on the basis of effects of hypothalamic lesions in male rats. Also consistent with this functional separation, preliminary studies in this laboratory have shown that relatively low doses of dexamethasone administered subcutaneously (11) or intracerebrally(12) to female rats selectively suppress "non-stress" levels of corticosterone without impairing acute plasma corticosterone responses to several types of stress. If separate mechanims regulate circadian and stress patterns of pituitary-adrenal functions, their respective contributions to total concentration of corticosterone in plasma are, under certain temporal conditions, essentially equal; approximately one-half of total circulating corticosterone existing 15 minutes after stress applied in the afternoon was present prior to the stress procedure.

Summary. Adult female rats were subjected to 3-minute ether or immobilization stress during the trough (08:30) or peak (16:30) of the circadian rhythm in adrenocortical function. Two samples of peripheral plasma were obtained from each rat for fluorometric determination of corticosterone concentrations; the first was collected in less than 3 minutes and the second at 15 minutes following initiation of stress. Adrenals were also collected 15 minutes after stress for determination of corticosterone levels. Corticosterone concentrations in the first or "non-stress" plasma samples collected in the afternoon were 2-3 times higher than those obtained in the morning. Nevertheless, stress-induced increments in steroid levels did not differ significantly at the two times of day. Although adrenal concentrations of corticosterone 15 minutes following onset of stress in the morning were similar to those following stress in the afternoon, the changes observed in plasma suggest that acute pituitary-adrenal responses to the types of stress used are not altered by marked diurnal variation in plasma corticosterone levels.

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The Influence of Acetyl-Salicylic Acid on Growth and Some Respiratory Enzymes in Broiler Chicks.* (32173)

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The enzyme, succinic dehydrogenase, was observed to be significantly inhibited by salicylate in *in vitro* studies(1). A serum salicylate level of 137 mg/100 ml had no effect on hexokinase, cytochrome oxidase or DPNH-cytochrome c reductase(2). *In vitro* studies with rat liver mitochondria indicated that malate and isocitric dehydrogenase activities were inhibited by salicylate(3,4). An additional study has demonstrated that acetyl salicylic acid fed at levels of 0.005-0.08% of the diet failed to produce a significant difference in growth rate or feed conversion in 3 chick studies(5).

New Hampshire X Delaware chicks were fed 0.3, 0.6 and 0.9% acetyl-salicylic acid (ASA) in addition to the basal diet (Table I). Each dietary treatment was replicated 5 times, and each replicate employed in the experiment consisted of 4 males and 4 females. All chicks were reared for 4 weeks in electrically-heated chick batteries with raised wire floors, and feed and tap water were supplied *ad libitum*. Feed and body weights were determined for each replicate group at the beginning and end of the experiment. At 4 weeks of age, whole blood samples, obtained by cardiac puncture, from 5 males and 5 females per treatment, were heparinized, centrifuged and frozen for analyses. The same birds were sacrificed; livers and kidneys were removed and wrapped in foil and quick-frozen. Isocitric dehydrogenase activities of the plasma were determined by the method of Wolfson and Williams-Ashman(6). Succinic dehydrogenase and cytochrome oxidase activities of the livers and kidneys were determined by the methods of Cooperstein *et al*(7) and Cooperstein and Lazarow(8). Enzyme activities were measured with a Beckman Model B spectrophotometer and the data recorded on a coupled Bausch and Lomb V.O. M-5 strip chart recorder. Data obtained from these tests were subjected to an analysis of variance as outlined by Snedecor(9), and differences between treatment means were tested by the multiple range test of Duncan(10).

TABLE I. Composition of Basal Diet.

Ingredients	%
Ground yellow corn	50.75
Dehydrated alfalfa meal (17% protein)	5.00
Soybean meal (45% protein)	15.00
Meat and bone scraps (50% protein)	5.00
Fish meal (65% protein)	7.50
Dried whey	5.00
Distiller's dried soluble	3.00
Hydrolyzed animal and vegetable fat	5.00
Calcium carbonate	.50
Dicalcium phosphate	.50
Salt (trace mineralized)	.25
Manganese sulfate pentahydrate (70%)	.20
Vitamin premix*	2.50
Total	100.20

* Vitamin mix supplied the following per kg of diet: 9,900 I.U. vit. A, 1,540 I.C.U. vit. D_3, 4.4 mg riboflavin, 27.5 mg niacin, 11.0 mg D-calcium pantothenate, 930.0 mg choline chloride, 13.2 μg vit. B_{12}, 5.5 I.U. alpha tocopheryl acetate, 2.2 mg menadione sodium bisulfite, 124.85 mg ethoxyquin (as a preservative) and 18.48 g soybean meal as carrier.

Average growth rates of both sexes were depressed stepwise with increasing level of ASA (Table II). The growth rate was significantly ($P<0.01$) depressed with either 0.6

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