Pharmacology Biochemistry & Behavior, Vol. 15, pp. 235–241, 1981. Printed in the U.S.A.

Pituitary-Adrenal Responses to Sub-Chronic Treatment with Phenobarbital and/or Phenytoin (Diphenylhydantoin) in Rats

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Received 6 October 1980

TORRELLAS, A., C. GUAZA, J. BORRELL AND S. BORRELL. *Pituitary-adrenal responses to sub-chronic treatment with phenobarbital and/or phenytoin (diphenylhydantoin) in rats.* PHARMAC. BIOCHEM. BEHAV. 15(2) 235–241, 1981.—The response of the pituitary-adrenal axis of the male rat to sub-chronic dose treatment with phenobarbital and/or phenytoin under basal and stress conditions was investigated. Plasma corticosterone levels were measured in rats sacrificed either in the morning or in the afternoon, subjected or not to 2 hours of immobilization stress. Phenobarbital did not seem to significantly affect the pituitary-adrenal activity under basal conditions or in the response to stress. Phenytoin induced a disruption of the corticosterone diurnal variation present in the rat under basal conditions and seemed to partially inhibit the pituitary-adrenal response to stress when applied in the morning. The combined treatment with phenobarbital and phenytoin affected the afternoon rise in corticosterone levels present under basal conditions, as well as the stress response at the same time of the day. The reported results agree with the hypothesis about the existence of mechanisms controlling ACTH release under basal conditions, dissociable from those controlling ACTH release in response to stress situations, and that phenytoin could influence some or others differently, depending on the animal's endocrine situation.

Corticosterone Stress Phenobarbital Phenytoin Corticosterone circadian variations

EXPERIMENTS in animals and observations in man indicate that treatment with anticonvulsant drugs can influence a variety of metabolic and endocrine events. However, discrepancies exist concerning the effects of some of these drugs upon the pituitary-adrenal axis.

Several studies have indicated that barbiturates are able to modify the pituitary-adrenal function under different experimental conditions [5, 9, 10, 11, 14]. On the other hand, it has been suggested that phenytoin has a stimulatory action upon the pituitary-adrenal axis [4, 6, 20]. Still other studies [3] indicate that rats treated with phenytoin fail to show the expected depletion of adrenal ascorbic acid following certain stimuli that normally elicit this depletion. It has even been suggested [19] that acute treatment with phenytoin in small doses results in stimulation, whereas large doses or chronic administration causes inhibition of the adrenal cortex.

The aim of the present study was to investigate the effects of sub-chronic dosing treatment with phenobarbital or phenytoin upon the pituitary-adrenal activity in rats sacrificed in the morning or in the afternoon, subjected or not to immobilization stress. Since therapy with both drugs has been widely used in the treatment of epilepsy and other convulsive disorders, experiments were also run in which the effects of the combined treatment of phenobarbital plus phenytoin were studied.

METHOD

Male wistar rats weighing 250–300 g were used. They were housed in groups of 4 and maintained under a controlled light-dark-schedule (the light was on between 7.00 and 19.00) in a temperature controlled room (25±2°C). Food and water were available ad lib. The sodium salts of phenobarbital (Merck, Germany), at doses of 50 mg/kg, and phenytoin (Diphenylhydantoin, Barcia, Spain), at doses of 60 mg/kg body weight, were prepared daily in a solution of 80% propylenglycol in ethanol. Drugs were injected subcutaneously in a volume of 0.5 ml and control animals received the same volume of vehicle.

The experimental design was as follows: sacrificing was performed at two times in the day, the first time corresponding to 3 hr after the switch to light (morning experiments), during the time of the diurnal trough in corticosterone levels as was previously determined in our laboratory (unpublished), and the second 2 hr before the switch to dark (afternoon experiments) at the time of the diurnal peak in the plasma levels.

Rats were injected daily for 8 days with phenobarbital and/or phenytoin at the doses mentioned above. Rats being sacrificed in the morning were injected daily at 18.00 hr; rats being sacrificed in the afternoon were injected at 9.00 hr.

