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Pineal Modulation of ACTH 1–17 Effect Upon Murine Corticosterone Production[1]

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Pineal Adrenal ACTH 1–17 Incubation Attenuation Amplification Feed-sideward

DEPENDING upon the circadian stage of isolated murine adrenal cells, their *in vitro* production of corticosterone, stimulated by a pituitary preincubation fluid, is attenuated, amplified or left unaffected by the addition of a pineal aqueous homogenate (APH) [31]. At certain circadian stages of the bisected murine adrenals (toward the middle of the dark span), the effect of APH is again an attenuation, amplification or no effect, depending on the circadian stage of the pineal and the pituitary [32]. The pineal, the pituitary and the adrenals are all characterized by variables that are rhythmic with several frequencies [2, 9, 10, 15, 22, 29, 31, 39]. The work here reported sought to gain insight concerning a possible mechanism for this "chronomodulation" by keeping one of the constituents constant, i.e., by replacing the pituitary preincubation medium with a synthetic short-chain ACTH 1–17 (Synchrodyn, Hoechst).

METHOD

Eighty female B6D2F$_1$ mice, obtained from the Jackson Laboratory, Bar Harbor, ME, 14 weeks of age, had been standardized for 7 weeks with food (Purina Laboratory Chow) and deionized water available ad lib, at 24±1°C and 50±3% relative humidity, on schedules of light and darkness alternating at 12-hr intervals, staggered by 4 hours, in 6 different Aminco chambers [18] as indicated in Fig. 1, with the pituitary effect represented by ACTH 1–17. Staggered lighting regimens were desirable for our experimental design: in order to stimulate bisected adrenals *in vitro* at different circadian stages with fresh APH also from different circadian stages, glands from the 6 different times needed to be available at any one time. It was known that the circadian rhythm in circulating corticosterone could be shifted by the manipulation of the lighting regimen [10]. Although there may have been some uncontrollable chamber effect [28], these effects are greatly outweighed by the circadian adrenal cycle [20].

On October 31, 1982, groups of 13–14 mice from each chamber were killed by cervical dislocation. During a total killing span of 70 minutes, decapitated heads (for pineals) were stored in ice and adrenals promptly removed at each of 6

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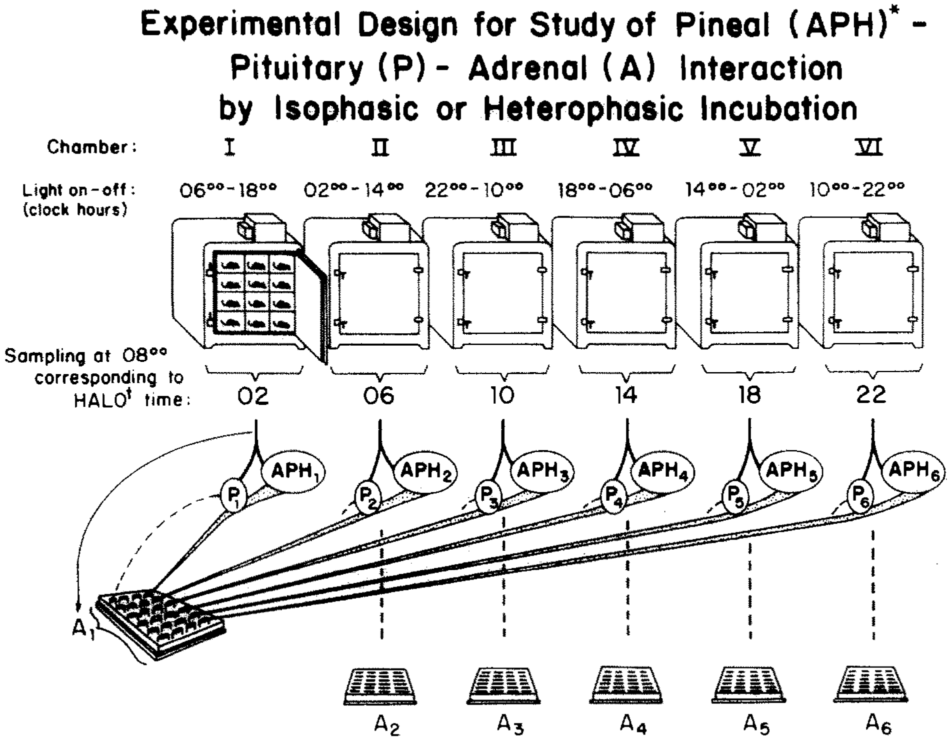

