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CONTROL MECHANISMS OF CIRCADIAN RHYTHMS IN BODY COMPOSITION:

IMPLICATIONS FOR MANNED SPACEFLIGHT

(NASA-CR-144413) CONTROL MECHANISMS OF
CIRCADIAN RHYTHMS IN BODY COMPOSITION:
IMPLICATIONS FOR MANNED SPACEFLIGHT Final
Report (Harvard Medical School, Boston,
Mass.) 173 p HC \$6.25

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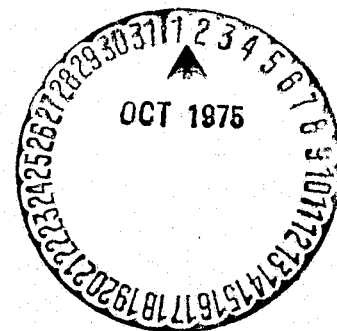
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Final Report on Contract NAS9-14249 of the
National Aeronautics and Space Administration
(Lyndon B. Johnson Space Center, Houston, Texas)

Held at the Department of Surgery
Harvard Medical School at the Peter Bent Brigham Hospital
Boston, Massachusetts

July 1, 1974 - June 30, 1975

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I. Introduction

This is the final report on NASA contract NAS9-14249 which was held by the Department of Surgery of Harvard Medical School at the Peter Bent Brigham Hospital, Boston, Massachusetts 02115 from July 1, 1974 - June 30, 1975.

The research conducted under this contract investigated the mechanisms that underlie the circadian (approximately 24 hour) variations in electrolyte content in body compartments, and examined the significance of these circadian oscillations for manned spaceflight. The research program utilized human volunteer subjects and an unanesthetized, chair-acclimatized monkey preparation which the principal investigator developed in the laboratories of the Department of Physiology, Harvard Medical School.

II. Background

Circadian rhythms in biological variables are one outward manifestation of an important evolutionary adaptation to life on a rotating planet: the ability to measure time. This capability enables organisms to predict the major changes in environmental conditions and the consequent alterations in food supply and predator activity which occur with a 24-hour periodicity because of the earth's rotation. Thus, for example, adaptive physiological and behavioral responses which may take several hours to be activated can be initiated in advance of the predicted environmental challenge, or events where timing may be critical for survival, such as emergence in flies, can be timed to occur at the point of maximum environmental advantage.

There is now considerable evidence to indicate that such circadian time measurement is the product of an oscillating system within the organism. The responses of this oscillating system to manipulations in environmental time cues are now well established, but current knowledge of the anatomical and physiological organization of the circadian timing systems within advanced multicellular organisms such as mammals is still very limited.

As man ventures out into space, it has become particularly important to investigate the control of the circadian oscillating system because of its important adaptive functions under earthbound conditions which no longer apply in space. The circadian oscillations in physiological functions are normally synchronized to a strict 24-hour period when man is on the surface of the earth. In space however the oscillating components of the earth's environment which contribute to the normal external and internal synchronization of the circadian system are no longer present, unless artificially supplied. It therefore becomes important to examine the effect of their absence, and to investigate the necessity for supplying circadian oscillations in the spacecraft environment to achieve optimal physiological functioning.

A system that is particularly important to study in this regard comprises the circadian oscillations in the body compartmental distribution of electrolytes and fluids. Fluid and electrolyte balance is subjected to major perturbations in space, and additional imbalances due to circadian internal desynchronization could have potentially dangerous consequences. The research performed under this contract investigated the extent of circadian oscillations in electrolyte distribution between body fluid compartments and studied the mechanisms which control the oscillations in order to investigate what effect internal desynchronization in such a system would have during manned spaceflight. The studies were performed in man, in both healthy subjects and in patients with specific clinical conditions, and in addition were supported by further studies into the control mechanisms using a primate preparation, the squirrel monkey (Saimiri sciureus).

III. Experimental Studies

A. Basic Studies in Man

Previous analyses of the intercompartmental distribution of potassium in man have assumed that the system is basically static with zero net fluxes between compartments except when definable events cause temporary loss of this steady state. However, the assumption that there is a homeostatically maintained steady state has now been challenged by studies of the circadian variation in urinary potassium excretion. Typically the

rate of urinary potassium excretion has a five-fold variation in each 24-hour day, with minimum rates in the early morning and maximum excretion around noon. Such a circadian variation might be thought to be a result of day-night differences in dietary intake, activity, posture and sleep. However, the circadian rhythm of urinary potassium excretion persists when all these variables are kept constant throughout day and night or are manipulated in a non-circadian pattern.

Accordingly, we examined the intercompartmental distribution of potassium in normal men when dietary intake, activity and posture were held constant throughout each 24-hour day.

Three healthy, normal volunteers were studied for up to 10 days in the Bartlett Intensive Care Unit of the Peter Bent Brigham Hospital. Posture, activity and dietary intake was maintained at constant levels throughout day and night. This was achieved by keeping the subjects on strict supine bedrest and providing a liquid diet divided into 8 equal aliquots which were taken at 3-hourly intervals through each 24 hour period. The room lighting was kept on between 08.00 and 23.00 hr and off from 23.00 to 08.00 hr daily (LD 15:9). After the subjects were equilibrated on this regimen, three hourly urine collections were obtained and blood samples were drawn from catheters inserted under local anesthesia into the radial artery and brachial vein of the non-dominant arm. For 48-hours these blood and urine samples were obtained with the subject continuing on his 3 hourly regimen of constant diet, activity and posture.

These studies demonstrated that there were marked fluxes of potassium in and out of body compartments over the course of the 24-hour day. A net flux of potassium was observed out of the body cell mass during the day and a reverse flux from the extracellular fluid into the body cell mass during the night. These fluxes were measured as reversing polarity arterio-venous differences across the resting forearm preparation in the volunteers, and also as changes in red blood cell potassium content. The

net fluxes were simultaneously estimated by following the content of potassium in the extracellular compartment using arterial plasma potassium concentration and Br⁸² extracellular fluid volume measurements to determine extracellular potassium content. (Fig. 1.)

These fluxes out of the body cell mass into the extracellular fluid were counterbalanced by a circadian variation in urinary potassium excretion which reached a maximum in the early afternoon and a minimum during the early morning hours. The counterbalancing of these two directional fluxes of potassium resulted in little net change in extracellular potassium content and in plasma potassium concentration.

The existence of these fluxes of potassium despite the maintenance of a constant level of posture, activity and dietary intake throughout day and night for up to 10 days indicates that these fluxes are an endogenous feature of the control of body potassium distribution. It, however, was apparent that if the two major fluxes of potassium with respect to the extracellular compartment (the fluxes in and out of the body cell mass) and the flux through the kidney (which is seen as urinary potassium excretion) became desynchronized from one another, that major fluctuations in extracellular potassium content and therefore plasma potassium concentration could occur. It was calculated that if these two fluxes came 180° out of phase that plasma potassium concentration could drop to a calculated value of 2.2 mEq/l instead of the normal approximately 4 mEq/l. (Fig. 2.) Such a transient hypokalemia could quite easily cause cardiac arrhythmias and it is well worth considering this as a possible cause of the cardiac arrhythmias that were seen during some of the Apollo missions. Internal desynchronization of circadian rhythms has been seen in space in the Biosatellite III mission and if such an event took place in human astronauts in the Apollo program this mechanism could perhaps account for those cardiac arrhythmias.

This work has been published in the Journal of Applied Physiology

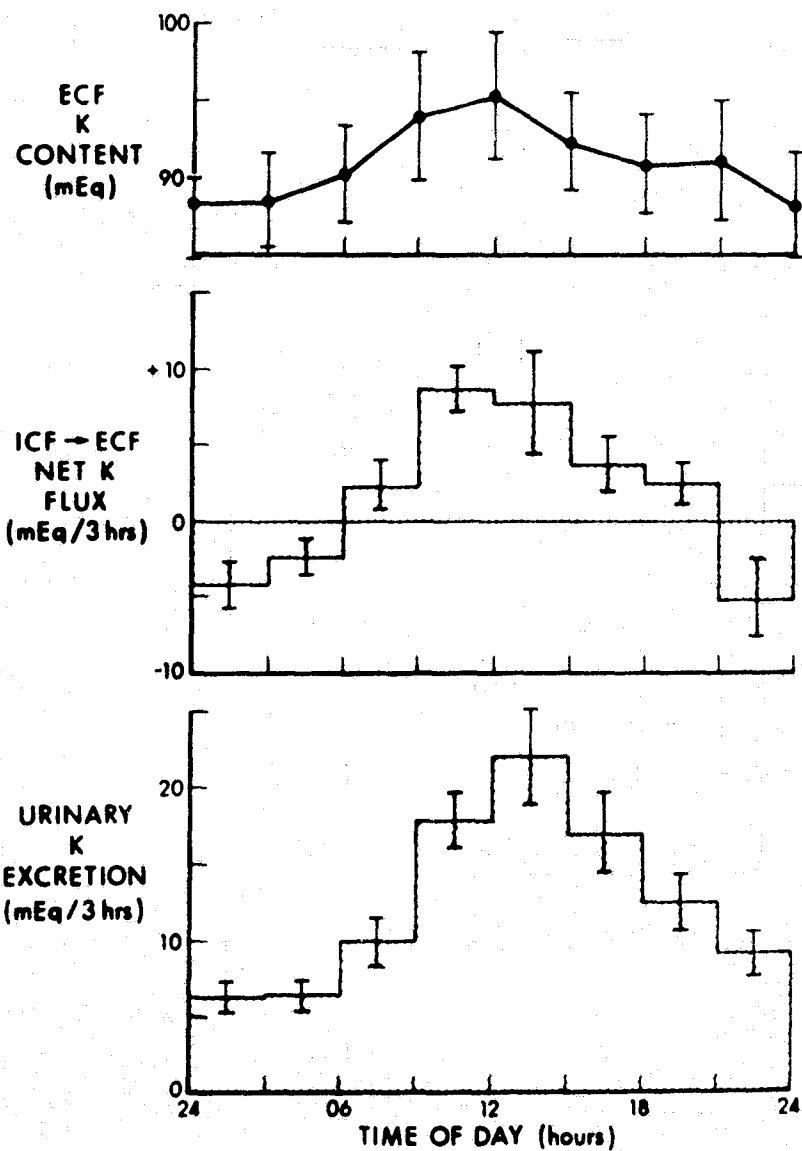


Fig. 1. Circadian variation in intercompartmental potassium fluxes in normal man in the absence of diurnal variation in posture, activity or feeding patterns.

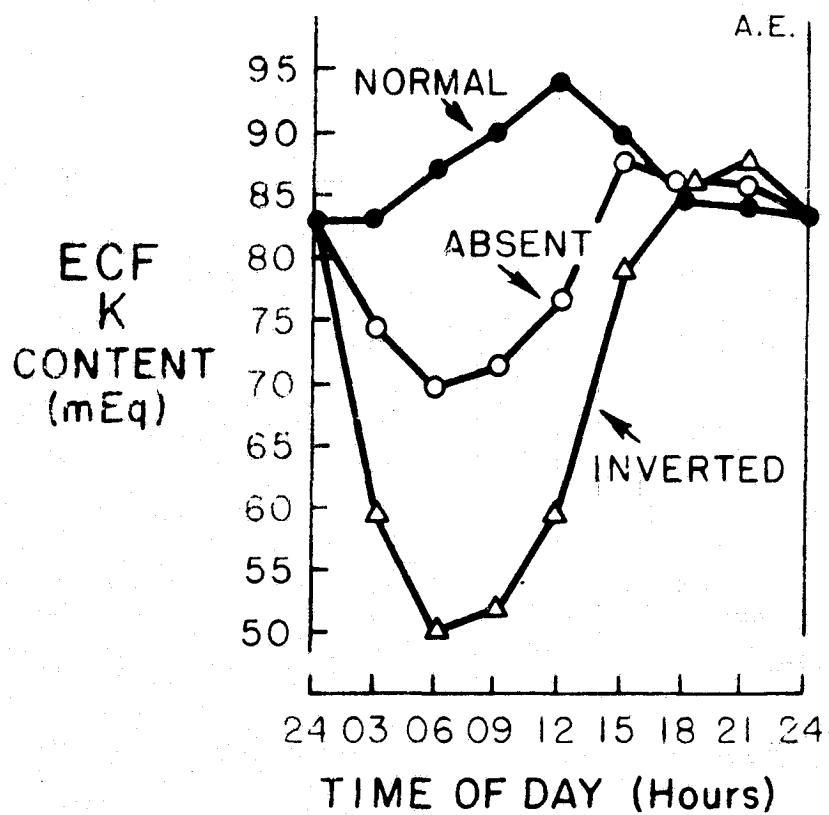


Fig. 2. Effect of changes in urinary potassium rhythm on circadian variation in ECF potassium content.

38; 163-170, 1975, a copy of which is attached.

B. Mechanisms of Control of Potassium Rhythms in the Squirrel Monkey

The studies that were undertaken in man of the circadian variation of intercompartmental potassium fluxes indicated that much more information was needed on the control of these circadian rhythms, and particularly the way in which they were synchronized with one another. To examine these questions an animal system was developed which utilized a chair-acclimatized, unanesthetized squirrel monkey preparation.

The circadian rhythm of urinary potassium excretion in the squirrel monkey (Saimiri sciureus) were found to have similar characteristics to those in man. Urinary potassium excretion rose to a maximum during the afternoon which was four times higher than the nocturnal minimum. The squirrel monkey is a diurnal animal and this circadian oscillation is normally synchronized with the environmental light-dark cycle and with the circadian oscillations of other variables within the animal.

We were particularly interested in the mechanisms of this internal synchronization. To explain such "internal synchronization", a single driving oscillator or "clock" in the brain has traditionally been postulated and attempts have been made to demonstrate control pathways from the driving oscillator to the circadian oscillations in peripheral tissues. The postulated control pathways have consisted of sets of oscillating variables in series, with the circadian oscillation in each variable passively dependent on the circadian oscillation in the preceding variable on the pathway. However, few such pathways have yet been convincingly demonstrated and it is therefore possible that the wrong model has been used. An alternative model of internal synchronization was therefore developed and its applicability demonstrated in studies of the synchronization of the circadian rhythm of urinary potassium excretion in the squirrel monkey.

The alternative model was based on the observation that circadian oscillations will persist in isolated tissues in vitro. The model postulated

that many different tissues in the body can act as spontaneous circadian oscillators. The circadian rhythm of urinary potassium excretion, for example, would be determined by a potentially independent circadian oscillation in potassium flux across the luminal membrane of the cells lining the renal distal tubule. Each such oscillator would then be synchronized with the other tissue oscillators, through nervous and hormonal mediators, by a process similar to that described for the synchronization of circadian oscillations by the light-dark cycle.

All experiments were conducted in unanesthetized, trained squirrel monkeys sitting in a metabolism chair within an isolation chamber. (Fig. 3.) Urine was collected in two-hourly fractions from a funnel between the animal's legs. With lights on (300 lux) from 08.00 - 20.00 hr and off (< 1 lux) from 20.00 - 08.00 hr daily, urinary potassium excretion in five monkeys on ad lib feeding rose to a maximum of $274 \pm 23 \mu\text{Eq/hr}$ (mean \pm SEM) at 17.00 hr and then fell to a minimum of $63 \pm \mu\text{Eq/hr}$ at 05.00 hr. Renal potassium excretion thus showed a regular fourfold circadian variation. (Fig. 4.) While on this ad lib schedule all feeding, drinking and activity occurred during the lights-on period of each 24 hours. Independence of the urinary potassium rhythm from these patterns of dietary intake and activity was established by a) depriving the monkeys of food and water for 24 hours, and b) training the monkeys to eat one gram of food pellets every two hours throughout each 24-hour period. Rhythm parameters remained unaltered in both circumstances.

In the next series of experiments, the synchronization of the circadian oscillation in renal potassium excretion by the light-dark cycle was examined. The circadian rhythm of potassium excretion had a 24-hour period when monkeys were exposed to a light-dark cycle of 12 hours light alternating with 12 hours of darkness. However, when a monkey was placed in isolation with constant light throughout day and night for three weeks the circadian oscillation in renal potassium excretion persisted but

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Figure 3: Chair-acclimatized squirrel monkey in metabolism chair within the isolation chamber. Urine is collected from a funnel between the monkeys legs, and passes down to the tubes in the fraction collector. Catheters and thermistor leads pass out from under the jacket to the outside of the chamber. Also note the lever the monkey operates to gain food pellets, and the ultrasound motion detector above the animal.

