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Circadian Effect of ACTH 1–17 on Mitotic Index of the Corneal Epithelium of BALB/C Mice

L. E. SCHEVING[2], T. H. TSAI, J. E. PAULY AND F. HALBERG*

Department of Anatomy, University of Arkansas for Medical Sciences and Chronobiology Laboratories University of Minnesota School of Medicine, Minneapolis, MN*

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SCHEVING, L. E., T. H. TSAI, J. E. PAULY AND F. HALBERG. *Circadian effect of ACTH 1–17 on mitotic index of the corneal epithelium of BALB/C mice.* PEPTIDES 4(2) 183–190, 1983.—The objective was to determine the effect of ACTH 1–17, an adrenocorticotropin analogue, on the mitotic index in the corneal epithelium of mice standardized in 12 hr of light alternating with 12 hr darkness. A question asked was whether the time of administration along the 24-hr time scale influenced any response found. The findings showed that ACTH 1–17 could, depending upon when it was administered, bring about a statistically significant decrease, an increase or even no such change in the mitotic index. The greatest responses found were increases, especially when ACTH 1–17 was administered during the dark span. Also the time after injection when the responses occurred varied. The greatest response recorded was at 12 hr after injection when ACTH 1–17 was given at 2 hr into the dark with a 641% and a 718% increase with a low (0.02 IU/kg) and a higher (20 IU/kg) dose, respectively. A 3-way analysis of variance supported the conclusion that the kind-of-treatment, time-of-treatment and treatment-to-kill interval (sampling time) are important factors when determining any response to ACTH 1–17 on the mitotic index.

Cornea Mitotic index ACTH 1–17 Circadian rhythm Placebo

A SHORT-CHAIN synthetic analogue, ACTH 1–17 (HOE 433=SYNCHRODYN®, has been extensively studied for the circadian-stage dependence of its effects in both human beings [2, 8–10] and rodents [1, 3, 4, 6, 7]. The analogue stimulates, in rather small doses, the secretion of aldosterone, cortisol and testosterone in human beings [2,9]. In experimental animals as well, it has been shown to stimulate corticosterone and aldosterone secretion. Such stimulation depends, in all species examined, upon circadian stage, and the time of maximal response to ACTH 1–17 differs for aldosterone and corticosterone, *in vitro* and *in vivo* [2,9].

ACTH 1–17 is also a molecule that has been found to achieve a so-called chronization, i.e., the presetting, to a known convenient time, of a tolerance of an anti-cancer drug comparable to that encountered at the peak of the circadian cycle in susceptibility and resistance. This goal of resetting has been achieved by giving ACTH 1–17 24 hr prior to doxorubicin to mice [4,11]. What is attractive for a potentially similar clinical use, the analogue can be given intranasally to stimulate the adrenal. Such an administration mode could represent a definite advantage: if a patient were to take the analogue 24 hr prior to chemotherapy, a sniff of ACTH 1–17 could be readily taken at home.

Finally, human psychophysiologic performance can be manipulated by ACTH 1–17 in dependence upon circadian stage [8].

By 1983, the time-dependence of pharmacologic effects can be anticipated rather generally for any agent, e.g., a hormone, a drug and even a placebo. Time-dependence is particularly pertinent to ACTH and corticoids [2,4]. Moreover, single changes in molecular structure may bring about statistically significant changes in the timing of a susceptibility rhythm [3]. Hence, relations along pertinent time scales must be mapped for each molecule. Since such relations have already been determined in the case of ACTH 1–17, along the scales of a day and of a year in healthy subjects, in patients with arthritis and in rodents, this molecule was chosen by us for further study on cell proliferation. Until proof is offered to the contrary from studies on adrenalectomized mice, it is assumed that the pervasive effects thus far reported for ACTH 1–17 are largely mediated by corticoids.

Irrespective of the extent to which corticoids account for chronization by ACTH 1–17, it was of interest to further explore for any effects of this analogue on cell proliferation in those tissues which are target organs of doxorubicin and many other anti-cancer agents [11–13] as well as in the tissues in which cell proliferation does not respond markedly to

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[2]Requests for reprints should be addressed to Dr. L. E. Scheving, University of Arkansas for Medical Sciences, Department of Anatomy, Slot 510, 4301 West Markham, Little Rock, AR 72205.

these same agents; the mitotic index in the corneal epithelium is one such example and this report deals with data obtained from this tissue.

METHOD

On 12/22/79, 800 $CD2F_1$ females mice arrived from the Simonson Laboratories, Gilroy, CA. At arrival they were approximately 6 weeks old. They were stratified by body weight and randomly allocated to 113 cages (7 mice per cage). Groups of either 4 or 5 cages (28 or 35 mice) were then placed in separate isolation chambers which differed only slightly in size. The fluorescent illumination within the chambers was programmed so that half of the animals (400) received light from 0600 to 1800 (CST) daily (LD12:12); and the remaining 400 mice were illuminated from 1800 to 0600 daily (DL 12:12). Food and water were available ad lib. Clean cages were replaced once each week on the same day; otherwise the animals were not intentionally disturbed until the beginning of the experiment. No animals were subjected to unscheduled bright light. A dim red light (of ~0.5 lux at the level of the mouse eye) was used when it was necessary to handle mice during the daily dark span.

