of 0.9% NaCl. The mice were decapitated on the 7th day, blood was collected, and the RNA content in 0.1 ml of erythrocytes was measured. Activity of the test material was determined from a calibration curve obtained with a known dose of erythropoietin (Fig. 3). As Fig. 3 shows, the first point (a dose of 0.05 unit of the erythropoietin standard) for the method with radioactive iron differs significantly from the control (0.49 ± 0.06%, $P < 0.05$). Smaller doses of the erythropoietin standard under the same conditions no longer differ significantly from the control. Consequently, the sensitivity of the method with radioactive iron is 0.05 unit of erythropoietin, in good agreement with data in the literature [5].

For the method of testing based on the RNA content in the erythrocytes, under the same conditions, the first point – a dose of 0.01 unit of erythropoietin – differs significantly under the same conditions from the control (219.8 ± 1.87 μg, $P < 0.01$). When the suggested method is used, sensitivity is thus increased fivefold.

Moreover the main advantage of the suggested method of testing erythropoietic activity is its simplification as a result of doing away with the need to work with a radioactive label, so that the method is capable of wide application in clinical and laboratory practice.

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EFFECT OF MORNING AND EVENING CORTISOL INJECTIONS ON CIRCADIAN RHYTHMS OF 11-HYDROXYCORTICOSTEROID EXCRETION IN RATS

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In modern life man frequently encounters factors which disturb the circadian rhythm of glucocorticoid secretion. Such factors include night work, flexible shift work, sleep disturbances, flights across time zones, steroid therapy, and so on. Investigations have shown a connection between the therapeutic effects of corticosteroids and the time of their administration [8, 11, 12]. However, the biorhythmologic aspects of the pharmacodynamics of glucocorticoids caused by their long-term administration or stimulated by stress, whose metabolic action depends essentially on relations with rhythms of excretion of other hormones [9], have received insufficient study.

This paper describes a study of circadian rhythms of 11-hydroxycorticosteroid (11-HCS) excretion with the urine during injection stress and administration of cortisol at different times (morning and evening).

EXPERIMENTAL METHOD

Male Wistar rats weighing 200 g were used. The animals were kept five in a cage at a temperature of 24 ± 1°C, with artificial lighting from 8 a.m. to 8 p.m. and with access to food and water ad lib. A microcrystalline suspension of hydrocortisone acetate (from Richter, Hungary) was injected intramuscularly once a day

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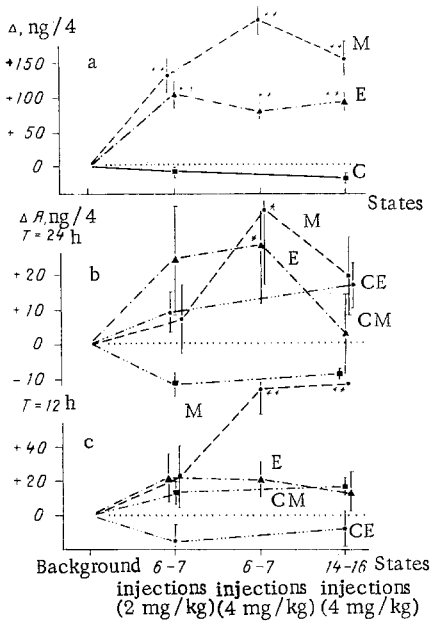

Fig. 1. Urinary 11-HCS excretion of rats: changes in mean 24-h level (a) and amplitudes (b, c) during long-term administration of cortisol or physiological saline (M ± m). "Cosinor" analysis. M) Morning injection of cortisol (8 a.m.), E) evening injection of cortisol (8 p.m.), C) control, morning or evening injection of physiological saline (CM + CE). Asterisk indicates that difference from background value is significant at $P \leq 0.05$ level, ** – the same at $P \leq 0.01$.

at 8 a.m. or 8 p.m. Urine was collected for 4 h during the 24-h period in individual metabolism cages after a 12-h period of adaptation.

The animals were studied in four states: initial, after 6-7 injections of cortisol in a dose of 0.2 mg/100 g, then after 6-7 and 14-16 injections of the hormone in a dose of 0.4 mg/100 g. Control rats were given injections of physiological saline at the same times of morning and evening in a volume of 0.1 ml/100 g, and morning and evening injection stress was simulated in them. The experiments were carried out in January.

Cortisol was chosen as exogenous glucocorticoid because it belongs to the 11-HCS group and has a longer half-circulation time in rats than corticosterone [13].

Analysis of the urine, reflecting the circadian dynamics of glucocorticoids, is a preferable method because automatic urine collection avoids interference by the experimenter in the animal's daily routine. The factor of randomness, present when the concentration of gluococorticoids secreted in short high-amplitude episodes is determined [5, 7] is eliminated under these circumstances. It has also been shown [6] that the circadian rhythm of corticosterone in the urine of rats accurately reflects the amplitude-phase characteristics of its dynamics in the blood.

The 11-HCS concentration in the urine was determined by a fluorometric method [2]. The results were analyzed by the "Cosinor" program [1, 5]. In the course of the 24-h period six measurements were sufficient to estimate parameters of the 12-h harmonic and the circadian rhythm. By keeping the individual composition of the groups unchanged throughout the experiment, the paired t test could be used to calculate the significance of differences between states.