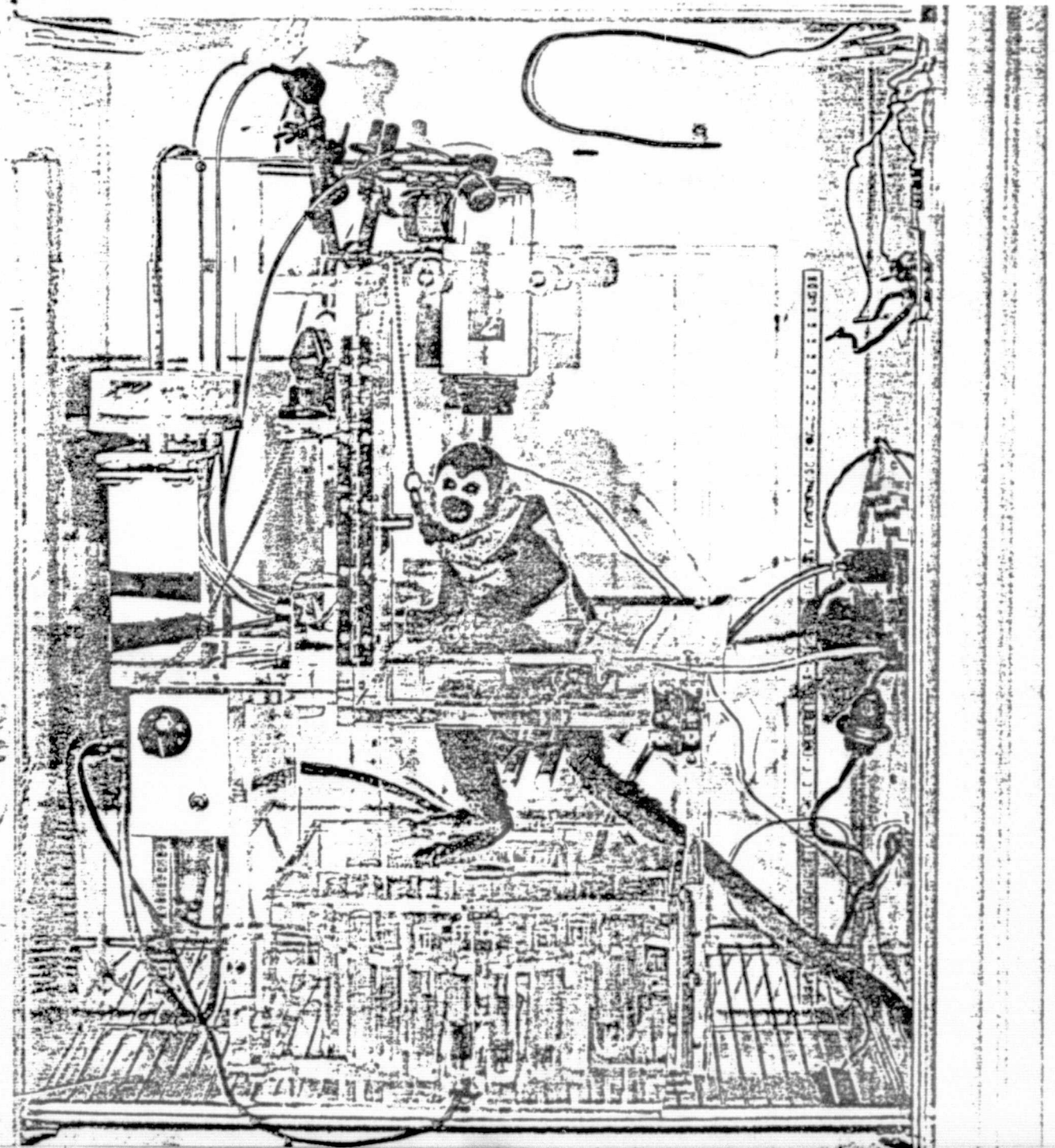
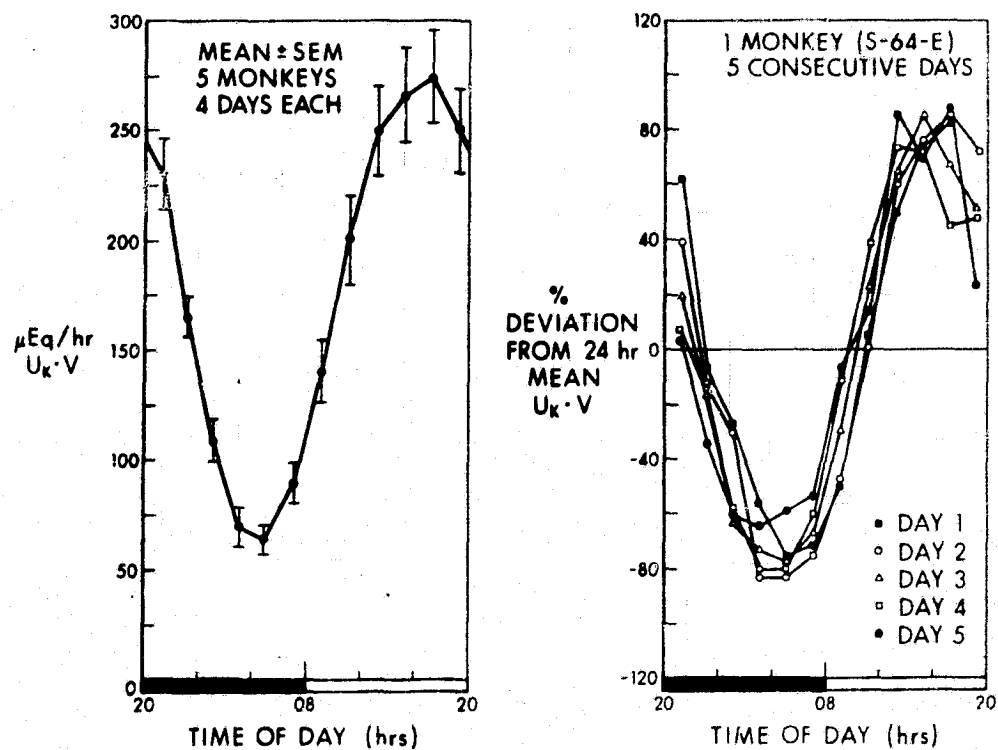


Fig. 4 URINARY POTASSIUM EXCRETION IN CHAIR-ADAPTED SQUIRREL MONKEYS



demonstrated a regular 25.1 hour free-running period. The renal potassium rhythm thus appeared to be normally constrained to a 24 hour period by the light-dark cycle.

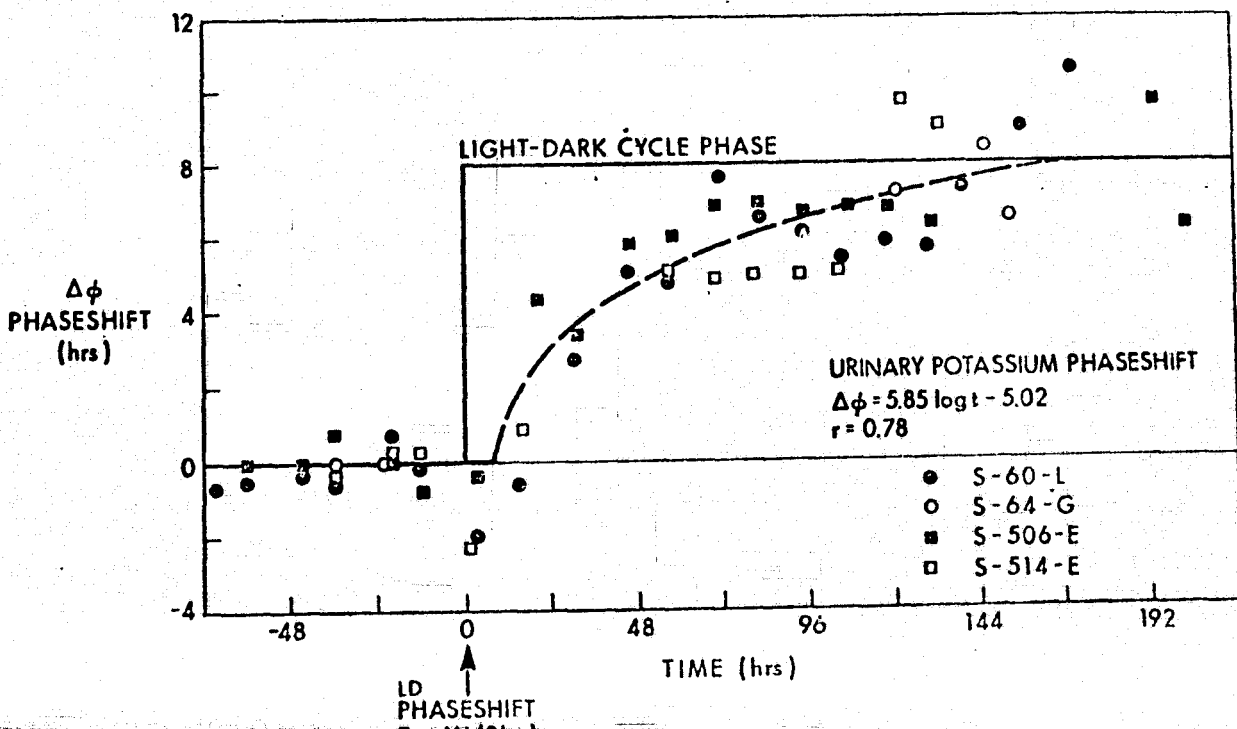
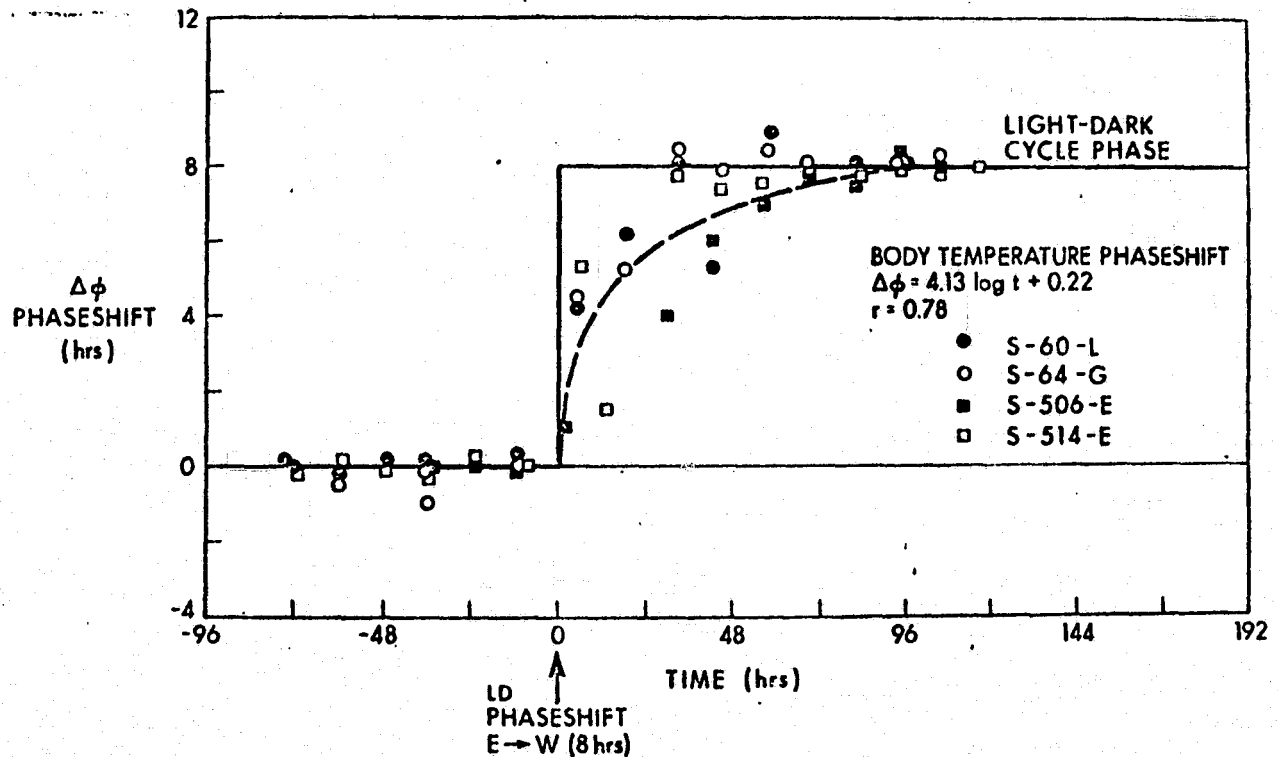
When four monkeys were each subjected to 36 hours of continuous light followed by 36 hours of continuous darkness, the urinary potassium oscillation continued with an approximately 24 hour period throughout this regimen ruling out any passive dependence of the rhythm on the light-dark cycle. However, the synchronization of the circadian oscillation in urinary potassium excretion by the light-dark cycle was confirmed in a further study in which four monkeys were subjected to an eight-hour phaseshift of the light-dark cycle. The urinary potassium oscillation gradually adjusted over a period of seven days to the new phase of the lighting regimen. The circadian oscillations in body temperature and activity however resynchronized in 3-4 days. (Fig. 5. and 6.)

To evaluate the mechanism by which synchronization pathways might operate it was necessary to identify one or more oscillating variables which mediated in the synchronization of the circadian rhythm of urinary potassium excretion by the light-dark cycle. The potential mediators chosen for further study were the adrenal steroids, cortisol and aldosterone. These hormones were selected because each is known to influence the rate of urinary potassium excretion and each has been demonstrated to have a circadian oscillation of concentration in the plasma which precedes the urinary potassium rhythm by a time period suggestive of a causal relationship.

Adrenalectomized monkeys were prepared with chronically indwelling arterial and venous catheters. By administering a daily intravenous infusion of 5 mg cortisol and 0.001 mg aldosterone through a catheter extending outside the isolation chamber, it was possible to reproduce in adrenalectomized monkeys the circadian patterns of cortisol and aldosterone secretion found in normal intact animals.

The circadian variation in potassium excretion in adrenalectomized

Figure 6. Comparison of the rate of resynchronization of the circadian rhythms of body temperature and urinary potassium excretion after an eight-hour phase-delay of the light-dark cycle. While the body temperature rhythm resynchronized within 3-4 days, the rhythm of urinary potassium excretion took 7 days. Thus there was a period of temporary internal desynchronization between these rhythms.



monkeys receiving cortisol and aldosterone at 08.00 hr daily was not significantly different from that found in untreated intact animals. However, when the same daily replacement dose of adrenal steroids was given as a continuous intravenous infusion throughout each 24 hours in three adrenalectomized monkeys, urinary potassium excretion continued to oscillate but lost its normally strict phase-relationship with the light-dark cycle.(Fig. 7.) The renal potassium rhythm oscillated with periods shorter or longer than 24 hours. The normal synchronization of the urinary potassium rhythm with the light-dark cycle thus appeared to be mediated by the circadian rhythm of adrenal steroid secretion.

Phaseshifts of the timing of adrenal steroid administration in adrenalectomized monkeys provided further evidence for the synchronization of renal potassium excretion by the rhythm of adrenal steroid secretion.(Fig. 8.) When the timing of cortisol and aldosterone administration was phaseshifted by eight hours in four monkeys, with the light-dark cycle unchanged, the circadian rhythm of urinary potassium excretion phaseshifted by between 4.0 and 9.1 hours. The urinary potassium oscillation, however, did not immediately respond to the phaseshift of adrenal steroid administration. The delay of several cycles before the final phase of the urinary potassium oscillation was reached was similar to the delay in the resynchronization of the urinary potassium rhythm after a light-dark cycle phaseshift.

These findings suggested that circadian rhythms of plasma adrenal steroid concentration are an important mediator in the synchronization of the circadian rhythm of renal potassium excretion with the light-dark cycle. While the responses of urinary potassium excretion to the changes in the pattern of adrenal steroid administration were predicted by the model of internal synchronization which we have proposed they were not compatible with the traditional model of the passive dependence of peripheral circadian oscillations on hormonal and nervous mediators.

To distinguish between the influences of the circadian rhythms in

Figure 7. Circadian rhythm of urinary potassium excretion in three adrenalectomized monkeys during two days with cortisol infused intravenously between 08.00 and 09.00hrs daily and then during the 7 subsequent days with the same dose of cortisol evenly distributed over each 24 hour day. When the circadian rhythm of plasma cortisol concentration was thus eliminated urinary potassium excretion began to oscillate with free-running periods which were significantly different from 24 hours.

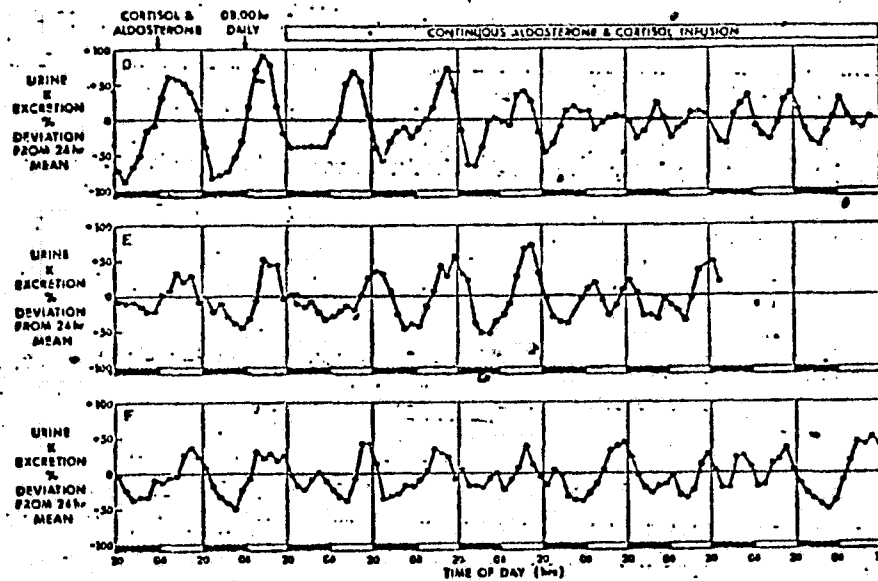
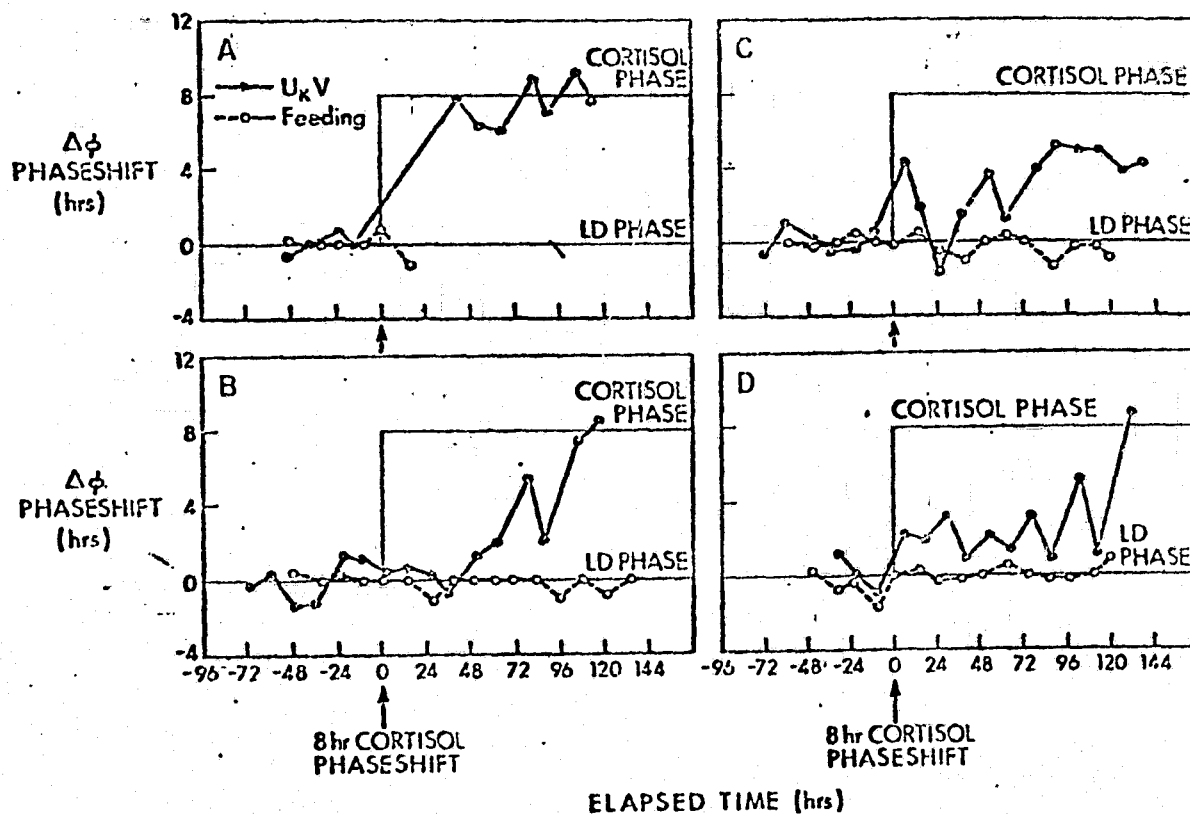


Figure 8.



