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Effect of ACTH 1–17 at Different Circadian Stages on [^{3}H]TdR Incorporation into DNA[1]

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SCHEVING, L. E., T. H. TSAI, J. E. PAULY AND F. HALBERG. *Effect of ACTH 1–17 at different circadian stages on [^{3}H]TdR incorporation into DNA*. PEPTIDES 5(3) 507–518, 1984.—The objective was to determine the effect of adrenocorticotropin (ACTH 1–17) on the incorporation of [^{3}H]TdR into DNA (DNA synthesis) in the tongue, esophagus and stomach of CD2F$_1$ mice standardized to 12 hours of light alternating with 12 hours of darkness. A question asked was whether the time of administration along the 24-hour time scale influenced any response found. The response was complex as ACTH 1–17 was capable of bringing about statistically significant increases in the incorporation of [^{3}H]TdR into DNA at certain times, decreases at other times, or no response at still another time. In general the most marked effects of 20 IU/kg of ACTH 1–17 when compared to controls, was to decrease DNA synthesis of as much as 60% 4 hours after administration at the end of the dark or beginning of the light span. A 2- and 3-way analysis of variance supported the conclusion that the kind-of-treatment, time-of-treatment and the interval-to-kill (Sampling time) as well as their interactions are important factors when determining any response of ACTH 1–17 or placebo.

ACTH 1–17 DNA Circadian Rhythm Placebo Tongue Esophagus Stomach

A short-chain synthetic analogue, ACTH 1–17 (HOE 433=SYNCHRODYN®), has been extensively studied for the circadian-stage (time of day) dependence of its effects in both human beings [2, 9–11] and rodents [1, 3–4, 6–7, 18–19]. The analogue stimulates, in rather small doses, the secretion of aldosterone, cortisol and testosterone in human beings [2–10]. 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* [2,10].

It also has been well documented that mice show a dramatic susceptibility-resistance rhythm to adryamycin (ADR) [7]. A predictable time of maximum and minimal toxicity has been demonstrated by administering this anti-cancer agent at frequent intervals along a 24 hr time scale, to different groups of mice who had been standardized to the same light-dark cycle. Moreover, it has been reported that when ACTH 1–17 was administered to mice 24 hr prior to giving the ADR at the expected circadian time of maximum toxicity there resulted a statistically significant reduction in host toxicity. However, when the ACTH 1–17 was given 24 hr prior to administering the ADR at the expected circadian time of greatest resistance (minimal toxicity) no reduction in toxicity was noted [7]. Of further interest was the finding that ACTH 1–17 can induce, when administered only at a certain circadian stage, a rhythm in the incorporation of [^{3}H]-TdR into DNA in the Harding-Passey melanoma of Balb/C female mice; normally this tumor does not show such a rhythm in either Balb/C of CD2F$_1$ mice [13]. It has also been reported that time of treatment as well as treatment-to-kill interval (sampling time) are important factors when determining any response that ACTH 1–17 has on the mitotic index of mouse corneal epithelium [17]. The same has been reported on the incorporation [^{3}H]TdR into DNA in thymus, bone marrow and spleen of mice [19].

The above studies suggest a potential role for ACTH 1–17 in improving the toxic-therapeutic ratio in cancer chemotherapy either by inducing a rhythm in cell proliferation in the tumor or by manipulating the natural endogenous rhythm in normal tissues, especially in anti-cancer tissues such as the digestive tract and bone marrow which are damaged by treatment with anti-cancer agents such as ADR and others. This paper represents data obtained on the circadian variation in the incorporation of [^{3}H]-TdR into DNA in the tongue, esophagus and glandular stomach and the response of the rhythms to ACTH 1–17.

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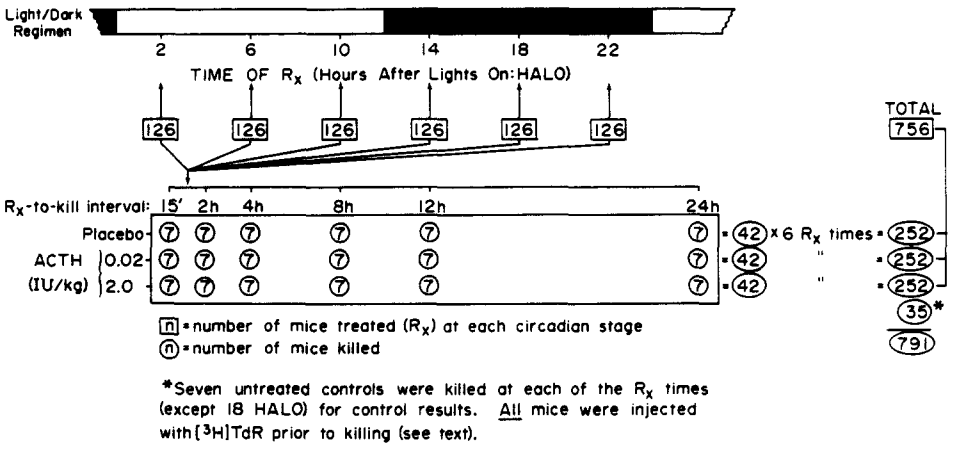

FIG. 1. Protocol of study.

METHOD

On 12/22/79, 800 CD2F$_1$ female mice arrived from the Simonson Laboratories, Gilroy, CA. At arrival they were approximately 6 week old. They were randomly distributed among 113 cages, 7 mice to a cage. After stratification by weight, four or five cages (28–35 mice) were placed into each of 23 separate isolation chambers; the chambers were of slightly different size. The programmed fluorescent illumination within the chambers subjected half of the animals (400) to light (L) from 0600 to 1800 (CST) daily and dark (D; from 1800 to 0600 (LD 12:12); the remaining 400 mice were illuminated from 1800 to 0600 daily (DL 12:12). Food and water were freely available. Clean cages were replaced once each week on the same day; otherwise the animals were not intentionally disturbed until the beginning of the experiment. A dim red light (of ~0.5 lux at the level of the mouse eye) was used for handling of the mice during the daily dark span.

At 0800 on 1/22/80, after standardization for 31 days, 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 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 ml/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 the 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 tritiated-thymidine [^{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 identical procedure was carried out at 1200 on another 252 mice from the LD and DL schedules (mice on 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 the 0400 time point). In summary, over the course of the working day, by manipulating the light-dark schedules, we injected 6 sets of 126 mice, three during the light span and three during darkspan. We do not express time in "clock hours" in the Results and Discussion sections; instead, we let the beginning of light=0 hr and everything is then referenced to this; thus 2 *h*ours *a*fter *l*ights *o*n=2 HALO, 14 *h*ours *a*fter *o*n=14 HALO (or *h*ours *a*fter *l*ights *o*ff). Thus, from each of the 6 HALO injection times, subgroups of 7 mice from each of the 3 treated groups were killed at 15 min, 2, 4, 8, 12 and 24 hr after R_x.