**APH* = *aqueous pineal homogenate,* *A* = *bisected adrenals or isolated cells*
†*HALO* = *Hours After Light On*

FIG. 1. Tissue harvest for incubations (cf. Figs. 2 and 3) at the same clock-hour but in 6 different circadian stages. Note that ACTH 1–17 replaces pituitary in these experiments.

different timepoints; 2, 6, 10, 14, 18 and 22 *h*ours *a*fter *l*ight *o*nset (HALO). At each timepoint, all pineals were pooled in a Petri dish with a central well containing 0.2 ml of 0.9% saline. The dish was kept in an ice bath until the last pineal was added. With a siliconized pipette, all pineals were transferred into a tube (also kept in the ice). The tube contained 1 pineal/0.1 ml of 0.9% saline. The pineals were homogenized in this tube, surrounded by ice, for about 3 minutes. The homogenate was then transferred into a plastic tube, one aliquot of 1 ml being kept on ice until use for incubation, the other aliquot of 0.1 ml being stored at −90° until melatonin RIA. Adrenals were bisected, placed on filter paper in a Petri dish (kept in an ice bath) containing Krebs-Ringer buffer with 0.01 M glucose and 0.1% albumin added (KRG) for *in vitro* bioassay [30].

From the overall pool at any one circadian stage, 3 halves were picked at random and immersed into plastic wells, each containing 0.5 ml of KRG. The plates were pre-incubated for 1 hour in 95% O_2 and 5% CO_2 at 37±1°C in a shaking (Dubnoff) incubator. The preincubation fluid was stored and KRG replaced for a 2-hour incubation of the adrenal halves with ACTH 1–17 (0.05 IU) alone or with 0.025 ml APH added to the medium 15 minutes earlier. The medium was harvested and APH, KRG and ACTH 1–17 replaced for another 2-hour incubation. The fluids from the two consecutive 2-hr incubations of adrenals stimulated by the ACTH 1–17, alone or after the addition of APH, were stored separately at −20°C until they were radioimmunoassayed for corticosterone.

From each circadian adrenal harvest stage, 2 wells were used for incubation with ACTH 1–17 alone, and additional wells for incubation with ACTH 1–17 and APH: one pair of wells contained ACTH 1–17 and APH from the same circadian HALO stage as the test adrenals (isophasic incubation, Fig. 2), while 5 other pairs of wells contained ACTH 1–17 and APH harvested at the 5 other circadian stages (heterophasic incubation, Fig. 3). Thus, say for the harvest at 0200 HALO, (1) 2 corticosterone determinations were made with adrenals stimulated by ACTH 1–17 alone (for the first 2 hours of incubation); (2) 4 determinations were made for the isophasic incubation (using APH from 0200 HALO), 2 for each of the two consecutive 2-hour incubations; and (3) 4 determinations were made on each of the 5 heterophasic incubations (using APH from either 0600, 1000, 1400, 1800 or 2200 HALO), 2 for each of the two consecutive 2-hour incubations. The same applies to the adrenals harvested at each of the 5 other circadian stages (see original data, expressed in ng/ml incubation fluid/hr in Table 1). Melatonin was radioimmunoassayed [8]. Intraassay estimates of a rat serum pool were 30.3±0.9 pg/ml (cv=6.7%, N=5) and interassay estimates of another pool were 143.0±11.2 pg/ml (cv=17.4, N=5). Six rat serum samples assayed by radioimmunoassay and by gas chromatography mass spectrometry [26] yielded a correlation coefficient (r)=0.983.

The data were examined by analysis of variance and cosinor methods [12,32].

RESULTS

The circadian response of the adrenals to the stimulation by ACTH 1–17 alone is very prominent, as can be seen from the data reported in Table 1: at 18 HALO, corticosterone