After standardization for 31 days, at 0800 on 1/22/80, we began administering a single dose of ACTH 1–17 or placebo to animals from the LD chambers in the following manner: 42 mice were injected subcutaneously (SC) with 20 IU/kg of body weight of ACTH 1–17, 42 mice were injected with 0.02 IU/kg of body weight of ACTH 1–17; and 42 mice were injected with a placebo (which was the carrier substance of ACTH 1–17). The total aliquot administered was 0.2 mg/mouse (the mice averaged 20±2 g). The same numbers of mice from the DL standardized group were given identical injections. Thus, at 0800 CST, we assumed that animals from the DL environment simulated biologically mice whose circadian systems were at 2000 hr, or just 2 hr after the beginning of their daily activity span; whereas the animals from LD environment were 2 hr into their daily rest span. A total of 252 mice from the two light-dark environments were injected within a span of 30 min beginning at 0800.

One cage of 7 mice from each of the ACTH 1–17 injected groups and from the placebo group were killed by rapid cervical dislocation at 15 min, 2, 4, 8, 12 and 24 hr after treatment (R_x). Thirty min prior to being killed, each mouse was given an intraperitoneal (IP) injection of 25 μCi of [^{3}H]TdR (25 Ci/mmole). Those mice that were killed 15 min after the treatment, were given the [^{3}H]TdR right after they received the ACTH 1–17 or placebo; thus for this group about 14 min elapsed between [^{3}H]TdR injection and killing.

This same identical procedure was carried out at 1200 on another 252 mice from the LD and DL schedules (mice on the latter schedule now simulating a 2400 time point). This was repeated on a comparable group of 252 mice at 1600 (the DL mice now simulating a 0400 time point). In summary, over the course of the working day, we injected 6 sets of 126 mice, three sets during the light span and three during darkness. From each of these sets subgroups of 7 mice from each of the 3 treated groups (high dose ACTH 1–17, low dose ACTH 1–17 or placebo) were killed at 15 min, 2, 4, 8, 12, 24 hr after R_x. In the Results and Discussion sections we do not express time in the "clock hours" but instead let the beginning of light=0 hr and then refer everything to this; thus, 2 *h*ours *a*fter *l*ights *o*n=2 HALO, 14 *h*ours *a*fter *l*ights *o*n=14 HALO (or 2 hours after lights off).

Figure 1 illustrates the protocol followed. Groups of control mice (7 mice each) were killed at 2, 6, 10, 14 and 22 HALO; the controls received no ACTH 1–17 but were injected as described above with [^{3}H]TdR 30 min piror to killing. Since there were no additional animals available for 6 control groups, since the 7 mice/cage arrangement was to be maintained and since 5-time points/cycle studies have yielded satisfactory results [6] one control group (at the 18 HALO time point) was omitted.

After the mice were killed, the thoracoabdominal cavity was opened and the carcasses were fixed in 10% buffered formalin solution for 2 weeks. Studies on the effect of ACTH 1–17 on the incorporation [^{3}H]TdR into DNA were investigated in several parts of the digestive tract (tongue, esophagus, stomach, duodenum, colon and rectum) and in the spleen, thymus and bone marrow. In addition the effect of ACTH 1–17 on total RNA and DNA in the spleen was investigated (results of all these studies are to be reported elsewhere). The mitotic index in the corneal epithelium of these same animals was evaluated in a manner previously described [14] and it is this aspect of the investigation that is being reported in this paper.

The data were subjected to a conventional *t*-test and 2-and 3-way analysis of variance (R_x-kind, R_x-timing and R_x-to-kill interval). In addition, all data were analyzed by the cosinor method, based on the least-squares fitting of the data to a 24-hr cosine curve [5] which provides the following information: (1) a *p* value which indicates the significance of the fit of the cosine curve to the data. If the *p* value is 0.05 or less, the fluctuation of the variable studied is presumed to be cyclic and not random); and (2) estimates of three rhythm parameters and their dispersions: these are designated as the mesor (M), acrophase (ϕ), and amplitude (A).

The mesor (M) is the cosinor-determined rhythm-adjusted mean; this is equivalent ot the 24-hr arithmetical mean if sampling is equidistant and covers an integral number of cycles. The amplitude (A) is defined as one-half the total excursion characterizing the data of the cosine best approximating the data. It represents the difference between the mesor and the crest of the cosine function used to approximate the rhythm. Both amplitude and mesor are expressed in the original unit which in this case is the number of mitoses encountered/1000 cells counted, rounded to the nearest integer.