Groups of control mice (7 mice each) were killed at 2, 6, 10, 14 and 22 HALO; the controls received no ACTH but were injected, as described above, with [^{3}H]TdR 30 min prior 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 the 5-time points/cycle studies have yielded satisfactory results [2] one control group (at the 18 HALO time point) was omitted. Figure 1 illustrates the protocol followed.

After the mice were killed, the thoraco-abdominal cavity was opened and the carcasses were fixed in 10% buffered formalin solution for 2 weeks. Pieces of the tissues studied were then removed and the DNA was extracted by the method of Ogur and Rosen [8], with the modification that the RNA hydrolysis was carried out in a 1N NaOH solution at 60°C for 18 hr. Although more tissues were analyzed, only the upper part of the digestive tract (tongue, esophagus and stomach) is being reported in this paper; the rest of the data have been or are to be published elsewhere, see Introduction. Specifically the other tissues in which the incorporation of [^{3}H]TdR into DNA (DNA synthesis) was studied include the: duodenum, colon, rectum and spleen, thymus and bone marrow. In addition, total RNA and DNA content was measured in the spleen and the mitotic index was determined in the corneal epithelium.

The data were subjected to conventional *t*-tests and a 2-

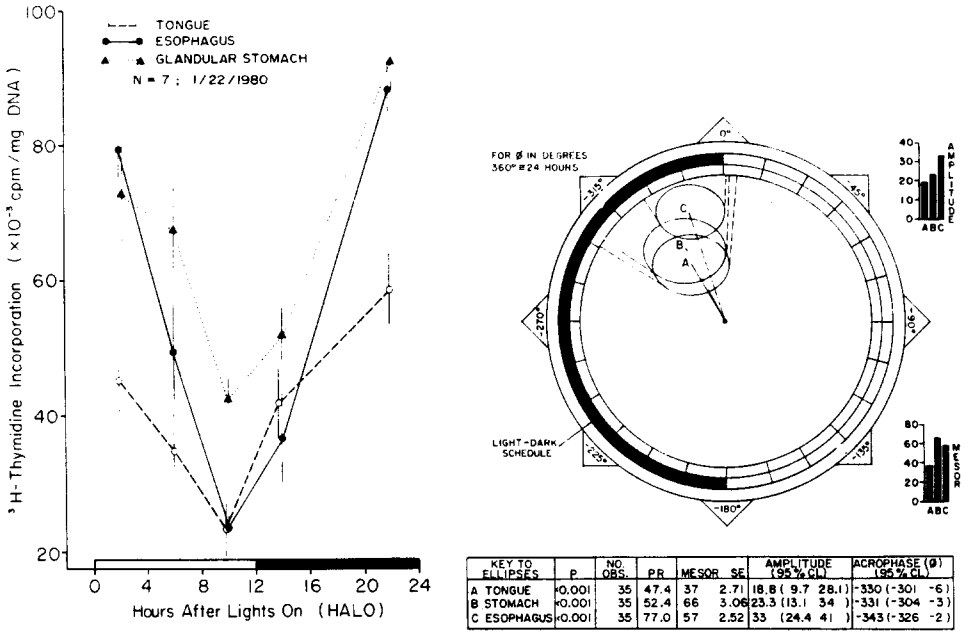

FIG. 2. Chronograms, rhythmometric summary (as determined by the cosinor analysis), and a display of the latter results in a polar plot. Note the remarkably similarity in the phasing of the rhythms of the three tissues.

and 3-way analysis of variance (R_x-kind, R_x-timing and R_x-to-kill interval). In addition, all data on [³H]TdR incorporation by a given tissue as a function of R_x-time—or, in the case of controls, kill-time—were analyzed by the cosinor method [5] which provides the following information: (1) a p-value indicating the statistical significance of the fit of a 24-hr 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 parameters are designated as the mesor (M), amplitude (A) and acrophase (ϕ).

The mesor (M) is the cosinor-determined rhythm-adjusted mean; this is equivalent to the 24-hr arithmetical mean only if the data points are equidistant. The amplitude (A) is defined as one-half the total cosine excursion best approximating the rhythm. It represents the distance between the mesor and the crest or the trough of the cosine function used to approximate the rhythm; this is in keeping with mathematical convention. Both amplitude and mesor are given in the original units, which in this case is the incorporation of [³H]TdR into DNA expressed as counts/min/mg DNA ($\times 10^{-3}$), rounded to the nearest integer; we believe that this represents DNA synthesis.

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. The reference point chosen in this study was lights on. Frequently, the acrophase is expressed in degrees rather than hours. If 360°≡24 hr, then 15°=1 hr. Thus, in this case the reference point of lights on=0°; therefore one would add 15° for each hour past this, and 0100 would be −15°. In the cosinor plot of Fig. 2 of this study 0° to 180° represents the light (rest) span, whereas 180° (1800) to 360° (2400) represents the dark (active) span. The minus signs preceding the number of degrees indicate lag from the reference point (lights on).

RESULTS

Controls

The data illustrated in Fig. 2 clearly illustrate the typical high amplitude circadian rhythm in the incorporation of [³H]TdR into DNA in the tongue, esophagus and glandular stomach; the data are summarized on the left as chronograms (time plots) and the cosinor results are displayed in a polar plot (on the right). Since these results are self-explanatory and confirm earlier findings by us [15] we will not discuss them in detail.

Experimental

We shall, however, first compare the data obtained from the two 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.

The degree of response seen in the effect of the two different doses of ACTH 1-17 on the incorporation of [³H]TdR into DNA when compared with the placebo treated animals is summarized in Table 1 and the waveform of the rhythms in each tissue are shown in Figs. 3, 4 and 5. Admittedly such a large amount of data can be somewhat overwhelming, thus we have summarized only our major observations while rec-

TABLE 1

PERCENT INCREASE OR DECREASE (↓) IN THE INCORPORATION OF [^{3}H]TdR INTO DNA WHEN PLACEBO TREATED ANIMALS ARE COMPARED TO ANIMALS INJECTED WITH THE SMALLER (S) OR LARGER (L) DOSE OF ACTH AT SPECIFIC INTERVALS AFTER TREATMENT

	Interval from Treatment to Killing											
	15 min		2 hr		4 hr		8 hr		12 hr		24 hr	
R_X-Time tissue	S	L	S	L	S	L	S	L	S	L	S	L
2 hr Tongue	74*	274	44↓†			42↓		31↓				42↓
Esophagus	66	206	26↓		26↓	22↓			51↓	67↓		44↓
Stomach	54	212			19↓	28↓		28↓	32↓	33↓		
6 hr Tongue			86									
Esophagus	41↓	36↓	43						45↓			
Stomach					26							
10 hr Tongue		44↓		47	74					31	48	
Esophagus			29↓		114	19↓						36↓
Stomach		34↓	29		28						27	
14 hr Tongue	19					21↓		36				50
Esophagus	48					39↓			153	72		61
Stomach	39					29↓		32↓	34			47
18 hr Tongue		11↓	21↓	20↓	21↓	39↓		33↓	No data			
Esophagus						35↓		19↓			53	
Stomach			27↓	18↓		24↓						33↓
22 hr Tongue			36↓	51↓		37↓						26↓
Esophagus					25↓	40↓			36↓			
Stomach						41↓			36↓			

*Percent difference between mean absolute values of control and experimental animals.
†When an ↓ follows a number it implies a decrease rather than an increase; all values listed above were found to be statistically significantly different ($p<0.05$) when compared to placebo controls.

ognizing that all differences such as changes in waveform etc., that might be evident from the illustrated data are not necessarily commented on in detail; they are, however, documented.