Phaseshifts of the rhythms of urinary potassium excretion (solid line) and feeding (interrupted line) in response to an eight-hour phase-delay of the time of cortisol administration in adrenalectomized squirrel monkeys. The light-dark cycle phase was kept unchanged throughout the experiment. All animals continued to feed with a rhythm synchronized to the light-dark cycle, but the rhythm of urinary potassium excretion resynchronized with the new phase of cortisol administration.

aldosterone and cortisol administration, adrenalectomized monkeys were given the same daily 08.00 hr dose of cortisol (5 mg) but with no aldosterone. The circadian oscillation in urinary potassium excretion was indistinguishable from that observed when both cortisol and aldosterone were administered. Similarly, an eight hour phaseshift in the time of cortisol administration alone resulted in a phaseshift in the circadian rhythm of renal potassium excretion which was similar to that seen when cortisol and aldosterone were both phaseshifted by eight hours. It was concluded that the synchronization of the circadian oscillation in urinary potassium excretion is mediated by the circadian rhythm in cortisol secretion and that aldosterone does not play an essential role in this process.

The circadian oscillation in urinary potassium excretion was also shown not to be passively dependent on the circadian rhythm in cortisol secretion in intact monkeys. Four monkeys were given an infusion of 15 mg cortisol between 20.00 and 23.00 hr on one day. This provided a second peak of cortisol 12 hours after the normal endogenous peak of cortisol secretion. However, this cortisol infusion failed to induce a second peak in urinary potassium excretion.

These responses of the circadian oscillation in renal potassium excretion to the pattern of cortisol administration in adrenalectomized monkeys demonstrated that the synchronization of the urinary potassium rhythm is mediated through the circadian oscillation in cortisol secretion by the adrenal cortex. The mode of control, however, was not the traditionally assumed passive dependence of one oscillating variable on another. Instead the experimental results suggested that the renal distal tubular cells which control urinary potassium excretion, were acting as a spontaneous circadian oscillator which was synchronized by the circadian oscillations in the plasma concentration of cortisol. Since there is evidence that membrane potassium fluxes may be a fundamental component

of cellular circadian oscillators in many species, it is possible that the synchronization mechanisms described for the circadian potassium flux across renal distal tubular cells may be common to other systems. This work has been presented in a Symposium on "Physiological and Biochemical Aspects of Circadian Rhythms" at FASEB meetings in April, 1975, and has been submitted in 3 papers to American Journal of Physiology, Federation Proceedings and Science.

C) Development of Models of Internal Synchronization

As a result of this work we have formalized three models of the circadian timing system (These are presented in Figure 9.). Minor variants of these models, or combinations of their features are also possible, but the models presented here emphasize the contrasts between certain possible organizations of the circadian system.

Model I, which has been proposed by Mills but has been assumed in many other investigations of the circadian timing system, consists of a network of cellular systems (A,B,C,...,etc.) which passively oscillate as a forced response to a single self-sustained driving oscillator (D.O.). Where these cellular units are non-contiguous in a multicellular animal, the model requires that oscillating levels of physical or chemical mediators be postulated (a,b,c,...,etc.), with the period of D.O. but not necessarily the same phase. These mediating systems, which would presumably be nervous (neurotransmitter release) or endocrine (hormonal concentration), would transmit the forced oscillating response to D.O. to the various passively responding cellular units. The entire circadian system would be entrained by environmental time cues via exteroceptive sensory inputs to the driving oscillator.

Model II describes a network of cellular units which are each themselves self-sustained oscillators, able to maintain oscillations with an independent period in the absence of periodic inputs. One oscillator (D.O.) acts as a pacemaker and is entrained by exteroceptive sensory inputs

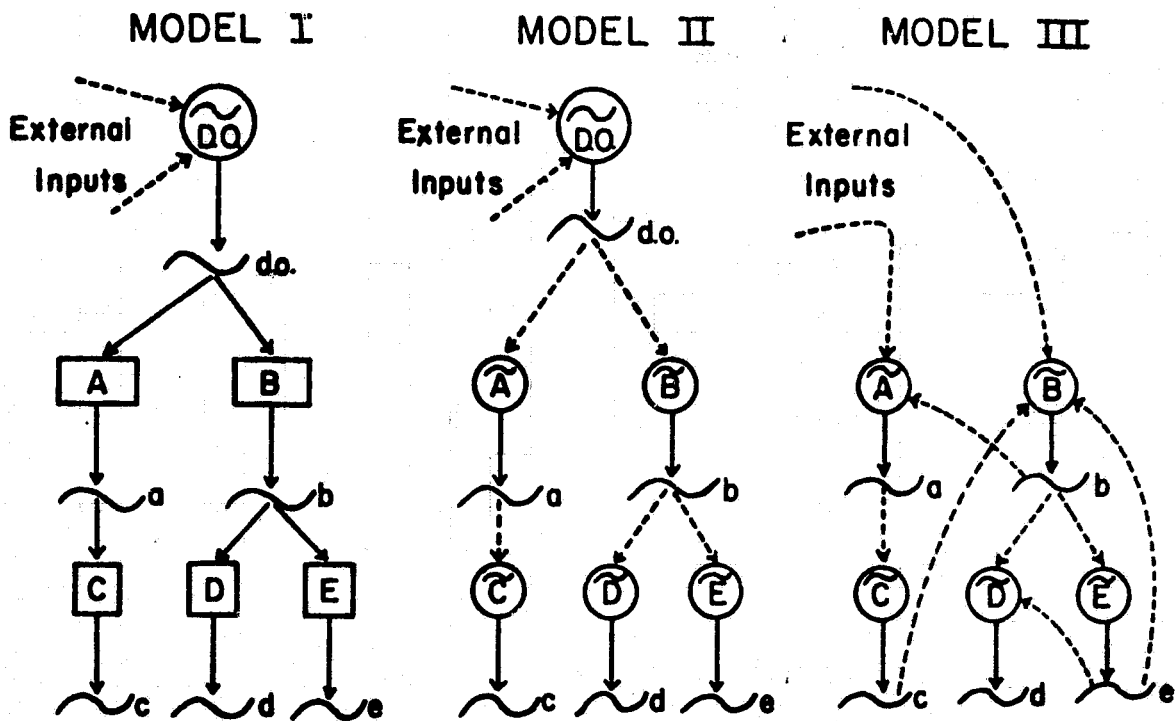


Figure 9. The symbol \odot represents an active cellular unit capable of maintaining a self-sustained oscillation with its own independent period; \square represents a cellular unit that responds passively to an oscillating driving force; \sim indicates the oscillating concentration of a chemical mediator; $--\rightarrow$ indicates the entrainment of a self-sustained oscillator by a phase-response mechanism; and \rightarrow is the direction of flow of passive responses to an oscillating driving force. Model I is therefore a single oscillator system whereas the other models are multioscillator systems arranged in a hierarchical (Model II) or non-hierarchical (Model III) manner.

from environmental time cues. As in Model I it is necessary to postulate oscillating nervous or endocrine mediators which maintain synchronization within the animal. However, the mediators in this model actively entrain the self-sustained cellular oscillators in a manner similar to the entrainment of the organism's circadian system by cycles of environmental illumination (3).

Model III also describes a multioscillator model but in this case no one oscillator consistently acts as a pacemaker. Instead the various exteroceptive sensory inputs entrain different oscillators. Internal synchronization within the system is maintained by the positive and negative feedback action of mediators (a,b,c,..., etc.) on the separate oscillating units (A,B,C,...,etc.). As in Model II, the mediators synchronize the oscillators by active entrainment.

The evidence we have gained from studies of our squirrel monkey preparation indicate that the circadian timing system is a multioscillator system (Model II or III) and not a single oscillator system (Model I). It therefore seems that there are multiple oscillators in the various tissues of the body which are synchronized by hormonal and nervous mediators. We however as yet have little evidence to clearly indicate whether the circadian timing system is hierarchical (Model II) or non-hierarchical (Model III).

There are several further pieces of evidence from other investigators which support our conclusion that the circadian timing system in advanced multicellular animals, such as mammals, is organized as a multioscillator system (such as Model II or III) rather than having only a single independent oscillator (Model I). Firstly, Aschoff and his colleagues have demonstrated that although internal synchronization was normally observed between a wide variety of circadian rhythmic functions in men studied under isolation conditions, 15% of their subjects demonstrated

internal desynchronization, with various monitored rhythmic variables oscillating with independent free-running periods within the same subject. This observation is incompatible with Model I, but is readily predicted from Model II or III, since this could occur whenever there was a loss of the circadian rhythm of a synchronizing mediator. Secondly, several investigators have been able to demonstrate free-running rhythms in isolated tissues maintained in vitro under constant conditions; presumably they have therefore isolated tissue containing self-sustained oscillators as would be predicted from Model II. Thirdly, it has been repeatedly observed in advanced multicellular animals that after the abrupt phase-shift of environmental time cues the various monitored circadian rhythms take different lengths of time to resynchronize with the new phase of environmental cues, so that temporary internal desynchronization occurs. Fourthly, rhythms in many different species have been observed to split in a manner suggestive of a multioscillator system under certain environmental lighting conditions and fifthly, the extensive data that has been accumulated on rephasing of the *Drosophila* eclosion rhythm by pulses of light can only readily be explained by postulating that there is more than one independent circadian oscillator in this organism.

Thus our work has shown that the circadian timing system in advanced multicellular animals, such as primates, appears to be organized as a set of multiple, potentially-independent oscillators which are normally synchronized with one another through chemical mediators. The further localization and characterization of these oscillators within the animal will be necessary for the understanding of the function of this timing system, and will form an essential base for examining the physiological roles which the timing system performs.

D) Applications in Man: Internal Desynchronization

With the information we gained from the squirrel monkey system we then moved back to study some of these systems in man. We were particularly

interested in applications that were relevant to spaceflight as well as those which had some clinical applicability.

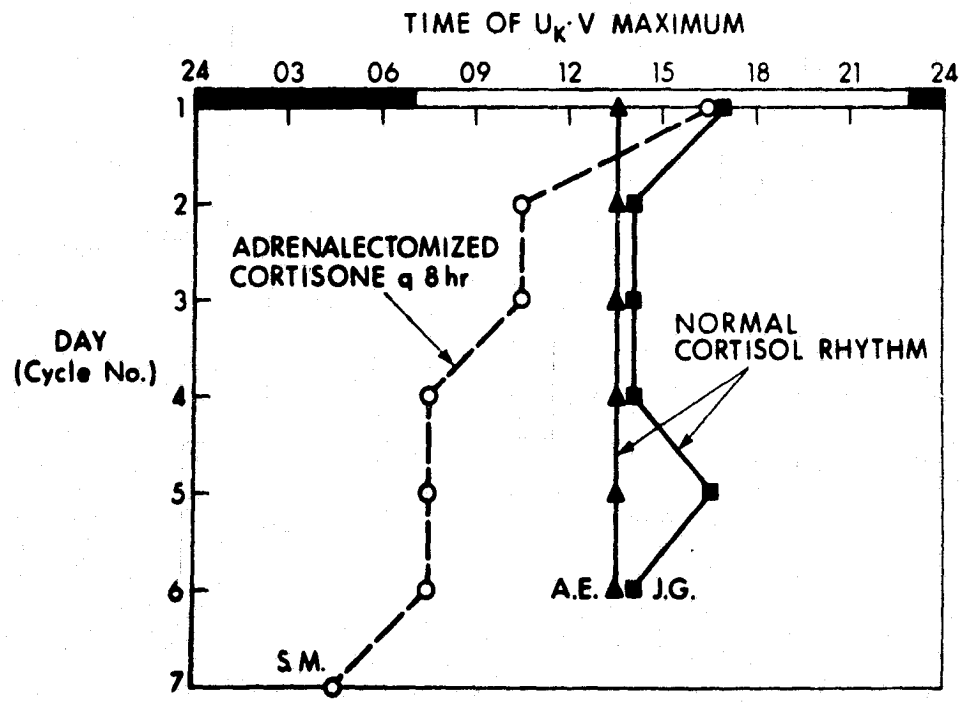
Some initial studies were conducted in adrenalectomized patients in whom we administered their replacement adrenal steroids evenly throughout the 24 hours in order to examine whether free-running rhythms of urinary potassium excretion were observed in adrenalectomized man as they were in adrenalectomized squirrel monkeys. In the two patients we have studied to date, our preliminary findings have shown that there are free-running rhythms in urinary potassium excretion in these subjects when they are administered an adrenal steroid replacement regime which provides no circadian rhythm in plasma cortisol concentration. (Fig. 10.) Thus, this appears to confirm that the observations that we have made in the squirrel monkey are relevant to man.

As has been discussed in section (A) one of the important pathophysiological events which would cause major changes in potassium balance and in plasma potassium concentration would be internal desynchronization of the fluxes of potassium which we have observed. Internal desynchronization of circadian rhythms has been observed in a number of situations which are correlated with emotional stress. It has been particularly observed in monkeys who have been subjected to stressful circumstances either in earth-based studies (Stroebel, 1969) or in space experiments such as the Biosatellite III experiment. In this orbiting monkey, internal desynchronization of the various monitored rhythmic functions was observed and it is possible that this was one feature of the pathophysiological developments which led to his early demise.

Our demonstration that the circadian timing system was a multioscillator system with various oscillators in peripheral tissues being synchronized with one another through circadian rhythms of hormonal and nervous mediators suggested to us that this might provide an explanation for the internal desynchronization that is seen in situations of emotional stress. Since

Fig. 10

URINARY POTASSIUM CIRCADIAN RHYTHM WITH AND WITHOUT NORMAL PLASMA CORTISOL RHYTHM



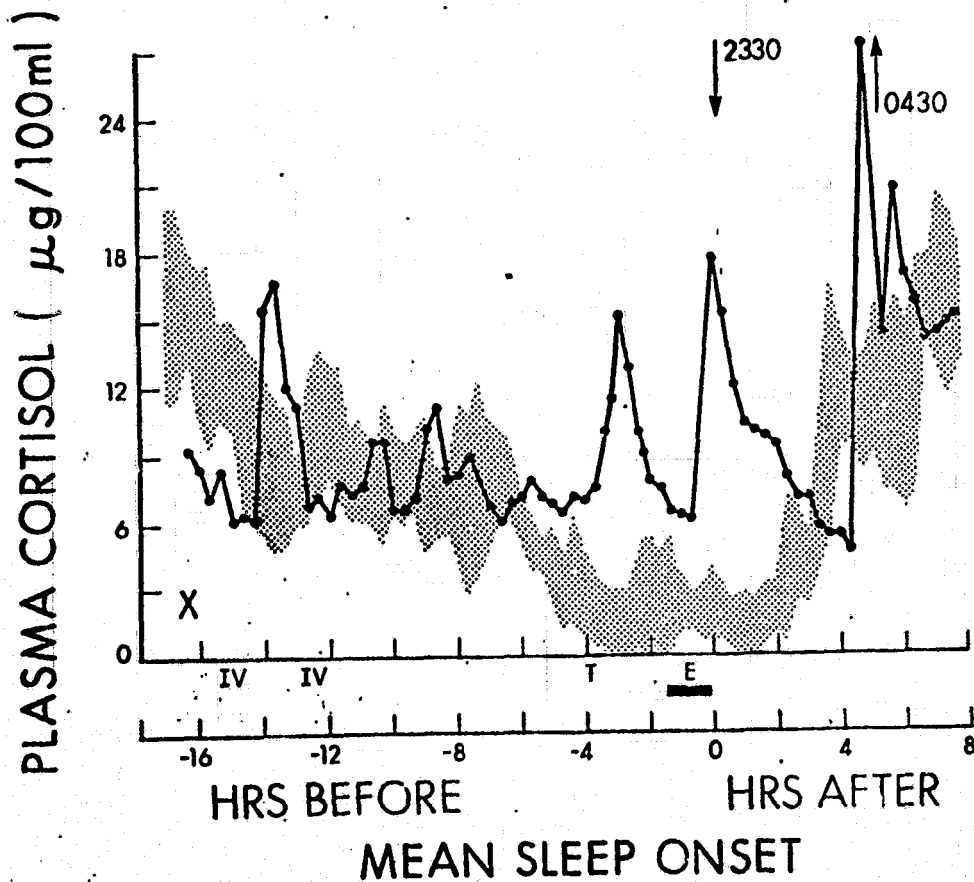
"stressful" events are known to cause elevations of plasma cortisol concentration, it was postulated that intermittent stresses inducing intermittent bursts of adrenal cortisol secretion might obliterate the circadian rhythm of plasma cortisol concentration thus inducing those oscillating functions which are dependent on the circadian rhythm of plasma cortisol concentration for their synchronization, to become desynchronized. We have proposed this explanation for circadian internal desynchronization in a paper that will be presented at the International Society for Chronobiology Meeting in Washington, D.C. this month. The paper is entitled "Circadian Internal Desynchronization: Causation by Circadian Arrhythmias in Hormonal Mediators?".

We have tested the proposition that there is a loss in the circadian rhythm of plasma cortisol concentration in human subjects subjected to emotional stress by examining the plasma cortisol pattern in patients during the 24 hours immediately prior to major cardiac surgery and have compared this pattern with that from normal volunteer subjects with no imminent anxiety-causing event, but otherwise studied under similar conditions. We found that in the pre-surgical patients there was a loss of the circadian rhythm of plasma cortisol concentration as we had predicted and thus, this would provide an explanation for the internal desynchronization that is seen in situations of emotional stress. (Fig. 11.)

Because of the great importance of the pathophysiological consequences of internal desynchronization, particularly as they relate to desynchronization of potassium fluxes in man, we consider it important to pursue these investigations further. It is a problem particularly relevant to space-flight because of the already increased risk of internal desynchronization due to confused time cues in the isolated environment of space.

