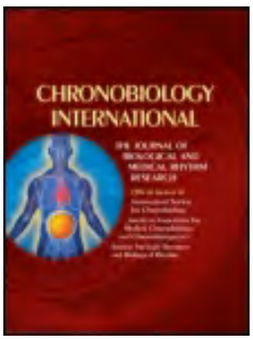

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CIRCADIAN-STAGE DEPENDENT ACTH 1–17 EFFECT ON DNA SYNTHESIS IN MURINE DUODENUM, COLON AND RECTUM*

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Abstract—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 duodenum, colon and rectum of CD2F$_1$ mice standardised to 12hr of light alternating with 12hr of darkness. A question asked was whether the difference in times of administration along the 24-hr 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. A generalization that can be made from all these tissues is that ACTH 1–17 had a greater influence in bringing about a decrease in DNA synthesis when it was administered around the time of transition from dark to light. A similar finding was made earlier for the ACTH 1–17 effect upon the tongue, esophagus and stomach.

A 2- and 3-way analysis of variance supports our 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.

Key words index—ACTH 1–17, DNA, circadian, rhythm, placebo, duodenum, colon, rectum.

Introduction

A short-chain synthetic analogue, ACTH 1–17 (HOE 433 = SYNCHRODYN®), has been extensively studied for the circadian-stage (rather than time of day) dependence of its effects in both human beings (1, 2–4) and rodents (5, 6, 7, 8, 9, 10, 11, 12). In rather small doses, the analogue stimulates the secretion of aldosterone, cortisol and testosterone in human beings (1, 3) and experimental animals. In all species examined, such stimulation depends upon the circadian stage administered, and the time of maximal response to ACTH 1–17 differs for aldosterone and corticosterone, both *in vitro* and *in vivo* (1, 3, 4).

Mice show a dramatic susceptibility–resistance rhythm to doxorubicin (DR) (9). Predictable times of maximal and minimal toxicity have been demonstrated by administering this anticancer agent at frequent intervals along a 24-hr time scale, to different groups of mice standardized to the same light-dark cycle. Moreover, when ACTH 1–17 was administered to mice 24 hr prior to DR, a statistically significant reduction in host toxicity was found at the expected circadian time of maximal toxicity. When the ACTH 1–17 was given 24 hr prior to administering the DR at the expected circadian time of greatest tolerance (minimal toxicity), no reduction in toxicity was noted (9). Of further interest was the finding that ACTH 1–17 can induce, only when administered 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; whereas otherwise this tumor does not show such a rhythm in either Balb/C or CD2F$_1$ mice (13). Moreover, the time-of-treatment as well as treatment-to-kill interval (sampling time) determine any effects of ACTH 1–17 on the mitotic index of mouse corneal epithelium (10).

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The same has been reported on the incorporation [^{3}H]TdR into DNA in thymus, bone marrow and spleen of mice (11) as well as at into metaphyseal bone (12).

These studies suggest a potential role for ACTH 1-17 in improving the toxic-therapeutic ratio of cancer chemotherapy either by inducing a rhythm in tumor cell proliferation or by manipulating the natural endogenous rhythm in host tissues, especially in tissues such as the digestive tract and bone marrow which are damaged by treatment with anti-cancer agents such as DR. This paper documents circadian variations in the incorporation of [^{3}H]-TdR into DNA of the duodenum, colon and rectum and the response of these rhythms to ACTH 1-17.

Materials and Methods

On 12/22/79, 800 CD2F$_1$ female mice, approximately 6 weeks old were received from the Simonson Laboratories, Gilroy, CA. They were randomly distributed among 113 cages, seven mice to a cage. 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 darkness (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 on the same day each week; otherwise the animals were not intentionally disturbed until the beginning of the experiment. A dim red light (of ~0.5 lx at the level of the mouse eye) was used for handling of the 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 IUK/kg of body weight of ACTH 1-17 and 42 mice were injected with a placebo (which was the vehicle of ACTH 1-17). The total aliquot administered was 0.2 ml per mouse (the mice were averaged 20±2 g). The same numbers of mice from the DL standardized group were given identical injections. We believe that it is reasonable to assume that most animals from the DL environment simulated biologically at 0800 CST mice with circadian systems comparable to those at 2000 hr, or just 2 hr after the beginning of their daily activity span; whereas most of the animals from the LD environment at 0800 CST 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.

Seven 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 (i.p.) 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 the latter schedule now simulating a 0400 time point). This was repeated on a comparable group of 252 mice at 1600 (the DL mice now simulating the 0400 time point). We injected over the course of the regular working day by properly altering the light-dark schedules in the different chambers, six sets of 126 mice, three during the light span and three during dark span. 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 zero time; 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). Following each of the 6 HALO injection times, subgroups of seven mice from each of the three treated groups were killed at 15 min, 2, 4, 8, 12 and 24 hr after R_x.

Control mice (7) were also 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 prior to killing. We wanted to

maintain the seven mice per cage treatment arrangement, but sufficient animals were not available for six control groups; however, previously we had shown that a five-time point/cycle study with ACTH 1–17, yielded satisfactory results [7] thus one control group (at the 18 HALO time point) was omitted. Figure 1 illustrates the protocol.

After killing, the thoraco-abdominal cavity was opened 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 (16), 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, we here report only on the lower part of the digestive tract (duodenum, colon and rectum); other results have been published elsewhere (10–12, 14). The other tissues in which the incorporation of [^{3}H]TdR into DNA (DNA synthesis) was studied round-the-clock include the tongue, esophagus, glandular stomach (14) and spleen, thymus and bone marrow (11) and in metaphyseal bone (12). In addition, total RNA and DNA content was measured in the spleen (11), and the mitotic index determined in the corneal epithelium (10).

