sealed vaginal membrane of the ovariectomized female pig was established. One-half microgram given as a single dose can induce the first histologic changes in the vaginal membrane described by Kelly *et al*(1). This dose is far greater than the amount shown by Hartman(6) to break down the membrane of the immature guinea pig when administered directly into the vulva. As noted, 2.0 mg of progesterone given to an animal primed with 1.0 μg of estradiol dipropionate reduces the duration of vaginal opening to 4 days. This approximates the natural state in cycling guinea pigs. The questionable effect of LH may be coincident with, and be masked by the effect of estrogen in the intact animal.

Demonstration of relaxin as an effective agent for opening the closed vaginal orifice has not been reported previously. Approximately 9 times more of the Warner-Chilcott preparation was required than of the Frieden preparation, a finding compatible with heterogeneity. It has been noted by Zarrow (7) that the maximum concentration of relaxin in the serum of the pregnant female guinea pig is reached around day 28 of gestation. The present findings suggest that this increase could account for the opening of the sealed vaginal membrane reported by Ford(8) to occur most commonly around day 26 or day 27 of gestation and attributed by those authors to a deficiency of progesterone.

Summary. The minimum effective dose of estradiol dipropionate, 0.5 μg as a single subcutaneous injection, which opened the closed vaginal membrane of the ovariectomized guinea pig has been determined. Vaginal opening occurred approximately 96 hours later and persisted for 4-7 days. Estrogen induced vaginal openings of 9 days duration can be shortened to the normal 4 days with 2 mg of progesterone. The effectiveness of several relaxin preparations in opening the closed vaginal membrane is reported. It is suggested that relaxin may be involved in the opening of the vaginal membrane observed in the pregnant guinea pig during the fourth week of gestation.

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Circadian Rhythmic Pituitary Adrenocorticotropic Activity, Rectal Temperature and Pinnal Mitosis of Starving, Dehydrated C Mice.* (29861)

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Certain circadian rhythms are described in terms of amplitude and timing in mice deprived of all food and water. Phase relations among these rhythms at different levels of organization are demonstrated as being similar to those recorded on comparable fully-fed animals. Such information was sought on the functions under study with a view

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toward the standardization of their use as endpoints in bioassays carried out under conditions of starvation and dehydration. The same results serve to indicate a high degree of independence of circadian system structure from intake of food and water.

Materials and methods. Female C strain mice, 3 to 4 months of age, were used. The strain had been maintained at the University of Minnesota Medical School by brother-to-sister mating for more than 12 years. A first group of 72 mice constituted Group A and a second group of 112 mice studied several weeks later constituted Group B. An additional 500 mice served as adrenal donors for incubation of pituitary glands from Groups A and B, as described below.

All animals were kept singly housed in temperature controlled (± .5°C) "periodicity rooms" during a 6-day standardization period, as well as during the subsequent sampling periods. The rooms were illuminated exclusively by fluorescent lights automatically turned on at 0600 and off at 0800 by an electric switch.

Purina Dog Chow and tap water were continuously available to Group A mice and to the adrenal donor mice up to time of sacrifice. Sampling of the mice in the A-Group was done on subgroups sacrificed at 4-hour intervals, starting at 0400 of one day and ending at 2400 of the same day. Food and water were available to Group B mice during standardization but were removed from all cages of this group at about 0500 (midpoint) on the first day of the study, 3 hours prior to sacrifice of the first subgroup. Sampling on the Group B mice started at 0800 of one day and was continued at 4-hour intervals until 2000 of the next day. The Group B mice were thus studied during a period of progressive starvation and dehydration. The period of complete deprivation of food and water covered over 1½ days for the last subgroup sacrificed.

