diamond were unaffected while the graphite transformed to diamond. The mixture of graphite and hexagonal boron nitride transformed to a composite of diamond and cubic boron nitride. The mixture of graphite and nickel powder was converted to a composite of diamond in which the nickel particles were entrapped. The pencil "lead," which is a mechanical mixture of graphite and clay, became converted to diamond, stishovite (*7*), and small amounts of other unidentified materials.

The quantitative results of the experiments are shown in Fig. 2. The upright triangles indicate the pressures and temperatures at which the graphite-to-diamond reaction started. The open circles show the maximum temperatures reached at a given pressure in those experiments in which there was no reaction. Squares indicate the onset of melting of graphite. The inverted triangles show the conditions at which rapid complete graphitization of diamond took place. The cluster of upright triangles establishes quite clearly a threshold line or band at which graphite transformed very rapidly to diamond.

On the same diagram are shown points of pressure and temperature taken directly or indirectly from the data in the publications of DeCarli and Jamieson and of Alder and Christian (*3*). All of these data points, together with my own work on the melting line of graphite (*8*) and the published work on the diamond graphite equilibrium line (*9*), rather well establish the phase diagram for carbon. By analogy with the behavior of indium antimonide (*10*), silicon, and germanium (*11–13*) at high pressure it is proposed here that around 600 to 700 kilobars the diamond melting line terminates in a triple point which joins liquid, diamond, and a third solid phase of carbon which is 15 to 20 percent more dense than diamond, and is metallic. Because the density of this proposed "carbon III" would be slightly greater than the liquid, its melting temperature showed increase with pressure, in the manner shown.

The pressure temperature line which marks the threshold of the spontaneous transformation of graphite to diamond is of interest in at least two respects. First, it appears to be closely related to the extension of the graphite melting line, or what may be considered to be the melting line of metastable graphite. This line, together with the data of Alder and Christian, plotted on a pressure volume diagram, predicts that graphite should, at room temperature, transform spontaneously to diamond at a pressure of about 400 to 450 kilobars. The fact that such a room-temperature transition has not yet been reported indicates that pressures of this magnitude have not yet been attained in static pressure apparatus.

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12 September 1962

## Circadian Rhythm in the in vitro Response of Mouse Adrenal to Adrenocorticotropic Hormone

*Abstract.* Adrenal corticosterone production resulting from adrenocorticotropic hormone (ACTH) stimulation in vitro depends upon the time of gland removal. This rhythm in adrenal reactivity to ACTH is out of phase with the corticosterone rhythm in serum of the mice used as donors of adrenals. The responsiveness of the gland to exogenous ACTH is highest when serum corticosterone levels are lowest.

In the mouse, the circadian (*circa*, about; *dies*, day) rhythm in corticosterone of serum (*1*) and adrenal (*2*) can be studied in vivo under conditions standardized with respect to the physical environment and the history of the animals, including their genetic background. On a regimen providing for light from 0600 to 1800, alternating with darkness, corticosterone levels in mouse serum and adrenal are highest at about 1600 and lowest at about 0400. The occurrence of peak levels in adrenal and blood corticosteroid in this nocturnally active rodent differs from that recorded for blood and urinary steroid levels in diurnally active men, but in mice, as well as men, spontaneous adrenal activation occurs prior to the onset of daily activity (*1*).

The adrenal cycle is not a mere direct and immediate reaction to extrinsic stimuli acting upon the gland via ACTH stimulation. In the face of fluctuating pituitary or central nervous system stimulation which influences the state of the adrenal at any one time, periodic changes in the metabolism of the mouse adrenal itself may contribute critically to the gland's intrinsic circadian cycle. Our in vitro studies, initiated to define any inherently periodic contribution of the adrenal itself to its reactive behavior and to correlate these effects with spontaneous changes in adrenal activity observed in vivo, document quite clearly the need for defining the state of an organ or organism studied in terms of its circadian rhythms (*3*).

Inbred male C (Bagg albino) mice, 3 months of age, were housed singly for 1 week prior to the study in three experimental rooms kept at 24°±0.5°C, with light from 0600 to 1800, alternating with darkness. Purina Fox Chow and tap water were available to the mice from weaning until sacrifice. Additional conditions for circadian periodicity analysis have been described elsewhere (*4*). Seven groups, each composed of 60 mice, were sacrificed every 4 hours, starting at 0800 on the day of study and ending at 0800 the next day. At each time point, 12 pools of blood, each from five mice, were obtained for determination of serum corticosterone.