The data were subjected to conventional *t*-tests at each time point investigated, only to indicate the dangers of incomplete, if not spurious inferences based upon conventional approaches focusing only upon 'decreases' or 'increases' at often-arbitrarily selected single time points. To distance ourselves from the limited heuristic value of such increases and decreases, we place them in quotation marks. In addition, all data on [^{3}H]TdR incorporation by a given tissue as a function of R_x time (or, in the case of controls, kill times) were analyzed by the cosinor method (15) which provides the following information:

(1) A *P*-value from the test of the 'no-rhythm' (zero-amplitude) assumption by 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, equalling the 24-hr arithmetical mean only if the data points are equidistant and cover an integral number of cycles. 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

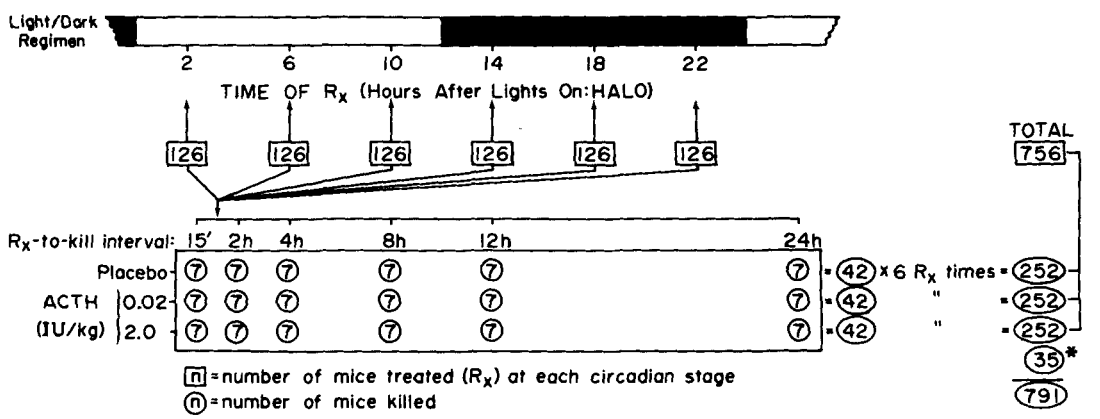


Figure 1. Protocol of study.

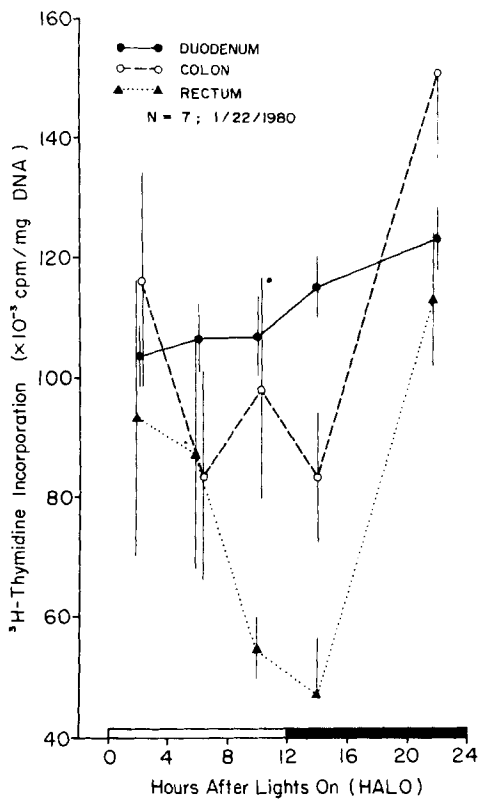
in keeping with mathematical convention. Both amplitude and mesor are given in the original units, which in this case is the incorporation of [^{3}H]TdR into DNA expressed as counts/min mg DNA (X 10^{-3}), rounded to the nearest integer; this value describes DNA synthesis and thus estimates cell proliferation (17).

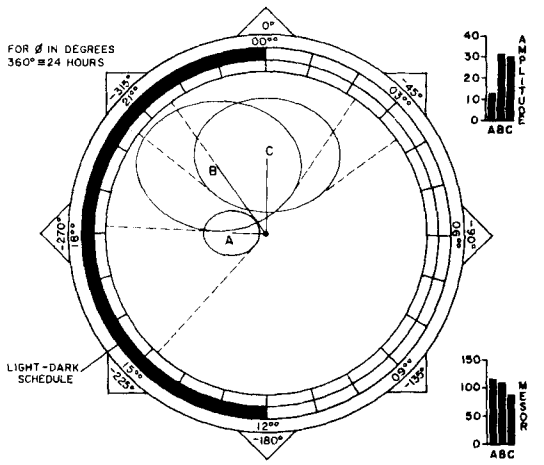
The acrophase (ϕ) represents the crest of the fitted cosine curve in relation to some arbitrarily selected reference point along the 24-hr time scale. Usually, the acrophase corresponds to the time when the data values are, on the average, highest; however, it should be noted that the acrophase is not 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 Figure 2 of this study 0° to 180° represents the light (rest) span, whereas 180° (1800) to 360° (0000) represents the dark (active) span. The minus signs preceding the number of degrees indicate a lag from the reference point (lights on). It cannot be overemphasized that the cosinor results represent only a first approximation. With denser data covering several cycles with the period investigated, a more rigorous approximation of the waveform in conjunction with regression diagnostic tests is advocated (18).

Results

Controls

The data in Figure 2 illustrate circadian variation in the incorporation of [^{3}H]TdR into DNA in the duodenum, colon and rectum; they are shown on the left as chronograms (time plots)





KEY TO ELLIPSES	P	NO. OBS.	PR	MESOR	SE	AMPLITUDE (95% CL)	ACROPHASE (Ø) (95% CL)
A DUODENUM	0.019	33	23.3	114	2.56	12.4 (1.81 23.0)	-271 (-222 -324)
B COLON	0.017	35	22.4	110	7.7	31.0 (4.7 59)	-324 (-272 -34)
C RECTUM	0.004	34	29.8	79	6.8	30.2 (8.8 52)	-1 (-308 -54)

Figure 2. Chronograms, rhythmometric summary (as determined by cosinor analysis) and a display of the same data in a polar plot.

while the cosinor results are displayed in a polar plot (on the right). The no-rhythm assumption is rejected in each case, in keeping with our earlier findings (19, 20).

Experimental

We shall first compare the data obtained from the two ACTH 1–17 groups with those 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 corresponding data from the untreated controls.

The kind and extent of response seen in the incorporation of [^{3}H]TdR into DNA for two different doses of ACTH 1–17 as compared with responses to placebo are summarized in Table 1. The waveform and other changes in each tissue are shown in Figures 3–5. Admittedly such a large amount of data can be somewhat overwhelming, thus we have summarized only our major observations while recognizing that all differences such as change in waveform, etc. that might be evident from the illustrated data are not necessarily commented on in detail; they are, however documented.

Duodenum

Out of the 72 data sets relative to the duodenum, 21 sets listed in Table 1 showed a 'statistically' significant response ($P<0.05$). Fourteen sets showed a 'decrease' and seven an 'increase' in the incorporation of [^{3}H]TdR into DNA when compared to the placebo group (Table 1, Figure 3). There was also a time (following the 14-HALO injection) when only one statistically significant response was recorded with the small dose.