Under this time schedule of drug treatment, rats sacrificed in the morning received the last dose of drug approximately 16 hr before killing, while rats sacrificed in the afternoon received the last dose 8 hr before killing. This time schedule was used with the aim that animals treated during the time of the diurnal trough in corticosterone levels were sacrificed during the diurnal peak, and inversely for animals sacrificed during the time of the diurnal trough. Immobilization stress was effected by securing a rat in the prone position to individual wire nets. The immobilization duration was 2 hr. Groups of drug-treated rats, as well as control rats, were submitted to this stress immediately before killing. The animals were sacrificed by decapitation with the aid of a guillotine. Trunk blood samples were collected in heparin tubes and centrifuged; afterwards, the plasma was pipetted off and stored. The adrenal glands were promptly removed, weighed, kept on ice, and homogenized in water. Samples were stored at −20°C until assay. Adrenal and plasma corticosterone levels were measured according to the procedure described by Matsumura *et al.* [12]. An Aminco-Bowman spectrophotofluorometer was used.

Results are expressed as means±SEM of 7–10 animals per group. In addition to plasma and adrenal corticosterone concentration values, the relative increments induced by stress in both parameters were also calculated from the difference between non-stressed and stressed values for each time of the day studied. The student's *t*-test was used to establish the significance of difference between two means [15]. Groups were considered significantly different at a level of $p<0.05$.

RESULTS

Non-Stressed Rats

Figure 1 shows the plasma and adrenal corticosterone levels of control, phenobarbital and/or phenytoin-treated rats sacrificed in the morning or in the afternoon without being subjected to immobilization stress. In control animals, plasma and adrenal corticosterone values were significantly higher in the afternoon than in the morning ($p<0.01$ and $p<0.01$, respectively). This was a consequence of the circadian rhythm of the pituitary-adrenal axis. The relative increase in plasma and adrenal corticosterone levels, as calculated from the difference between morning and afternoon values, is also shown in Fig. 1. We used this measure in order to better evaluate drug effects on the diurnal variations of plasma and adrenal corticosteroids presented under basal conditions.

Animals submitted to phenobarbital treatment and sacrificed in the morning or in the afternoon showed plasma and adrenal corticosterone levels, as well as the relative increases between morning and afternoon hormone values, not significantly different from the corresponding control values.

Phenytoin-treated rats sacrificed in the morning showed plasma and adrenal corticosterone levels significantly higher than in the corresponding controls. Phenytoin-treated animals sacrificed in the afternoon showed plasma and adrenal hormone levels not significantly different from the corresponding controls. It is remarkable that plasma and adrenal corticosterone levels in the phenytoin-treated rats sacrificed in the afternoon were within the same range as the corresponding values obtained in the group of animals treated with the drug and sacrificed in the morning; as a consequence, the relative increases between morning and afternoon plasma and adrenal hormone values in the phenytoin-treated rats were significantly lower than in control animals.

In animals subjected to the combined treatment of phenobarbital plus phenytoin, morning plasma and adrenal corticosterone levels were not significantly different from those measured in the corresponding controls or phenobarbital-treated rats; but they were significantly lower than those detected in phenytoin-treated rats sacrificed at the same time of the day. Plasma and adrenal hormone levels in rats subjected to the combined treatment and sacrificed in the afternoon, were significantly lower than the corresponding values detected in control, phenobarbital, or phenytoin-treated animals sacrificed at the same time of the day. There were no significant differences between the plasma or adrenal corticosterone levels of phenobarbital plus phenytoin-treated animals sacrificed in the morning and in the afternoon. The relative increases between morning and afternoon plasma and adrenal corticosterone levels in rats treated with phenobarbital plus phenytoin were significantly lower than the corresponding values detected in controls or phenobartial-treated rats with regard to phenytoin-treated animals, a significantly higher value in this parameter in the animals subjected to the combined therapy was observed.

Stressed Rats

Non-drug treated rats sacrificed in the morning or in the afternoon immediately after being submitted to 2 hr immobilization stress (Table 1) showed plasma and adrenal corticosterone levels significantly higher than in the corresponding unstressed control animals. It is remarkable that the increments of the hormone levels resulting from a stress situation differed significantly in the morning from those observed in the afternoon, but the final levels of the hormone measured after immobilization did not differ significantly between morning and afternoon. For this reason, in addition to plasma and adrenal corticosterone concentrations, the relative increments in both parameters, as calculated from the difference between non-stressed and stressed values for each time of the day studied, were also calculated. These measures allow a closer evaluation of the drug effects on the pituitary-adrenal response to the stress situation.