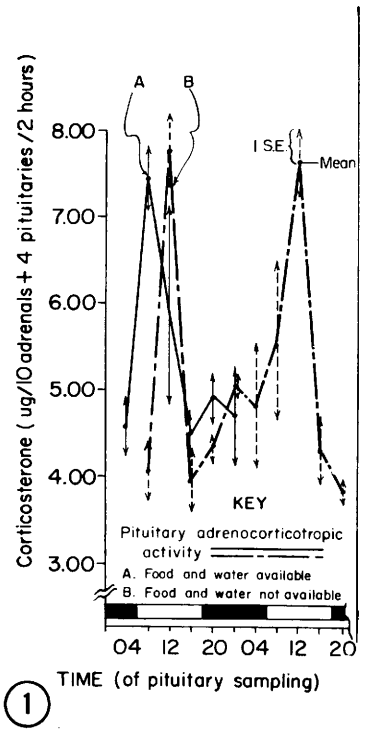
The sampling times indicated with the results in the Figures are midpoints. Actual sampling at any one time-point extended over a period of about one hour. At intervals of several minutes, an animal was removed from the periodicity room to an adjacent laboratory and its rectal temperature was measured by a thermistor-bridge circuit(1). Immediately thereafter, the animal was decapitated with scissors and sampling was carried out by a team of 4 individuals with assembly line procedures.

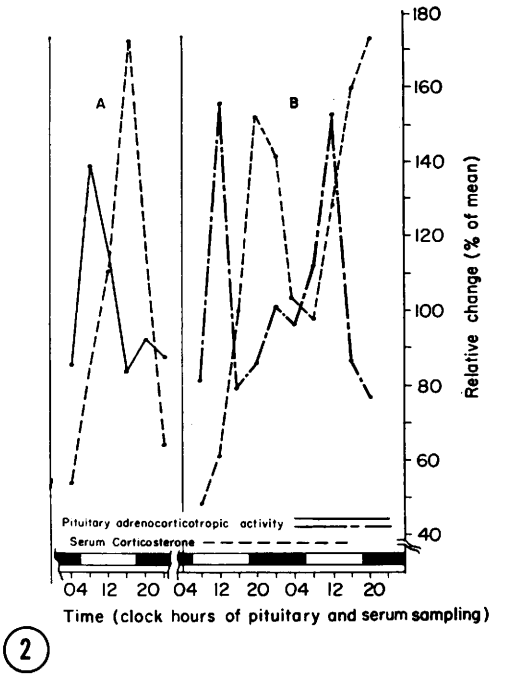
Blood was allowed to drain from the neck into a centrifuge tube. Adrenal glands were removed and frozen on dry ice. Pools of blood, each obtained from 6 animals, were allowed to clot. The serum was separated by centrifugation and frozen. Corticosterone in serum and in pairs of adrenals from individual mice was determined by the fluorometric method of Silber *et al*(2). The specificity of this method and the usefulness for periodicity analysis of data obtained by this procedure have been discussed elsewhere(3,4). The ear of each mouse was removed with scissors at time of sacrifice and fixed in Bouin's solution. Three thousand nuclei in ear epidermis of each of the 112 Group B mice were examined on microscopic sections stained with Heidenhain's iron hematoxylin. Group-A ears were not evaluated, but earlier charts of mitotic rhythm in pinnal epidermis of fully fed mice are available(5).

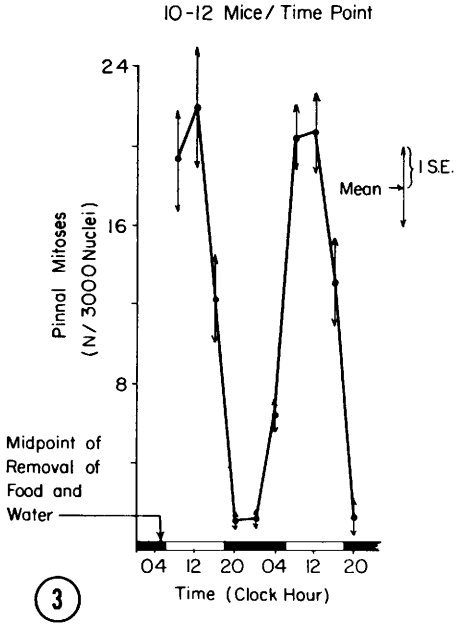
The pituitary glands of both the A and B Group mice were removed and pools of 4 glands were frozen in a buffer solution(6) and stored for subsequent determination of adrenocorticotropic activity(7).