Colon

Out of 72 data sets, 23 showed a statistically significant response; 15 data sets showed a

Table 1. Percentage 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 1–17 at specific intervals after treatment

		Interval between R_x and killing											
		15 min		2 hr		4 hr		8 hr		12 hr		24 hr	
R_x-Time	Tissues	S	L	S	L	S	L	S	L	S	L	S	L
2 hr	Duodenum	338*	162				22↓			13↓	21↓	38↓	
	Colon	136	206			24↓	44↓			44↓	56↓		39↓
	Rectum	114	284							45↓	48↓		56↓
6 hr	Duodenum	38					17↓					45↓	
	Colon						30↓						
	Rectum									70	67		
10 hr	Duodenum	33↓				15		39↓				35	
	Colon	29↓	48↓		27↓	199	105				42		
	Rectum					59			89				
14 hr	Duodenum	13											
	Colon						40↓	40		106	49		
	Rectum	85	171			26↓			50	150	218	45↓	
18 hr	Duodenum	13↓		25↓			30↓						
	Colon		32↓										
	Rectum								24↓	51			
22 hr	Duodenum							13↓	15↓		24↓		19
	Colon				18↓						28↓	39↓	31
	Rectum	72				39↓					39	42↓	

*Percentage difference between mean absolute values of placebo treated control and experimental animals.
†When a ↓ 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.

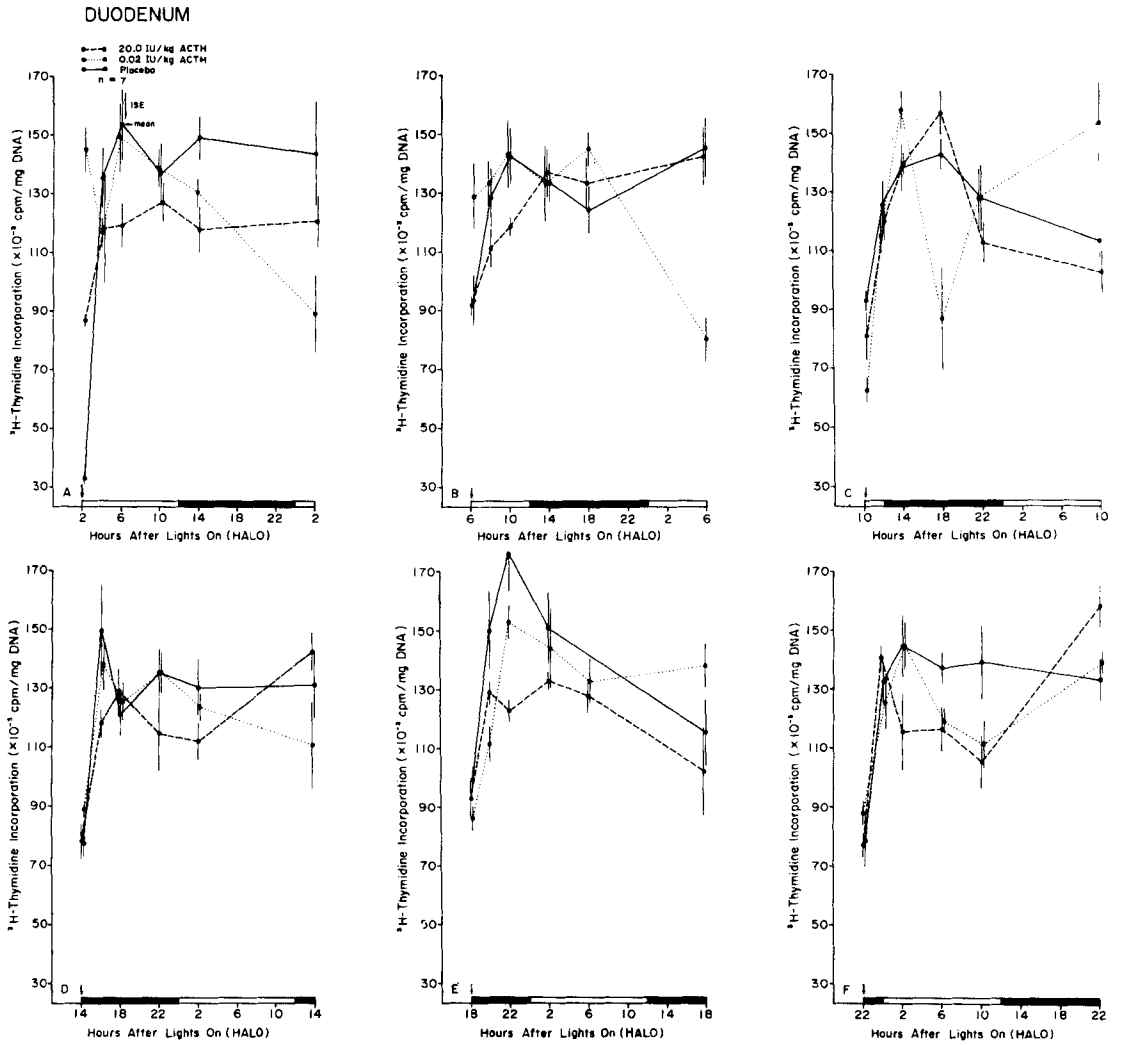


Figure 3. Data from duodenum plotted in a conventional manner to show the variation in response at different intervals after each of the six treatment times representing different circadian stages of the mouse (see arrows and their relationship to the light–dark cycle). The abscissa indicates that the animals were subjected to 12 hr of light (white bar) alternating with 12 hr of darkness (black bar). Each time point is given by 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 on). The kill times shown on the abscissa were 15 min, 2, 4, 8, 12 and 24 hr after each treatment. Thus there were 18 sets of data generated for each of the 3 treatment groups or a total of 54 sets.

'decrease' and seven an 'increase' in the incorporation of [^{3}H]TdR into DNA. Ten out of the 15 'decreases' occurred when ACTH 1–17 was administered at 22, and 2–HALO, a time of transition from dark to light (Table 1; Figure 4). In the colon DNA synthesis was remarkably stimulated at 4 hr after the 10–HALO injection and at 12 hr after treatment given at 14–HALO.

Rectum

Out of 72 data sets 22 showed a statistically significant response with eight out of the 22 sets showing a 'decrease' and 14 an 'increase' in the incorporation of [^{3}H]TdR into DNA. Again, as mentioned above for the colon, there was a remarkable stimulation at 12 hr following the 14–HALO treatment (Table 1; Figure 5).