The results of the study of pre-operative patients were presented at the American Psychosomatic Society Meeting, New Orleans on March 22, 1975 in a paper entitled "The Effect of Psychological Stress in the Pre-operative Period on the Episodic 24-Hour Plasma Cortisol Secretion

Figure 11. The nycthemeral pattern of plasma cortisol concentration in a patient during the 24 hours prior to major elective cardiac surgery. Time is plotted as hours before and after the time of mean sleep onset for the preceding week. Events during the pre-operative day are shown: T=pre-operative teaching, IV=intravenous puncture, E=enema and the black bar is the time of pre-operative shaving. Also shown (shaded pattern) is the mean \pm SD of the plasma cortisol pattern in five hospitalized control subjects with no expectation of surgery (Reproduced from CZEISLER *et al.*).



Pattern". This paper has also been submitted to the Journal of Clinical Endocrinology and Metabolism and has been accepted subject to some minor revisions. The Journal of Clinical Endocrinology and Metabolism paper is entitled "Episodic 24-Hour Cortisol Secretory Pattern in Patients Awaiting Elective Cardiac Surgery".

In summary, therefore, during this 12 month contract we have (1) documented in man major circadian fluxes of potassium ions between body compartments, (2) have demonstrated the potential for the causation of transient hypokalemias if these potassium fluxes became desynchronized, (3) have demonstrated in a squirrel monkey preparation the role of the circadian rhythm of plasma cortisol concentration in synchronizing circadian rhythms of potassium flux, (4) have developed models to describe the functioning of this circadian system, (5) have demonstrated a potential cause of the desynchronization of potassium fluxes involving the elimination of the plasma cortisol rhythm, (6) have demonstrated the applicability of these findings in adrenalectomized man, (7) and have studied human patients under the influence of environmental stresses to demonstrate that the circadian rhythm of plasma potassium concentration is indeed lost under conditions associated with internal desynchronization.

IV. List of Publications

The work performed under this contract has been published or will be published in the following papers. Reprints are enclosed.

1. Moore Ede, M.C., Brennan, M.F., and Ball, M.R.: Circadian variation of intercompartmental potassium fluxes in man. J Appl Physiol 38: 163-170, 1975.
2. Moore Ede, M.C., and Herd, J.A.: Renal electrolyte circadian rhythms: independence from feeding and activity patterns. Submitted to Am J Physiol, 1975.
3. Czeisler, C.A., Moore Ede, M.C., Regestein, Q.R., Kisch, E.S., Fang, V.S., and Ehrlich, E.N.: Episodic 24-hour cortisol secretory pattern in patients awaiting elective cardiac surgery. Conditional acceptance, J Clin Endocrinol Metab, 1975.
4. Moore Ede, M.C., Schmelzer, W.S., Kass, D.A., and Herd, J.A.: Internal organization of the circadian timing system in multicellular animals. Submitted to Fed Proc, 1975
5. Moore Ede, M.C., Schmelzer, W.S., Kass, D.A., and Herd, J.A.: Circadian timing system: organization of multiple oscillators synchronized by chemical mediators. Submitted to Science, 1975.
6. Czeisler, C.A., Moore Ede, M.C., Regestein, Q.R., Kisch, E.S., Fang, V.S., and Ehrlich, E.N.: Effect of psychological stress in the presurgical period on the episodic 24-hour plasma cortisol secretory pattern. Presented at the Annual Meeting of the American Psychosomatic Society, New Orleans, Louisiana, March 22, 1975.
7. Moore Ede, M.C.: Internal synchronization of spontaneous circadian oscillators: The identification of the hormonal mediator synchronizing a renal oscillator. Presented at the Symposium on "Physiological and Biochemical Aspects of Circadian Rhythms: at the 59th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April 15, 1975.
8. Moore Ede, M.C., Czeisler, C.A., Schmelzer, W.S., and Kass, D.A.: Circadian internal desynchronization: causation by circadian arrhythmias in hormonal mediators? Presented at the Annual Meeting of the International Society for Chronobiology, Washington, D.C., August, 1975. (Abstract)
9. Moore Ede, M.C., Schmelzer, W.S., and Herd, J.A.: Plasma cortisol oscillations synchronize the circadian rhythm of renal potassium excretion in the squirrel monkey. Presented at the International Congress on "Rhythmic Functions in Biological Systems", Vienna, Austria, September 1975. (Abstract)

V. EQUIPMENT PURCHASED BY NASA GRANT 9610

Pellet Dispensers (5), Ralph Gerbrands Co.	\$ 642.00
Model 20 Incubator (Modified) (3), Forma Scientific Co.	2079.60
Harvard Cumulative Recorder (1), Ralph Gerbrands Co.	770.00
6-Pen Event Recorder (3), Ralph Gerbrands Co.	500.00

\$4211.60

VI. MONTHLY REPORTS

1st monthly report	August 1, 1974
2nd monthly report	September 1, 1974
3rd monthly report	October 1, 1974
4th monthly report	November 1, 1974
5th monthly report	Presented verbally at meeting with Dr. J.A. Rummel
6th monthly report	January 1, 1975
7th monthly report	February 1, 1975
8th monthly report	Presented verbally at meeting with Dr. J.A. Rummel
9th monthly report	April 1, 1975
10th monthly report	May 1, 1975
11th monthly report	Presented verbally at meeting with Dr. J.A. Rummel
12th monthly report	Included in Final Report

William E. Hassan, Jr., Ph.D.
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Leroy D. Vandam, M.D.
Anesthesiologist-in-Chief

PETER BENT BRIGHAM HOSPITAL

721 Huntington Avenue, Boston, Massachusetts 02115 (617) 734-8000

August 1, 1974

John A. Rummel, PhD
Chief, Environmental Physiology Branch
National Aeronautics and Space Administration
Lyndon B. Johnson Space Center
Houston, Texas 77058

Dear John,

This letter will serve as the first monthly report of the progress of our NASA contract NAS9-14249 which started on July 1, 1974.

We have now completed the studies of normal volunteer male subjects as were outlined in Sections 4.1-4.3 in the statement of work. A paper has been prepared for publication and has been submitted to the Journal of Applied Physiology. The paper is titled "Circadian Variation of Intercompartmental Potassium Fluxes in Man". A copy of the submitted manuscript is enclosed.

The studies on the squirrel monkey preparation are also proceeding well. Preliminary evidence suggests that the circadian rhythm in plasma cortisol concentration may act as the hormonal mediator in the synchronization of the rhythm of urinary potassium excretion in the squirrel monkey to the light-dark cycle. I will give you a fuller report on the squirrel monkey work in my next monthly report.

If there is any further information you require at any time please do not hesitate to let me know.

With best wishes,

Sincerely



Martin C. Moore Ede, M.B., B.S.

MCME/sr
Enclosures



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PETER BENT BRIGHAM HOSPITAL

721 Huntington Avenue, Boston, Massachusetts 02115 (617) 734-8000

September 1, 1974

John A. Rummel, Ph.D.
Chief, Environmental Physiology Branch
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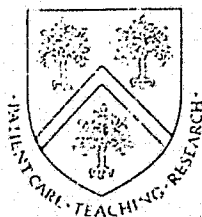
Dear John

This letter will serve as the second monthly report of the progress of our NASA contract, NAS9-14249.

Studies are progressing well with the squirrel monkey model. Using squirrel monkeys with chronically indwelling arterial and venous catheters which extend outside the isolation chamber, in which the monkey and metabolism chair are placed, we have demonstrated that it is possible to reproduce the natural circadian patterns of cortisol and aldosterone secretion in adrenalectomized monkeys by the infusion of these hormones through the venous line. The monkeys are quite unaware of the timing of infusions since these are all conducted from outside the isolation chamber. With this preparation it can be demonstrated that the circadian oscillation in urinary potassium excretion in these animals is synchronized by the circadian oscillation in plasma cortisol concentration. When the timing of the artificial circadian rhythm in plasma cortisol concentration is phase-shifted by 8 hours this induces a similar phase-shift in the timing of the urinary potassium excretory rhythm. The circadian oscillation in plasma aldosterone concentration, however, does not appear to play an essential role in this synchronization process.

It is noteworthy that circadian variation in other variables such as feeding and activity are not influenced by the phase-shift in the artificial plasma cortisol concentration rhythm. Thus, we have induced an artificial desynchronization between the urinary potassium rhythm and the rhythm of food intake.

However, the urinary potassium circadian rhythm does not appear to be passively dependent upon the circadian oscillation in plasma cortisol concentration. After the eight hour plasma cortisol phase-shift in adrenalectomized monkeys, the circadian oscillation in urinary potassium excretion does not immediately phase-shift and in fact takes some three or four days to resynchronize with the new phase of the plasma cortisol rhythm.



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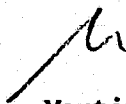
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Similarly, when all circadian oscillations in plasma cortisol and aldosterone are eliminated by continuously infusing these substances at a constant level which is equal to the mean 24 hour secretory rate the urinary potassium rhythm continues to oscillate but now has a free-running period which is quite independent from the period of the light-dark cycle to which the animal is subjected. Thus, in this situation, artificial desynchronization between the urinary potassium rhythm and the light-dark cycle and the rhythms which are dependent upon the light-dark cycle, has also been induced.

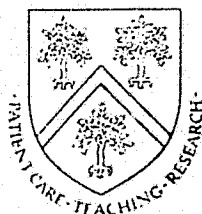
These findings have lead us to develop a new model of internal synchronization of circadian oscillations. It resolves some of the experimental findings which are incompatible with the classical model of internal synchronization such as that proposed by Mills (Mills, J.N.: Transmission processes between the clock and manifestations. In: Biological Aspects of Circadian Rhythms, edited by Mills, J.N., New York: Plenum Press, 1973, p. 27-84.) *Out* This model of internal synchronization enables us to predict situations in which internal synchronization will occur. I will give you a more detailed explanation of this model and the experimental findings which support it in the next monthly report.

Do let me know when it is time to gather material together for your next funding review. This squirrel monkey model is proving most valuable for studies of internal synchronization mechanisms in a way not possible with previous preparations. If there is any further information you require at any time, please do not hesitate to let me know. With best wishes.

Sincerely yours


Martin C. Moore Ede, M.B., B.S.

MCME/sr



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PETER BENT BRIGHAM HOSPITAL

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October 1, 1974

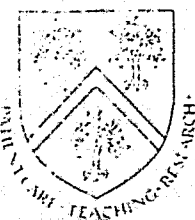
John A. Rummel, Ph.D.
Chief, Environmental Physiology Branch
National Aeronautics and Space Administration
Lyndon B. Johnson Space Center
Houston, Texas 77058

Dear John

This letter will serve as the third monthly report of the progress of our NASA contract, NAS9-14249.

The squirrel monkey studies on the control of circadian urinary electrolyte oscillations by the circadian rhythms in plasma cortisol and aldosterone concentration progress well. Interestingly, all the adrenal steroids can be produced with cortisol administration alone at physiological doses, and it does not seem to make any difference whether aldosterone is infused as well. It, thus, appears that cortisol plays the more important role in the synchronization of these oscillations. This is not entirely surprising since Vagnucci and colleagues (J Appl Physiol 26: 720-731, 1969) have shown that urinary aldosterone and potassium excretion are poorly correlated while urinary potassium and 17-OHCS excretion are relatively well correlated. This is, of course, in conflict with the classical view that aldosterone is by far the most important adrenal steroid in the control of renal potassium excretion. It may be that while aldosterone plays a role in the modulation of potassium excretion in response to homeostatic disturbances the underlying circadian oscillation is synchronized by the rhythm of plasma cortisol concentration.

This leads me to comment on your interesting paper on "Temporal Relationships of Urinary Constituents Before and After Long Duration Space Flight" which you gave to me when I was visiting Houston. It is possible that your failure to demonstrate desynchronization between the urinary variables which you studied before and after the Skylab missions was due to the selection of variables which are not normally desynchronized from one another even when other circadian rhythms are desynchronized within an individual. Our studies in the squirrel monkey are showing that phaseshifts in the timing of hydrocortisone administration in adrenalectomized animals results in an equivalent phaseshift in the rhythms of sodium, potassium and water excretion. All the variables, while remaining synchronized with one another, became desynchronized from such variables as feeding and activity patterns. This suggests that different control pathways may be involved in the synchronization process and that you may have studied in the Skylab mission oscillating variables which do not



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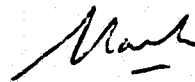
normally become desynchronized from one another.

In order to detect desynchronization in space flight, I believe it essential that multiple variables should be monitored which have circadian oscillations which can be readily shown to desynchronize in the laboratory. An animal preparation such as our squirrel monkey would make such an investigation feasible. As I mentioned in last month's report, we are now getting a better understanding of internal synchronization mechanisms and can induce desynchronization between oscillating physiological variables in a reproducible manner.

We currently have journal articles in preparation on this subject and will inform you as soon as they are submitted. We look forward to seeing you in Boston soon. Do let me know if there is any further information which would be helpful at this stage.

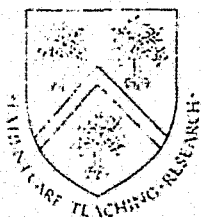
With best wishes.

Sincerely yours



Martin C. Moore Ede, M.B., B.S., Ph.D.

MCME/sr



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PETER BENT BRIGHAM HOSPITAL

721 Huntington Avenue, Boston, Massachusetts 02115 (617) 734-8000

November 1, 1974

John A. Rummel, Ph.D.
Chief, Environmental Physiology Branch
National Aeronautics and Space Administration
Lyndon B. Johnson Space Center
Houston, Texas 77058

Dear John

This letter will serve as the fourth monthly report of the progress of our NASA contract, NAS9-14249.

The studies of the effect of cortisol phaseshifts and the continuous infusion of cortisol in adrenalectomized monkeys procede according to plan. We aim soon to have four monkeys who have been exposed to an eight hour phaseshift in cortisol administration, four monkeys who have been exposed to a phase-shift of both aldosterone and cortisol and four monkeys in whom the daily dose of cortisol and aldosterone has been given as a constant infusion spread evenly over each 24 hour period. The data from these studies will probably be presented some time in the spring, probably at the FASEB meeting,

I think that you will also be interested to know that Woodland Hastings and I are planning a symposium to be held at the Federation meetings in Atlantic City in April of next year. This will be concerned with the recent studies of the control and synchronization of circadian oscillators. I think it will bring together some interesting work and give a good review of where we are in this field right now.

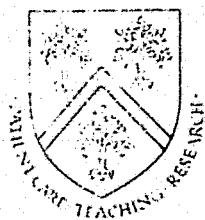
I will be grateful for your guidance as to when is the time to put together an application for continued support beyond June 30, 1975. We can of course let you have any further information or material whenever this is appropriate. I am sure we should talk more about the potentialities of the squirrel monkey preparation for space flight, but perhaps this is best done when you come up to visit us.

With best wishes.

Sincerely yours

Martin C. Moore Ede, M.B., B.S., Ph.D.

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721 Huntington Avenue, Boston, Massachusetts 02115 (617) 734-8000

January 1, 1975

John A. Rummel, Ph.D.
Chief, Environmental Physiology Branch
National Aeronautics and Space Administration
Lyndon B. Johnson Space Center
Houston, Texas 77058

Dear John,

This letter will serve as the sixth monthly report of the progress of our NASA contract NAS9-14249.

Our studies of the synchronization of the urinary potassium rhythm in squirrel monkeys by the circadian rhythm of plasma cortisol concentration continue as scheduled. We have now extended the studies to adrenalectomized patients using women who have been adrenalectomized for breast cancer. We find in the first pilot experiment that the urinary potassium rhythm persists when cortisone replacement therapy is evenly spread throughout the 24 hours but the urinary potassium rhythm appears to have a free-running period of approximately 22 hours so that the peak of urinary potassium excretion sequentially moves earlier each day. By the end of the seven day study the peak of urinary potassium excretion was 3AM instead of approximately 3PM.

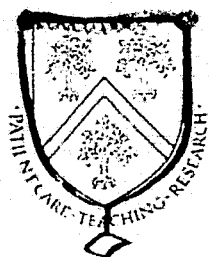
We are most encouraged by these results which suggest that the conclusions that we are reaching with our squirrel monkey preparation are applicable to man, that the squirrel monkey is an excellent model for studying the synchronization and desynchronization of adrenal controlled circadian rhythms in man. If we can reproduce these findings in subsequent studies we will have to investigate whether the circadian rhythms in inter-compartmental potassium flux desynchronize in such a situation as was predicted in our Journal of Applied Physiology paper. This paper, incidentally, is due to be published in this month's Journal of Applied Physiology.

Other activities this month have centered around the teaching of a course on the cardiovascular and body fluid adaptations to weightlessness in which our medical students here have shown great interest. We are trying out as a class exercise the design of experiments for space shuttle missions. I will let you know if anything of interest develops here.

With best wishes.

Sincerely yours

Martin C. Moore, Ed. M.B., B.S., Ph.D.



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PETER BENT BRIGHAM HOSPITAL

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February 1, 1975

John A. Rummel, Ph.D.
Chief, Environmental Research Division
National Aeronautics and Space Administration
Lyndon B. Johnson Space Center
Houston, Texas 77058

Dear John

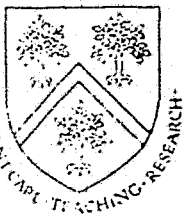
This letter will serve as the seventh monthly report of the progress of our NASA contract NAS9-14249.

As you know, our studies in the chair-acclimatized squirrel monkey preparation have shown that when the normal circadian rhythm of plasma cortisol concentration is eliminated by the continuous infusion of cortisol in adrenalectomized monkeys the rhythm of urinary potassium excretion demonstrates an apparently free-running period. We have now started to examine this phenomenon more closely since we believe it represents some sort of self-sustaining oscillator in the kidney. We have brought in as a consultant Professor Richard E. Kronauer, who is professor of Engineering and Applied Physics at Harvard and has considerable experience in the analysis of oscillating systems. We have one of his students working with us for a thesis project and we are examining the influences of different cortisol input patterns in order to determine the characteristics of the putative renal oscillator. I think we are really now beginning to get a grip on the mechanisms of internal synchronization and the potential causes of desynchronization. As you know, desynchronization was one of the major problems of the biosatellite III monkey. It is perhaps significant that when we desynchronize our monkeys' activity and urinary potassium rhythms from each other that we get a significant number of fatalities while the monkeys are in this desynchronized condition. This observation is based on too few animals to permit a statistical analysis but it is in line with the observations of mortality induced in other species by repeated light-dark phaseshifts--which presumably also cause temporary internal desynchronization. For these reasons I am sure we should plan to have an experiment which can investigate changes in the circadian system during space flight in the Space Shuttle program. We must get together and talk about the studies that should be done.