Tongue

Out of the 72 sets of data relative to the tongue 26 showed a statistically significant response ($p<0.05$) and they are listed in Table 1. Sixteen sets showed a decrease and 10 an increase in the incorporation of [^{3}H]TdR into DNA when compared to the placebo group. Fourteen out of the 16 decreases occurred when ACTH was administered at either 18, 22 and 2 HALO (dark and early light span); there was no such effect at 6 HALO (Table 1; Fig. 3). Of the 10 responses recorded during the 6, 10 and 14 HALO (latter part of light and early dark) 8 were increases and resulted from both low and high doses. Clearly maximal response resulted from the administration of ACTH 1–17 during either late dark or early light and the higher dose was the most effective, specifically at about 4 hr after the administration of ACTH 1–17 (Table 1).

Esophagus

Out of the 72 sets of data, 27 showed a statistically significant response. Eighteen sets showed a decrease and 9 an increase in the incorporation of [^{3}H]TdR into DNA. Eleven out of the 16 decreases occurred when ACTH was administered at 18, 22, and 2 HALO (Table 1; Fig. 4). The strongest consistent inhibitory effect was associated with those animals who received the large dose 4 hr after the administration, especially when administered at 10, 14, 18, 22 and 2 HALO (that is during the dark and first part of the light span). There was no such effect at 6 HALO (Table 1).

Stomach

Out of 72 sets of data 23 showed a statistically significant response with 14 out of the 23 sets showing a decrease and 9 showing an increase in the incorporation of [^{3}H]TdR into DNA. Eleven out of the 14 increases occurred when ACTH was administered either at 18, 22 and 2 HALO. Of the 10 responses recorded at 6, 10 and 14 HALO 7 were increases and resulted from both the low and the high doses of ACTH 1–17. Clearly maximal responses resulted from the administration of ACTH 1–17 during 22 and 2 HALO, the high dose was the most effective, specifically at about 4 hr after its administration (Table 1; Fig. 5).

Comparisons of the Circadian Mesors of All Four Experimental Groups at Fixed Intervals After Treatment

Figures 6, 7 and 8 illustrate the variation in the circadian mesor of incorporation of [^{3}H]TdR into DNA in each of the

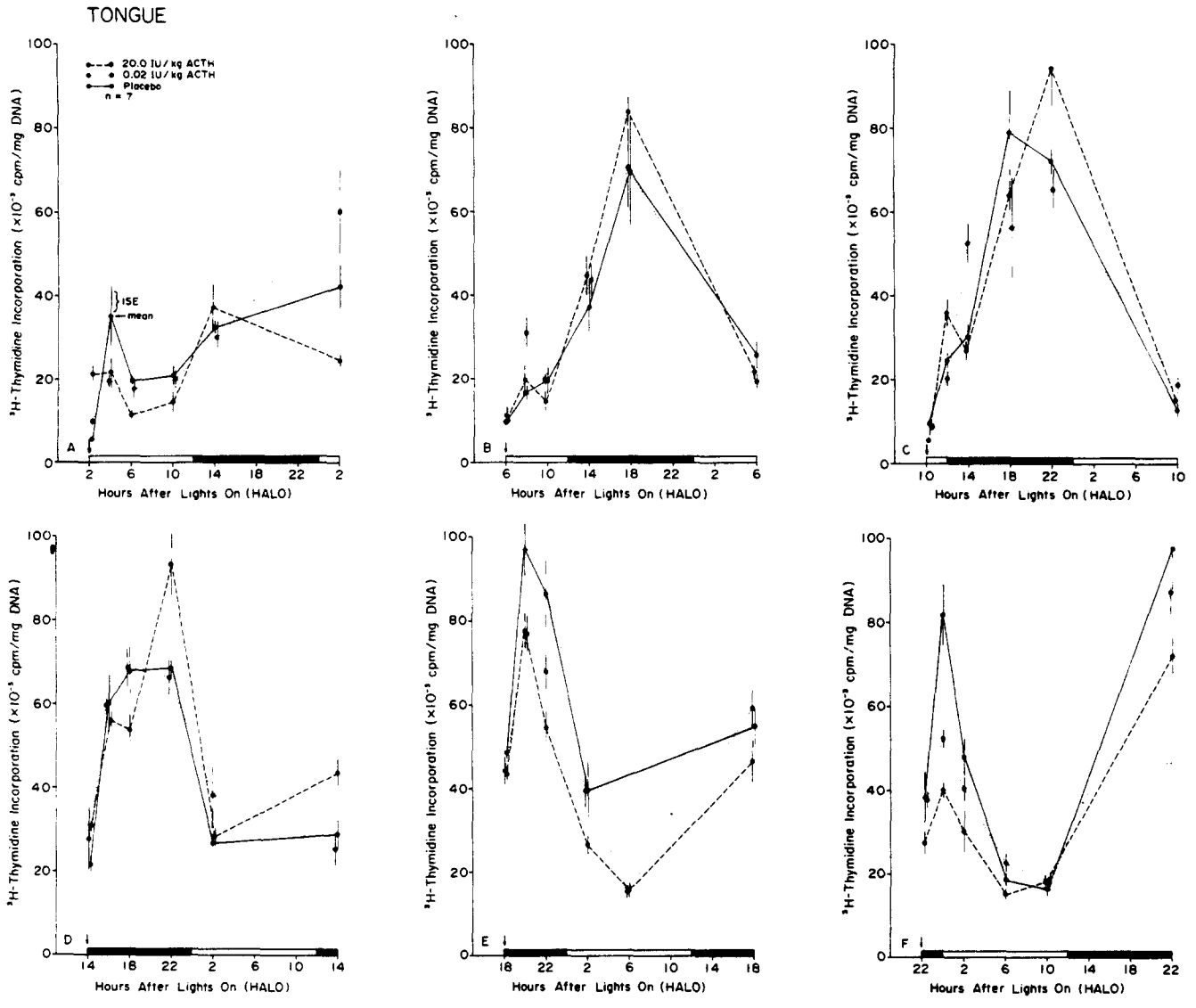

FIG. 3. Data plotted in a conventional manner to show the variation in response at different intervals after each of the 6 treatment times representing different circadian stages of the mouse; see arrows and their relationship to the light-dark cycle). The abscissa indicates that the animals were subjected to 12 hours of light (white bar) alternating with 12 hours of darkness (black bar). Every time point is in reference to "lights on"; thus 0 hours is the beginning of light. 2 *H*ours *A*fter *L*ights *O*n is expressed as 2 HALO. 14 HALO represents 14 hours after lights on (or in this case 2 hours after lights off). The kill times shown on the abscissa were 15 min, 2, 4, 8, 12 and 24 hours after each treatment. Thus there were 18 sets of data generated for the 3 treatment groups or a total of 54 sets.

three different experimental groups at different intervals after treatment and compares them with the mesors determined from the data obtained on untreated control animals.