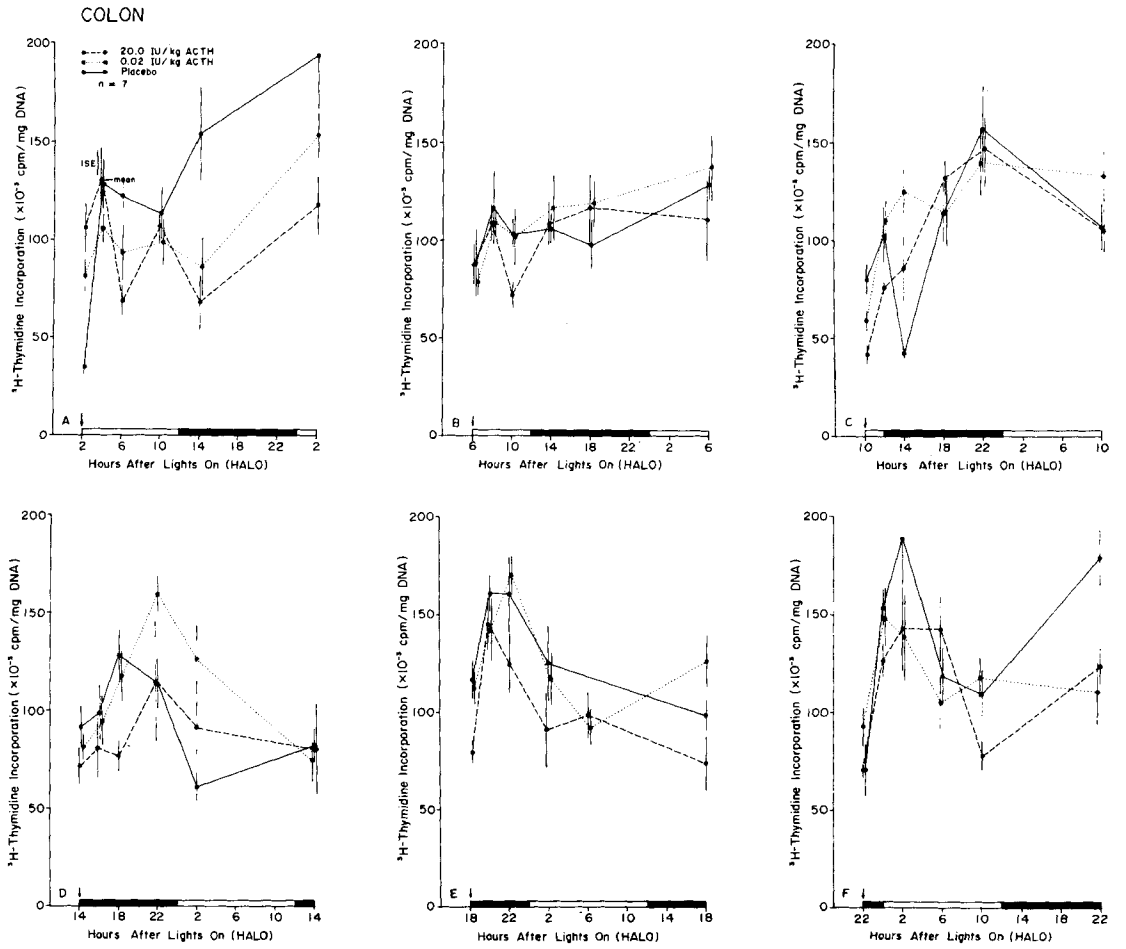


Figure 4. See explanation given in Figure 3 for data from the colon.

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 three different experimental groups at different intervals after treatment and compare these with the mesors determined from the data obtained on untreated control animals. Such an averaging aims to isolate consistent overall effects, yet necessarily obscures the circadian-stage dependence of effects.

Duodenum

15 min post-treatment. In this case (Figure 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 [^{3}H]TdR into DNA was 'decreased' when compared to the mesor of the untreated controls by 31, 8 and 26% in the placebo, the low and high doses of ACTH 1–17, respectively. Only the 'decreases' for the placebo and high dose group were statistically significant with $P<0.0001$ (Figure 6).