Immediately after decapitation the adrenal glands were removed, defatted, quartered (*5*), and placed in 25-ml erlenmeyer flasks. Each flask contained 10 adrenals in 2.0 ml of a Krebs-Ringer bicarbonate buffer, *p*H 7.4 containing glucose (2 g/liter). Ovine ACTH (Parke, Davis and Co.) in doses of 0.04, 0.4, and 4.0 International Units, was added at the start of incubation. At each of the seven time points, additional adrenals were incubated without ACTH or frozen for direct extraction of steroid. An additional series of flasks containing progesterone-4-$C^{14}$ in the incubation fluid served to explore the extent of conversion of this material to other adrenal steroids (*6*). All flasks were run in duplicate. The incubations were carried

out in a Dubnoff incubator at 37°C for 2 hours with a gas phase of 95 percent oxygen and 5 percent carbon dioxide.

The corticosterone values in serum, adrenal, and incubation fluid were determined by the fluorometric procedure of Silber *et al.* (*7*) with slight modifications depending upon the volume and type of tissue analyzed. The qualified usefulness of the measurement of the corticosterone fluorescence peak at 520 m$\mu$ in mouse serum has been previously demonstrated by comparing fluorometry with a double isotope derivative technique (*1*). Cortisol which fluoresces under the assay conditions employed has not been detected in the mouse. However, at least six steroids other than corticosterone and deoxycorticosterone have been found in mouse adrenal incubations (*6*). Their contribution to the fluorescence measurements is minor. Nonetheless, the results of the analyses are presented to indicate relative changes in levels with time and do not necessarily represent absolute corticosterone values. Values are expressed in micrograms of corticosterone which was used as a standard for the assay. Corticosterone standards in 1-$\mu$g amounts were routinely carried through the entire extraction procedure with each set of determinations.

The corticosterone values obtained in 2-hour adrenal incubations are plotted against time of adrenal removal in Fig. 1. The three curves at the bottom of this figure represent respectively the values of the corticosterone content of adrenal glands extracted directly, control incubations without added ACTH, and incubations with the smallest dose (0.04 unit) of ACTH tested. The larger doses of ACTH used in this study, represented by the two top curves, significantly increased the production of corticosterone by the quartered adrenal gland preparation.

The magnitude of the adrenal response to ACTH added in vitro is a function of time. The response to 4 units of ACTH was much greater at 0400 than at 1600. Reactivity to ACTH also may be examined as a function of all three dose levels employed. For this purpose, but only as a first approximation to the relation of dose and reactivity to ACTH, the slope of log dose-response curves is plotted against time of adrenal removal as a broken line in Fig. 2. As a whole, the data demonstrate a rhythm in the adrenal's responsiveness to ACTH. Previous results obtained in vivo on a rhythm in the adrenal's reactivity to exogenous ACTH (*8*) and on the timing of this reactivity rhythm in relation to the lighting regimen on which the mice were kept are in accord with our in vitro study.

Factors influencing the state of the adrenals at the time of their removal must be considered in viewing our results. The level of ACTH in the adrenal as a result of prior stimulation has been suggested as a determinant of the response to exogenous ACTH administered in vivo (*9*). It has been suggested that the adrenal glands' response to ACTH might be influenced also by the level of adrenal corticosterone which could act via a negative feedback mechanism directly on the enzymatic systems involved in cortico-