Starting several weeks later, 6 different subgroups of the total of 500 adrenal donor mice were standardized under conditions designed to be identical with those used for standardizing the mice of Groups A and B. Each subgroup was sacrificed in quick succession by a team of 6 individuals between 0700 and 0800 of the same day. They provided adrenals from a defined circadian system-phase (fixed physiologic time) for incubation with 1) the pools of pituitary glands from A or B mice, removed earlier at 4-hour intervals during a period of 20 or 36 hours, as previously indicated, or with 2) 0.4 International Units of ACTH, or with 3) pituitaries of some of the adrenal donor mice themselves.

Incubations were carried out at 37°C for 2 hours in 25 ml Erlenmeyer flasks, contain-







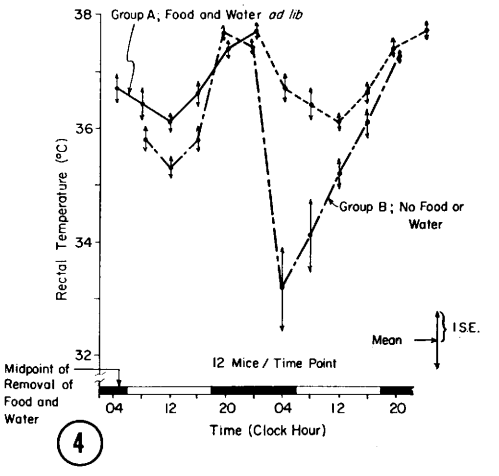


FIG. 1. Circadian rhythm in pituitary adrenocorticotropic activity of groups of mice investigated under conditions of light-dark synchronized periodicity analysis(5).

FIG. 2. Timing of circadian rhythm in pituitary adrenocorticotropic activity in relation to serum corticosterone rhythm of groups investigated under conditions of light-dark synchronized periodicity analysis.

FIG. 3. Mitotic counts of C female mice deprived of food and water, studied under conditions of light-dark synchronized periodicity analysis.

ing 2.0 ml of a Krebs-Ringer bicarbonate buffer, pH 7.4, with glucose (2 g/liter). Consistently, pools of 10 adrenals were incubated with pools of 4 pituitary glands or with ACTH. In any one series of incubations, pituitary pools were included from each of the timepoints chosen for sacrifice of either the A or the B Group mice. The final corticosterone content in incubation medium and glands was determined fluorometrically, as previously described(7). The values obtained with 0.4 International Units of ACTH or with pituitary glands of the adrenal donor mice were within the range of the corresponding values obtained with the incubation of previously frozen pituitaries, and thus in keeping with the assumption that adrenocorticotropic activity was not lost during storage in the frozen state.

Results. Rhythm in pituitary ACTH content. Fig. 1 and 2 document two basic points. The persistence, during starvation, of the recently disclosed circadian rhythm in pituitary adrenocorticotropic activity can be seen in Fig. 1. Equally interesting is the maintenance of the physiologic time relation of this pituitary rhythm to that in serum corticosterone(8), demonstrated in Fig. 2.

In Fig. 1, the Group A data describing pituitary glands from animals that were allowed to feed and drink *ad libitum* show the circadian rhythm in pituitary adrenocorticotropic activity, with a crest at 0800 for these animals standardized in light from 0600 to 1800, alternating with darkness. The timing of this crest is in keeping with other data on this rhythm obtained recently in the same laboratory and on comparable mice kept on the same lighting regimen, the latter data revealing, as they do, an adrenocorticotropic crest between 0800 and 1200. In addition, a new main point can be supported by the plot of means and standard errors of the Group B data in Fig. 1: A circadian rhythm stands out clearly over a 36-hour observation period. An analysis of variance substantiates the effect of time of pituitary removal from the starved, dehydrated mice as statistically significant below the 1% level.