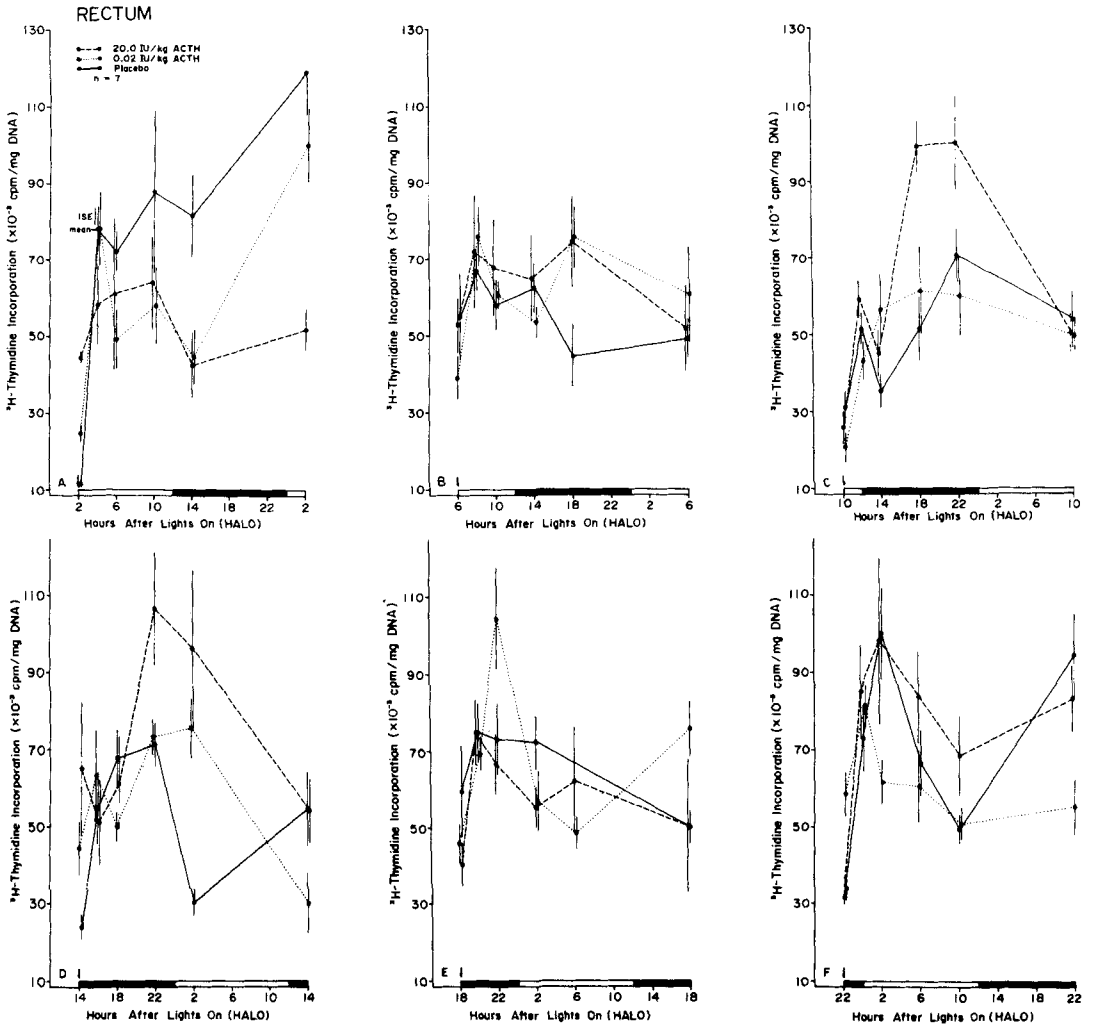


Figure 5. See explanation given in Figure 3 for the data from rectum.

2, 4, 8, 12 and 24hr post-treatment. At 2 and 4hr there was a remarkable rebound in the incorporation of [^{3}H]TdR into DNA for all three experimental groups. The strongest effect appeared to be associated with the placebo treated group because the increase remained above the value of the untreated controls throughout the 24hr span. The highest percentage 'increase' for the placebo group was 27% at 4hr post-treatment; the highest percentage 'increase' in response to the low dose of ACTH 1–17 was 26% and occurred also at 4hr post-treatment. The highest percentage 'increase' (15%) for the large ACTH 1–17 dose occurred at 8hr. The above mentioned increases were all statistically significant ($P<0.01$). The incorporation of [^{3}H]TdR into DNA synthesis in the ACTH 1–17 treated groups also remained higher than that of untreated controls, at least through 2, 4 and 8hr where all increases were significant when compared to the untreated controls ($P<0.01$ in all cases).

Colon

15 min post-treatment. As in the duodenum, the mesor of [^{3}H]TdR incorporation was 'decreased' when compared to the mesor of the untreated animals by 27, 24 and 17% in groups

receiving the placebo, the low, and high doses of the ACTH 1–17, respectively. All these 'decreases' were statistically significant ($P<0.006$ in all cases) (Figure 7).

2, 4, 8, 12 and 24 hr post-treatment. Again, as in the duodenum, there was a rebound in the level of incorporation of [^{3}H]TdR into DNA at 2 hr with only the placebo-treated group exceeding the level in the untreated controls by 15%; this 'increase' over the controls was significant ($P<0.023$). At no other time point were the untreated controls statistically significantly different from any of the experimental groups. The response in animals who received the higher dose of ACTH 1–17 was remarkably similar to that of the untreated controls (Figure 7).

Rectum

15 min post-treatment. The incorporation of [^{3}H]TdR into DNA 'decreased' when compared to the mesor of the untreated animals by 54, 52 and 46% in the placebo, the low and high doses

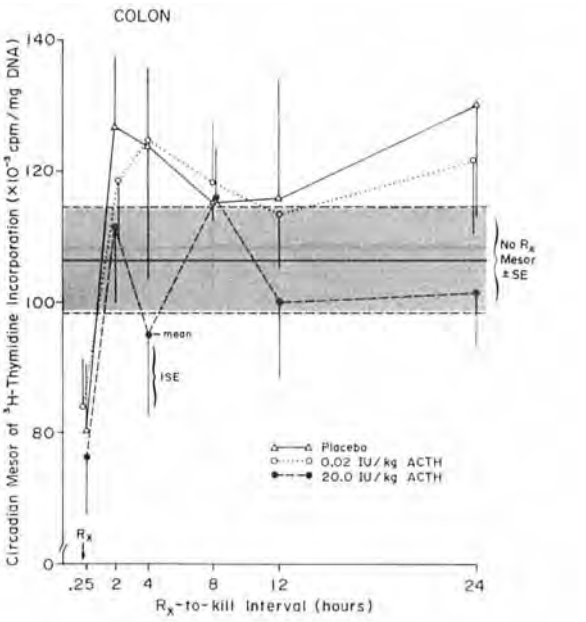


Figure 7. Comparison of the mesors of the three treated groups with the mesor of the untreated controls for DNA labelling in the colon.

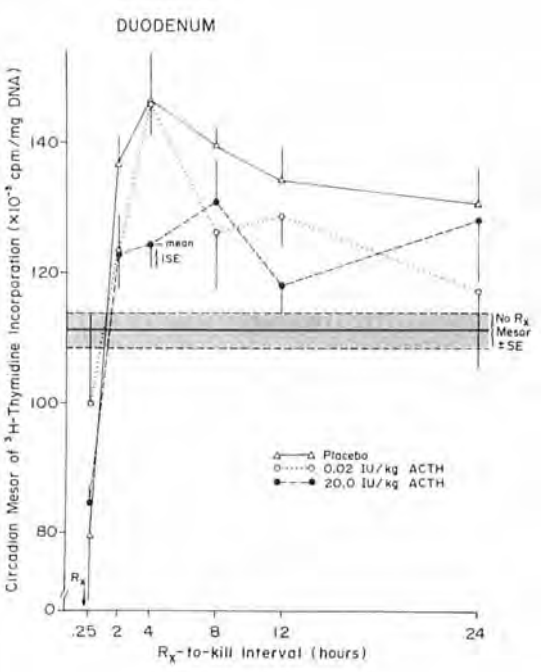


Figure 6. Comparison of the mesors of the three treated groups with the mesor of the untreated controls for DNA labelling the duodenum.