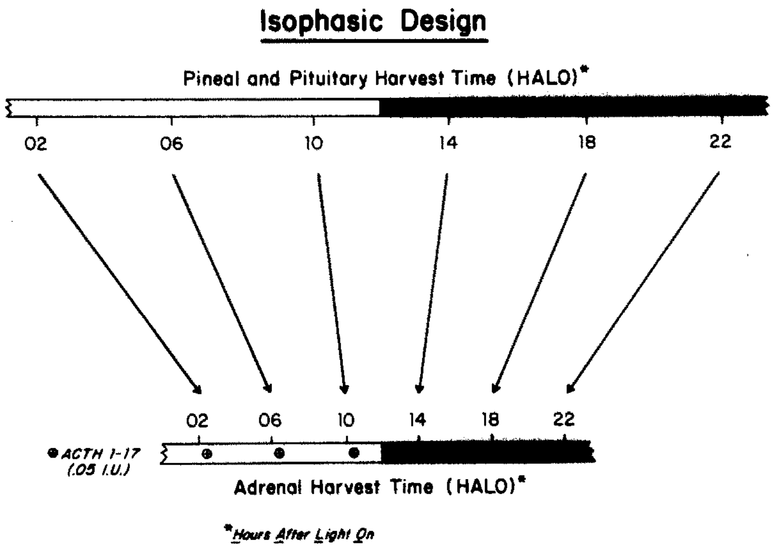

FIG. 2. Work (sketched in Fig. 1) with tissues harvested at the same circadian stage (note that tissues from the 6 different circadian stages were obtained at the same clock-hour, with animals in different chambers kept in staggered lighting conditions). In these studies, ACTH 1-17 was used instead of pituitary preincubation fluid.

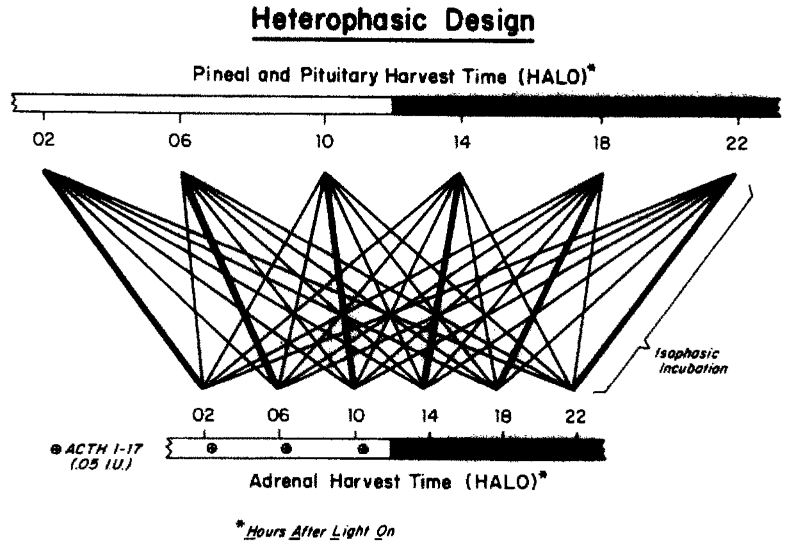

FIG. 3. Work (sketched in Fig. 1) at each of 6 circadian stages with tissues not only from the same (Fig. 2) but also from the 5 other stages. In these studies, ACTH 1-17 was used instead of pituitary preincubation fluid.

TABLE 1

CORTICOSTERONE PRODUCTION (ng/ml/hr) BY BISECTED ADRENALS STIMULATED BY ACTH 1–17 (0.05 IU) WITH OR WITHOUT AQUEOUS PINEAL HOMOGENATE

Adrenal harvest time (HALO)*	ACTH 1–17 alone	Circadian Stage of Pineal Harvest (HALO)*					
		0200	0600	1000	1400	1800	2200
0200a†	284.0	217.6	237.0	310.0	312.8	297.0	245.0
	312.0	355.5	286.6	225.0	186.0	343.0	424.0
0200b†		198.0	212.0	273.0	234.0	266.0	200.0
		254.0	340.0	337.0	209.0	184.0	178.0
0600a	247.0	209.0	215.0	426.0	320.0	213.0	303.0
	309.6	266.3	310.0	406.0	297.0	245.0	242.0
0600b		107.0	126.0	342.0	190.0	167.0	257.0
		98.0	150.0	320.0	125.5	132.6	385.0
1000a	186.5	76.1	196.3	138.5	84.0	71.6	135.0
	160.0	157.5	135.3	136.2	140.0	159.0	141.2
1000b		123.0	193.0	174.0	106.0	139.6	109.0
		152.0	135.0	146.3	138.0	172.6	146.0
1400a	180.0	365.2	178.0	320.0	286.0	243.0	118.0
	220.0	240.3	264.0	238.1	227.1	205.6	156.0
1400b		197.0	141.3	303.0	304.3	340.0	286.1
		120.0	155.0	265.5	364.5	236.4	228.2
1800a	63.4	149.4	123.2	102.6	90.0	134.0	120.0
	89.1	130.0		80.0	81.0		131.3
1800b		228.0	258.3	311.0	274.0	213.0	177.0
		167.0		141.0			
2200a	220.0	137.4	231.0	204.6	251.2	223.0	123.7
	260.0	264.0	238.0	356.1	190.7	222.2	268.2
2200b		88.0	103.0	90.0	224.0	201.0	193.4
		156.0	168.0	216.0	167.7	181.0	114.1