Tongue

Fifteen min post-treatment. In this case (Fig. 6) the mesor for each experimental group represents the pooled data from all sampling performed at 15 min following the injection of placebo or ACTH 1–17 at each of the six different circadian stages. Incorporation of [³H]TdR into DNA was decreased when compared to the mesor of the untreated controls by 46%, 48% and 51% in the placebo, the low, and high doses of ACTH, respectively. All these decreases were highly statistically significant with $p<0.0001$ for all 3 sets of data (Fig. 6).

Two hr post-treatment. The mesor in the incorporation of [³H]TdR into DNA in the three experimental groups approached the mesor of the untreated controls, with the placebo data showing a statistically significant increase of 24% over the untreated controls ($p=0.0261$) (Fig. 6).

Four hr post-treatment. In this case the data from animals receiving the high doses of ACTH 1–17 showed as 25% reduction in the incorporation of [³H]TdR into DNA ($p=0.0453$) (Fig. 6).

Eight, 12 and 24 hr post-treatment. At these intervals, none of the mesors from the experimental groups were

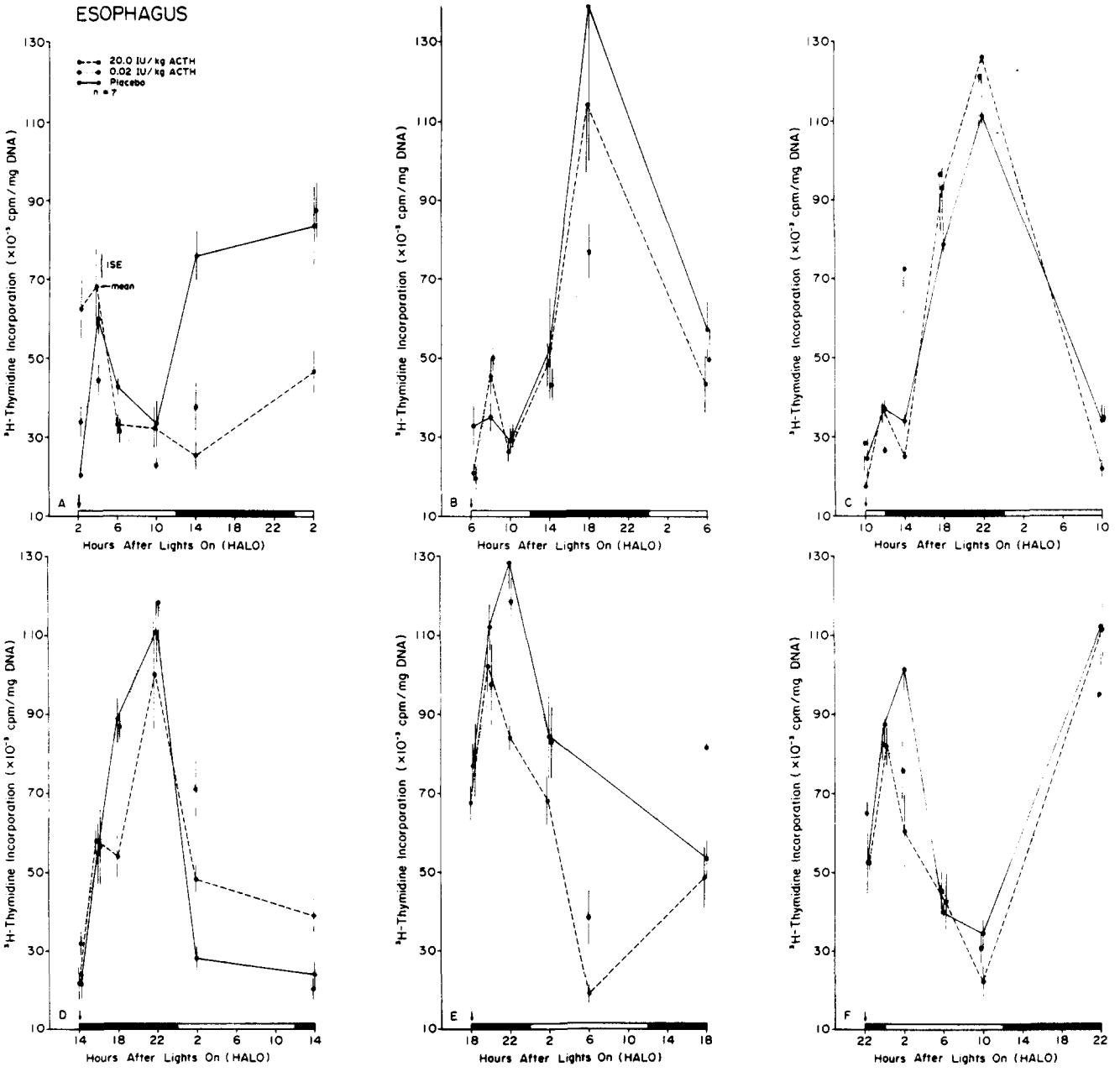

FIG. 4. See explanation given in Fig. 3 for data from the esophagus.

statistically significantly different from the mesors obtained on the untreated controls (Fig. 6).

Esophagus

Fifteen min post-treatment. As in the tongue, the mesor of [^{3}H]TdR incorporation was decreased when compared to the mesor of the untreated animals by 34%, 25% and 30% in groups receiving the placebo, the low, and high doses of the ACTH 1–17, respectively. All these decreases were statistically significant ($p<0.03$ in all cases) (Fig. 7).

Two hr post-treatment. Two hours after ACTH 1–17 treatment the mesor of [^{3}H]TdR incorporation in the three experimental groups approached the mesor in the untreated controls; in fact the high dose and placebo were increased somewhat above the level of the mesor of the untreated animals (Fig. 7). However, none of these differences were statistically significant.

Four hr post-treatment. Similar again to the situation in the tongue, the mesor in [^{3}H]TdR incorporation into DNA was decreased by 18% when the high dose of ACTH was used but this decrease was not statistically significant ($p=0.10$). There was a 24% and 22% increase in the placebo and the low dose groups respectively ($p=0.027$ in both cases) (Fig. 7).