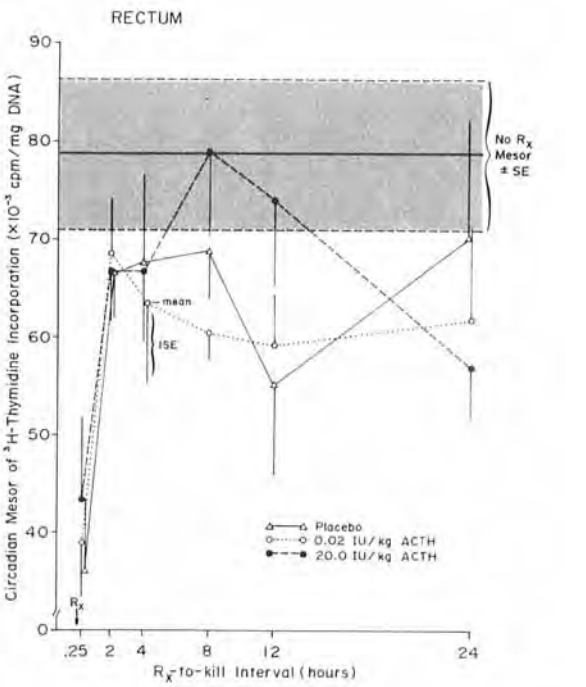


Figure 8. Comparison of the mesors of the three treated groups with the mesor of the untreated controls for DNA labelling in the rectum.

of ACTH 1–17, respectively. All these 'decreases' were highly significant ($P<0.0001$ in all cases) (Figure 8).

2 and 4 hr post-treatment. The data of all three experimental groups approached the control values, but were not statistically significantly different from the control data.

8 and 12 hr post-treatment. The mean for the smaller dose was significantly 'decreased' ($P<0.01$) by 23% at 8 hr. The means for the placebo and lower dose of ACTH 1–17 were 'decreased' at 12 hr by 44 and 25%, respectively ($P<0.0007$ and <0.008).

24 hr post-treatment. Only the mean of the group treated with the high dose was significantly 'decreased' by 28% ($P=0.006$) when compared to that of the untreated controls (Figure 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. The 2-way interactions also were highly statistically significant indicating, for example, that the effect of treatment kind varies with treatment time (Table 2).

Rhythmometric Summary of All Data

Each of the 57 sets of data on [^{3}H]TdR incorporation into DNA as a function of kill-time, for the different R_x-to-kill intervals and for the three tissues, along with the three sets of

Table 2. Three-way analysis of variance summary of [^{3}H]TdR incorporation into DNA source of variation

	Degrees of freedom	Mean square	F	P
	Duodenum			
Main effects	12	16603.8	31.9	0.001
Kind of treatment (K)	2	5224.8	10.0	0.001
Time of treatment (T)	5	1394.0	2.6	0.021
Sampling time (S)	5	36515.9	70.2	0.001
2-way interactions	45	1784.3	3.4	0.001
K × T	10	638.7	1.2	0.269
K × S	10	3637.2	6.9	0.001
T × S	25	1519.3	2.9	0.001
	Colon			
Main effects	12	17113.8	14.0	0.001
Kind of treatment (K)	2	17101.9	14.0	0.001
Time of treatment (T)	5	12547.3	10.2	0.001
Sampling time (S)	5	21686.8	17.7	0.001
2-way interactions	45	5656.4	4.6	0.001
K × T	10	2609.1	2.1	0.020
K × S	10	3820.0	3.1	0.001
T × S	25	7610.4	6.2	0.001
	Rectum			
Main effects	12	8124.5	14.5	0.001
Kind of treatment (K)	2	3099.1	5.5	0.004
Time of treatment (T)	5	3726.7	6.6	0.001
Sampling time (S)	5	14427.3	25.8	0.001
2-way interactions	45	2185.8	3.9	0.001
K × T	10	1418.1	2.5	0.005
K × S	10	1804.6	3.2	0.001
T × S	25	2699.4	4.8	0.001

Table 3. Rhythomometric summaries of data obtained from duodenum, colon and rectum

	Treatment-to-kill-interval	*N*	*P*	Mesor* ± S.E.	Amplitude* ± S.E.	Acrophase (95%) confidence (Int.)
No treatment						
Duodenum		33	<0.019	114.2 ± 2.6	12.3 ± 4.1	-271 (-243, -299)
Colon		35	<0.017	110.1 ± 7.7	30.1 ± 10.5	-324 (-283, -5)
Rectum		34	<0.004	78.6 ± 6.8	30.1 ± 9.6	-1 (-320, -42)
Placebo						
Duodenum	15 min	41	<0.005	78.8 ± 3.8	18.2 ± 5.3	-204 (-171, -237)
Colon	15 min	40	<0.005	80.1 ± 5.2	26.1 ± 7.5	-224 (-193, -256)
Rectum	15 min	40	<0.685	35.8 ± 3.9	4.8 ± 5.6	-231
Duodenum	2 hr	41	<0.248	136.8 ± 4.4	10.7 ± 6.3	-289
Colon	2 hr	42	<0.002	126.8 ± 5.5	29.4 ± 7.8	-351 (-321, -21)
Rectum	2 hr	41	<0.023	66.6 ± 3.1	13.0 ± 4.5	-20 (-342, -58)
Duodenum	4 hr	40	<0.148	145.5 ± 4.7	13.6 ± 6.8	-28
Colon	4 hr	<41	<0.001	124.7 ± 6.3	59.0 ± 9.0	-12 (-355, -21)
Rectum	4 hr	<41	<0.001	68.0 ± 3.4	25.1 ± 4.7	-23 (-2, -45)
Duodenum	8 hr	41	<0.633	139.5 ± 3.4	4.6 ± 4.8	-15
Colon	8 hr	41	<0.700	115.3 ± 5.7	6.9 ± 8.2	-37
Rectum	8 hr	41	<0.249	66.1 ± 2.9	6.9 ± 4.1	-65
Duodenum	12 hr	33	<0.421	134.8 ± 5.2	8.7 ± 6.6	-159
Colon	12 hr	33	<0.053	111.4 ± 9.3	37.5 ± 15.1	-250
Rectum	12 hr	34	<0.226	43.9 ± 3.5	8.6 ± 5.1	-316
Duodenum	24 hr	40	<0.156	130.2 ± 4.6	13.0 ± 6.6	-43
Colon	24 hr	<40	<0.001	131.5 ± 5.8	54.5 ± 8.2	-16 (-359, -33)
Rectum	24 hr	41	<0.056	60.6 ± 4.0	14.0 ± 5.6	-326
Low-dose ACTH						
Duodenum	15 min	41	<0.001	104.8 ± 3.3	35.4 ± 4.7	-2.7 (-12, -41)
Colon	15 min	39	<0.001	83.3 ± 3.5	20.9 ± 5.0	-303 (-227, -330)
Rectum	15 min	38	<0.001	37.7 ± 2.6	14.5 ± 3.6	-304 (-275, -332)
Duodenum	2 hr	41	0.169	121.2 ± 3.1	8.3 ± 4.3	-181
Colon	2 hr	41	<0.022	118.4 ± 5.1	21.0 ± 7.2	-342 (-304, -21)
Rectum	2 hr	40	<0.012	68.8 ± 3.4	14.9 ± 4.7	-24 (-348, -60)
Duodenum	4 hr	41	<0.656	143.7 ± 3.7	4.8 ± 5.3	-154
Colon	4 hr	41	<0.009	124.6 ± 6.2	28.8 ± 8.8	-325 (-291, -359)
Rectum	4 hr	41	<0.068	63.0 ± 4.1	14.2 ± 5.9	-334
Duodenum	8 hr	42	<0.983	120.8 ± 5.7	3.8 ± 8.1	-49
Colon	8 hr	40	<0.019	119.2 ± 5.6	23.5 ± 7.9	-319 (-218, -357)
Rectum	8 hr	40	<0.412	60.5 ± 3.2	6.0 ± 4.5	-335
Duodenum	12 hr	42	<0.421	130.9 ± 3.0	3.5 ± 4.2	-258
Colon	12 hr	41	<0.083	113.4 ± 5.8	19.0 ± 8.3	-342
Rectum	12 hr	41	<0.029	59.2 ± 3.3	13.0 ± 4.7	-335 (-226, -14)
Duodenum	24 hr	40	<0.039	120.1 ± 5.7	21.9 ± 8.3	-247 (-206, -288)
Colon	24 hr	40	<0.013	122.1 ± 6.0	26.3 ± 8.4	-50 (-14, -86)
Rectum	24 hr	40	<0.009	62.0 ± 4.8	22.5 ± 6.9	-12 (-339, -46)