The acrophase (ϕ) represents the crest of the fitted cosine curve in relation to some arbitrarily selected reference point along the 24-hr time scale. Usually, the acrophase corresponds to the time when the data values are, on the average, highest; however, it should be noted that the acrophase is not necessarily the time when the peak value was recorded. Frequently, the acrophase is expressed in degrees rather than hours. If 360°≡24 hr, then 15°=1 hr. In this study the reference point is the time of lights-on, or 0°; therefore one would add 15° for each successive hr, and one HALO would be −15°. In the cosinor plot of Fig. 2 of this study 0° to 180° represents the light (usual rest) span whereas 180° (1800) to 360° (2400) represents the dark (usual active) span. The negative acrophase stem from the desire to deal with trigometric functions in a clock-wise fashion, in keeping with mathematical definition.

RESULTS

Control

Figure 2 illustrates the typical rhythm in the mitotic index

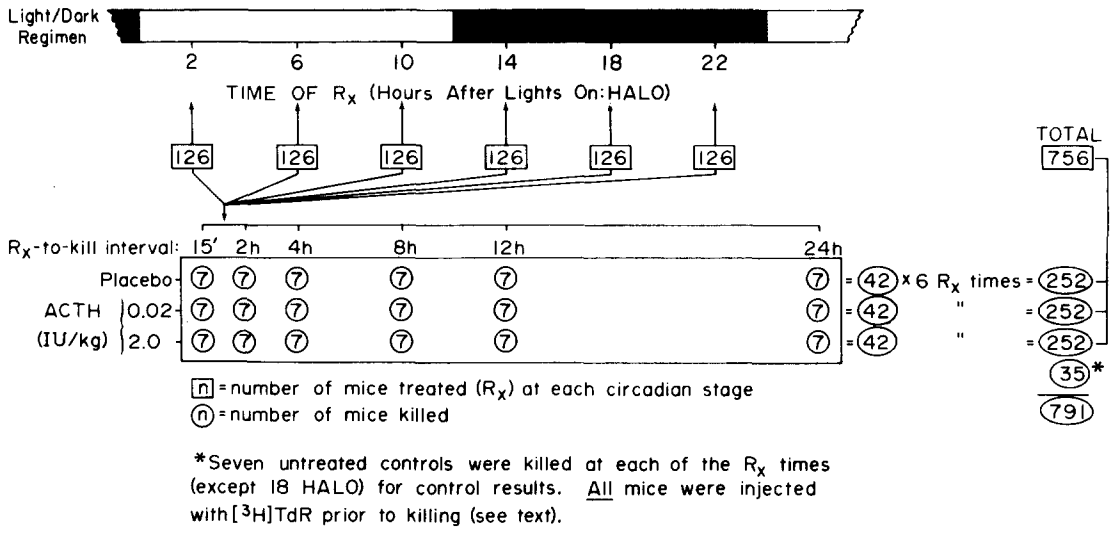

FIG. 1. Protocol of study.

of the corneal epithelium of the rodent [14]. The peak (11.1±0.6 mitoses/1000 cells counted) occurred at 2 HALO and the trough (0.3±0.1) at 2 hr after lights off (14 HALO). A cosinor analysis indicated a statistically significant circadian rhythm ($p<0.001$). Since these result are self-explanatory and confirm earlier work by us and others [12,14], we will not describe them in any detail.