Figure 2 shows data obtained on the effects of drug treatment upon plasma and adrenal corticosterone response to immobilization. Phenobarbital-treated rats showed, at both times of the day, plasma and adrenal corticosterone values, as well as the corresponding relative increments in response to stress of both parameters, that were not significantly different from the values obtained in the corresponding immobilized control group of rats.

Phenytoin-treated rats showed morning plasma and adrenal corticosterone levels within the same range as those detected in the corresponding control rats. However, the relative increments induced by immobilization in both plasma and adrenal corticosterone levels were significantly lower in phenytoin-treated rats than in controls. Plasma corticosterone levels in phenytoin-treated rats sacrificed in the afternoon were not significantly different from those detected in the corresponding controls. However, adrenal corticosterone concentration in the same group of animals was significantly lower than in the control groups. No significant differences between phenytoin-treated and control animals were detected concerning plasma and adrenal corticosterone increments induced by immobilization in the afternoon. It must be pointed out that, in phenytoin-treated rats, adrenal

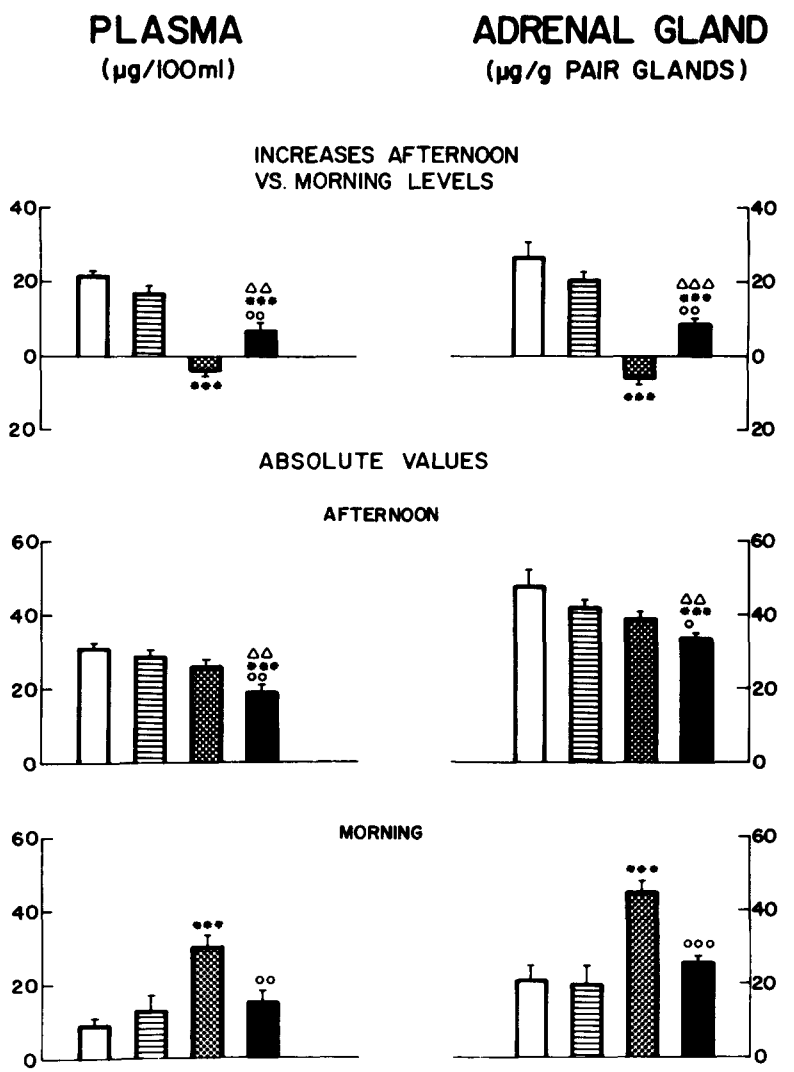

FIG. 1. Effects of sub-chronic drug treatment on plasma and adrenal corticosterone levels in non-stressed rats sacrificed in the morning or in the afternoon. Results are expressed as means ±SEM. Significance of differences as compared with the corresponding (1) Control group, $^{***}p<0.001$; (2) Phenobarbital-treated group, $^{\Delta\Delta}p<0.005$, $^{\Delta\Delta\Delta}p<0.001$; (3) Phenytoin-treated group, $^{\circ}p<0.05$, $^{\circ\circ}p<0.005$, $^{\circ\circ\circ}p<0.001$. □ Controls ☰ Phenobarbital ▩ Phenytoin ■ Phenobarbital plus phenytoin

corticosterone levels were significantly lower in the afternoon than in the morning ($p<0.02$), compared to the control values mentioned above.

In rats subjected to the combined treatment of phenobarbital plus phenytoin and then sacrificed in the morning, plasma corticosterone concentration had the same range as those detected in controls or phenobarbital-treated rats; a significantly higher value for this parameter was observed in the drug combined treated rats group compared with phenytoin-treated animals. A similar pattern of response was detected in the same group of animals concerning plasma corticosterone increments in response to stress. The morning adrenal corticosterone concentration in