Internal timing of two hormonal rhythms. Once the significance of circadian rhythm is demonstrated, one may examine two aspects of its timing(9). On the one hand, one can relate certain phases of the rhythm to the local clock-hour if one also specifies the particular lighting regimen or synchronizer used. This aspect is the so-called *external* timing of a circadian rhythm. On the other hand, one can explore the phase relations of a given circadian rhythm to other such rhythms, *i.e.*, the so-called *internal* timing of a circadian rhythm. The time relations among the circadian rhythms themselves can indeed be evaluated as a phase difference under conditions of synchronization with a 24-hour cycle of 12 hours of light, alternating with 12 hours of darkness(9). In the study of the timing of rhythms, external or internal, from plots such as those in Fig. 1 and 2, respectively, one may use one or several indices, *e.g.*, the crest, slope and/or trough of a rhythm.

Crests of pituitary adrenocorticotropic activity on 2 consecutive days of study occurred at about 1200 for the Group B mice. The corresponding crest for the A Group mice was at 0800. It does not seem justified to attribute any significance to this slight inter-study difference. However, there remains the added question of the internal timing of the pituitary rhythm as it relates to the serum corticosterone rhythm in Group A or Group B mice. For C mice fed and watered *ad libitum* and studied under similarly standardized conditions in the same laboratory, it has previously been reported that the adrenocorticotropic activity rhythm leads in phase the rhythm in serum corticosterone by 4 to 8 hours(7). The Group A data on the 2 rhythms plotted as percent of mean, on the left in Fig. 1, confirm this earlier report.

The corresponding Group B data plotted in the right third of Fig. 1 demonstrate a similar internal timing of these rhythms in starved and dehydrated mice. On 2 occasions available for comparison, 24 hours apart, the ascending stage, as well as the crest of the rhythms in pituitary adrenocorticotropic ac-

FIG. 4. Circadian rhythms in rectal temperature of groups investigated under conditions of light-dark synchronized periodicity analysis. Points for the second day of study on Group A mice, connected by dashed line, are re-plots at corresponding times of actual data for the first day.

tivity, precedes the corresponding stage of the rhythm in serum corticosterone. Under the standardized conditions of these studies, the external timing of the pituitary and serum rhythms differed in Group A and B mice by about 4 hours, while the internal timing of rhythms in pituitary adrenocorticotropic activity and in serum corticosterone was maintained throughout a considerable period of food and water deprivation with a phase-difference corresponding roughly to that in freely feeding and drinking mice.

These findings are of interest to students of bioassay. In evaluating, among many other endpoints, pituitary adrenocorticotropic activity, it must be kept in mind that rhythmic changes of the organism continue whether or not food and water are removed, as is often done in vain with the aim of "eliminating rhythms." Of theoretical interest is the scope of much earlier work(10-12) extended by the present data on the persistence of several endocrine circadian rhythms during starvation and dehydration. Finally, Fig. 1 and 2 as a whole suggest the maintenance of the physiologic time relation between 2 hormonal rhythms under defined environmental conditions. The basic feature of circadian functional integration in time(13) appears to reside in plastic, yet detectable, phase-differences among rhythms. In the present data on 192 mice, the internal timing of a pituitary rhythm, in relation to an adrenal temporal index, appears to be at least as stable, if not more so, than the timing of either rhythm in relation to external factors.

Rhythm in mitotic count of ear epidermis. Fig. 3 presents a plot of means and standard errors of mitotic counts in ear epidermis of the animals in Group B. These serially independent data describe separate subgroups, each composed of 10-12 mice. Since food was removed from the cages of all of these subgroups 3 hours prior to the start of the sampling period, the data cover a period of 3-39 hours of starvation and dehydration. These mitotic counts are analyzed in Table I. The pinnal mitotic rhythm is a significant feature of temporal organization, persisting in absence of food and water intake, and it is not simply a "feeding phenomenon," as is often stated. While data on Group A mitoses are not available, ample earlier work on the mitotic rhythm of C mice fed and watered *ad libitum* shows an external timing which agrees closely with the Fig. 3 data(13).