Let me know when you think we need to get together again; you are welcomed to take up the invitation to visit Boston any time, of course. With best wishes.

Sincerely yours


Martin C. Moore Ede, M.B., B.S., Ph.D.



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PETER BENT BRIGHAM HOSPITAL

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April 1, 1975

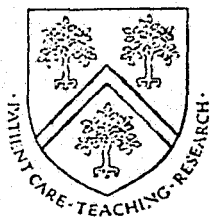
John A. Rummel, Ph.D.
Chief, Environmental Research Branch
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Houston, Texas 77058

Dear John

This letter will serve as the ninth monthly report on the progress of our NASA contract NAS9-14249.

This month, I would like to bring you up to date with the publications that have come out of our NASA contracted research during this past 9 months.

1. The paper describing the analysis of intercompartmental potassium fluxes in normal volunteers was published in the Journal of Applied Physiology in January, 1975. The title was Circadian Variation of Intercompartmental Potassium Fluxes in Man by Moore Ede, Brennan, and Ball, J Appl Physiol 33: 163-170, 1975. Reprint copies are enclosed.
2. A paper in which disruptions of the circadian pattern of plasma cortisol concentration under conditions of stress were studied in pre-operative patients was presented at the American Psychosomatic Society in New Orleans on Saturday, March 22, 1975. This paper was entitled "The Effect of Psychological Stress in the Preoperative Period on the Episodic 24-Hour Plasma Cortisol Secretion Pattern". A copy of this paper is enclosed.
3. A lecture will be given by myself at the Symposium on Physiological and Biochemical Aspects of Circadian Rhythms at the FASEB meeting in Atlantic City on April 15th. This will be entitled "Internal Synchronization of Spontaneous Circadian Oscillators: the Identification of the Hormonal Mediators Synchronizing the Renal Oscillator". Besides being presented at the symposium, this paper will be published in Federation Proceedings within the year. As soon as a final copy is available, I will send this on to you.
4. A paper entitled "Circadian Internal Desynchronization: Causation by Circadian Arrhythmias in Hormonal Mediators?" will be presented by me at the International Society of Chronobiology meeting in Washington, D.C. in August. The abstract of this paper is enclosed and this abstract will be published in Chronobiologica within the next few months.
5. A paper entitled "Plasma Cortisol Oscillations Synchronize the Circadian Rhythm of Renal Potassium Excretion in the Squirrel Monkey" will be presented at the International Congress on Rhythmic Functions in Biological Systems in September this year. An abstract of this paper is also enclosed with this letter.



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6. A paper entitled "Renal Electrolyte Circadian Rhythms in Squirrel Monkey: Independence from Feeding and Activity Patterns" will shortly be submitted to the American Journal of Physiology.

7. A paper entitled "Synchronization of Renal Electrolyte Circadian Rhythms By the Light-Dark Cycle" is also in preparation and will shortly be submitted to the American Journal of Physiology.

8. A paper entitled "Cortisol Mediated Synchronization of Urinary Potassium Circadian Rhythm in the Squirrel Monkey" is also in preparation and will probably be submitted to the American Journal of Physiology.

9. A paper entitled "Episodic 24-Hour Cortisol Secretory Pattern in Patients awaiting Elective Cardiac Surgery" by Czeisler, Moore Ede, Regestein, Kisch, Fang and Ehrlich has been prepared and is being submitted to the Journal of Clinical Endocrinology and Metabolism.

In our current research, we are now moving very rapidly in the mathematical analysis of the characteristics of the renal oscillator. We are developing some very potent tests of the coupling function but are still needing to develop our methods of Fourier analysis of the oscillations in the data.

We would therefore much welcome your help with the program you have designed and would be grateful if it were possible to have this so that we can use it as a guide, of course, with full acknowledgement to you. * Arrived today, many thanks.

I look forward to hearing from you soon and for your advise on submission of our next annual contract.

With best wishes.

Sincerely yours



Martin C. Moore Ede, M.B., B.S., Ph.D.
Assistant Professor of Physiology

MCME/ser

P.S. I enclose for your interest my reply to Chuck Winget when he asked for my answers to his set of questions as a follow-up to his recent NASA conference.

HARVARD MEDICAL SCHOOL
DEPARTMENT OF PHYSIOLOGY



25 Shattuck Street
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May 1, 1975

John A. Rummel, Ph.D.
Chief, Environmental Research Branch
National Aeronautics and Space Administration
Lyndon B. Johnson Space Center
Houston, TX 77058

Dear John

This letter will serve as the tenth monthly report of the progress of our NASA contract NAS9-14249.

This month we have been continuing our studies of the synchronization process by which the putative renal oscillator is synchronized with other circadian rhythms within the animal. We have been developing the computer programs necessary to analyze the data in more detail and have been greatly aided by your program which you kindly sent to us. We are currently having to revise some of our computer systems to make it possible to undertake some of this processing. In addition, we have been designing a revised edition of our isolation chamber which enables greater isolation of the animal from experiment-induced disturbances.

I would welcome your guidance on when we should be proceeding with my research contract renewal which will be due at the end of June. We will have to allow some extra time in this process above that we allowed last year because I will be transferring the contract from the Peter Bent Brigham Hospital to the Harvard Medical School and this involves jumping some administrative hurdles which all have inbuilt delays of up to several weeks.

I have also recently received ^{a copy of the} invitation to participate in planning the NASA Life Sciences Program in Space which was just sent out by NASA HQ in Washington. As you know, this is asking for suggestions for experiments ^{for Shuttle} and encourages investigators to put their names forward as part of the review process. I would much appreciate you advise on how I should be responding to this and one whether the internal organizational processes in NASA have sorted out how the whole Shuttle Program is going to be organized.

I look forward to hearing from you soon and to getting some clear direction on these two matters.

With very best wishes.

Sincerely yours,

Martin C. Moore Ede, M.B., B.S., Ph.D.
Assistant Professor of Physiology

Circadian variation of intercompartmental potassium fluxes in man

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MOORE EDE, MARTIN C., MURRAY F. BRENNAN, AND MARGARET R. BALL. *Circadian variation of intercompartmental potassium fluxes in man.* J. Appl. Physiol. 38(1): 163-170. 1975.—Circadian rhythms of plasma potassium concentration and urinary potassium excretion persisted in three normal volunteers when diurnal variations in activity, posture, and dietary intake were eliminated for 3-10 days. Measurements of the arteriovenous difference in plasma potassium concentration across the resting forearm and of erythrocyte potassium concentration suggested that there is a net flux of potassium from ICF to ECF in the early morning and a reverse net flux later in the day. The total net ICF-ECF fluxes were estimated from the diurnal variations in extracellular potassium content corrected for dietary intake and urinary potassium loss. The net fluxes between ICF and ECF were found to be counterbalanced by the circadian rhythm in urinary potassium excretion. Desynchronization of these rhythms would result in marked fluctuations in extracellular potassium content. These findings suggest that some revision is required of the concept of basal state in potassium homeostasis.

circadian rhythms of plasma and urinary potassium; forearm arteriovenous plasma potassium difference; erythrocyte potassium concentration; extracellular fluid, ⁸²Br volume; intercompartmental potassium fluxes; potassium homeostasis

A FUNDAMENTAL CONCEPT underlying the principle of homeostasis is the intrinsic steady state, or basal level to which physiological systems return when there are no externally induced perturbations. Thus in the analysis of the intercompartmental distribution of potassium in man (10, 12, 20, 21) it has been assumed that the system is basically static with zero net fluxes between compartments, except when definable events cause temporary loss of this steady state. The assumption of a basically constant distribution during the course of any 24-h period has now been challenged by studies of the circadian variation in urinary potassium excretion. Typically the rate of urinary potassium excretion has a fivefold variation in each 24-h day, with minimum rates in the early morning and maximum excretion around noon (9, 17). Such a circadian variation might be thought to be a result of day-night differences in dietary intake, activity, posture, and sleep. However, the circadian rhythm of urinary potassium excretion persists when these variables are kept constant throughout day and night or are manipulated in a noncircadian pattern (18, 19, 22, 30).

These studies suggest that there can be no single steady

state of compartmental potassium distribution even when environmental and behavioral variations are eliminated. We have examined this proposition and have analyzed the circadian variation in intercompartmental potassium movements in normal man when dietary intake, activity, and posture are held constant throughout each 24-h period.

METHODS

Three healthy, normal volunteers were studied from 3 to 10 days in the Bartlett Intensive Care Unit of the Peter Bent Brigham Hospital. Normality was established by clinical history, physical examination, and biochemical screening. Signed informed consent was obtained.

Except where otherwise indicated, posture, activity, and dietary intake were maintained at constant levels throughout day and night. This was achieved by keeping the subject on strict supine bed rest and by providing a liquid diet divided into eight equal aliquots which were taken at 3-h intervals throughout each 24-h period. The diet provided 100 meq potassium, 150 meq sodium, 500 mg calcium, 70 g protein, 260 g carbohydrate, 80 g fat, 2,000 calories, and 3,000 ml fluid per 24 h. In one subject (HH) the diet was altered to give 50 meq potassium and 200 meq sodium per 24 h but was otherwise kept the same. The room lighting was on between 0800 and 2300 h and off from 2300 to 0800 h (LD 15:9). Low illumination was used for brief periods during sample collection at night. The first subject (HH) was allowed to equilibrate on this regimen for 12 h. The other subjects, however, were given 4 days to equilibrate so as to minimize the transients induced by postural changes and dietary adjustment. During the equilibration period 3-h urine collections were made. Each collection contained all urine voided during the 3-h period, including a voluntary bladder emptying at the end of the period.

At the end of the equilibration period catheters were inserted under local anesthesia into the radial artery and brachial vein of the nondominant arm. These were kept open by a 0.05 ml/min infusion of normal (0.9%) saline through a Sorenson Intraflo system (Sorenson Research Corp., Salt Lake City, Utah). A 48-h period of study was then commenced with the subject continuing on his previous regimen of constant diet, activity, and posture. Every 3 h blood samples were taken from both arterial and venous catheters after the forearm had remained at rest for at least 5 min. Urine collections were obtained as before. The 3-h

food aliquot was given after the sample taking had been completed.

In one subject (*AE*) the study was repeated under conditions of daytime activity and nocturnal bed rest. He consumed the same 3-h liquid diet as before. Every 3 h from the time of rising from bed (0800 h) until he retired (2300) he went through a moderate exercise routine. This involved sitting in a chair for the 1st h, cycling at a rate of 50–60 rev/min with a 2-kg load for 20 min on an exercise bicycle, resting on the bicycle for 20 min, followed by a further 20 min of cycling. The final hour was spent sitting quietly in a chair before the 3-h specimens were collected. This regimen was followed for an equilibration period of 24 h and then a 48-h study period. During this study period the same measurements were made as in the bed-rest studies.

Potassium estimations. Blood was drawn slowly from the catheter without tourniquet or fist pumping and carefully mixed with lithium heparin (1,000 U/100 ml blood) in potassium-free glassware. Plasma was obtained by spinning immediately for 20 min at 2,700 rpm at 13-cm radius, and respinning the supernatant plasma for 10 min.

The plasma was stored in a 4°C refrigerator until analysis within 5 days. Arterial and venous plasma potassium concentration was analyzed in triplicate in an IL flame photometer model 343 using an internal lithium standard. We have established that the precision of the determination of plasma potassium concentration by our standard procedure is high; a single sample of blood has a coefficient of variation of plasma potassium concentration of 0.39%. This includes the variance due to anticoagulation, plasma separation, and flame photometer estimation. Thus an estimated mean value of plasma potassium concentration of 4.00 meq/l would have a standard deviation of 0.015 meq/l due to errors in handling and analysis. Plasma concentrations were corrected to meq/l of plasma water. Plasma water was estimated by drying 1 ml of plasma at 90°C to constant weight (approximately 24 h).

Red blood cell potassium concentration was determined from an aliquot of the same blood sample used to estimate the plasma potassium concentration. This aliquot was mixed thoroughly by slow rotation for 5 min. It was divided into duplicate Wintrobe tubes and, by volumetric pipetting of 1-ml portions, into potassium-free test tubes. The Wintrobe tubes were centrifuged at 2,800 rpm at 15-cm radius for 55 min. Hematocrit readings were corrected for trapped plasma by the method of Chaplin and Mollison (8). The test tubes with 1 ml of whole blood in each were frozen at -20°C and then thawed at the time of analysis. Hemolysis was completed by diluting the whole blood with nine volumes of distilled water. Whole blood potassium concentration was estimated in the IL flame photometer in the same manner as plasma potassium concentration. Erythrocyte potassium concentration was calculated from the formula

$$R_K = \frac{100}{H} \left[W_K - \left(P_K \times \frac{100 - H}{100} \right) \right]$$

where R_K = erythrocyte potassium concentration (meq/l of cells), H = hematocrit (%), W_K = whole blood potassium concentration (meq/l), and P_K = plasma potassium

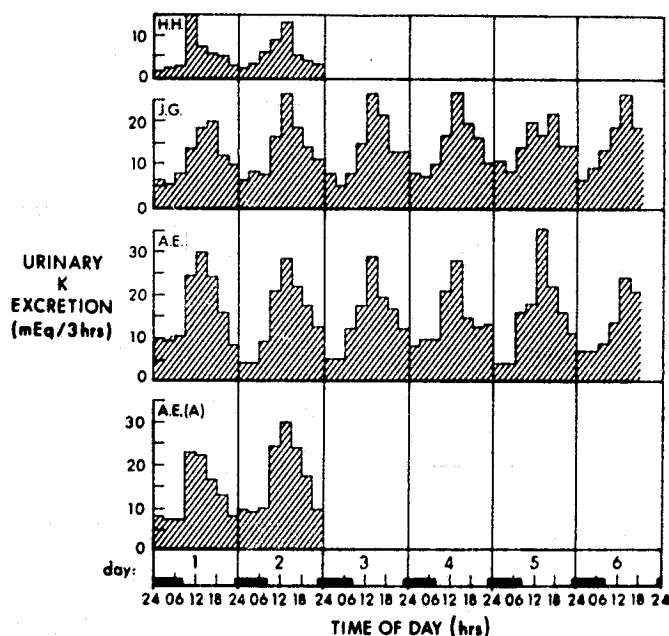


FIG. 1. Circadian variation in urinary potassium excretion (meq/3 h) in each subject during the constant bed-rest and 3-h meal regimen. Subject *AE* was restudied on a rest-activity regimen (*AE(A)*) while still consuming 3-h meals.

concentration (meq/l uncorrected for plasma water). In our laboratory the coefficient of variation of this method is 1.1%.

Urine potassium excretion was calculated from the urine volume and the urine potassium concentration. The volume of each 3-h urine collection was measured. A 20-ml aliquot was acidified with three drops of concentrated sulfuric acid and then refrigerated at 4°C within a few minutes of collection. Urinary potassium concentration was estimated on the IL flame photometer.

Other estimations. The extracellular fluid volume was estimated at 3-h intervals by ^{82}Br isotope dilution (21). A single injection of 50 μCi ^{82}Br in 50 ml normal saline was given 6 h prior to the start of each study period to ensure equilibration. Thereafter 3-h blood samples were taken and the separated plasma was counted in a deep-well scintillation counter. Aliquots of the 3-h urine samples were also counted and correction for excreted isotope was made. Because the sequential measurements of ^{82}Br volume were determined using the same initial ^{82}Br injection, errors in the estimation of changes in ^{82}Br volume exclude the major source of error in this determination—the initial injected dose error. The remaining sources of error which apply in this study have a coefficient of variation in this laboratory of 0.75%.

Arterial plasma aldosterone and cortisol concentrations were estimated at 3-h intervals for 48 h in one subject (*JG*). The assays¹ were done by displacement analysis (29).

Analysis of data. A circadian rhythm in a variable y , oscillating between a maximum value y_Z and a minimum value y_N may be described in terms of several basic parameters. These are the period of the rhythm, the cycle mean (M), the amplitudes of the maximum (A_Z) and minimum (A_N) points on the cycle, the phase angles of reference points such as the maximum (θ_Z) and minimum (θ_N) and the contour. The

¹ In the laboratory of Dr. Gordon Williams.

TABLE 1. Analyses of variance of data from 48-h intensive study period

A) Urinary Potassium Excretion	DF	SS	MS	F
Time of day	7	1,852.77	264.68	27.15*
Due to regression	1	1,704.57	1,704.57	174.83*
About regression	6	148.20	24.70	2.53†
Experiments	3	1,015.91	338.64	34.73*
Experiments × time of day	21	382.95	18.23	1.87
Replication (error)	32	311.95	9.75	
Total	63	3,563.58	56.56	

Regression maximum: 1200-1500 h collection
Proportion time of day SS unexplained: 8.0%

B) Venous Plasma Potassium Concentration	DF	SS	MS	F
Time of day	7	0.6971	0.0996	5.19*
Due to regression	1	0.6425	0.6425	33.46*
About regression	6	0.0546	0.0091	0.47
Experiments	3	0.5663	0.1888	9.83*
Experiments × time of day	21	0.7099	0.0338	1.76
Replication (error)	32	0.6157	0.0192	
Total	63	2.5890	0.0411	

Regression maximum: 1200 h
Proportion time of day SS unexplained = 7.8%

C) Plasma Potassium Arteriovenous Difference	DF	SS	MS	F
Time of day	7	0.1746	0.0249	1.47
Due to regression	1	0.1088	0.1088	6.44†
About regression	6	0.0658	0.0110	0.65
Experiments and days	4	0.0982	0.0246	1.46
Residual (error)	28	0.4741	0.0169	
Total	39	0.7469	0.0192	

Regression maximum: 2100 h
Proportion time of day SS unexplained: 37.7%

D) Red Blood Cell Potassium Concentration	DF	SS	MS	F
Time of day	7	34.58	4.94	3.21†
Due to regression	1	30.58	30.58	19.86*
About regression	6	4.00	0.67	0.44
Experiments	2	131.64	65.82	42.74*
Residual (error)	14	21.55	1.54	
Total	23	187.77	8.16	

Regression maximum: 0000 h
Proportion time of day SS unexplained = 11.6%

E) Extracellular Potassium Content	DF	SS	MS	F
Time of day	7	352.38	50.34	4.01*
Due to regression	1	296.46	296.46	23.64*
About regression	6	55.92	9.32	0.74
Experiments	3	4,827.83	1,609.28	128.33*
Experiments × time of day	21	470.76	22.42	1.79
Replication (error)	32	401.17	12.54	
Total	63	6,052.14	96.07	

Regression maximum: 1200 h
Proportion time of day SS unexplained = 15.9%

TABLE 1.—Continued

F) ICF-ECF Net Potassium Flux	DF	SS	MS	F
Time of day	7	1,571.10	224.44	9.99*
Due to regression	1	1,109.76	1,109.76	49.41*
About regression	6	461.34	76.89	3.42†
Experiments	3	83.31	27.77	1.24
Experiments × time of day	21	1,006.04	47.91	2.13†
Replication (error)	32	718.63	22.46	
Total	63	3,379.06	53.64	

Regression maximum: 0900-1200 h period
Proportion time of day SS unexplained = 29.4%

* Statistically significant at 0.01. † Statistically significant at 0.05.

symbols used are based on those proposed by Aschoff et al. (3). Because of the maintenance of a strict LD 15:9 regimen throughout this study, the circadian rhythms may be assumed to have a period indistinguishable from 24 h. The cycle mean is therefore a 24-h average of the data for any given 24-h day. Amplitudes are the maximum absolute deviations in either direction from the mean. Thus $A_z = |y_z - M|$ and $A_N = |y_N - M|$. In these terms the circadian variation in a variable may be expressed as the total amplitude relative to the mean $[(A_z + A_N)/M]$ %. Phase angles, with 24 h equaling 360° in this case, are calculated from the reference point midnight (2400 h = 0°). The contour is a statement on the shape of the rhythm.