phenobarbital plus phenytoin-treated rats was not significantly different from the values detected in control, phenobarbital, or phenytoin-treated rats. The relative increment in the level of the hormone in the adrenal gland of rats subjected to the combined treatment and sacrificed in the morning, was not different from control or phenobarbital-treated rats, but it was significantly greater than in phenytoin-treated rats. The plasma corticosterone concentration, as well as the corresponding relative increment in the hormone levels in response to stress, in rats subjected to the combined treatment and sacrificed in the afternoon were both significantly greater than in control, phenobarbital or phenytoin-treated rats sacrificed at the same time of day. Adrenal corticoste-

TABLE 1

LEVELS OF PLASMA AND ADRENAL CORTICOSTERONE IN NON-DRUG TREATED RATS SACRIFICED IN THE MORNING OR IN THE AFTERNOON, SUBJECTED OR NOT TO 2 HR OF IMMOBILIZATION STRESS

	Corticosterone							
	Plasma (μg/100 ml)			Adrenal (μg/g)			Increments in response to stress	
	Unstressed	Stressed	p*	Unstressed	Stressed	p*	Plasma (μg/100 ml)	Adrenal (μg/g)
Morning	8.5 ± 2.40	53.8 ± 2.86	<0.001	21.3 ± 4.40	62.9 ± 3.19	<0.001	45.3 ± 2.90	41.6 ± 3.09
Afternoon	30.7 ± 1.34	45.1 ± 2.43	<0.001	48.5 ± 4.00	60.3 ± 4.07	<0.05	14.4 ± 2.42	11.8 ± 4.06
p†	<0.01	ns		<0.01	ns		<0.001	<0.001

Results are expressed as means ± SEM of 7–10 animals per group.
*Significance of the differences vs corresponding unstressed group.
†Significance of the differences vs corresponding morning group.

rone concentration in the above-mentioned group of rats was not significantly different in control, phenobarbital or phenytoin-treated rats. The relative increment in the level of the hormone in the adrenal gland of rats subjected to the combined treatment and sacrificed in the morning was not different from control or phenobarbital-treated rats. The plasma corticosterone concentration, as well as the corresponding relative increment in the hormone level in response to stress, in rats subjected to the combined treatment and sacrificed in the afternoon, were both significantly greater than in control, phenobarbital or phenytoin-treated rats sacrificed at the same time of day. Adrenal concentration of corticosterone in the above-mentioned group of rats was not significantly different in control, phenobarbital or phenytoin-treated rats. The relative increment in the adrenal hormone level in response to stress in the same group of animals was in the same range as those measured in control or phenobarbital-treated rats, but it was significantly greater