Experimental

We shall however, first compare the data obtained from

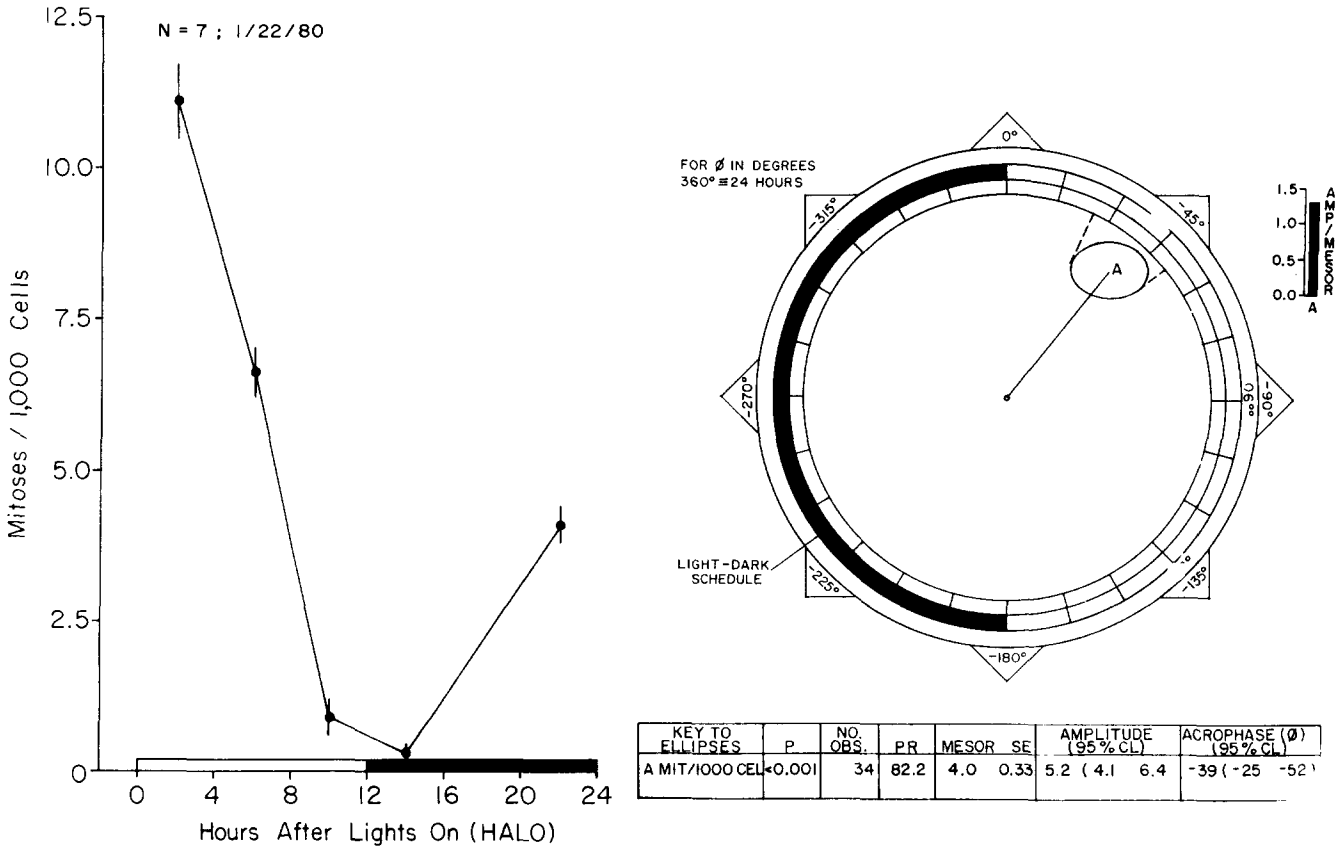

FIG. 2. Chronogram and polar plot of cosinor results on circadian rhythm of mitotic index in untreated control animals.