An analysis of variance (ANOVA) was performed for each variable to partition the variance between individuals, time of day and interaction effects and to examine the data for significant sources of variance. The time of day effect was fitted to a simple regression model described by the equation $y = at + b$, where y is the predicted value of the variable, t is time of measurement expressed in absolute hours difference from the time when the mean of y is at its maximum value, a is the slope of the line, and b is the predicted maximum value of y . This regression model describes a "zig-zag" approximation of the time of day effect consisting of the regular alternation of a positive slope for 12 h rising to a maximum and an equal, but negative, slope for 12 h falling to a minimum. The total time of day effect could then be subdivided into variance due to regression and variance about the regression line. In most instances a major portion of the time of day effect could be described by the regression model leaving only a small portion unaccounted for because of the limitations of the simple model used. F variance ratio tests were applied to determine which partitions were significant contributors of variance to the data, and specifically to test for the significance of variance attributable to circadian variation. Where there were two complete days of data from each experiment the replication variance was used as the denominator in the F test. However, when there were less data available the residual variance was used after other sources of variance had been subtracted from the total sum of squares (SS). The term "experiments" in the ANOVA refers to the four intensive study periods of 48 h each. Two of these "experiments" are from the one subject who was studied twice (AE and AE(A)).

RESULTS

Urinary potassium excretion demonstrated a marked circadian variation $[(A_z + A_N)/M = 120 - 180\%]$ in all subjects (Fig. 1) despite the elimination of diurnal variations in dietary intake, activity, and posture. Table 1A gives the results of the analysis of variance demonstrating a significant ($P < 0.01$) time of day effect which conformed well (92.0% of SS) to the "zig-zag" regression model with a maximum during the 1200-1500 h collection ($\phi_z = 203^\circ$). The significant between experiment effect ($P < 0.01$) is mostly due to a 60% reduction in 24-h mean in subject *HH* who was given a dietary potassium intake 50% lower than the other subjects. There was no change in the parameters of the circadian rhythm of excretion when subject *AE* was restudied with daytime activity and night time bedrest $[(A_z + A_N)/M = 120\%, \phi_z = 158-203^\circ, \phi_N = 45-90^\circ]$.

Plasma potassium concentration also exhibited a circadian rhythm $[(A_z + A_N)/M = 7.4-12.3\%]$ in all subjects

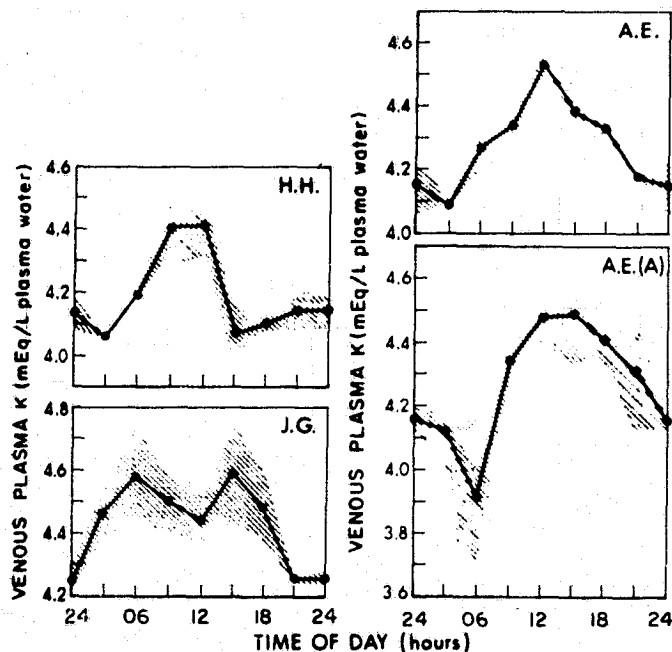


FIG. 2. Mean and range of venous plasma potassium concentration at 3-h intervals throughout day and night in each subject.

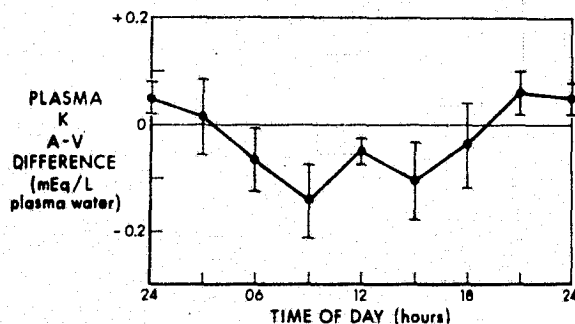


FIG. 3. Forearm arteriovenous difference in plasma potassium concentration (mean \pm SEM) across the resting forearm during the constant bedrest and 3-h meal experiments (*HH*, *JG*, *AE*).

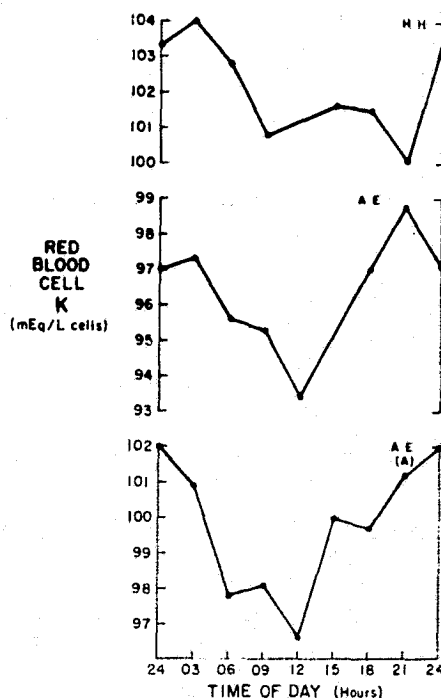


FIG. 4. Circadian variation in erythrocyte potassium concentration. No measurements were made from subject *JG*.

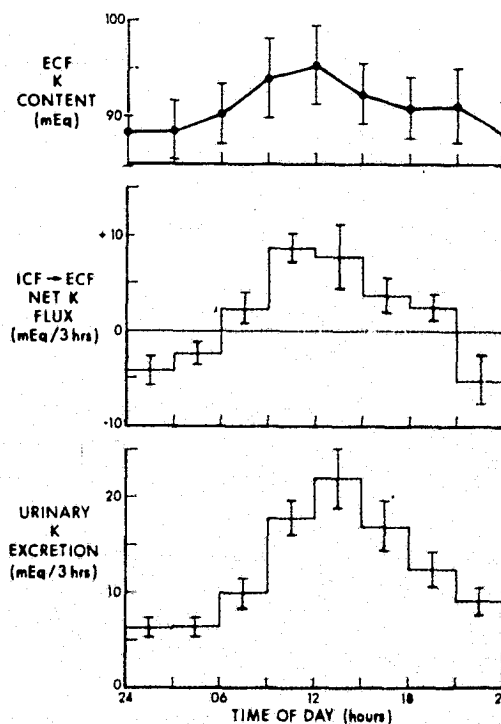


FIG. 5. Mean and SEM of extracellular potassium content, ICF-ECF net potassium flux and urinary potassium excretion from all four experiments (*HH*, *JG*, *AE*, and *AE(A)*).

despite the constant regimen (Fig. 2). The analysis of variance (Table 1B) determined that the time of day effect was significant ($P < 0.01$) and was well described (92.2% of SS) by the regression model with the maximum at 1200 h ($\phi_z = 180^\circ$) and the minimum at 0000 h ($\phi_N = 0^\circ$).

During the equilibration period, prior to the study proper, even greater amplitude circadian variations were seen. Subject *AE* for example, showed a 35% variation in one day with maximum and minimum concentrations of 4.19 and 3.08 meq/l, respectively. On each day of these studies there were similar patterns of plasma concentration with a rise in the early morning and a fall in the evening. However, as can be seen in Fig. 2, there were some individual differences in rhythm contour.

Since the rhythms of urinary potassium excretion and plasma potassium concentration are not inversely related, movements from other potassium containing compartments must be looked for to explain the circadian variations. Forearm arteriovenous plasma potassium difference demonstrated a circadian variation (Fig. 3) which could be fitted to the "zig-zag" regression model with a maximum (positive) value at 2100 h ($\phi_z = 315^\circ$) and a minimum (maximum negative) value at 0900 h ($\phi_N = 135^\circ$) (Table 1C). The circadian variance described by the regression model was significant ($P < 0.05$). Thus there was a net flux of potassium from the resting forearm during the morning and early afternoon, and a reverse net flux into the forearm during the evening hours. This was seen in each subject although there was some variation in the phase of this rhythm in the individual subjects with the timing of reversal from net efflux to net influx ranging between 1200 h ($\phi = 180^\circ$, subj *HH*) and 2000 h ($\phi = 300^\circ$, subj *JG*). This accounted for most of the 37.7% of time of day SS which were unexplained by the "zig-zag" regression model.

A further circadian variation in net potassium flux is apparent from Fig. 4. Red blood cell potassium concentration shows a circadian rhythm [$(A_z + A_N)/M = 3.8-5.6\%$] with a minimum in the middle of the day. The analysis of

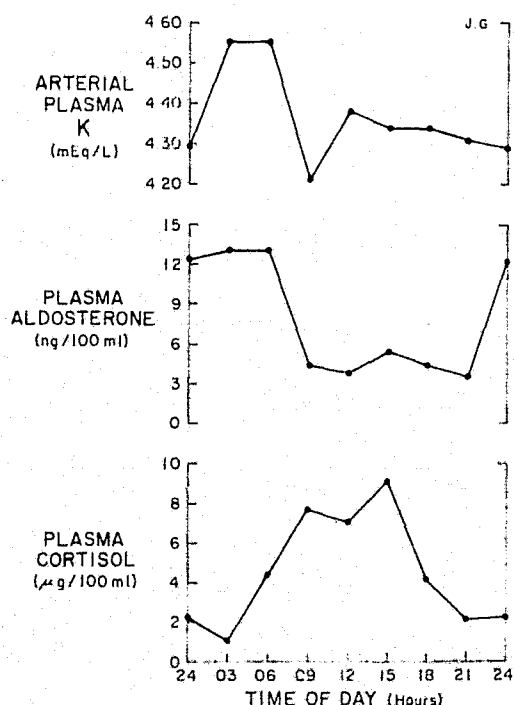


FIG. 6. Mean circadian rhythms of arterial plasma potassium, aldosterone, and cortisol concentrations in subject *JG* during the constant bed-rest and 3-h meal regimen.

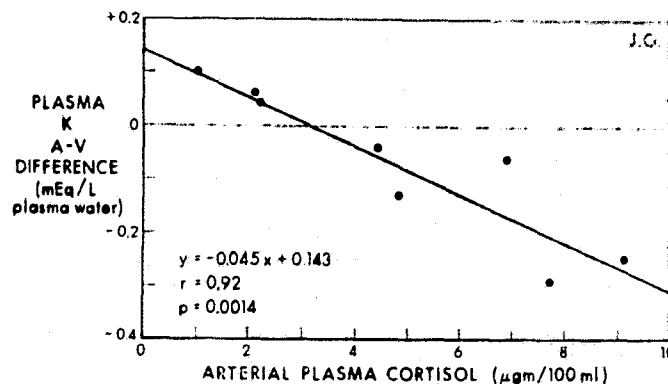


FIG. 7. Relationship between arterial plasma cortisol concentration and the resting forearm arteriovenous difference of plasma potassium concentration in subject *JG* during the constant bed-rest and 3-h meal regimen.

variance (Table 1D) demonstrated a time of day effect which was well described (88.4% of SS) by the regression model with a maximum at 0000 h ($\phi_z = 0^\circ$) and a minimum at 1200 h ($\phi_N = 180^\circ$). This effect was significant ($P < 0.01$). Thus during the morning there would appear to be a movement of potassium from erythrocytes to plasma and a net flux in the reverse direction later in the day. This pattern of potassium movement is similar to that observed from the forearm in timing and direction.

Serial measurement of the extracellular space with a ^{82}Br marker indicated a small circadian variation in the volume of the extracellular compartment [$(A_z + A_N)/M = 2.1-4.5\%$] with a maximum at midday ($\phi_z = 180^\circ$). A computed "extracellular potassium content" using the arterial potassium concentration demonstrated a circadian rhythm with a mean amplitude of 7.2 mEq (Fig. 5). The analysis of variance (Table 1E) demonstrated a significant time of day effect ($P < 0.01$) which could be fitted (84.1% of SS) to the regression model with a maximum at 1200 h ($\phi_z = 180^\circ$) and a minimum at 0000 h ($\phi_N = 0^\circ$).

The mean circadian variations of arterial plasma aldosterone and cortisol concentration are shown for one subject (*JG*) in Fig. 6. The plasma aldosterone concentration was approximately in phase with the circadian rhythm of arterial plasma potassium concentration, but the plasma cortisol concentration showed a peak phase delay of about 110° . However, plasma potassium forearm arteriovenous difference, correlated well with plasma cortisol concentration ($r = 0.92$, $P < 0.01$, Fig. 7) while there was no significant correlation ($r = 0.52$, $P = 0.18$) with plasma aldosterone concentration.

DISCUSSION

The circadian rhythms of plasma potassium concentration and urinary potassium excretion are clearly not secondary to diurnal variation in activity, posture, or dietary intake. They persisted in this study even though these behavioral variables were held strictly constant for up to 8 days. The sleep-wake cycle or the pattern of disturbances and noise are unlikely to be major determinants of the observed pattern since the subjects tended to nap throughout day and night, and noise and activity in the investigative unit was evenly spread over each 24 h.

The light-dark cycle (LD 15:9) of the room in which they were studied was, however, rigidly maintained throughout this study. Although the lighting regimen does not directly control circadian rhythms such as urinary potassium excretion (18) it acts as an important Zeitgeber (synchronizer) in man (4, 9, 24, 31) as well as animals (7, 25, 32). Thus when the light-dark cycle is shifted by several hours, as after east-west travel, the circadian rhythm of urinary potassium excretion persists with its original phase for several days before adjusting to the new environmental time (13, 28). This excretory rhythm also persists in subjects confined to isolation chambers in constant light and with no time clues; in such conditions it usually has a period slightly different from 24 h (2). These studies demonstrate some inherent stability in the urinary potassium rhythm, and it is often termed "intrinsic" or "endogenous" (9).

We found that the circadian rhythm of plasma potassium concentration and urinary potassium excretion were not 180° out of phase with each other. In the constant conditions of the experiments this means that potassium movements between body compartments must underlie the observed circadian variations in urinary and plasma potassium. An investigation of arteriovenous plasma potassium difference across the resting forearm indicated that there was an alternation between positive and negative differences during the course of a 24-h day. Andres et al. (1) have also noted the net movement of potassium out of the forearm in the early morning hours but did not observe the reverse tide since their studies ended at noon. Our direct measurement of erythrocyte potassium concentration, an accessible part of the intracellular compartment, supported the conclusion that there is an outward tide of potassium from intracellular to extracellular compartments during the early morning, and a reverse tide in the evening.

Estimations may be made of the extent of these tides using the extracellular potassium content calculated at 3-h intervals from the ⁸²Br extracellular volume and the arterial plasma concentration. The ICF-ECF net potassium flux can be calculated from the change in ECF potassium content over each 3-h period, after corrections have been made for urinary excretion of potassium and dietary potassium intake in the 3-h period. Figure 5 depicts the relationships between the extracellular potassium content, the ICF-ECF net potassium flux and urinary potassium excretion. Analyses of variance on these variables (Table I, A, E, F) determined that each had a significant ($P < 0.01$) circadian variation which was well described by the "zig-zag" regression model with a maximum at 0900–1200 h ($\phi_z = 158^\circ$) for ICF-ECF net flux, 1200 h ($\phi_z = 180^\circ$) for ECF potassium content and 1200–1500 h ($\phi_z = 203^\circ$) for urinary potassium excretion.

It is apparent that changes in ECF potassium content are minimized by the counterbalancing of the circadian variation in ICF-ECF net potassium flux by the rhythm of urinary potassium excretion. The synchronization of the circadian rhythm of urinary potassium excretion with the other intercompartmental potassium tides is thus essential to the maintenance of extracellular potassium homeostasis. The large relative amplitude $[A_z + A_N]/M$ % and constant phase angles (ϕ_z, ϕ_N) of this rhythm are consistently found under normal conditions (9, 17). However, after time zone

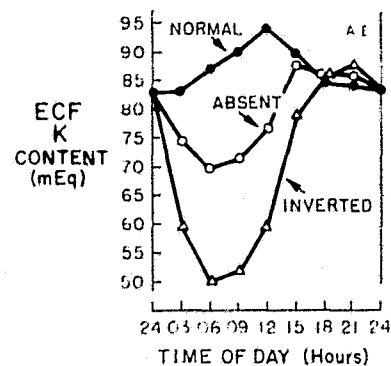


FIG. 8. Predicted circadian variation in extracellular potassium content for subject AE when urinary potassium is excreted with a) the observed circadian rhythm, b) no circadian variation, and c) a 180° inversion of the rhythm.

shifts or during isolation, the urinary potassium rhythm may become desynchronized from circadian rhythms in other variables such as body temperature and urinary sodium excretion (2, 13, 28). The possibility of such desynchronization occurring between the circadian rhythms in the various intercompartmental potassium fluxes has not yet been investigated. Nevertheless, it is important to examine the probable effects of such internal desynchronization, as the various potassium fluxes between the extracellular space and other body compartments are large compared to the extracellular potassium content. The predicted effect on the extracellular potassium content of abolishing the urinary potassium rhythm or phase-shifting it by 12 h (180°) has been calculated for subject AE (Fig. 8). With a 180° reversal of the urinary rhythm the extracellular potassium content could theoretically fall by 43% to a minimum value which represents a plasma concentration of 2.2 meq/l. Extracellular potassium content is unlikely to fall as far as this because of other homeostatic adjustments. However, if such desynchronization occurs, it could potentially cause serious disequilibrium in potassium distribution.