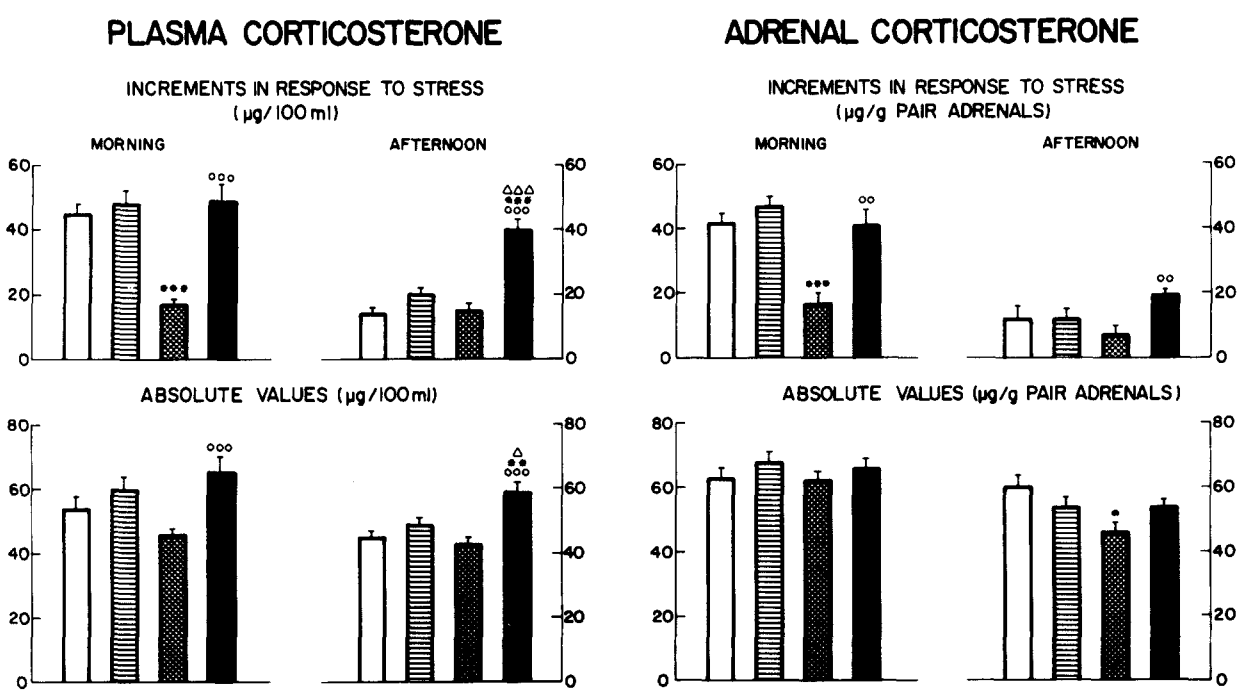

FIG. 2. Effects of sub-chronic drug treatment on plasma and adrenal corticosterone levels in response to 2 hr of immobilization stress on rats sacrificed in the morning or in the afternoon. Results are expressed as means±SEM. Increments in response to stress were evaluated as the differences between non-stressed and stressed values for each time of the day studied in the corresponding experimental groups. Significance of differences as compared with the corresponding (1) Control group, $*p<0.02$, $**p<0.005$, $***p<0.001$; (2) Phenobarbital-treated group, $\Delta p<0.01$, $\Delta\Delta p<0.005$, $\Delta\Delta\Delta p<0.001$; (3) Phenytoin-treated group, $\circ p<0.02$, $\circ\circ\circ p<0.001$. □ Controls ≡ Phenobarbital ▩ Phenytoin ■ Phenobarbital plus phenytoin