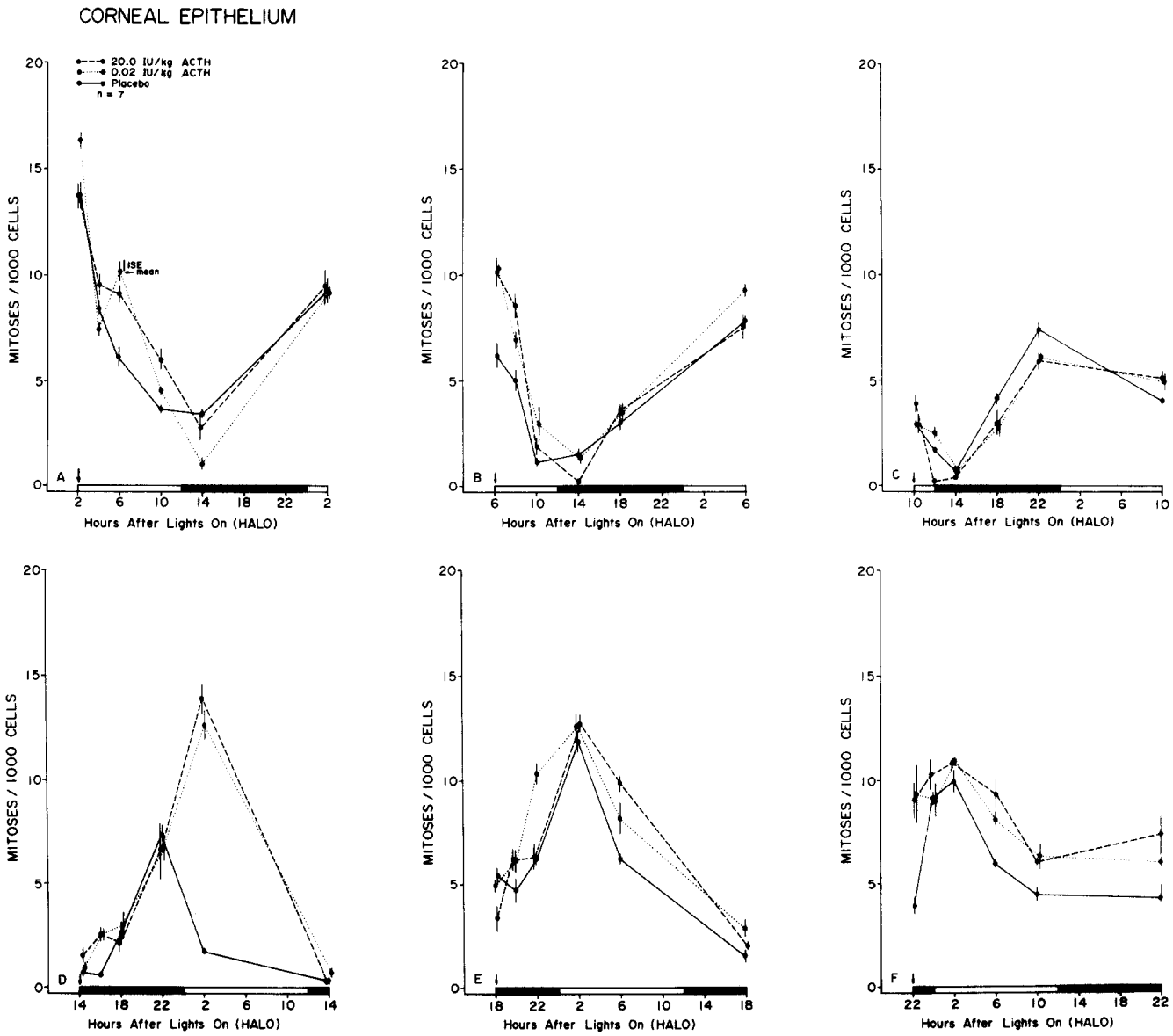

FIG. 3. Data summarized in a conventional manner to show variation in response at different intervals after each of the 6 treatment times representing different circadian stages of the mouse. The abscissa indicates that the animals were subjected to 12 hr of light (white bar) alternating with 12 hr of darkness (black bar). Every time point is referenced to "lights on." Thus, 0 hr=the beginning of light; 2 *h*ours *a*fter *l*ights *o*n is expressed as 2 HALO; 14 HALO represents 14 hr after lights on (or in this case 2 hr after lights off), etc. The arrows represent the times of treatment. The kill times were 15 min, 2, 4, 8, 12 and 24 hr after each treatment. Thus, there were 18 sets of data generated.

the ACTH 1–17 groups with the data obtained from the placebo-treated group and follow this by a comparison of the pooled data obtained from all three treated groups at fixed intervals after treatment with the data from the untreated controls.

Comparison of the Mitotic Index in the Corneal Epithelium of Placebo and ACTH 1–17 Treated Groups at Successive Intervals after Treatment Administered at Six Different Circadian Stages

Treatment—2 HALO. When the small dose of ACTH 1–17 (0.02 IU/kg) was administered at 2 hr into the light-span a statistically significant increase in the mitotic indices of 19%, 66% and 25% was recorded at 15 min, 4 and 8 hr, respectively when compared with data from the placebo group. The low dose caused a statistically significant decrease in the mitotic indices of 12% and 71% at 2 and 12 hr, respectively (Fig. 3A). Hereafter, unless otherwise qualified, when an increase or decrease is mentioned we always refer to the mitotic index, with no change implying that any change recorded was not statistically significant.

The large dose of ACTH 1–17 (20 IU/kg) was stimulatory at 2 hr (13%), 4 hr (49%) and 8 hr (67%); it had no effect on the mitotic index at the other intervals. The p values for all the above differences ranged from <0.04 to <0.000005.

Treatment—6 HALO. With the small dose of ACTH 1–17 there were increases in the mitotic indices of 66%, 38%, 164% and 18% at 15 min, 2, 4 and 24 hr, respectively.

The large dose of ACTH 1–17 caused increases of 64% and 70% at 15 min and 2 hr, respectively; there was a decrease of 88% at 8 hr (Fig. 3B). The p values ranged from <0.01 to 0.00003.

Treatment—10 HALO. When the ACTH 1–17 was administered at this time the small dose was associated with increases of 47% and 23% at 2 and 24 hr, respectively. There were decreases in the mitotic indices of 34% and 18% at 8 and 12 hr, respectively.

The high dose showed increases of 35% and 28% at 15 min and 24 hr, respectively. There were decreases of 88%, 43% and 21% at 2, 4 and 12 hr, respectively (Fig. 3C). The p values for the above changes ranged from <0.04 to <0.000005.

Treatment—14 HALO. When ACTH 1–17 was injected at 2 hr into the dark (14 HALO) the small dose showed increases in the mitotic indices of 317%, 641% and 133% at 2, 12 and 24 hr, respectively.

Increases in the mitotic indices of 114%, 317% and 718% were recorded with the large dose at 15 min, 2 and 12 hr, respectively (Fig. 3D). The P values for the above changes ranged from <0.03 to <0.000005.

Treatment—18 HALO. When ACTH 1–17 was administered at 6 hr into the dark (18 HALO) the small dose was associated with increases in the mitotic indices of 66%, 31% and 88% at 4, 12 and 24 hr, respectively.

With the large dose there were increases in the mitotic indices of 32% and 58% at 2 and 12 hr, respectively; there was a decrease of 39% at 15 min (Fig. 3E). The p values ranged for all the above differences from <0.01 to <0.0001.

Treatment—22 HALO. When ACTH 1–17 was administered 10 hr into the dark-span (22 HALO) the small dose showed increases in the mitotic indices of 139%, 37%, 41% and 43% at 15 min, 8, 12 and 24 hr, respectively.

With a large dose there were increases of 131%, 58%, 36% and 74% at 15 min, 8, 12 and 24 hr, respectively (Fig. 3F). The p values for all changes ranged from 0.01 to <0.0001.