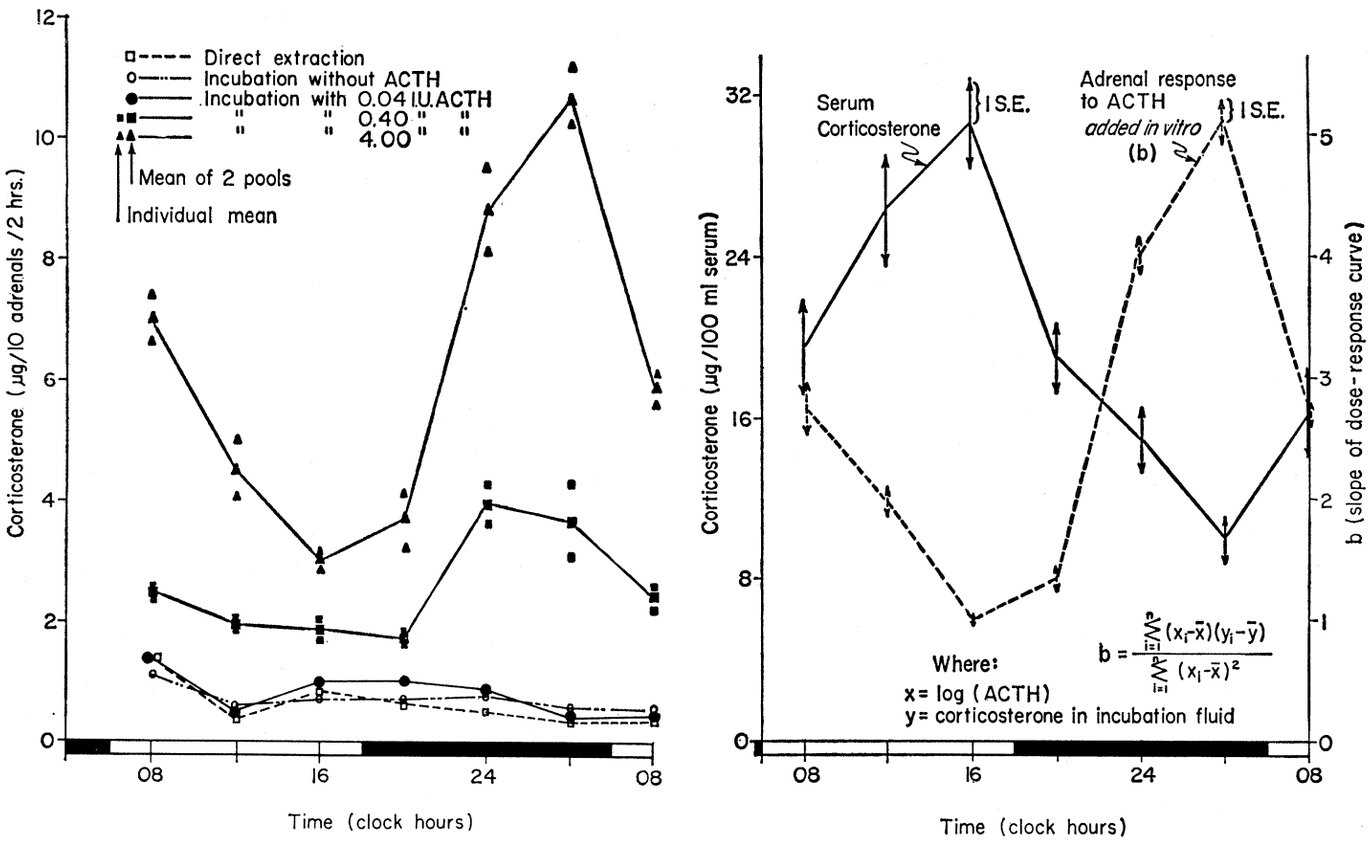


Fig. 1 (left). Circadian periodic response of mouse adrenals to ACTH added in vitro. Each point represents the means of duplicate flasks per treatment and time. Total of 700 C adrenals incubated. Fig. 2 (right). Phase relations of circadian rhythms in serum corticosterone and in adrenal responsivity to ACTH in vitro. (Broken line) slope of log dose-response relation of quartered adrenals to ACTH added in vitro—computed from Fig. 1 data. (Solid line) serum corticosterone; mean of 12 pools, each from five mice per time point. Total of 420 C mice.

sterone synthesis (*10*). A variation in the amount of precursor substrates or cofactors would be equally likely to influence the adrenal cycle.

The conversion of added substrate progesterone-4-$C^{14}$ to corticosterone is not constant but this conversion apparently follows a different time course from that of the in vitro ACTH response (*6*). Since a rhythm of adrenal response to ACTH added in vitro can be observed in the absence of the glands' nervous, hormonal or circulatory controls during the 2-hour incubation periods, use of this technique may further reveal the roles of intrinsic adrenal, pituitary, and other factors in the adrenal cycle.

The timing of the reactivity rhythm to ACTH, demonstrated in vitro, can be compared with that of the serum corticosterone rhythm of the adrenal donor mice, also shown in Fig. 2. Means and the standard errors are shown for each time of adrenal removal for the incubation study. Peak adrenal responsiveness to ACTH, added in vitro, is grossly out of phase with the peak of serum corticosterone found in the absence of all intentional external stimulation other than that by alternating 12-hour periods of light and darkness.