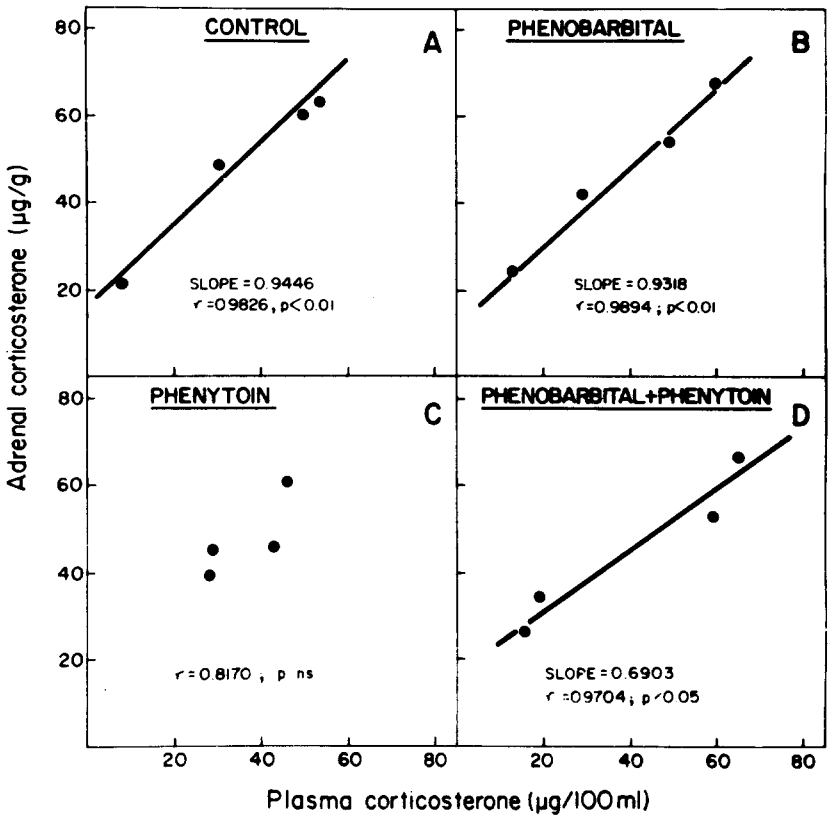

FIG. 3. Plot of mean values of adrenal corticosterone levels vs mean values of plasma corticosterone levels from unstressed or stressed rats subjected to drug treatment and sacrificed in the morning or in the afternoon. Each point represents the mean of values from 7 to 10 animals.

than in phenytoin-treated rats. It must be mentioned that, as was reported in phenytoin-treated animals, the adrenal concentration of the hormone in phenobarbital plus phenytoin-treated stressed rats was significantly lower ($p<0.05$) in the afternoon than in the morning.

In Figure 3 is presented the mean values of adrenal corticosterone levels plotted against the mean values of plasma corticosterone levels from unstressed or stressed rats subjected to drug treatments and sacrificed in the morning or in the afternoon; a significant positive correlation ($p<0.01$) between plasma and adrenal levels of corticosterone for the overall experimental groups studied was observed in control and phenobarbital treated rats (A and B, respectively). However, in phenytoin-treated rats (C) such correlation was not observed and in the group of animals subjected to the combined treatment with both drugs (D) the correlation, although significant ($p<0.05$), was less apparent than in control or phenobarbital-treated rats.

DISCUSSION

The study of the effects of phenobarbital and/or phenytoin sub-chronic treatments upon the pituitary-adrenal activity on basal and stress situations was the object of the present experiments. Several previous studies have indicated that barbiturates are able to modify the pituitary-adrenal function under different experimental conditions [5, 9, 10, 11, 14]. It has also been reported [13] that the resting plasma corticosteroid concentrations appear to be unchanged in barbitone-dependent animals, but that stress-induced increments in the concentration of corticosterone in plasma are less in dependent animals. Under the schedule of treatment followed in the present experiments, phenobarbital did not seem to significantly affect the pituitary-adrenal activity under basal conditions or in the response to immobilization stress. These results contrast with those reporting [9, 10, 14] a depressive effect of barbiturates on ACTH release. Nevertheless, it must be pointed out that in our experiments a sub-chronic treatment with sub-anesthetic doses of phenobarbital was performed, while in other studies acute administration of anesthetic doses of barbiturates were mainly used. Moreover, it has been reported [11] that, in rats, phenobarbital at low doses exerted an anti-stress effect, followed by significantly less effectiveness with medium doses, and good effectiveness with anesthetic doses. On the other hand, the present study used 2 hr of immobilization as a stress situation, which can be considered to induce a high activation of the pituitary-adrenal axis; it must be noted that

in other studies less potent stress situations were used, and this fact could also explain the differences in the results obtained.