*HALO=*h*ours *a*fter *l*ight *o*nset.

†a=First two hours of incubation; b=second two hours of incubation.

production is <90 ng/ml of incubation fluid per hour, whereas at 2 HALO, it is close to 300 ng/ml/hr. This result is in keeping with earlier work on the circadian stage dependence of ACTH [9, 22, 39] and ACTH 1–17 [23] effects.

The means and standard errors (SE) of corticosterone production relative to each adrenal and each APH harvest time are given in Table 2. Means and SE's relative to a given APH harvest time, irrespective of the circadian stage of the adrenals, are reported at the bottom of Table 2. Similarly, means and SE's relative to a given adrenal harvest time, irrespective of the circadian stage of the pineals, are reported on the right of Table 2. It is apparent from Table 2 that effects of adrenal harvest time (varying from 137.7 to 263.5 ng/ml/hr) are much more pronounced than those of pineal harvest time (varying from 185.7 to 244.2 ng/ml/hr). A 2-way ANOVA assessing the effects of adrenal and pineal harvest time indeed shows a highly significant effect of adrenal harvest time ($p<0.001$) and an effect of pineal harvest time of borderline significance ($p=0.06$), with no interaction ($p=0.207$).

These results are corroborated by a single cosinor analysis rejecting the zero amplitude (no rhythm) assumption, when analyzed as a function of adrenal harvest time ($p=0.009$), with the following rhythm characteristics: mesor=208±7 ng/ml/hr; amplitude (A)=30±10 ng/ml/hr; acrophase (ϕ)=$-42°\pm19$ (360°≡24 hr; 0°=light onset). Similarly, the results from the single cosinor analysis, when analyzed as a function of pineal harvest time, are of borderline statistical significance ($p=0.095$) with A=8.2±3.8 (ng/ml/hr) and $\phi=-171°\pm27$.

In viewing these results, one must consider the fact that the endpoint studied is corticosterone produced by the adrenals: thus, at a given adrenal harvest time, a separate group of mice (kept in the same chamber) is used, whereas mice from all chambers contribute adrenals at a given pineal harvest time. In other words, in the case of different adrenal harvest times, we confront only intra-chamber inter-animal variation, whereas for a summary as a function of pineal harvest time, both inter-animal and inter-chamber variation are contributing [28].

It is also clear from Table 2 that the addition of APH to ACTH 1–17 affects the *in vitro* corticosterone production by the bisected adrenals differently at different adrenal harvest times. When the adrenals are harvested between 22 and 10

TABLE 2

MEANS AND SE OF CORTICOSTERONE PRODUCTION *IN VITRO* BY MURINE BISECTED ADRENALS STIMULATED WITH OR WITHOUT AQUEOUS PINEAL HOMOGENATE HARVESTED AT EACH OF SIX DIFFERENT CIRCADIAN STAGES, PRIOR TO THE ADDITION OF ACTH 1–17 (0.05 IU)

Adrenal harvest time (HALO)*	ACTH 1–17 alone	Pineal harvest time (HALO)* Means ± SE (ng/ml incubation fluid/hr) 0200	0600	1000	1400	1800	2200	Mean ±SE
0200	298.00	256.27	268.90	286.25	235.45	272.50	261.75	263.52
	±14.00	±35.05	±28.32	±24.27	±27.58	±33.47	±55.85	± 7.00
0600	278.30	170.07	200.25	373.50	233.12	189.40	296.75	243.85
	±31.30	±40.77	±41.13	±25.28	±45.71	±24.79	±32.15	±31.64
1000	173.25	127.15	164.90	148.75	117.00	135.70	132.80	137.72
	±13.25	±18.62	±17.19	± 8.69	±13.48	±22.41	± 8.25	± 6.90
1400	200.00	230.62	184.57	281.65	295.47	256.25	197.05	238.81
	±20.00	±51.29	±27.54	±18.45	±28.29	±29.08	±36.07	±19.44
1800	76.25	168.60	190.75	158.66	148.33	173.50	142.83	163.78
	±12.85	±21.19	±67.55	±52.32	±62.89	±39.50	±17.38	± 7.19
2200	240.00	161.35	185.00	216.67	208.40	206.80	174.85	192.18
	±20.00	±37.10	±31.54	±54.49	±18.36	±10.00	±35.78	± 8.91
Mean	210.97	185.68	199.06	244.25	206.30	205.69	198.88	
±SE	±32.99	±19.63	±14.75	±35.16	±26.37	±20.99	±26.98	