The rhythms of serum levels as well as adrenal content of corticosterone should reflect, in part, spontaneous cyclic adrenal activity. If so, these results demonstrate a significant difference between the timing of endogenous cyclic adrenal activity under basal conditions and the timing of the glands' ability to respond to increasing doses of ACTH. Extrapolation of a phase difference between rhythms in adrenal activity and the glands' reactivity to ACTH beyond the inbred C mouse and the dose range of the ovine rather than murine ACTH studied would not be justified without additional evidence. From a methodologic viewpoint, the demonstration of differences in the in vitro response of an organ coincident with predictable changes in physiologic state emphasizes the usefulness of circadian rhythm analysis as a powerful tool for biological investigation, particularly for bioassay, and in the general area of endocrinology (*11*).

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## Conditioning of Extrasystoles in Humans with Respiratory Maneuvers as Unconditional Stimulus

*Abstract.* Extrasystoles are known to occur in some healthy individuals during common respiratory maneuvers such as holding the breath. Evidence is presented which shows that under controlled laboratory conditions this kind of extrasystole can be conditioned. Conditional stimuli signaling inspiration and expiration acquired the properties of the respiratory maneuvers by inducing extrasystoles during normal breathing.

Recent studies in the field of cardiovascular conditioning have revealed that changes in the configuration of the electrocardiogram can occur as a conditional reflex. Bykov and collaborators (*1*) have shown that electrocardiographic changes induced by morphine, strophanthin, or nitroglycerin can be conditioned after a given conditional signal, such as the sound of a trumpet, has been paired with the injection of one of these drugs. Perez-Cruet and Gantt (*2*) have shown conditioning of the changes in amplitude of the T-wave on the electrocardiogram to a tone previously paired with an intravenous injection of bulbocapnine.

Most investigations on conditioning of the electrocardiogram have been performed in dogs. In humans evidence of this basic principle is not available. Some knowledge of electrocardiographic conditioning in animals provided the basis for the design of the present study, whose main purpose it was to investigate some of the mechanisms by which the brain can induce extrasystoles through conditional reflexes.

One hundred and five volunteers with negative history of cardiac disease were studied. There were 91 males and 14 females, ranging in age from 17 to 43 years (average age was 26). The subjects were placed in a recumbent position on a ballistocardiograph or sitting on a chair. They were instructed to look at a rectangular board (3 by 4 feet) on which were mounted eight lights in serial arrangement. All subjects were instructed that when a given set of three lights was on they should take a deep breath and hold it until another set of eight lights was on, at which time they should exhale slowly and completely, and resume normal breathing again when the eight lights were off.

In 13 subjects the lights plus the respiratory maneuvers induced one isolated extrasystole in one out of ten trials. In six other subjects the mean values (41 to 93 trials) of unconditional extrasystoles per trial induced by respiratory maneuvers were: $S_1$, 5.2; $S_2$, 1.9; $S_3$, 0.6; $S_4$, 1.0; $S_5$, 0.3; and $S_6$, 0.2. The percentages of trials with unconditional extrasystoles during the same conditions were: $S_1$, 92.7; $S_2$, 69.0; $S_3$, 61.0; $S_4$, 46.5; $S_5$, 23.7; and $S_6$, 15.1. Extrasystoles occurred during the inspiratory and expiratory phases of respiratory maneuvers, except in three subjects where extrasystoles occurred only at the beginning of expiration. Almost all extrasystoles were of ventricular origin, but some premature auricular beats were observed in young subjects. Figure 1*A* illustrates an example of unconditional extrasystoles induced by the respiratory maneuvers in subject 4.

In view of the high incidence of extrasystoles during respiratory maneuvers in subjects 1 to 6, these six individuals were selected for the attempted conditioning of extrasystoles. The conditioning techniques consisted in pairing the same lights signaling inspiration and expiration, namely, the conditional stimuli, with the respiratory maneuvers. Average duration of the conditional stimuli was 39 seconds. Subjects were allowed to breathe normally after each trial for at least 38 seconds (intertrial interval). After the subjects had been exposed to the lights plus the respiratory maneuvers 15 or more times, instructions were