EXPERIMENTAL RESULTS

The following parameters of the rhythm of 11-HCS excretion were determined in 24 animals in the background state. The mean level for the 24-h period was 124 ± 4.3 ng/4 h, the amplitude of the 24-h harmonic

TABLE 1. 11-HCS Excretion with the Urine of Rats after Injections of Physiological Saline or Cortisol during Morning and Evening (grouped Cosinor analysis. Positions of acrophases, h; 95% confidence interval)

Experimental conditions (n = 24)	Number of injections	Harmonics	
		$T=24$h	$T=12$h
		23,4 (22,3—0,5)	10,5 (8,5—0,5)
Physiological saline at 8 a.m. (n = 4)	7	0,3	10,1
	23	3,0	11,2
Physiological saline at 8 p.m. (n = 4)	7	0,2 (20,1—5,5)	7,9
	23	1,2 (21,2—5,9)	6,8
Cortisol at 8 a.m. (n = 8)	6, each of 2 mg/kg	18,0	2,0
	6, each of 4 mg/kg	14,1 (11,8—20,3)	1,5 (0,8—2,9)
	14, each of 4 mg/kg	17,1	1,8 (0,2—3,3)
Cortisol at 8 p.m. (n = 8)	7, each of 2 mg/kg	1,9 (21,0—4,3)	2,0
	7, each of 4 mg/kg	3,4 (1,2—5,9)	0,4
	16, each of 4 mg/kg	3,8 (1,3—6,3)	11,2

Legend. Confidence intervals of acrophases given only for significant rhythms at the $P \leq 0.05$ level.

(with a 95% confidence interval) was 50.4 (37.6-63.3) ng/4 h, and the amplitude of the 12-h rhythm was 16.5 (1.6-29.4) ng/4 h.

Injection of physiological saline under both experimental conditions did not change the levels of hormone excretion (Fig. 1a). Evening injections led to an increase in the 24-h amplitude and a very small decrease in the ultradian harmonic (Fig. 1b, c). The opposite picture was observed after morning injections: extinction of the circadian rhythm was accompanied by an increase in amplitude of the 12-h variation. Although deviations of amplitude from the basal level were very small, the fact that changes in the groups compared were opposite in direction led to significant differences between them in each state.

Injection of cortisol in a dose of 0.2 mg/100 g, as might be expected, caused an increase in the mean 24-hourly level of excreted hormones (Fig. 1a). Doubling the dose caused a significant further increase in excretion after morning, but not after evening injections. This may be evidence that during cortisol loading at the end of the period of daylight compensatory processes aimed at rapid destruction of the exogenous hormone to physiologically inactive derivatives, not detectable in the 11-HCS group, develop more actively. Another possibility is activation of other, not lateral, pathways of glucocorticoid elimination.

The curves shown in Fig. 1 are evidence of a greater rise in the urinary 11-HCS level after morning administration of cortisol. Meanwhile numerous investigations have demonstrated the stronger inhibitory action of exogeneous glucocorticoids on the adrenal cortex when injected at the minimum of secretory activity [3, 10], which is attributable to increased sensitivity of the negative feedback mechanisms to increased concentrations of hormones.

This apparent contradiction disappears if the following explanation is adopted. Increased 11-HCS excretion during the morning against a background of depressed corticosterone secretion is evidence of the "unreadiness" of the metabolic systems to degrade increased quantities of glucocorticoids during the period of daylight – the phase of rest in rats, and as a result marked hypercortisolemia and increased excretion of the hormone through the kidney are observed.

Injection of cortisol into rats at the approach of darkness, superposed on a rise of corticosterone secretion, caused a significant increase in amplitude of the circadian rhythm. When the number of injections reached 23 (seven at 0.2 and 16 at 0.4 mg/100 g) the amplitude of the circadian rhythm returned to its background values (Fig. 1b) on account of activation of the mechanisms counteracting external interference with the hormonal status and reducing the nocturnal increase in excretion. These changes in the 24-h harmonic took place against the background of absence of a significant 12-h rhythm. A small increase in absolute values of the amplitude of the ultradian harmonic (Fig. 1c), observed in only five of eight individuals, was not statistically significant.

Six morning injections of cortisol in a dose of 0.2 mg/100 g caused arrhythmia – disappearance of significant fluctuations with both periods (Table 1). A daytime rise in excretion appeared, and later it increased significantly with doubling of the dose. In this state (six injections each of 0.4 mg/100 g) the considerable predominance of the diurnal over the nocturnal peak, the well-marked separation of the maxima, and the 12-hourly

interval between them caused, first, an increase in the amplitudes of both harmonics with a leading rise in the 12-h harmonic (Fig. 1b, c), and second, inversion of the circadian cycle – a shift of the acrophase to daytime (Table 1). The procedure described above also led to marked synchronization of the rats with respect to the 12-h harmonic, as shown both by visual analysis of scatter of the maxima of individual rhythms and by the small confidence interval for the acrophase (0.8-2.9 h).

In the next state (20 injections) the development of processes aimed at reducing the artificial diurnal hypercortisolemia and restoring the depressed evening secretion led to equalization of the diurnal and nocturnal maxima of 11-HCS excretion, disappearance of the significant 12-h harmonic, and a reduction in its amplitude (Fig. 1b).

This investigation showed that injection stress is a synchronizer of the secretory activity of the adrenal cortex; morning injections of physiological saline lead to an increase in the relative contribution of the 12-h harmonic to the rhythmic structure of 11-HCS excretion, evening injections lead to an increase in amplitude of the circadian rhythm. Long-term cortisol injections in the morning and evening have a similar action on excretion. During simulation of hypercorticism without any corresponding increase in demand for the hormone in the body, processes aimed at reducing the level and restoring the usual amplitude and phase characteristics of the rhythmic structure develop or are activated. After evening injections of cortisol normalization of the rhythm takes place more rapidly, although the parameters were not completely restored to normal during the period of the experiment.

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