Eight hr post-treatment. As was the case in the tongue,

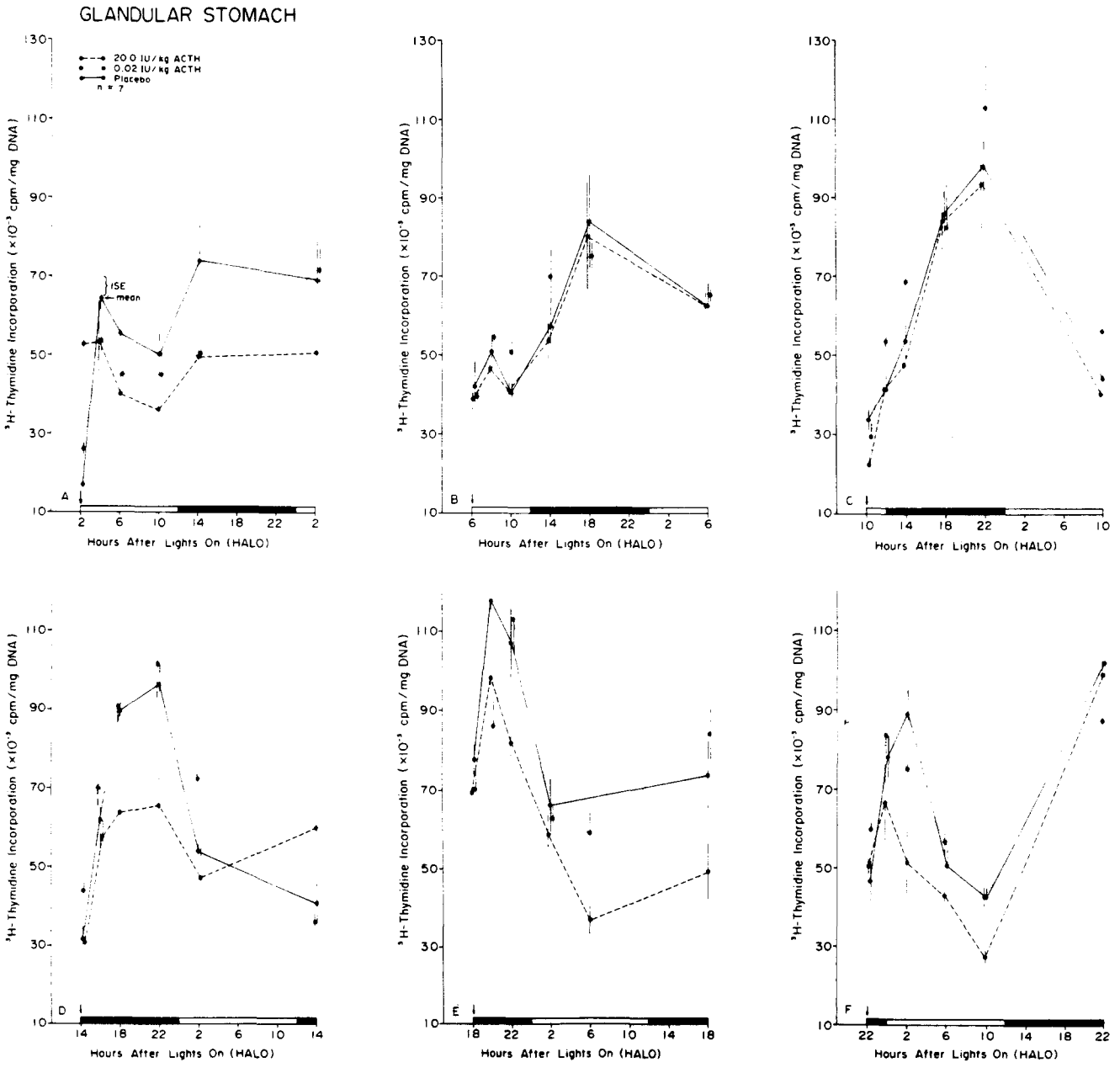

FIG. 5. See explanation given in Fig. 3 for the data from stomach.

there was a rebound in the mesor in the incorporation of [^{3}H]TdR into DNA in the group receiving the high dose, so that all three experimental groups approached the control mesor but still they represented an increase above the control of 16%, 22% and 12% in the placebo, low and high doses of ACTH 1–17, respectively (Fig. 7).

Twelve and 24 hr post-treatment. None of the mesors for the placebo, low and high doses differed statistically from those of the untreated controls (Fig. 7).

Glandular Stomach

Fifteen min post-treatment. The incorporation of [^{3}H]TdR into DNA was decreased when compared to the mesor of the untreated animals by 39%, 33% and 34% in the placebo, the low and the high doses of ACTH 1–17, respectively. All these decreases were statistically highly significant ($p<0.0001$ in all cases) (Fig. 8).

Two, 4, 8, 12 and 24 hr post-treatment. By two hr after treatment the data from the placebo and the lower dose of ACTH 1–17 animals approached values very near the untreated controls. The high dose of ACTH was still lower than the untreated controls by 8% but this was not statistically significant. The data from the animals receiving the high dose remained low at 4, 8 and 12 hr being decreased by 18%, 15% and 15%, respectively. Two of the decreases repre-

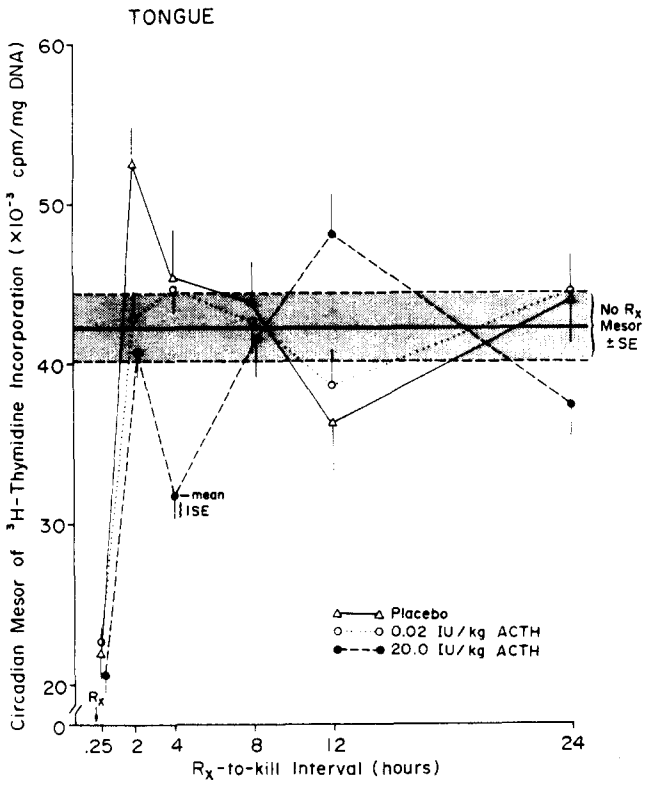

FIG. 6. Comparison of the mesors of the three treated groups with that of the untreated controls, in the tongue.