Continued on next page

Table 3 (continued)

	Treatment-to-kill-interval	*N*	*P*	Mesor* ± S.E.	Amplitude* ± S. E.	Acrophase (95%) confidence (Int.)
High-dose ACTH						
Duodenum	15 min	41	<0.822	84.7 ± 2.4	2.1 ± 3.4	-53
Colon	15 min	41	<0.006	91.8 ± 4.4	21.1 ± 6.2	-115 (-83, -147)
Rectum	15 min	38	<0.840	42.2 ± 3.5	2.9 ± 4.9	-178
Duodenum	2 hr	38	<0.052	123.6 ± 3.3	11.3 ± 4.5	-335
Colon	2 hr	39	<0.621	123.5 ± 6.1	8.6 ± 8.8	-26
Rectum	2 hr	40	<0.257	67.2 ± 4.5	10.7 ± 6.4	-20
Duodenum	4 hr	42	<0.075	124.3 ± 2.9	9.8 ± 4.2	-236
Colon	4 hr	41	<0.014	97.6 ± 5.9	25.7 ± 8.3	-346 (-310, -22)
Rectum	4 hr	42	<0.120	66.0 ± 5.9	17.1 ± 8.4	-29
Duodenum	8 hr	41	<0.038	131.3 ± 3.4	13.0 ± 4.9	-253 (-209, -296)
Colon	8 hr	41	<0.025	99.8 ± 7.5	30.2 ± 10.6	-99 (-59, -138)
Rectum	8 hr	40	<0.068	79.1 ± 4.9	16.4 ± 6.8	-308
Duodenum	12 hr	38	<0.774	118.6 ± 3.4	3.3 ± 4.7	-279
Colon	12 hr	38	<0.714	87.8 ± 5.3	6.2 ± 7.6	-360
Rectum	12 hr	38	<0.006	73.6 ± 5.2	25.3 ± 7.4	-358 (-325, -30)
Duodenum	24 hr	39	<0.293	128.5 ± 5.0	11.2 ± 7.0	-2
Colon	24 hr	37	<0.063	99.5 ± 7.0	24.1 ± 9.9	-25
Rectum	24 hr	38	<0.144	57.9 ± 4.0	11.2 ± 5.5	-325

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

untreated control data, were fitted to a 24-hr cosine curve. The results of the cosinor analyses are summarized in Table 3. As mentioned above, the control data for the three tissues showed a statistically significant rhythm. Four out of 18 sets from the duodenum, 13 of the 18 sets from the colon, and seven of the 18 sets from the rectum allowed the rejection of the no-rhythm assumption; stated as the null hypothesis. That more of the sets of data did not show a statistically significant rhythm was not surprising since the data were rather limited for this type of analysis because they were collected at unequal intervals of sampling and, in particular there was no sampling between 12 and 24 hr (Table 2; Figures 3–5). The rhythm almost certainly was altered by handling and/or treatments (see below). Earlier we repeatedly have shown that rhythmicity characterizes each of these three tissues and results in a statistically significant fit to a 24-hr cosine curve when sampling is at equidistant intervals (20, 21). It should be mentioned that every set of data obtained from the tongue, esophagus and stomach of these same animals (13) showed a statistically highly significant rhythm fit to a 24-hr cosine curve, even with the limitations of the sampling mentioned above. The circadian rhythm in the three tissues described in this report are somewhat 'noisier' than those found in the first part of the intestinal tract. This is an observation that we have made in a number of earlier studies (20).

Discussion

Control data

The control data reveal that the incorporation of [^{3}H]TdR into DNA of the duodenum, colon and rectum undergoes circadian variation, with the lowest amplitude rhythm characterizing the duodenum; this confirms earlier findings (19, 20). Much of our previous data derive from studies done on epidermal growth factor (EGF) which we have found to have a profound effect on DNA synthesis in the same three tissues as well as in other parts of the gut (19). It remains an

important task to determine how EGF and ACTH 1-17 (as well as other peptides or growth factors) interact in the time domain.