Comparison of the Circadian Mesors of All Three Experimental Groups with Untreated Controls

The results are best understood by referring to Fig. 4. There were no statistically significant differences recorded when the six mesors of the placebo treated groups were compared with the mesor of the untreated controls.

At 15 min the low and the high doses of ACTH 1–17 caused a 61% and 28%, respectively increase when compared to the untreated controls. Both differences were statistically significant (p<0.007 and 0.01). At two hours the increases with the lower and higher doses were 24% and 35%, respectively; only the increase from the high dose was statistically significantly different from the untreated controls (p<0.04). At 4 hr only the low dose caused a statistically significant increase of 37% (p<0.04). At 8 hr there was a statistically significant increase of 37% for the high dose (p<0.04). At 12 hr the increases caused by the small and large doses were 37% and 55%, respectively (p<0.03 and 0.01). The changes seen at 24 hr with the low and high doses of ACTH were not statistically significantly different from the untreated controls.

Rhythmometric Summary of the Cosinor Analysis

The 19 sets of data shown in Table 1 represent the results obtained after fitting by least-squares to a 24 hr cosine curve the control and each set of experimental data. The control data exhibited highly statistically significant rhythm as did all the data obtained from the three treated groups with the one exception of the 12-hr-interval placebo group. Such results serve as objective evidence that the variable under investigation was rhythmic and not random. Moreover, they also provide estimates of the three rhythm parameters (mesor, amplitude and acrophase) and their dispersions.

Analysis of Variance (ANOVA)

Summarized in Table 2 are the results from a three-way analysis of variance. The remarkable degree of statistical significance supports the conclusion that the kind-of-treatment, the time-of-treatment and the treatment-to-kill interval (sampling time) are important factors when determining any response to ACTH 1–17 or placebo. All 2 -way interactions were also statistically significant indicating, for example, that the effect of treatment kind varies with treatment time.

DISCUSSION

Control Group

The mitotic index in corneal epithelium is characterized by a high-amplitude rhythm. The rhythm is strongly synchronized to the ambient light-dark cycle to which the animals were subjected; however, it is not generated by the light-dark cycle [14]. The mitotic index in this particular tissue, unlike some tissues such as the duodenum, reaches zero or almost zero during a predictable time along the 24-hr day in synchronized animals as in those of this study.

The cyclic nature of the data, in addition to confirming earlier findings [14], help us to understand why any perturbation capable of affecting this rhythm might bring about a different response when administered at different stages of the rhythm, such as its trough, ascending limb, peak or descending limb. All of these phases occur at different times of the day or night. We describe the multitude of interrelated rhythmic physiological and metabolic rhythms, all of which are acting in harmony in the healthy organism, as a circadian system; the way we speak of a nervous, circulatory, or digestive system. When administering a stimulus such as ACTH 1–17 at different times of the day or night ot determine its effect on a particular rhythmic variable such as the mitotic index, we really are perturbing the host at different stages of its circadian system, and therefore we speak of the variations in response seen as being circadian-stage-dependent and not as a "time-of-day" dependent response.

Experimental Groups

Placebo versus ACTH 1–17 treated animals. An examination of the results in Fig. 3 and Table 1 shows clearly that the responses to either the small or large dose of ACTH 1–17, when compared to the placebo-treated group, were strongly circadian-stage dependent. For example, when the ACTH 1–17 was administered at 2 HALO, both doses caused a rather large statistically significant increase at 4 and 8 hr (Fig. 3). There was, however, a 71% decrease at 12 hr with the small dose, but not with the large dose; since we are suspicious of the latter finding it deserves further comment. Our suspicion arises because the decrease occurred at a time when the