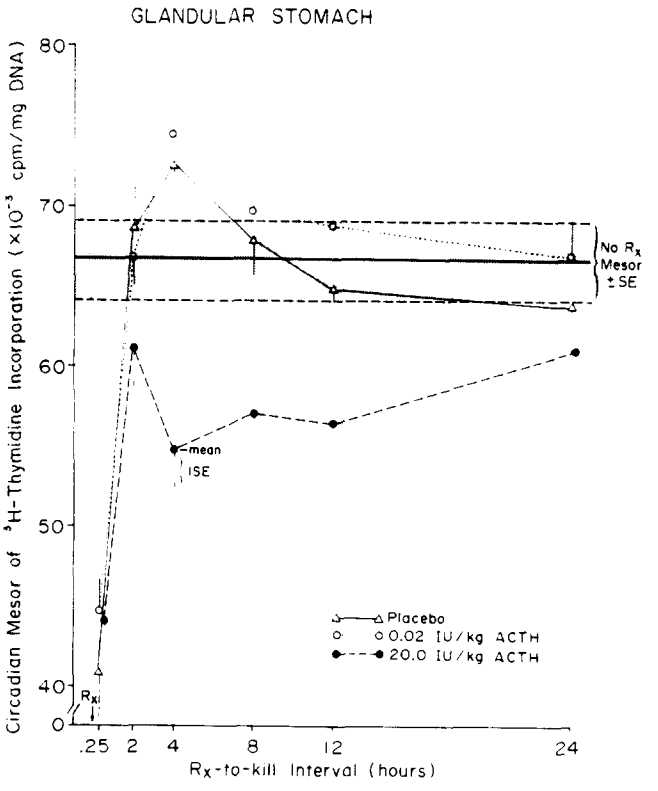

FIG. 8. Comparison of the mesors of the three treated groups with that of the untreated controls, in the stomach.

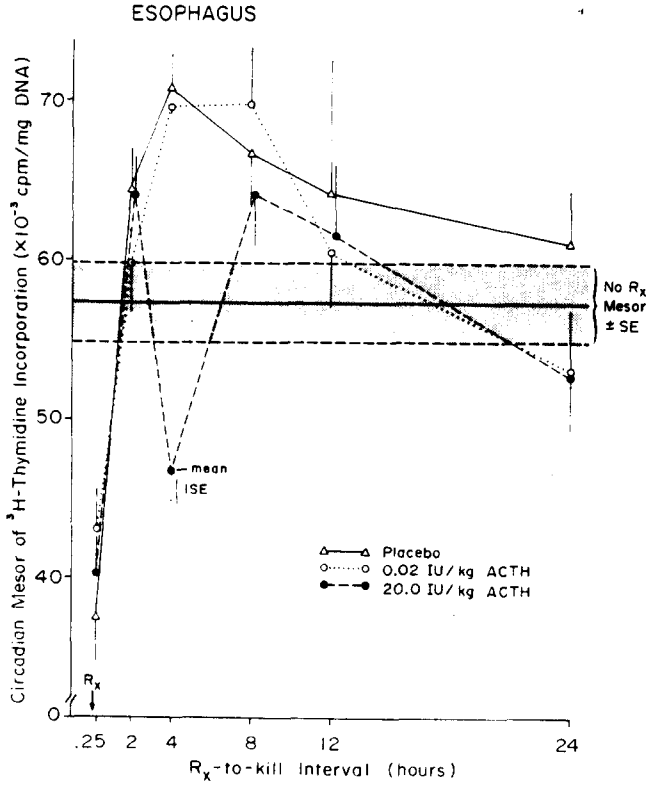

FIG. 7. Comparison of the mesors of the three treated groups with that of the untreated controls, in the esophagus.

sented statistically significant decreases ($p<0.009$ for the 4 hr and <0.04 for the 8 hr; the 12 hr decrease was of borderline significance ($p<0.09$).

The data strongly suggest that the large dose of ACTH 1–17 strongly inhibited the incorporation of [³H]TdR into DNA at 4 and 8 hr but it was approaching control levels at 12 and 24 hr (Fig. 8).

Statistical Analysis of the Data

Analysis of variance. 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-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 (Table 2).

Rhythmometric Summary of All Data

Cosinor analyses indicated a remarkable degree of circadian rhythmicity in the incorporation of [³H]TdR into DNA for all 57 sets of data with $p<0.001$ in all cases. This is best illustrated in Table 3.

One reason for including Table 3 is to show the dramatic statistical significance ($p<0.001$ in all cases) of the data obtained from both the control and experimental animals. Such is convincing evidence that there is a strong sinusoidal cir-

TABLE 2

THREE-WAY ANALYSIS OF VARIANCE SUMMARY ON [^{3}H]TdR INCORPORATION INTO DNA SOURCE OF VARIATION

	Degrees of Freedom	Mean Square	F	p
Tongue				
Main Effects				
Kind of treatment (K)	2	1838.8	12.1	0.001
Time of treatment (T)	5	12630.8	83.4	0.001
Sampling time (s)	5	8827.4	58.3	0.001
2-Way Interactions				
K × T	10	758.1	5.0	0.001
K × S	10	681.6	5.7	0.001
T × S	25	10343.5	68.3	0.001
Residual*	650	151.5		
Esophagus				
Main Effects				
Kind of treatment (K)	2	5049.6	11.6	0.001
Time of treatment (T)	5	18593.7	42.6	0.001
Sampling time (s)	5	12393.1	28.4	0.001
2-Way Interactions				
K × T	10	2068.1	4.7	0.001
K × S	10	1550.5	3.6	0.001
T × S	25	18167.2	41.7	0.001
Residual*	670	436.0		
Stomach				
Main Effects				
Kind of treatment (K)	2	6933.0	28.7	0.001
Time of treatment (T)	5	10050.2	41.5	0.001
Sampling time (s)	5	10078.3	41.6	0.001
2-Way Interactions				
K × T	10	599.7	2.5	0.002
K × S	10	722.3	3.0	0.001
T × S	25	7360.2	30.4	0.001
Residual*	674	242.0		

*Residual includes within-group and 3-way interaction terms.

cadian variation in all 3 tissues when the incorporation of [^{3}H]TdR into DNA is monitored. However, when comparing mesors (level) of the 3 different tissues it should be kept in mind that the mesor may depend on local pool size, permeability, availability of the label at the tissue, etc. For this reason we have refrained from commenting, as much as one might, on the differences between tissues in mesors. However, along this line it is of interest that a recent study by Rubin *et al.* [12] was done, on the tongue and esophagus of mice, to compare the circadian variation obtained using the incorporation of [^{3}H]TdR technique with the flow cytometry (FCM) technique, which does not depend on the above mentioned variables. The results showed a remarkable similarity in the rhythm characteristics, irrespective of the technique used. Thus the incorporation technique used by us is very reliable for estimating rhythmic parameters such as acrophase, amplitude and possibly even the mesor, at least in the tongue and esophagus.

DISCUSSION

Control Group

A high amplitude circadian rhythm was found to characterize the incorporation of [^{3}H]TdR into DNA in the tongue, esophagus and glandular stomach; this confirms earlier findings [14–15]. Much of our previous data has involved studies done on epidermal growth factor (EGF) which we have found to be a powerful stimulator of DNA synthesis in the same three tissues as well as in other parts of the gut [14]. It remains an important task to determine how EGF and ACTH 1–17 (as well as other peptides or growth factors) are interacting in the time domain.