*HALO=*h*ours *a*fter *l*ight *o*nset.

HALO, APH attenuates the response to ACTH 1–17; when the adrenals are harvested at 14 and 18 HALO, however, APH enhances the response to ACTH 1–17 of the murine adrenal. Thus, for the nocturnally-active mammal, there is a stimulatory effect of APH at a time when there may be a greater demand on a response to ACTH 1–17, i.e., when the endogenous response to ACTH 1–17 is relatively small.

This alternation between inhibitory and stimulatory effects of APH on the response to ACTH 1–17 of the *in vitro* production of corticosterone by the adrenals is best illustrated by calculating the difference in response. Thus, the values in Table 3 were obtained by subtracting the mean ACTH 1–17 response at each adrenal harvest time from the data on corticosterone production corresponding to the stimulation by both ACTH 1–17 and APH. A one-way ANOVA carried out on this difference in response shows highly significant differences as a function of adrenal harvest time ($F=12.78$, $p<0.001$): Bonferronized t-tests performed at each adrenal harvest time show that, by the addition of APH to the incubation medium, corticosterone production in response to ACTH 1–17 is attenuated at 1000 and 2200, while it is amplified at 1800 ($p<0.05$). Figure 4 visualizes the rhythmic sequence of attenuation, no-effect and amplification, interpreting the data in the penultimate column of Table 3 as a so-called feed-sideward.

Melatonin concentration was determined for the pineals harvested at the 6 different circadian stages [5]. Melatonin was not detected by RIA at 02, 06 and 22 HALO; only traces were detectable at 10 HALO, whereas at 14 and 18 HALO, large amounts of melatonin were observed (2007 and 200 pg/pineal, respectively). Melatonin is found in the pineal at the time when APH stimulates the effect of ACTH 1–17 upon the *in vitro* corticosterone production by the adrenals.

DISCUSSION

Pineal melatonin concentration was also determined for pineals used in a previous experiment. Again, detectable amounts of melatonin were only found around 14 HALO (170 pg/pineal). Considering the results shown in Table 3 and Fig. 4, it is apparent that the inhibitory/stimulatory effects of APH upon the ACTH 1–17 effect remain grossly the same, whatever the circadian harvest time of APH. Whether or not melatonin is playing any role, the chronomodulatory effects of APH might involve circadian changes in the adrenal. This hypothesis remains to be tested, with focus, among others, on any role played by second messengers and possible melatonin receptors.

Results from four other experiments also show that the addition of APH to ACTH 1–17 modulates the adrenal response in a rhythmic fashion. Results from these "isophasic" experiments—the pineals were harvested at the same circadian stage as the adrenals—are compared in Table 4 to those of the "isophasic" part of the experiment reported herein. Despite differences, among others, in the dose of ACTH 1–17 and the amount of adrenal tissue incubated per well, maximal stimulation by APH of the adrenal response to ACTH 1–17, on the average, occurs around 14 HALO, as summarized in Fig. 5.

These results are also corroborated, but only in part, by another "heterophasic" study wherein adrenal cells from each of 3 circadian harvest times were incubated with a pituitary (anterior lobe only) preincubation fluid with or without APH, both harvested at one of 6 circadian stages. Again, APH was found to be inhibitory on adrenal cells harvested at 08 HALO, to have no effect on adrenal cells harvested at 20 HALO and to be partly stimulatory on adrenal cells har-

TABLE 3

DIFFERENCE IN CORTICOSTERONE PRODUCTION (ng/ml/hr) BY BISECTED ADRENAL GLANDS STIMULATED WITH ACTH 1–17 (0.05 IU) PLUS PINEAL HOMOGENATE WITH RESPECT TO STIMULATION WITH ACTH ALONE