It is now well established that urinary potassium excretion is principally a function of potassium reabsorption and secretion by the cells of the renal distal tubule (15). The synchrony between the circadian rhythms of ICF-ECF net potassium flux and urinary potassium excretion suggests that the renal distal tubular cells may be functioning in a similar manner to the rest of the body cell mass. During the morning while potassium moves from the general cell mass into the general ECF, potassium would appear to be moving from the distal tubular cells into a specialized compartment of the ECF—the distal tubular fluid—resulting in an increase in urinary potassium excretion. Similarly in the late evening when there is a net potassium flux into the body cell mass, a net reabsorption of potassium by the distal tubular cells from the distal tubular fluid is probably responsible for the observed reduction in urinary potassium excretion. The attraction of this hypothesis is that it suggests that common regulatory mechanisms could control the circadian net fluxes of potassium from both the distal tubular cells and the body cell mass in general, thus minimizing desynchrony between the two major determinants of ECF potassium content.

The mechanisms of control of these intercompartmental

fluxes are far from clear. Diurnal variations in sodium-potassium activated ATPase activity have been reported in various tissues (5) and could play a role in these rhythms. The circadian rhythm in plasma cortisol concentration may be an important determinant. Cortisol promotes the movement of potassium out of the body cell mass (especially muscle) (11) and causes a rise in plasma potassium concentration and increase in urinary potassium excretion (6, 16). In subject JG the early morning rise and evening fall in plasma cortisol concentration correlated well with the net movement of potassium to and from the resting forearm preparation (Fig. 7). However, limited studies in adrenalectomized or Addisonian patients have shown that the normal circadian rhythm of urinary potassium excretion is still observed when their replacement steroids and food intake are evenly spread over the 24-h day (14, 23). It is therefore not possible to postulate the simple dependence of all the circadian intercompartmental potassium fluxes on the plasma cortisol rhythm. Whatever the mechanisms of control it appears that such intercompartmental potassium fluxes may be fundamental to many systems for they also play an important role in diurnal leaf movements in plants (26, 27).

Traditionally homeostasis is viewed as the maintenance of an internally constant milieu despite the variations in the external environment. Studies of plants, animals, and man have shown that when environmental circadian variation is minimized or absent many circadian rhythms persist, usually

with a period not greatly different from 24 h (2, 7). Thus responses that would be "appropriate" for the environmental change usually occurring at a certain time of day still are exhibited when that environmental variation is suppressed. The rise in urinary potassium excretion in the middle of the day might be considered a response to the daytime increase in potassium intake from the diet and the potassium released during daytime muscular activity. The observation that the rhythm of potassium excretion persists despite the apparent absence of "causal" environmental variation rules out this conclusion. The concept of homeostasis must be refined to include cyclical variations in physiological set points, for the observed circadian rhythms do not appear to be externally produced perturbations of a homeostatically protected base line.

The authors thank Dr. Francis D. Moore for his support and encouragement of this work. They are indebted to Dr. James Warram for his advice on the statistical analyses. They are also grateful for the assistance of Dr. G. H. Williams, Dr. P. Klovekorn, Miss Maureen MacBurney, Mrs. Caryl Boyden, Mrs. Lourdes Holcjkio, and the staff of the Bartlett Unit.

This work was supported in part by National Aeronautics and Space Administration Contract NAS 9-14249, by National Institutes of Health Grant HL-13872-15, and by Army Research Contract DA49-193-MD-2337.

M. C. Moore Ede was the recipient of a NATO Research Studentship.

Received for publication 30 July 1973.

REFERENCES

- ANDRES, R., G. CADER, P. GOLDMAN, AND K. L. ZIERLER. Net potassium movement between resting muscle and plasma in man in the basal state and during the night. *J. Clin. Invest.* 36: 723-729, 1957.
- ASCHOFF, J. Circadian rhythms in man. *Science* 148: 1427-1432, 1965.
- ASCHOFF, J., K. KLOTTER, AND R. WEVER. Circadian vocabulary: a recommended terminology with definitions. In: *Circadian Clocks, Proceedings of the Feldafing Summer School*, edited by J. Aschoff. Amsterdam: North Holland, 1965.
- ASCHOFF, J., E. POPPEL, AND R. WEVER. Circadiene Periodik des Menschen unter dem Einfluss von licht-dunkel Wechseln unterschiedlicher Periode. *Pfluegers Arch.* 306: 58-70, 1969.
- BAKKEREN, J. A., J. A. VAN DER BEEK, AND S. L. BONTING. Daily rhythmic changes in sodium-potassium activated adenosinetriphosphatase activities in rat liver and kidney. *Pfluegers Arch.* 325: 77-84, 1971.
- BARTTER, F. C., AND P. FOURMAN. The different effects of aldosterone-like steroids and hydrocortisone-like steroids on urinary excretion of potassium and acid. *Metabolism* 11: 6-19, 1962.
- BUNNING, E. *The Physiological Clock* (3rd ed.). New York: Springer-Verlag, 1973.
- CHAPLIN, H., AND P. L. MOLLISON. Correction for plasma trapped in the red cell column of the hematocrit. *Blood* 7: 1227-1238, 1952.
- CONROY, R. T. W. L., AND J. N. MILLS. *Human Circadian Rhythms*. London: Churchill, 1970.
- CORSA, L., JR., J. M. O'NEV, JR., R. W. STEENBURG, M. R. BALL, AND F. D. MOORE. The measurement of exchangeable potassium in man by isotope dilution. *J. Clin. Invest.* 29: 1280-1295, 1950.
- DANOWSKI, T. S., AND J. R. ELKINTON. Exchanges of potassium related to organs and systems. *Pharmacol. Rev.* 3: 42-58, 1951.
- EDELMAN, J. S., AND J. LIEBMAN. Anatomy of body water and electrolytes. *Am. J. Med.* 27: 256-277, 1959.
- ELLIOT, A. J., J. N. MILLS, D. S. MINORS, AND J. M. WATERHOUSE. The effect of real and simulated time-zone shifts upon circadian rhythms of body temperature, plasma 11-hydroxycorticosteroids and renal excretion in human subjects. *J. Physiol., London* 221: 227-257, 1972.
- GARROD, O., AND R. A. BURSTON. The diuretic response to ingested water in Addison's disease and panhypopituitarism and the effect of cortisone thereon. *Clin. Sci.* 11: 113-128, 1952.
- GIEBISCH, G. Renal potassium excretion. In: *The Kidney*, edited by C. Rouiller and A. P. Muller. New York: Academic, 1971, vol. 3, p. 329-382.
- KNIGHT, R. P., D. S. KORNFIELD, G. H. GLASER, AND P. K. BONDY. Effects of intravenous hydrocortisone on electrolytes of serum and urine in man. *J. Clin. Endocrinol. Metab.* 15: 176-181, 1955.
- LOBBAN, M. C. The entrainment of circadian rhythms in man. *Cold Spring Harbor Symp. Quant. Biol.* 25: 325-332, 1960.
- MILLS, J. N., AND S. W. STANBURY. Persistent 24-hour renal excretory rhythm on a 12-hour cycle of activity. *J. Physiol., London* 117: 22-37, 1952.
- MILLS, J. N., S. THOMAS, AND P. A. YATES. Reappearance of renal excretory rhythm after forced disruption. *J. Physiol., London* 125: 466-474, 1954.
- MOORE, F. D., J. M. HARTSUCK, R. M. ZOLLINGER, JR., AND J. E. JOHNSON. Reference models for clinical studies by isotope dilution. *Ann. Surg.* 168: 671-700, 1968.
- MOORE, F. D., K. H. OLESEN, J. D. McMURRAY, H. V. PARKER, M. R. BALL, AND C. M. BOYDEN. *The Body Cell Mass and Its Supporting Environment. Body Composition in Health and Disease*. Philadelphia, Pa.: Saunders, 1963.
- MOORE EDE, M. C., M. H. FAULKNER, AND B. E. TREDRE. An intrinsic rhythm of urinary calcium excretion and the specific effect of bedrest on the excretory pattern. *Clin. Sci.* 42: 433-441, 1972.
- NABARRO, J. D. N. The adrenal cortex and renal function. In: *Modern Views on the Secretion of Urine*, edited by F. R. Winton. Boston, Mass.: Little, Brown, 1956.
- ORTU, D. H., AND D. P. ISI AND. Light synchronization of the circadian rhythm in plasma cortisol (17-OHCS) concentration in man. *J. Clin. Endocrinol. Metab.* 29: 479-486, 1969.
- RICHTER, C. P. Inherent twenty-four hour and lunar clock of a primate the squirrel monkey. *Conn. Behav. Biol., Part A* 1: 305-332, 1968.

26. SATTIER, R. L., AND A. W. GALSTON. Potassium flux: a common feature of *Albizia* leaflet movement controlled by phytochrome or endogenous rhythm. *Science* 174: 518-520, 1971.
27. SATTIER, R. L., P. MARINOFF, AND A. W. GALSTON. Phytochrome controlled nyctinasty in *Albizia julibrissin*. II. Potassium flux as a basis for leaflet movement. *Am. J. Bot.* 57: 916-926, 1970.
28. SHARP, G. W. G. Reversal of diurnal rhythms of water and electrolyte excretion in man. *J. Endocrinol.* 21: 97-106, 1960.
29. UNDERWOOD, R. H., AND G. H. WILLIAMS. The simultaneous measurement of aldosterone, cortisol and corticosterone in human peripheral plasma by displacement analysis. *J. Lab. Clin. Med.* 79: 848-862, 1972.
30. VAGNUGLI, A. M., A. P. SHAPIRO, AND R. M. McDONALD. Effects of upright posture on renal electrolyte cycles. *J. Appl. Physiol.* 26: 720-731, 1969.
31. WEVER, R. Autonome circadiane Periodik des Menschen unter dem Einfluss verschiedener Beleuchtungsbedingungen. *Pflügers Arch.* 306: 71-91, 1969.
32. ZUCKER, I. Light-dark rhythm in rat eating and drinking behavior. *Physiol. Behav.* 6: 115-126, 1971.



RENAL ELECTROLYTE CIRCADIAN RHYTHMS:
INDEPENDENCE FROM FEEDING AND ACTIVITY PATTERNS

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ABSTRACT

The interrelationships between urinary electrolyte circadian rhythms and rhythms of feeding, drinking and activity were studied in six conscious chair-acclimatized squirrel monkeys (Saimiri sciureus) kept in temperature-controlled isolation chambers on a light-dark (LD) 12:12 hour cycle. With lights on (600 lux) from 08.00-20.00 hr and off (<1 lux) from 20.00-08.00 hr, renal potassium excretion in monkeys fed ad lib fell to a daily minimum of 64 ± 6 $\mu\text{Eq/hr}$ at 05.00 hr and rose to a maximum of 274 ± 23 $\mu\text{Eq/hr}$ at 17.00 hr. Sodium excretion fell to a minimum of 13 ± 2 $\mu\text{Eq/hr}$ at 10.00 hr and rose to a maximum of 43 ± 6 $\mu\text{Eq/hr}$ at 21.00 hr, while water excretion fell to a minimum of 869 ± 63 $\mu\text{l/hr}$ at 05.00 hr and rose to a maximum of 2307 ± 222 $\mu\text{l/hr}$ at 17.00 hr. Feeding, drinking and activity occurred only during the lights-on period. Independence of the urinary rhythms from diurnal variations in feeding, drinking and activity was established a) by depriving monkeys of food for 24 hours, b) by depriving monkeys of water for 24 hours, and c) by training monkeys to perform a two-hourly schedule of feeding, drinking and activity throughout day and night. None of these three regimens resulted in reductions of the amplitude, or changes in the phase of the circadian rhythms of urinary electrolyte or water excretion. These findings indicate that the circadian rhythms of urinary potassium, sodium and water excretion are controlled by mechanisms which are independent of the behavioral patterns of feeding, drinking and activity.

Index terms

Circadian internal synchronization, behavioral rhythms, urinary potassium, sodium and water excretion, squirrel monkey.

Many physiological functions have been shown to oscillate spontaneously with a period of 24 ± 4 hours when an animal is isolated from environmental time cues (1,2). There is now considerable evidence to suggest that these circadian rhythms are generated by one or more oscillators within the organism (3,4). Such oscillators appear to serve a variety of timing functions which include the control of developmental sequences (5), the initiation of adaptive changes before the occurrence of a periodic environmental challenge (6) and the time-compensation of sun orientation mechanisms (7).

When circadian rhythms in several physiological variables are monitored simultaneously in an individual animal they are usually found to have identical periods and constant phase-relationships. This "internal synchronization" can be demonstrated whether the rhythms are synchronized with environmental time cues or are free-running under constant environmental conditions (1,8,9). The internal synchronization of circadian oscillations in mutually interdependent processes appears to be an important feature of physiological organization (10), and events which promote internal desynchronization are often associated with deteriorations in psychological and physiological function (11-14).

Little is known about the mechanisms responsible for internal synchronization in higher animals. This is partly because the extent to which oscillating physiological variables are dependent upon one another has not been adequately examined. The experiments described in this paper were designed to investigate the interdependency of two sets of endogenous circadian rhythms--the behavioral rhythms of activity and dietary intake and the renal rhythms of potassium, sodium and water excretion. There is evidence to suggest that all these rhythms are endogenously generated (1,2). However, the internal synchronization that is seen between these rhythms could be achieved by any of three possible mechanisms. Firstly, internal synchronization could be a result of the simple passive dependence of renal excretory rhythms on endogenous rhythms in those variables which influence electrolyte excretion, such as dietary intake (15),

and muscular activity (16). Secondly, the behavioral and renal rhythms could both be passively dependent upon one central driving oscillator. Thirdly, the behavioral and renal rhythms could be generated by potentially independent separate oscillators which are normally kept in synchrony with one another. These questions have been studied using unanesthetized, chair-acclimatized squirrel monkeys (Saimiri sciureus).

MATERIALS AND METHODS

The studies were performed using six adult male squirrel monkeys (Saimiri sciureus) weighing 600-900 gm. For periods of up to three weeks, continuous urine collections were obtained from unanesthetized monkeys, conditioned to sit in a specially designed metabolism chair. Environmental illumination, temperature and auditory stimuli were controlled by conducting experiments within an isolation chamber.

Metabolism Chair. The design of this chair (Figure 1) was based upon the squirrel monkey chairs used in the behavioral experiments of Kelleher and Morse (17). The monkey sat on a bar and was restrained by a plexiglass sheet which served as a table around its waist. The space between the table and the monkey was sealed by a soft rubber waist cuff. The monkey had freedom of movement about the waist. Below the plexiglass table, it could either squat with its feet on a footrest or sit on the perch.

A lever was provided which the animal could operate to obtain food pellets. Pellets were delivered into a tray directly front of the animal from a pellet dispenser (Model 11-1, Gerbrands Co., Arlington, Massachusetts). A row of colored light bulbs were used to train the monkey to perform certain desired feeding schedules. Drinking water was provided from a calibrated water bottle.

A padded funnel, placed between the monkey's legs, enabled the collection of urine samples uncontaminated by feces and food debris. Urine passed from the funnel into test tubes within a specially designed automatic fraction

collector. The apparatus which contained slots for 24 test tubes (100 x 15 mm) was rotated every two hours by a stepping motor (Ledex 24-step digimotor, Ledex, Inc., Dayton, Ohio). The fraction collector was covered by a sheet of plexiglass which both prevented particles from falling into the test tubes and served as a foot rest for the monkey.

Isolation Chamber. The monkey, chair and fraction collector were housed in a temperature-controlled isolation chamber (Forma Scientific, Models 12 or 20, Marietta, Ohio). The chamber temperature was monitored by a continuously recording thermometer (Bacharach Instrument Co., Pittsburgh, Pennsylvania). To provide ventilation, the fan on the heating-cooling unit was used to provide a circulation with air from outside the chamber.

A light source within the chamber, yielding approximately 600 lux of white light, was switched on each day from 08.00-20.00 hr and off from 20.00-08.00 hr. When the light was off there was less than 1 lux of illumination in the chamber. The animals were thus subjected to a 24-hour light-dark cycle (LD 12:12; 600:<1).

The isolation chambers partially attenuated extraneous sounds and a white noise source was used in addition to provide further muffling. The white noise was generated by a Grason-Stadler Noise Generator (Model 901-B, West Concord, Massachusetts). Activities outside the chamber had no discernable effects on the animal's behavior.

Experimental Control and Recording Systems. The timing and control of the experimental system were accomplished by an automatic switchboard. One section of the switchboard was controlled by a clock which operated switches in electrical circuits every two hours thus activating the stepping motor of the fraction collector, the timing record on the continuous paper recorders, and the counter and switch which controlled the illumination cycle of the isolation chamber. Another part of the switchboard controlled the food pellet delivery to the monkey. The number of lever operations to gain a

pellet was controlled by a counter and the time between pellet deliveries was controlled by an adjustable timer.

Feeding, drinking and movements in the chair were recorded from each monkey continuously using Harvard C-3 cumulative and 6-pen recorders (Gerbrands, Arlington, Massachusetts). Physical activity was monitored by an ultrasound motion detector (Alton Electronics Co., Gainesville, Florida). Drinking from the water bottle was detected by closure of an electrical circuit between the perch, the monkey and the water bottle spout. The volume of water consumed each day was determined by measuring the fluid level according to calibrations on the water bottle.

Food pellet lever responses and food pellets obtained were also recorded. Electrical pulses were generated from the automatic switchboard by the food lever countdown devices and these were used to activate the recorders. An additional cumulative counter was used to record the total pellets obtained. The twenty-four hour food intake could be read from this counter. By adjusting the number of responses required to gain a pellet it was possible to ensure that the monkey would eat all of the food pellets delivered.

Once the monkeys were conditioned they tolerated studies lasting two to three weeks, and showed no ill effects or loss of agility upon return to their cages. While in the metabolism chair they behaved normally and maintained body weight.