Comparison of ACTH 1–17 and Placebo Treated Groups

The experimental data showed for the first time (for tongue, esophagus and stomach) that exogenous ACTH 1–17 administered at different circadian stages effect, at different intervals of time, the incorporation of [^{3}H]TdR into DNA in different ways when compared to the placebo group. When ACTH 1–17 was administered at certain circadian stages no statistically significant effect was recorded using either the higher or lower dose of ACTH 1–17 at a fixed interval after the administration. When, however, the ACTH 1–17 was administered at other circadian stages the same dose caused a statistically significant decrease at the same fixed interval, or conversely when administered at still another circadian stage the effect of ACTH on the incorporation of [^{3}H]TdR into DNA appeared to be stimulatory when compared to the treated animals. For examples of these three possible responses simply compare the results obtained on the tongue at 2 hr after injection (Fig. 3 and Table 1). Following a large dose of ACTH 1–17 at 2 or 6 HALO (Fig. 3a and b), there is no statistically significant difference between the ACTH 1–17 treated and placebo group. After injection at 10 HALO, results reveal a statistically significant increase of 47% (Fig. 3C). Results in Fig. 3F (R_x at 22 HALO), on the other hand, indicate a statistically significant decrease of 105%. An examination of Table 1 or Figs. 4–5 reveals similar examples of diverse responses for the esophagus and stomach.

Moreover, the results demonstrated that the interval of time between administration of ACTH 1–17 and tissue sampling, as well as the circadian stage at which ACTH 1–17 was administered, determines the magnitude of the response.

The most consistent effect of the higher dose of ACTH 1–17 when compared to the placebo group occurred for all three tissues at four hr after treatment, with 13 out of 18 sets of data showing a statistically significant decrease. An exception was when ACTH 1–17 was administered at 6 HALO (Table 1), clearly at 4 hr post-injection the effect was strongest when ACTH 1–17 was administered during the dark span or in the first part of the light span.

Comparison of the Mesors of ACTH 1–17 and Placebo Treated Groups with Untreated Controls

When the data were pooled from all six injection times and then were compared to the mesor obtained from the untreated controls the responses in all three tissues were similar in that they all showed a statistically significant decrease in the incorporation of [^{3}H]TdR into DNA at 15 min post-injection. One difference in experimental design which made the groups of animals killed at 15 min different from all other groups was that [^{3}H]TdR was administered shortly after treatment was given and thus the animals were sac-

TABLE 3

RHYTHMOMETRIC SUMMARIES OF DATA OBTAINED FROM TONGUE, ESOPHAGUS AND STOMACH ($p<0.001$ IN EACH CASE)

	Treatment-to-Kill Interval	N	Mesor ±S.E.	Amplitude ±S.E.	Acrophase (95% Confidence Int.)
No Treatment					
Tongue		31	37.0 ± 2.7	18.8 ± 2.9	−330° (−301, −6)
Esophagus		35	57.2 ± 2.5	32.5 ± 3.1	−343° (−330, −357)
Stomach		34	66.6 ± 2.5	23.3 ± 3.2	−331° (−304, −3)
Placebo					
Tongue	15 min	38	22.0 ± 1.6	20.6 ± 2.3	−283° (−271, −295)
Esophagus	15 min	41	37.7 ± 3.0	20.2 ± 4.3	−296° (−273, −320)
Stomach	15 min	41	40.9 ± 2.4	15.4 ± 4.1	−271° (−242, −301)
Tongue	2 hr	42	52.5 ± 2.3	41.5 ± 313	−313° (−304, −322)
Esophagus	2 hr	41	64.4 ± 2.5	37.1 ± 3.5	−326° (−315, −337)
Stomach	2 hr	42	68.6 ± 2.9	30.3 ± 4.2	−320° (−305, −336)
Tongue	4 hr	42	45.3 ± 2.1	34.4 ± 2.9	−315° (−306, −325)
Esophagus	4 hr	42	70.8 ± 2.1	52.5 ± 2.9	−337° (−331, −343)
Stomach	4 hr	42	72.5 ± 2.1	33.6 ± 3.0	−331° (−321, −341)
Tongue	8 hr	42	43.9 ± 2.4	31.1 ± 3.4	−298° (−286, −310)
Esophagus	8 hr	41	66.6 ± 3.2	37.5 ± 4.5	−327° (−313, −341)
Stomach	8 hr	41	67.8 ± 2.3	23.8 ± 3.2	−311° (−295, −326)
Tongue	12 hr	33	36.3 ± 3.4	31.5 ± 5.1	−298° (−283, −314)
Esophagus	12 hr	35	64.1 ± 8.3	68.8 ± 13.1	−277° (−260, −294)
Stomach	12 hr	35	64.8 ± 3.7	30.1 ± 5.7	−290° (−272, −308)
Tongue	24 hr	40	44.0 ± 2.8	36.2 ± 3.9	−324° (−311, −336)
Esophagus	24 hr	41	61.1 ± 3.2	39.7 ± 4.4	−357° (−344, −9)
Stomach	24 hr	42	63.8 ± 3.0	23.3 ± 4.3	−342° (−321, −3)
Low-Dose ACTH 1–17					
Tongue	15 min	39	22.6 ± 1.0	20.0 ± 1.4	−286° (−278, −293)
Esophagus	15 min	41	43.1 ± 2.6	27.8 ± 3.6	−298° (−283, −313)
Stomach	15 min	40	44.7 ± 1.9	19.1 ± 2.7	−286° (−270, −302
Tongue	2 hr	40	42.7 ± 1.8	27.9 ± 2.6	−295° (−285, −305)
Esophagus	2 hr	40	59.7 ± 3.0	29.4 ± 4.3	−327° (−310, −343)
Stomach	2 hr	41	66.8 ± 1.8	18.7 ± 2.6	−312° (−297, −328)
Tongue	4 hr	42	44.6 ± 1.5	29.1 ± 2.1	−291° (−283, −299)
Esophagus	4 hr	42	69.5 ± 2.9	41.6 ± 4.1	−326° (−313, −339)
Stomach	4 hr	42	74.5 ± 2.6	32.8 ± 3.2	−310° (−299, −321)
Tongue	8 hr	41	42.6 ± 2.0	29.5 ± 3.0	−299° (−286, −312)
Esophagus	8 hr	41	69.7 ± 3.6	44.0 ± 5.1	−326° (−313, −339)
Stomach	8 hr	42	69.6 ± 3.0	24.0 ± 4.3	−307° (−287, −327)
Tongue	12 hr	41	38.6 ± 2.2	29.5 ± 3.0	−299° (−287, −311)
Esophagus	12 hr	42	60.3 ± 3.5	44.7 ± 4.9	−334° (−321, −346)
Stomach	12 hr	42	68.7 ± 2.7	29.8 ± 3.9	−333° (−319, −348)
Tongue	24 hr	39	44.5 ± 2.2	35.3 ± 3.1	−329° (−319, −338)
Esophagus	24 hr	42	53.0 ± 3.8	28.7 ± 5.3	−322° (−301, −343)
Stomach	24 hr	41	66.9 ± 2.5	20.3 ± 3.6	−345° (−325, −5)
High-Dose ACTH 1–17					
Tongue	15 min	39	20.6 ± 1.2	15.7 ± 1.6	−301° (−289, −313)
Esophagus	15 min	41	40.2 ± 2.3	26.0 ± 3.2	−331° (−317, −345)
Stomach	15 min	42	44.1 ± 1.8	18.3 ± 2.5	−326° (−311, −342)
Tongue	2 hr	38	40.7 ± 1.5	27.3 ± 2.1	−285° (−277, −294)
Esophagus	2 hr	40	64.0 ± 2.4	30.5 ± 3.3	−331° (−318, −344)
Stomach	2 hr	39	61.1 ± 2.5	22.6 ± 3.3	−318° (−300, −336)
Tongue	4 hr	40	31.8 ± 1.5	23.8 ± 2.1	−302° (−292, −312)
Esophagus	4 hr	41	46.7 ± 2.3	27.1 ± 3.3	−334° (−320, −347)
Stomach	4 hr	42	54.3 ± 2.1	19.2 ± 3.0	−314° (−297, −332)