Adrenal harvest time (HALO)*	Pineal harvest time (HALO)* 0200	0600	1000	1400	1800	2200	Mean ±SE	*t*
0200	−80.4	−61.0	+12.0	+14.8	−1.0	−53.0	−34.5 ±13.3	2.6†
	+57.5	−11.4	−73.0	−112.0	+45.0	+126.0		
	−100.0	−86.0	−25.0	−64.0	−32.0	−98.0		
	−44.0	+42.0	+39.0	−89.0	−114.0	−120.0		
0600	−69.3	−63.3	+147.7	+41.7	−65.3	+24.7	−34.5 ±19.6	1.7
	−12.0	+31.7	+127.7	+18.7	−33.3	−36.3		
	−171.3	−152.3	+63.7	−88.3	−111.3	−21.3		
	−180.3	−128.8	+41.7	−152.8	−145.7	+106.7		
1000	−97.1	+23.0	−34.7	−89.2	−101.6	−38.2	−35.5 ± 6.5	5.5‡
	−15.7	−37.9	−37.0	−33.2	−14.2	−32.0		
	−50.2	+19.7	0.7	−67.2	−33.6	−64.2		
	−21.2	−38.2	−26.9	−35.2	−0.6	−27.2		
1400	+165.2	−22.0	+120.0	+86.0	+43.0	−82.0	+40.9 ±14.8	2.8†
	+40.3	+64.0	+38.1	+27.1	+5.6	−44.0		
	−3.0	−58.7	+103.0	+104.3	+140.0	+86.1		
	−80.0	−45.0	+65.5	+164.5	+36.4	+28.2		
1800	+73.1	+46.9	+26.3	+13.7	+57.7	+43.9	+85.5 ±16.2	5.3‡
	+53.7	+182.0	+3.7	+4.7	+136.7	+55.0		
	+151.7		+234.7	+197.7		+100.7		
	+90.7		+64.7					
2200	−102.6	−9.0	−35.4	+11.2	−17.0	−116.3	−47.8 ±13.1	3.7‡
	+24.0	−2.0	+116.1	−49.3	−17.8	+28.2		
	−152.0	−137.0	−150.0	−16.0	−39.0	−46.6		
	−84.0	−72.0	−24.0	−72.3	−59.0	−125.9		

*HALO=*h*ours *a*fter *l*ight *o*nset.

†$p<0.05$ (single test); ‡$p<0.05$ (adjusting for multiple testing).

vested at 16 HALO [31]. At the latter adrenal harvest time, APH inhibits, stimulates or does not affect corticosterone production by the adrenal cells stimulated by the pituitary preincubation medium, depending on the harvest time of the pituitary and the pineal.

In still another "isophasic" experiment [32], wherein bisected adrenals were incubated with a pituitary preincubation medium with or without APH, a reversed effect of APH was observed. Namely, APH was found in this experiment to be stimulatory during the light span and inhibitory during the dark span. Further work is needed to explain these differences, perhaps in terms of infradian modulation (by rhythms with a period longer than 28 hours) or in terms of hormonal content of the pituitary. In this experiment, the posterior and intermediate pituitary lobes reportedly rich in hormones capable of stimulating corticosterone production by the adrenals, were also used to prepare the preincubation medium.

Without extrapolating beyond the scope of a short series of experiments [31–34], a major inference from this study relates to the fact that stimulation, inhibition or no effect seem to be the rule rather than the exception. Thus, one may view work with melatonin on cultured human mammary tumor cells, yielding "very inconsistent results, namely stimulation, inhibition or no effect" [4]. If a phenomenon such as pituitary-adrenal interaction is predictably rhythmic [23, 31, 40] and is influenced by another rhythmic interaction [27], such as that by the pineal [31–34], a seeming inconsistency becomes consistent, as soon as chronobiologic methods are applied. Such methods resolve statistically significant rhythmic interactions, called feed-sidewards [16, 19, 31]. The time-dependent pineal, pituitary and adrenal interaction affects all those many phenomena influenced by ACTH and/or adrenal corticoid. Mitotic activity is a case in point [17,35]. Findings of altered melatonin rhythms, not only in women with breast cancer [37] but also in relation to a heightened risk of developing breast cancer [13], also come to mind, as does, apart from any rhythms, the role of the pineal or melatonin in relation to cancer [1, 3, 4, 6, 7, 21, 24, 25, 36–38].