Several studies have reported that phenytoin causes hypertrophy of the adrenal glands in rats and mice [4, 6, 17]. Other investigators [20] found an increase in plasma corticosteroids in rats treated for 12 days with the drug. On the other hand, reported results on the effect of phenytoin treatment upon the response to stress situations [3] indicated that rats treated with the drug fail to show the expected depletion of adrenal ascorbic acid following certain stimuli that normally elicit this depletion. The results here reported seem to support the suggestion that different effects of phenytoin upon the pituitary-adrenal axis is a function of the endocrine status of the animals. Thus, in non-stressed animals the drug seems to activate the pituitary-adrenal function in the morning, when the resting levels of both plasma and adrenal corticosterone are lower; however, in the afternoon, when the resting levels of the hormone are higher, phenytoin does not modify the pituitary-adrenal activity. This fact points to a disruption of the corticosteroids diurnal variation present in rats under basal conditions. This finding is at odds with that reported on man [1] that the drug did not appear to modify the diurnal rhythm of plasma corticosteroids.

In the response of the pituitary-adrenal axis to immobilization stress, a different effect of phenytoin from that in non-stressed drug-treated animals is observed. Although morning plasma and adrenal corticosterone levels in the group of rats treated with the drug and subjected to immobilization were in the same range as those measured in controls, it is remarkable that stress response, evaluated by the relative increments in the hormone levels as calculated from the difference between non-stressed and stressed values, was significantly less in phenytoin-treated rats than in controls. This fact points to the possibility that phenytoin-treatment could influence the pituitary-adrenal response to stress when it is applied in the morning. This fact may seem to be in contradiction to that mentioned above concerning the ACTH releasing effect of phenytoin in unstressed rats. It might be considered that the morning plasma corticosterone increase, induced by the drug in the non-stressed animals, may cause sufficient steroid feedback action to influence subsequent ACTH release in response to stress immobilization. However, several reports indicated that some psychotherapeutic drugs increase pituitary-adrenal activity in non-stressed rats, but decrease the ACTH response to different stress situations [9, 11, 18]. This points to the notion that the neuroendocrine mechanisms controlling the circadian variations of the pituitary-adrenal activity are dissociable from those controlling the release of ACTH in response to stress immobilization.

The effects of the combined treatment with phenobarbital plus phenytoin are more difficult to interpret. Under non-stress situations the combined treatment seems to affect the pituitary-adrenal function in such a way that no differences in the plasma and adrenal levels between morning and afternoon are detected in the treated rats. This effect resembles that observed in non-stressed phenytoin-treated rats. However, it must be pointed out that, in this latter-mentioned experiment, disruption of the corticosteroid diurnal variation was due to increases in the morning hormone levels; while in the phenobarbital plus phenytoin treated animals the same effect seems to be more accurately ascribed to decreases in the afternoon corticosteroids levels. On the other hand, results obtained in the group of rats subjected to the combined treatment and sacrificed in the afternoon showed an increased plasma corticosterone response to the immobilization stress. That is, under this endocrine situation, in which the single treatment with phenobarbital or phenytoin did not seem to modify the pituitary-adrenal activity, the combined treatment seemed to be able to increase such activity.

It is interesting to note that statistical analysis of the correlation between adrenal and plasma corticosterone levels for the overall experimental groups studied, shows a positive correlation between both parameters in control and phenobarbital treated-rats; however, in phenytoin-treated rats such correlation was not observed, and in the group of animals subjected to the combined treatment with both drugs the correlation was less apparent than in control or phenobarbital-treated rats. This observation once again indicates that, under our experimental conditions, phenytoin is able to alter in a significant way the pituitary-adrenal axis, while phenobarbital does not seem to exert a clear effect.

Taken together, the results reported here agree with the hypothesis mentioned above [8,21] that, although the mechanisms were related, the one controlling basal ACTH secretion might be dissociable from that controlling ACTH release in response to stress and that phenytoin could influence one or the other differently, depending on the animal endocrine situation. It is known that several central neurotransmitters are involved in the control of the pituitary-adrenal activity, and a direct effect of the studied drugs on such neurotransmitters must also be considered because the depressant and anticonvulsant properties of these drugs are directly related to neurotransmission effects on several brain regions [2, 7, 16].

ACKNOWLEDGEMENTS

The technical assistence of M. Vargas and A. Ortiz is gratefully acknowledged.

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