Continued

TABLE 3

RHYTHMOMETRIC SUMMARIES OF DATA OBTAINED FROM TONGUE, ESOPHAGUS AND STOMACH ($p < 0.001$ IN EACH CASE)

Tongue	8 hr	39	41.5 ± 2.4	37.0 ± 3.5	−300° (−289, −310)
Esophagus	8 hr	40	64.0 ± 3.2	35.3 ± 4.6	−315° (−301, −330)
Stomach	8 hr	41	57.1 ± 2.4	20.9 ± 3.4	−300° (−282, −318)
Tongue	12 hr	36	48.0 ± 2.5	41.9 ± 3.4	−297° (−287, −307)
Esophagus	12 hr	38	61.6 ± 4.3	58.0 ± 5.8	−309° (−296, −321)
Stomach	12 hr	38	56.4 ± 3.4	31.8 ± 4.7	−308° (−290, −325)
Tongue	24 hr	37	37.4 ± 2.0	23.5 ± 2.9	−297° (−384, −349)
Esophagus	24 hr	39	52.9 ± 3.6	33.1 ± 5.0	−332° (−314, −349)
Stomach	24 hr	39	61.0 ± 3.4	16.8 ± 4.5	−335° (−304, −6)

*All units expressed as counts/min/mg of DNA ($\times 10^{-3}$).

rificed after about 14 min rather than the customary 30 min. Our first reaction was that this short interval of 14 min, as contrasted to a 30 min span allowed for all other post-treatment intervals, might be the cause of this. However, we are not convinced of this explanation because some other tissues from the same animals did not show such dramatic decreases. Moreover, extensive studies have since been done by us showing that it makes little difference in the values obtained whether one injects the [^{3}H]TdR 15 or 30 min prior to killing, due to the short half-life of the isotope in the circulation. Further work is needed to determine why this decrease in the incorporation of [^{3}H]TdR into DNA was frequently found at 15 min. (If ACTH 1–17 was involved, clearly it must have been a response to endogenous ACTH.)

Interestingly, the placebo was capable of influencing the incorporation of [^{3}H]TdR into DNA when compared to the untreated controls. Such results demonstrate that not only the placebo but also the untreated controls are necessary when evaluating the effect of ACTH 1–17 on the incorporation of [^{3}H]TdR into DNA. Such has not been the conventional way of carrying out experiments, where frequently the placebo or carrier substance serves as the only control.

When the mesors of all 4 groups and all 3 tissues were compared at certain intervals after treatment the higher dose clearly decreased the incorporation of [^{3}H]TdR into DNA at 4 hr after treatment when compared to the other 3 groups. It is emphasized that such a conclusion can be made only when the circadian mesors are compared. For example, if one examines the effects of this same large dose after treatment at different specific circadian stages there were certain times when ACTH did not inhibit the incorporation of [^{3}H]TdR into DNA at 4 hr but was more effective at other intervals after administration (Figs. 3–5). One generalization that can be made is that the greatest inhibition of the incorporation of [^{3}H]TdR into DNA occurs at 4 hr subsequent to administering the higher dose between mid-dark and early light and this holds true for all three tissues.

The response to the higher dose of ACTH 1–17 was remarkably similar in both the tongue and esophagaus, but it was considerably more prolonged in the stomach because the prominent decrease was sustained up to 12 hr after injection (Fig. 8).

Clearly, the results emphasize the necessity in any experimental design of considering the temporal organization [17]. To ignore such dramatic rhythmic changes can only lead to ambiguity as to what effect ACTH has on the incorporation of [^{3}H]TdR into DNA. Clearly time of administration may be as important a factor to consider as is the dose. If we are ultimately to understand normal as well as abnormal cell proliferation we cannot ignore the basic oscillatory nature of the system under study.

Recognition of such complexity in response is a first step toward determining what might be the biological significance of such variation. Certainly variation in response is not something one can simply control by sampling at one time of day.

As mentioned above the variation in response to anti-cancer agents, especially those that are cell-cycle specific, might be used to improve the cancer chemotherapeutic ratio. It is quite likely that the susceptibility-resistance cycle to such agents in non-tumor bearing animals are due to circadian cell metabolism and/or proliferation in susceptible tissues such as the digestive tract and bone marrow which are critical for host tolerance and survival. An ideal situation for timed treatment (chronotherapy) would be if a chemotherapeutic agent could be administered at a time when these susceptible tissues of the host, which limit the application or dosage of the agent, are at the most resistant stage of their proliferative cycle, while the tumor is at the same time at its most sensitive one. In other instances, the susceptible host tissues may be circadian periodic but the tumor may not. In such a case, treatment can be timed according to the susceptibility-resistance cycle of the host, with the drug given at the most resistant stage of the circadian cycle of the critical host tissues. Experimentally [16,17], there already has been considerable success with this approach. Also as mentioned above it has been reported that ACTH 1–17 can induce a statistically significant circadian rhythm in DNA synthesis the Harding-Passey melanoma in Balb/C female mice [13]. Interestingly, such can only be demonstrated if the injection of ACTH 1–17 is given at the beginning of the light span to which the animals are standardized (which was the same as used in this present study). Against the above background ACTH 1–17 as a synchronizer of rhythms of cell proliferation in tumors and by its ability to manipulate rhythm in susceptibility-resistance to anti-cancer agents such as adrimycin could be important, to consider in cancer chemotherapy and needs to be explored further.

Finally it should be mentioned that the analogue ACTH 1–17 was selected for study for several reasons. Among them

was the fact that considerable data has already accumulated documenting its circadian-stage dependence in rodents, in healthy subjects and in patients with arthritis. It is assumed, until proof is offered to the contrary from studies on adrenalectomized mice, that the pervasive effects thus far reported for ACTH 1–17 are largely mediated by corticoids. What is attractive for a potential use clinically is that it can be given intranasally to stimulate the adrenal [4]. This administration mode represents a definite advantage: if a patient were to take ACTH 1–17, 24 hr prior to chemotherapy with the aim of reducing toxicity such that has been experimentally demonstrated for adrymycin, a sniff of ACTH 1–17 could readily be taken at home.

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