The pineal feed-sideward also deserves scrutiny in relation to the as-yet sparsely documented role of the hypothalamus in carcinogenesis [11]. It will be mandatory to examine the contributions to chronomodulation of interactions in-

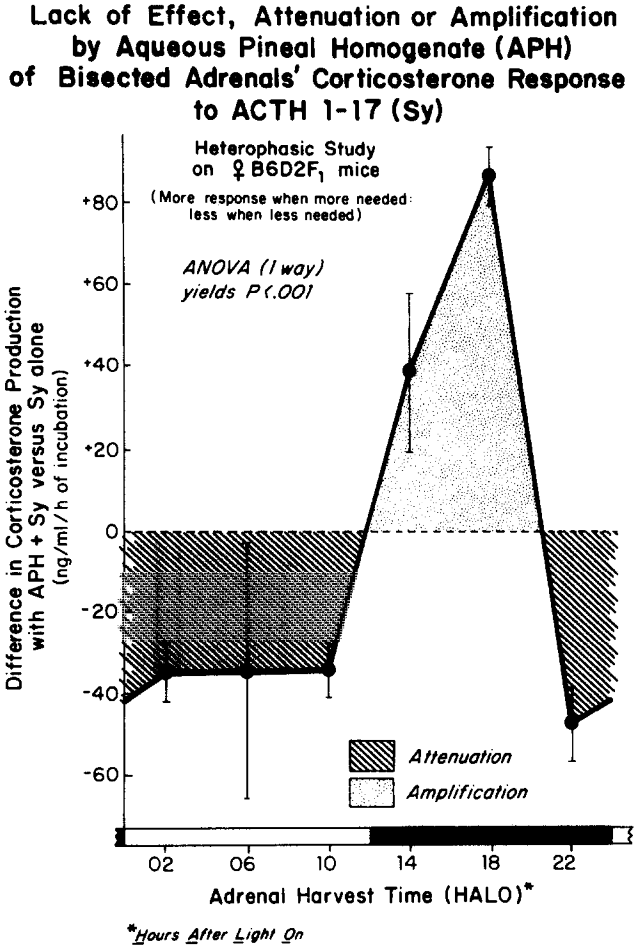

FIG. 4. Feed-sideward—circadian rhythmic pineal interaction with ACTH 1-17 effect upon the bisected adrenal, documented with the heterophasic scheme shown in Fig. 3.

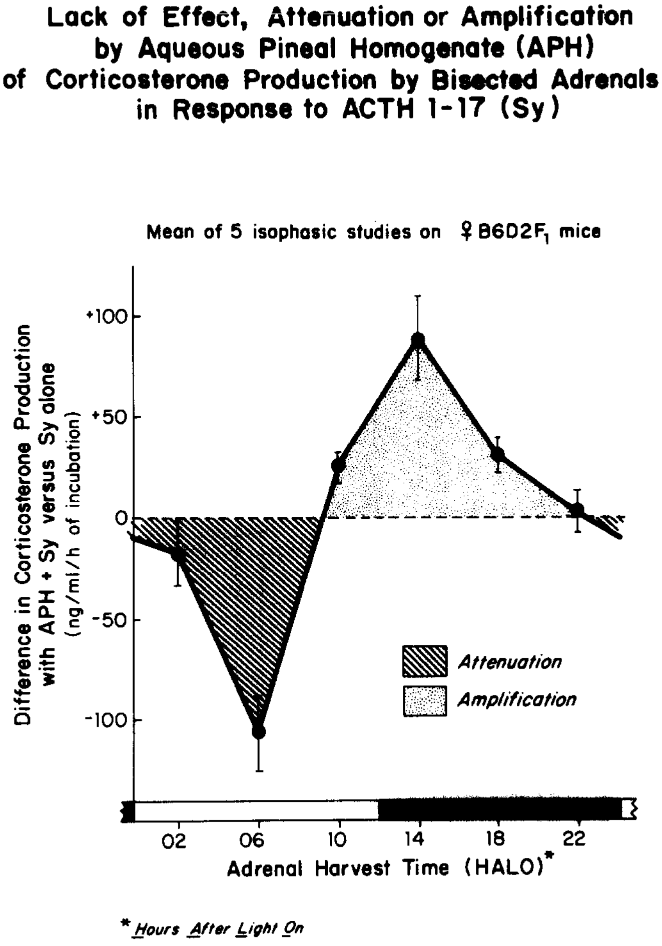

FIG. 5. Feed-sideward—circadian rhythmic pineal interaction with ACTH 1-17 effect upon the bisected adrenal, documented with the isophasic scheme shown in Fig. 2.