Comparison of ACTH 1-17 and placebo treated groups

The plethora of data generated in this study showed that the response to both the placebo and the ACTH 1-17 stimulus differed and that the effects found depended upon the circadian stage of the perturbation. A surprising finding was the response to ACTH 1-17 seen in all three tissues at 15 min post-injection; a similar response to the placebo at 15 min may be due to the release of endogenous ACTH. From only cursory examination of the chronograms in Figures 3-5 and that data in Table 1 it might be concluded that at certain circadian stages there were statistically significant changes between the data obtained from placebo and ACTH 1-17 treated animals. For example, if Figures 3A, 4A and 5A are compared the placebo data at 15 min were always lower when compared to the ACTH 1-17 data. If however, any of the sets of data resulting from other treatment times, are compared such a conclusion cannot be drawn. Generally at 15 min there was a decrease in the incorporation of [^{3}H]TdR into DNA with all three treatments and the degree of response varied depending upon the circadian stage at which the ACTH 1-17 and placebo were administered. Such a pattern of response was very complex and this undoubtedly was due in large part to the underlying oscillating nature of the variable being measured.

From the data obtained for all three tissues one could claim, depending upon the circadian-stage analyzed, that ACTH 1-17, when compared to placebo, was capable of bringing about a statistically significant increase at one time, a decrease at another time or no such change at another time. For example, in the colon when the mice were treated at 2 HALO (Figure 4A) it would be reasonable to assume that the data obtained from animals receiving the higher dose of ACTH 1-17, when compared to the placebo data, had a tendency to decrease the incorporation of [^{3}H]TdR into DNA, especially at 4-12 hr after injection. An opposite conclusion could be made from the data obtained 4 hr after treatment given toward the end of the light (10 HALO) (Figure 4C). Four hours after the 18-HALO injection (Figure 4E), no statistically significant change was recorded. The data for all 3 tissues show that ACTH 1-17 had a greater influence in bringing about a decrease in the incorporation of [^{3}H]TdR into DNA if it was administered around time of transition from dark to light. A similar finding was made earlier for the ACTH 1-17 effect upon tongue, esophagus and stomach (14).

Comparison of the mesors of ACTH 1-17 and placebo treated groups with untreated controls

When the overall 24-hr means (mesors) of the data from placebo and ACTH 1-17 treated animals were compared with the data from the untreated control animals an overall pattern in response seemd to emerge. From examinations of Figures 6, 7 and 8 it was evident that both ACTH 1-17 and the placebo treatment brought about a decrease in the incorporation of [^{3}H]TdR into DNA at 15 min (compared to controls). This decrease cannot be ascribed to exogenous ACTH 1-17, but could be due to endogenous ACTH which is known to undergo circadian change (22). In the case of the duodenum the conclusion might be drawn from data obtained from those time points subsequent to the 15 min decrease that all three kinds of treatment brought about an increase in the incorporation of [^{3}H]TdR into DNA (Figure 6); however, the higher dose of ACTH 1-17 would seem to depress somewhat the tendency to increase especially at 12 hr. From a similar examination of the data obtained from the colon there was no effect from any of the different treatments (Figure 7). In the case of the rectum, the placebo and the smaller dose of ACTH 1-17 were inhibitory at 12 hr (Figure 8). It seems reasonable to suggest that handling is responsible for many of the changes observed and that in such studies untreated controls are essential in separating out not only the effect of exogenous ACTH 1-17, but to define also the effect of the placebo. Indeed, the circadian-stage dependence of an ACTH effect has been documented in human being studies of ACTH 1-17 (3).

A similar examination of the mesors in the tongue and esophagus of these same animals (14) reveals that the higher dose of ACTH 1-17 brought about a statistically significant reduc-

tion in the incorporation of [^{3}H]TdR into DNA at 4 hr in the tongue and esophagus and even a more dramatic decrease in the stomach where the higher dose of ACTH 1–17 brought about a sustained statistically significant decrease at both 4 and 8 hr and a decrease of borderline statistical significance was recorded even at 12 hr, but not at 24 hr after treatment. From all data collected in this study relative to the digestive tract we conclude that the strongest overall response to ACTH 1–17 was in the stomach (14) and that any response seen in this or any other region of the gut was circadian-stage dependent. On the other hand, the data show that the colon was least responsive to ACTH 1–17 when compared to all other parts of the intestinal tract studied.

In the tongue, esophagus and stomach (14) every set of data showed a statistically highly significant rhythm usually (<0.001), in 18 out of 18 sets of data for each tissue. Such a finding was not surprising even though the data were collected at unequal intervals and there was no sampling between 12 and 24 hr. The response to both the placebo and ACTH 1–17 was remarkably sinusoidal in the first part of the gut and greatly altered and non-sinusoidal in the duodenum, colon and rectum. This observation fits with previous studies using other peptides. Certainly in any region of the intestinal tract (including the duodenum) one should expect to find with adequate sampling, a statistically significant rhythm in standardized animals (19, 20).

Clearly the data show the necessity to consider temporal organization in any experimental design (19, 20). To ignore such dramatic rhythmic change can only lead to ambiguity as to what effect ACTH 1–17 has on [^{3}H]TdR incorporation into DNA. Time of administration is as important a factor to consider as is the dose. If our goal is to understand healthy and abnormal growth, we cannot ignore its basic oscillatory nature. Certainly variation in response is not something one can simply control by sampling at the same single arbitrarily selected time of day. Such an approach can only produce an unrealistic picture of what is actually happening.

As mentioned at the outset the variation in response to anti-cancer agents, especially to those that are cell cycle specific, might be used to improve the chemotherapeutic ratio. It is quite likely that the susceptibility–resistance cycle to anti-cancer agents in non-tumor bearing animals are due to an underlying circadian cell metabolism and/or proliferation in susceptible tissues such as the digestive tract and bone marrow which are known to be 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 highly susceptible tissues of the host, which limit the dosage of an 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 another situation, the susceptible host tissues, but not the tumor, may be circadian-stage dependent. 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 (21, 23, 24), 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 the DNA synthesis of the Harding–Passey melanoma in Balb/C female mice (13). Interestingly, such an effect can only be demonstrated if the injection of ACTH 1–17 is given at the beginning of the light span for 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 DR could be important, to consider in cancer chemotherapy and needs to be explored further.

Finally, the analogue ACTH 1–17 was selected for study for several reasons. Among them was the fact that considerable data have 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 of ACTH 1-17 clinically is that it can be given intranasally to stimulate the adrenal (7). 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 (a possibility already experimentally demonstrated for DR) (7, 9), a sniff of ACTH 1-17 could readily be taken at home. This approach presupposes, however, that the desired total ACTH 1-17 dose can be reliably given intranasally, a point which remains to be documented.

Even if there was no practical application, such as we suggest above, the recognition of rhythms still remains a major challenge for science and a *sine qua non* on the path of exploring the periodic mechanisms underlying growth.

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