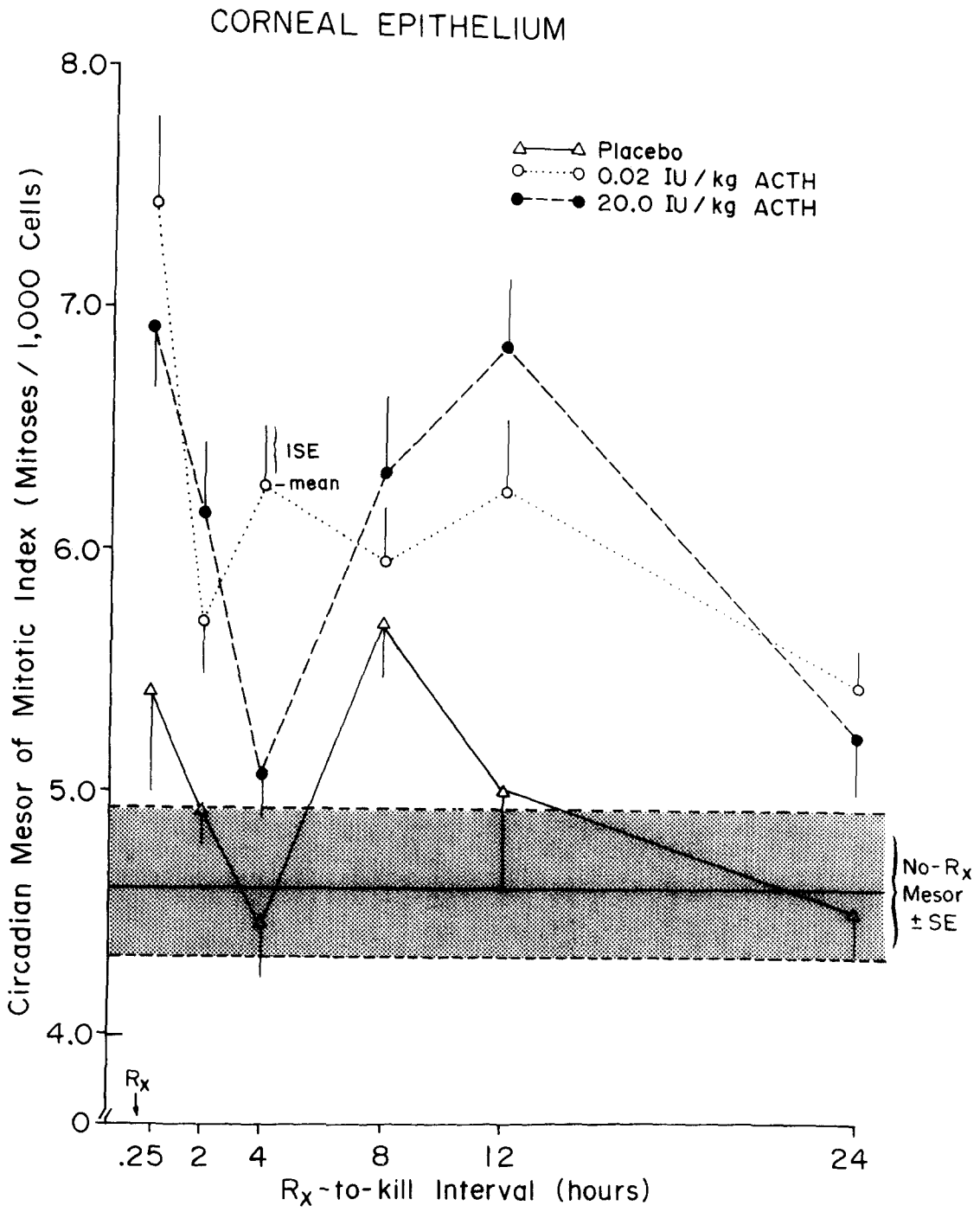

FIG. 4. Comparison of the circadian mesors of the mitotic indices of the three treated groups with the mesors of the untreated controls.

mitotic activity in the corneal epithelium of normal animals is virtually zero, thus even the presence of 1 or 2 stray mitotic figures could generate a large percentage increase or decrease when compared to a group having no mitotic figures. Moreover, from the over-all general response in this study we frequently found that, if effective at all, both low and high doses were effective; thus in this case we might have expected that if the response was biologically significant, a similar effect might have been seen with the larger dose. Thus we do not attribute, in spite of the statistical significance, much biological significance to such a change; of course, this explanation has not been proven. When the ACTH 1–17 was administered at 6 HALO, or simply 4 hr later, the largest repsonses were seen at 15 min and 2 hr.

TABLE 1
RHYTHMOMETRIC SUMMARIES OF DATA OBTAINED ON THE MITOTIC INDEX OF THE CORNEAL EPITHELIUM

Treatment	Treatment-to-kill Interval	N	*p*	Mesor* ±S.E.	Amplitude* ±S.E.	Acrophase (95%) Confidence Limits
None		34	0.0001	4.6 ± 0.3	5.21 ± 0.5	−39° (−29, −49)
Placebo	15 min	41	0.0001	5.4 ± 0.4	4.7 ± 0.6	−33° (−18, −48)
	2 hr	42	0.0001	4.9 ± 0.2	4.4 ± 0.2	−32° (−26, −37)
	4 hr	42	0.0001	4.5 ± 0.2	4.5 ± 0.3	−24° (−17, −31)
	8 hr	42	0.0001	5.7 ± 0.2	4.4 ± 0.3	−23° (−15, −31)
	12 hr	42	0.59	4.4	0.5	−36° (,)
	24 hr	42	0.0001	4.5 ± 0.2	4.4 ± 0.2	−54° (−47, −60)
Low-Dose ACTH	15 min	42	0.0001	7.4 ± 0.3	7.1 ± 0.4	−31° (−24, −39)
	2 hr	41	0.0001	5.7 ± 0.2	3.3 ± 0.3	−30° (−19, −41)
	4 hr	42	0.0001	6.3 ± 0.2	5.8 ± 0.3	−30° (−23, −36)
	8 hr	42	0.0001	5.9 ± 0.2	5.1 ± 0.3	−40° (−34, −47)
	12 hr	42	0.0001	6.3 ± 0.3	4.8 ± 0.4	−46° (−37, −56)
	24 hr	42	0.0001	5.5 ± 0.1	4.3 ± 0.2	−51° (−45, −57)
High-Dose ACTH	15 min	42	0.0001	6.9 ± 0.3	6.0 ± 0.4	−38° (−32, −45)
	2 hr	42	0.0001	6.2 ± 0.3	4.9 ± 0.4	−34° (−25, −44)
	4 hr	42	0.0001	5.1 ± 0.2	5.4 ± 0.3	−38° (−33, −44)
	8 hr	41	0.0001	6.3 ± 0.3	5.6 ± 0.4	−47° (−39, −56)
	12 hr	38	0.0001	6.7 ± 0.3	4.9 ± 0.4	−52° (−43, −61)
	24 hr	42	0.0001	5.3 ± 0.3	4.4 ± 0.4	−43° (−33, −52)