TABLE I. Analysis of Variance Summary of Mitotic Counts in Ear Pinna. Groups of 10-12 C mice deprived of food and water, studied at consecutive 4-hour intervals during a 36-hour period.

Source of variation	DF	Sum of squares	Mean square	F	P
Time	9	7847.8	871.98	23.2	<.005
Residual	104	3909.9	37.59		

Rectal temperature rhythm. Fig. 4 reveals the rectal temperature rhythm in mice of the 2 groups studied. The Group A data shown by the continuous line, covering a 24-hour period of sampling, are extended by the dashed line to cover a period comparable in length to the Group B series on starving mice. A statistically highly significant rectal temperature rhythm ($P<0.01$) persists clearly in these mice deprived of all food and water for over 1½ days. The external timing of this rhythm in Group B mice corresponds to that in the Group A mice allowed food and water *ad libitum*. The amplitude of the temperature rhythm increases during the period of study in the starving and dehydrated Group B mice, in keeping with earlier work on other species(10,11).

Discussion. Fig. 5 summarizes the internal timing of the rhythms in pinnal mitosis and in rectal temperature of starving and dehydrated mice in relation to the corticosterone rhythm in the adrenal gland of the same ani-

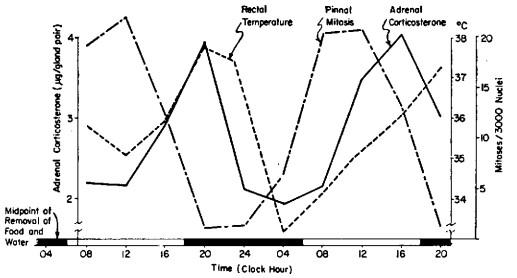


FIG. 5. Timing of rhythms in pinnal mitosis and rectal temperature in relation to rhythm in adrenal corticosterone of mice deprived of food and water. Note persistence of differences in phase among circadian rhythms during a period of progressive starvation.

mals. The corticosterone rhythm in the serum—with an external timing similar to that in the gland—has, in its turn, served as a time marker for the internal timing of the rhythm in pituitary ACTH content (Fig. 2).

Fig. 2 and 5 as a whole support the suggestions that: 1) circadian functional integration usually involves differences in phase that may be positive or negative as well as zero, and 2) the internal timing of the 5 circadian rhythms investigated is maintained in animals deprived of all food and water for a day and a half. These circadian maps in Fig. 2 and 5 do not necessarily imply causal relations among all functions charted. However, they constitute indispensable information for a complete description of such rhythmic functions under physiologic or pathologic conditions. The functions here mapped were chosen with this point in mind as temporal reference standards in an investigation of the effect of cerebral ablations upon the circadian system of a mammal.

It is of further interest that the present and earlier(7) *in vitro* demonstration of a circadian rhythm in adrenocorticotropic activity of the pituitary gland of mice can be complemented by the finding of a circadian rhythm in pituitary prolactin concentration, also tested *in vitro*(14) as well as *in vivo*(15) in the rat.

Summary. Circadian rhythms in pituitary adrenocorticotropic activity, pinnal mitosis and rectal temperature, as well as in the corticosterone content of serum and adrenal, persist in inbred Bagg albino (C) mice deprived of food and water. A lead in phase of the rhythm in pituitary ACTH content over the circadian rhythm in serum corticosterone—an aspect of internal timing in a mammal—is apparent in starved and dehydrated mice as well as in mice with food and water available *ad libitum*. The rhythms in pinnal mitosis and rectal temperature, compared with each other or with the rhythms in adrenal and serum corticosterone, also show statistically significant differences in phase that characterize circadian systems in the absence, as well as presence, of food and water.

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