volving the hypothalamus, pineal, pituitary and adrenal. Similarly, the work reported herein will have to be extended so that the influence of infradian rhythms such as the circannual and circaseptan ones [13, 14, 19, 41] can also be studied. Such interactions may be particularly pertinent to chronoimmunopharmacology [19]; they may account for those possibly neuroendocrine mechanisms that underlie the enhancement or the inhibition of a malignant growth by the same total dose of a given agent, the only known difference being the schedule of administration along the scales of a day and a week of the immunomodulator [19]. Following his demonstration of a 7-day synchronized circaseptan rhythm in a pineal enzyme, 5-HIOMT [41], Vollrath notes [42] ". . . that the curves (for HIOMT) exhibited distinct drops in the Friday-Saturday region, independently of whether the experiment was started on a Monday or a Wednesday." Such unqualified reports imply, of course, that there is no endogenous component and may be (mis)interpreted to mean that there is only an imposed exogenous circaseptan rhythm. The exogenous effect is hardly surprising. The major task is to dissect an endogenous component in dealing with circadian-circaseptan intermodulation. In this context, Vollrath recently found, circaseptan variation in N-acetyltransferase activity (personal communication), which upon analysis reveals a period differing slightly and with statistical significance from precisely 7 days. The circadian interactions documented herein will have to be scrutinized along circaseptan and longer time scales before the phenomenon of a pineal feed-sideward can be better understood.

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TABLE 4

DIFFERENCE IN CORTICOSTERONE PRODUCED (ng/ml/hr/ADRENAL) BY BISECTED ADRENAL GLANDS STIMULATED WITH ACTH 1-17 (1) WITH AND (2) WITHOUT PINEAL HOMOGENATE (PINEAL HARVESTED AT SAME CIRCADIAN STAGE AS ADRENALS)

	Means ± SE Adrenal harvest time (HALO)†					
Experiment*	0200	0600	1000	1400	1800	2200
(1) September 81a	65.42	−244.96	40.42	251.00	59.79	13.29
	±51.75	±14.71	±15.07	±19.79	± 5.19	±21.94
(2) September 81b	−68.21	−154.84	13.75	−12.12	−15.83	55.33
	± 7.77	±13.08	± 8.45	±25.39	±15.89	±18.20
(3) January 82	−58.50	−50.00	47.50	74.50	11.75	2.00
	± 2.21	± 3.67	±14.93	±21.05	± 8.50	± 1.39
(4) October 82a	9.61	−37.94	43.95	28.89	59.00	7.00
	± 7.75	± 5.79	±12.08	±12.52	± 4.44	± 9.37
(5) October 82b‡	−27.82	−52.12	−16.32	63.65	64.80	−43.43
	±23.37	±27.45	± 5.78	±18.86	±26.33	±23.86

Results from cosinor analysis§

	Percent rhythm (%)	p-value (Ho: A=0)	Mesor ± SE	Amplitude ± SE	Acrophase (95% CI) (360°=24 hr; 0°=light-on)		
(1)	43.2	0.003	34.2 ± 24.0	135.9 ± 34.0	−243°	(−215;	−271)
(2)	37.8	0.007	−30.4 ± 12.5	62.9 ± 17.6	−266°	(−235;	−298)
(3)	72.9	<0.001	4.6 ± 5.9	62.4 ± 8.3	−214°	(−199;	−229)
(4)	49.7	0.006	18.4 ± 6.1	33.4 ± 8.7	−241°	(−212;	−270)
(5)	46.9	0.002	−3.8 ± 9.8	57.0 ± 14.0	−233°	(−206;	−260)

*On ♀ B6D2F$_1$ mice (1) 8–10 weeks old with 0.04 IU ACTH 1–17; (2) 8–10 weeks old with 0.4 IU ACTH 1–17; (3) 8–10 weeks old with 0.05 IU ACTH 1–17; (4) and (5) 12–14 weeks old with 0.05 IU ACTH 1–17.

†*H*ours *a*fter *l*ight *o*nset.

‡Isophasic part of the study.

§For terminology, see [32].

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