*All units expressed as mitoses/1000 cells counted.

TABLE 2
THREE WAY ANALYSIS OF VARIANCE SUMMARY FOR CORNEAL MITOTIC INDEX SOURCE OF VARIATION

	Degrees of Freedom	Mean Square	F	*p*
Main Effects	12	262.1	97.644	0.001
Kind of treatment (K)	2	123.1	45.877	0.001
Time of treatment (T)	5	541.0	201.560	0.001
Sampling time (S)	5	38.1	14.202	0.001
2-Way Interactions	45	142.0	52.960	0.001
K × T	10	14.3	5.345	0.001
K × S	10	10.4	3.865	0.001
T × S	25	246.0	91.640	0.001

Again, it should be pointed out that an increase of 164% was found at 4 hr, but this increase occurred only with the small dose of ACTH 1–17 and furthermore, it also occurred at a time when there was virtually no mitotic activity (Fig. 2). Additional examples of variable changes occurring at different circadian stages are evident from Fig. 2.

The greatest stimulatory responses to ACTH 1–17 were seen when it was administered during the dark span, especially at 14 HALO. On of the doses of ACTH 1–17 administered at three different times during the dark, 8 out of 20 of the subsequent statistically significant responses showed over a 100% increase in the mitotic index over those of the placebo-treated group, and two showed increases as high as 641% and 718% at 12 hr with the low and high dose, respectively. Interestingly, all but one of the statistically significant responses in the mitotic index from the three different times when ACTH 1–17 was administered during the dark span, were increases.

The least response to ACTH 1–17 was associated with the three times when ACTH 1–17 was administered during the light span since none of the 24 statistically significant responses recorded in the mitotic index were over 100%. Moreover, 8

of the 24 responses represented decreases in the mitotic index when compared to the placebo controls. Five of the 8 decreases occurred subsequent to the single injection of ACTH 1–17 given at the end of the light span (10 HALO). Two of the 5 appeared to be a real biological change since they were found with both doses and occurred 12 hr after treatment (see Fig. 3D; Table 1). The 3 other decreases in the mitotic index seen at this time were suspect, since as described above they occurred at a circadian stage when mitotic activity was minimal.

It is concluded that one or both doses of ACTH may bring about a statistically significant response in the mitotic index, the response may be a decrease or an increase depending on the circadian stage of treatment as well as the treatment-to-kill interval. Obviously the far greater tendency was to increase the mitotic index. The largest increases in the mitotic index could not be attributed to any specific time of treatment other than to say that the greater responses occurred subsequent to giving ACTH 1–17 during the dark span.

Consideration of the Mesors of the Three Treated Groups With the Mesor of the Untreated Controls

As mentioned above, when the mesors (24-hr means) of each set of data, that is the 15 min, 2, 4, 8, 12 and 24 hr sets, are compared with the data from the untreated controls, the following conclusions can be drawn: (1) Threre was no statistically significant difference between the mesors of the placebo and the untreated control animals. This was contrary to the findings in these same animals when the incorporation of [^{3}H]TdR into DNA was measured; with this particular endpoint, frequently the mesor of the untreated and the placebo treated controls were statistically significantly different. Such results were obtained, however, on tissues other than the cornea and are to be published elsewhere. (2) In general, the tendency was, as mentioned earlier for both the large and the small doses of ACTH 1–17, to increase the mitotic index over and above either the placebo treated or the untreated controls. The largest responses when mesors are compared were those seen at 15 min and at 12 hr after treatment. It is of interest that the greatest response, in those tissues that did respond to ACTH 1–17 when we measured the mesor incorporation of [^{3}H]TdR into DNA, usually occurred at 4 to 8 hr.

The results emphasize the necessity in any experimental design of considering the rhythmic structure; to ignore such dramatic changes as demonstrated can only lead to ambiguity and false interpretation as to what really are the effects of ACTH 1–17 on the mitotic index or on any other endpoint measured. With single time point sampling, the conventional way of doing experiments, one could not fully appreciate the complexity of the response such as we have demonstrated here. Clearly time of administration may be as important a factor to consider as is the dose. If we are ultimately to fully understand normal as well as abnormal cell proliferation, we must consider its underlying temporal organization which is the basis of an ordered structure in the living organism [12].

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