Control Ad Lib Feeding and Drinking. Five of the conditioned monkeys were studied for a period of seven days while seated in the metabolism chair within the isolation chamber. The monkeys were provided with food and water ad lib and were maintained on the LD 12:12 hour light-dark cycle, with lights on from 08.00 hr to 20.00 hr daily. Two days were allowed for the monkeys to achieve stable patterns of feeding, drinking, activity and urinary excretion. The following five days served as the experimental period. Urine collections were obtained throughout the seven days of the experiment as described above.

Urine samples were removed from the chamber once every one or two days at varied times during the animal's activity period.

24-Hour Food Deprivation. Four of the conditioned monkeys were seated in the metabolism chairs within the isolation chambers as described for the control experiments. Two days were allowed for acclimatization as before. They were then studied during one control day of ad lib feeding followed by a 24 hour period when they were deprived of food. Drinking water remained available on an ad lib basis. Throughout the experiment the monkeys remained on the same light-dark schedule (LD 12:12) as in the control experiments.

24-Hour Water Deprivation. After two days of acclimatization in the isolated metabolism chair, four of the conditioned monkeys were studied during a control day of ad lib feeding. This was then followed by a 24 hour period with the water bottle removed from the chair. Food was available ad lib throughout. Urine collections and other procedures were carried out as previously described.

Two-hourly (q2h) Feeding Regimen. Four monkeys from the original control group were trained to operate a lever to gain food pellets whenever a small green signal light came on. The signal light was mounted in front of the monkey and provided approximately 5 lux of green illumination. Once every two hours the green light was turned on for a 10 minute session when the monkey could obtain up to four pellets (1 g) of food. Operation of the food pellet lever between sessions did not produce pellets. After two or three days on this schedule, the monkeys pressed the lever to obtain food only during the 10 minute periods when the signal light was on. Water drinking and movements within the chair mostly occurred during these feeding sessions.

Once the monkeys were fully conditioned to this schedule, they were placed in the metabolism chair in isolation. Three days were allowed to re-establish the two-hourly pattern of feeding, drinking and activity. Then, for the next two days urine collections were obtained while the monkey followed

the two-hourly feeding schedule. The light-dark cycle (LD 12:12) and other experimental procedures were conducted as before.

Urine Analyses. After the urine samples were removed from the fraction collector they were acidified with two drops of 25% sulfuric acid and refrigerated at 4°C. Analyses were completed within one week. The volume of urine in each tube was measured, and sodium and potassium concentrations were analyzed by flame photometry (Instrumentation Laboratories, Lexington, Massachusetts). Urine excretion rates ($\mu\text{Eq/hr}$) were then calculated for each electrolyte from the volume of each sample, the concentration of the electrolyte and the length of time over which the sample was collected.

Data Processing. The urinary data was expressed as a smoothed three-point running mean. This was done by averaging the excretory rate during each two-hourly period with the excretory rates of the two neighboring two-hourly collections. This procedure reduced the influence of the monkey's irregularly timed micturitions on the excretory pattern without significantly affecting the amplitude of any circadian periodicity in the data.

For certain experiments the urinary data was then expressed as percentage deviation from a running 24 hour mean. The 24 hour mean was calculated from the excretory values for 12 hours on either side of each data point. This procedure enabled any circadian periodicities to be separated out from occasional longer term trends in the data. The various computations involved in these procedures were performed on a Hewlett-Packard 2116B computer.

RESULTS

Control Ad Lib Feeding and Drinking. The five squirrel monkeys showed marked circadian variations in potassium, sodium and water excretion (Figure 2). The rate of potassium excretion fell to minimum of $64 \pm 6 \mu\text{Eq/hr}$ (mean \pm SEM) at 05.00 hr daily and then rose to a maximum of $274 \pm 23 \mu\text{Eq/hr}$ at 17.00 hr. This represented a 115% circadian variation about the 24 hour mean. The reproducibility of the pattern of potassium excretion is illustrated in Figure 3;

five consecutive days of data from one monkey are shown.

Sodium excretion fell to a minimum value of $13 \pm 2 \mu\text{Eq/hr}$ between 09.00 hr and 11.00 hr and rose to a maximum of $43 \pm 6 \mu\text{Eq/hr}$ at 21.00 hr; this was a 113% circadian variation. There was considerably more fluctuation from day to day and more variation between monkeys in the sodium pattern than that of potassium. Sodium excretion was on average four hours phase-delayed from the urinary potassium rhythm. However, individual subjects had maxima of sodium excretion that occurred at various times between 17.00 hr and 07.00 hr.

Urinary water excretion also had a marked circadian rhythm reaching a minimum of $869 \pm 68 \mu\text{l/hr}$ at 05.00 hr and then rising to a maximum of $2307 \pm 222 \mu\text{l/hr}$ at 17.00 hr; an 85% circadian variation. The urinary water rhythm was approximately in phase with the urinary potassium rhythm, although it had a smaller amplitude. Urinary water excretion did not show the variability between subjects that was seen with the sodium excretion pattern.

Feeding, drinking and activity occurred only during the light period of the 24 hour cycle. Figure 4 shows a representative pattern of food and water intake from one monkey. The total 24 hour electrolyte intakes are shown in Table 1.

24-Hour Food Deprivation. The excretory patterns of potassium, sodium and water from four monkeys are shown in Figure 5 during a control day of normal ad lib feeding which was followed by a 24 hour period when the monkey was deprived of food. Despite the absence of feeding and therefore of potassium or sodium intake, the excretion of potassium and sodium rose to a peak during the light period with a similar pattern to that observed during control day. The rhythm of urinary water excretion was also unchanged.

24-Hour Water Deprivation. Figure 6 presents the excretory patterns of the four monkeys who were studied during a control day of ad lib feeding and drinking, and then during a day of water deprivation. The patterns of urinary potassium and sodium excretion were unchanged, but the urinary water rhythm

was damped during the 24 hours of water deprivation. The effects of restoring the drinking water supply after 36 hours of water deprivation are shown in Figure 7. This had little effect on the rhythm of urinary potassium excretion although the daily maximum urine flow rates during the four days of this experiment ranged from 0.6 ml/hr during water deprivation to 12.0 ml/hr on the day that drinking water was restored.

Two-hourly Feeding Regimen. The four monkeys adapted fully to the two-hourly feeding regimen. In addition, most of their drinking and movements in the chair occurred during the ten minute feeding periods in every two hours. Thus, the normal circadian rhythms of feeding, drinking and activity were eliminated by the behavioral conditioning. Figure 8 shows that the circadian rhythms of urinary potassium, sodium and water excretion were not damped by the altered behavioral patterns of feeding, drinking and activity. There was no change in the pattern of potassium excretion and the rhythms of sodium and water excretion were increased in amplitude as compared to the control regimen.

DISCUSSION

All monkeys demonstrated regular circadian rhythms in the rate of urinary potassium, sodium and water excretion and in the behavioral patterns of feeding, drinking and activity. Although the monkeys in the control studies had food and water continuously available throughout day and night they undertook most of their feeding, drinking and movements in the chair during the lights-on period of each 24 hour day. These findings confirmed previous reports that the behavior of these animals is strictly diurnal, both in their natural habitat (18) and in the laboratory (19). The circadian rhythms of potassium and water excretion also reached their maximum rates during the lights-on period. Sodium excretion, however, rose to a daily maximum during the first part of the dark period, on average four hours later than the maximum for potassium and water excretion.

This study was designed to investigate whether the circadian rhythms

of urinary electrolyte excretion are to any extent determined by the diurnal patterns of feeding, drinking and activity. These urinary rhythms are normally internally synchronized with the behavioral rhythms with a phase-relationship suggestive of a direct causal dependence (2,9,20,21). In the rat (20) and the dog (Moore Ede, Drake and Hotez, unpublished observations) the urinary electrolyte rhythms are either eliminated or have greatly reduced amplitudes when food intake is evenly distributed throughout day and night. However, in man (10,22-24) and in the present studies in the squirrel monkey, the circadian rhythms of potassium, sodium and water excretion were unaffected when food and water were withheld for 24 hours, or when the circadian rhythms of dietary intake and activity were eliminated in regimens where small identical meals were given at two or three hourly intervals throughout day and night. Thus, the renal excretory rhythms in human and non-human primates appear to be independent of the circadian rhythms in the behavioral patterns of feeding, drinking and activity. The internal synchronization which is normally observed between these behavioral and urinary rhythms cannot be explained by any direct dependence of renal function on behavioral patterns.

Two alternate mechanisms by which circadian internal synchronization could be achieved in primates must therefore be considered. Firstly, the behavioral rhythms and the renal rhythms could both be passively dependent upon a central circadian oscillator to which they are separately linked. Alternatively, the various behavioral and renal rhythms could be controlled by separate potentially-independent oscillators which are normally kept in synchrony with one another.

Two main lines of evidence make the latter mechanism of internal synchronization the most probable. Firstly, Aschoff and his colleagues (9) have demonstrated that while internal synchronization is normally seen in human subjects studied in the absence of environmental time cues, internal desynchronization can occasionally occur. In 15% of their subjects the various monitored rhythms spontaneously begin to free-run with different periods, so that the rhythmic

functions became desynchronized from one another. This indicates that there must be more than one self-sustaining circadian oscillator within an advanced multicellular animal such as man. A similar conclusion must also be reached from the studies that have been conducted in isolated tissues from multicellular animals. Persistent circadian oscillations have been shown to occur in various isolated tissues maintained in constant conditions. The preparations in which free-running circadian rhythms have been demonstrated in vitro include hamster adrenal glands (25-27), cardiac muscle (28) and the eye of *Aplysia* (29,30).

These two sets of observations strongly suggest that the circadian timing mechanism is organized as a system of multiple, potentially-independent oscillators in the various tissues of an animal. Internal synchronization would presumably be achieved in this system through oscillations in hormonal and nervous mediators. This laboratory is currently engaged in the identification of the anatomical locations and the mechanisms which synchronize these putative oscillators. The definition of the properties of these oscillators is important to the understanding of physiological processes such as renal electrolyte excretion, in which the phase of the circadian cycle is a major determinant of physiological function.

ACKNOWLEDGEMENTS

The authors are grateful for the support and encouragement of Dr. A.C. Barger and Dr. F.D. Moore and for the assistance of Ms. Wendy Schmelzer, Mr. Jonathan Russo, Mr. Harvey Kaufman, Ms. Susan Grose, Ms. Lourdes Holejko, Ms. Margaret Ball and Ms. Susan Ruane.

This work was supported in part by National Aeronautics and Space Administration Contract NAS9-14249, and by National Institutes of Health Grant HL-13872 and SCOR Grant HL-14150. MOME was the recipient fo a NATO Research Studentship during part of this work.

REFERENCES

1. Bünning, E. The Physiological Clock. New York: Springer-Verlag, 3rd edition, 1973.
2. Conroy, R.T.W.L., and J.N. Mills. Human Circadian Rhythms. London: J and A Churchill, 1st edition, 1970.
3. Pittendrigh, C.S. Circadian oscillations in cells and the circadian organization of multicellular systems.. In: The Neurosciences, edited by F.O. Schmidt and F.G. Worden, Cambridge: M.T.T.Press, 1974, p. 437-458.
4. Menaker, M. Aspects of the physiology of circadian rhythmicity in the vertebrate central nervous system. In: The Neurosciences, edited by F.O. Schmidt and F.G. Worden, Cambridge: M.I.T. Press, 1974, p. 479-489.
5. Saunders, D.S. Circadian control of larval growth rate in Sarcophaga argyrostoma. Proc. Natl. Acad. Sci. USA 69: 2738-2740, 1972.
6. Cloudsley-Thompson, J.L. Rhythmic Activity in Animal Physiology and Behavior. New York: Academic Press, 1st edition, 1961.
7. Kramer, G., and U.V. Saint Paul. Stare (Sturnus vulgaris L.) lassen sich auf himmelsrichtungen dressieren. Naturwissenschaften 37: 526-527, 1950.
8. Wever, R. Autonome circadiane periodik des menschen unter dem einfluss verschiedener beleuchtungs-bedingungen. Pfluegers Arch 306: 71-91, 1969.
9. Aschoff, J., U. Gerecke, and R. Wever. Desynchronization of human circadian rhythms. Jap. J. Physiol. 17: 450-457, 1967.
10. Moore Ede, M.C., M.R. Brennan, and M.R. Ball. Circadian variation of inter-compartmental potassium fluxes in man. J. Appl. Physiol. 38: 163-170, 1975.
11. Klein, D.E., H. Bruner, H. Holtmann, H. Rehme, J. Stolze, W. Steinhoff, and H. Wegmann. Circadian rhythm of pilots efficiency and effects of multiple time zone travel. Aerosp. Med. 41: 125-132, 1970.
12. Taub, J., and R. Berger. Acute shifts in the sleep-wakefulness cycle: Effects on performance and mood. Psychosom. Med. 36: 164-173, 1974.
13. Simpson, H.W. Human body temperature, 17-hydroxycorticosteroid excretion and performance tests on a 21-hour day. Clin. Trial. J. 7: 29-44, 1970.
14. Aschoff, J., U.V. Saint Paul, and R. Wever. Die lebensdauer von fliegen unter dem einfluss von zeit-verschiebungen. Naturwissenschaften 58: 574, 1971.
15. Black, D.A.K. Essentials of Fluid Balance. Oxford: Blackwell Scientific Publications, 4th edition, 1967.
16. Castenfors, J. Renal function during exercise. Acta Physiol. Scand. Suppl. 293: 1-44, 1967.
17. Kelleher, R.T., and W.H. Morse. Escape behavior and punished behavior. Fed. Proc. 23: 808-817, 1964.

18. Thorington, R.W., Jr. Observations of squirrel monkeys in a Columbian forest. In: The Squirrel Monkey, edited by L.A. Rosenblum and R.W. Cooper, New York: Academic Press, 1968, p. 69-85.
19. Richter, C.P. Inherent twenty-four hour and lunar clocks of a primate-- the squirrel monkey. Comm. Behav. Bio. Part A 1: 305-332, 1968.
20. Cohn, C., L. Webb, and D. Joseph. Diurnal rhythms in urinary electrolyte excretion by the rat: Influence of feeding habits. Life Sci. 9: 803-809, 1970.
21. Hawking, F., and M.C. Lobban. Circadian rhythms in Macaca monkeys (physical activity, temperature, urine and microfilarial levels). J. Interdiscipl. Cycle Res. 1: 267-290, 1970.
22. Mills, J.N., and S.W. Stanbury. A reciprocal relationship between K^+ and H^+ excretion in the diurnal excretory rhythms in man. Clin. Sci. 13: 177-186, 1954.
23. Stanbury, S.W., and A.E. Thompson. Diurnal variation in electrolyte excretion. Clin. Sci. 10: 267-293, 1951.
24. Moore Ede, M.C., M.H. Faulkner, and B.E. Tredre. An intrinsic rhythm of urinary calcium excretion and the specific effect of bedrest on the excretory pattern. Clin. Sci. 42: 433-445, 1972.
25. Andrews, R.V., and G.E. Folk, Jr. Circadian metabolic patterns in cultured hamster adrenal glands. Comp. Biochem. Physiol. 11: 393-409, 1964.
26. Andrews, R.V. Temporal secretory responses of cultured hamster adrenals. Comp. Biochem. Physiol. 26: 179-193, 1968a.
27. Andrews, R.V., and R. Shiotsuka. In vitro adrenal studies in relation to cyclic reproductive success. In: Biorhythms and Human Reproduction, edited by, M. Ferin, F. Halberg, R. Richart, and R. Vande Wiele, New York: John Wiley and Sons, 1974, p. 591-603.
28. Tharp, G.D., and G. E. Folk, Jr. Rhythmic changes in rate of the mammalian heart and heart cells during prolonged isolation. Comp. Biochem. Physiol. 14: 255-273, 1965.
29. Eskin, A. Phase-shifting a circadian rhythm in the eye of Aplysia by high potassium pulses. J. Comp. Physiol. 80: 353-376, 1972.
30. Jacklett, J.W., and J. Geronimo. Circadian rhythm: population of interacting neurons. Science 174: 299-302, 1971.

FIGURE LEGENDS

- Figure 1. Squirrel monkey seated in metabolism chair within isolation chamber. Urine passed from a funnel between the animal's legs to the test tubes in the fraction collector. Food was delivered from the pellet dispenser in response to operations of the lever in front of the animal. The colored light bulbs above the lever were utilized in conditioning the animal to certain feeding schedules. Movements in the chair were detected by an ultrasound activity meter above the animal and water intake was monitored by a drinkmeter circuit.
- Figure 2. Circadian variation in urinary potassium, sodium and water excretion during the control ad lib feeding studies in LD 12:12 . Mean \pm SEM of urinary data from five monkeys each studied for four days is plotted on the left side of the figure as $\mu\text{Eq/hr}$ or $\mu\text{L/hr}$, and on the right side as % deviation from a running 24 hour mean.
- Figure 3. Circadian rhythm of urinary potassium excretion in one representative monkey plotted over five consecutive days of a control ad lib feeding experiment. The pattern of excretion was highly reproducible in all animals studied.
- Figure 4. Pattern of ad lib food and water intake in a monkey. A continuous paper tape recorded events associated with feeding and drinking with each event recorded as a single stroke of the pen. This paper tape was cut into 24 hour strips and the 24 hour strip from each day pasted under the previous day's strip for eight consecutive days. The black bars represent periods of darkness. It can be seen that all the events associated with feeding and drinking occurred in the lights-on period of the 24 hours.

Figure 5. Urinary excretory rhythms during acute food deprivation. The circadian rhythms of potassium, sodium and water excretion of four monkeys (mean \pm SEM) are plotted during a control day of ad lib feeding and a day when the animals were deprived of food, but were given free access to water. Despite no dietary intake of potassium and sodium the circadian rhythms in the excretion of these electrolytes were unchanged.

Figure 6. Urinary excretory rhythms during acute water deprivation. The circadian rhythms of potassium, sodium and water excretion of four monkeys (mean \pm SEM) are plotted during a control day of ad lib feeding and drinking followed by a 24 hour period when the water bottle was removed from the chair but food remained available ad lib. The circadian rhythms of potassium and sodium excretion were unaffected by water deprivation. The amplitude of the circadian rhythm in water excretion was decreased from 101% to 72% relative to the 24 hour mean.

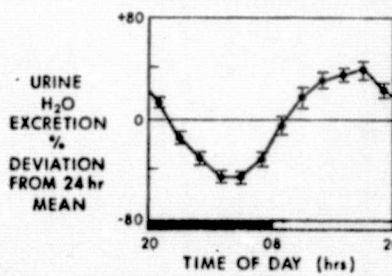
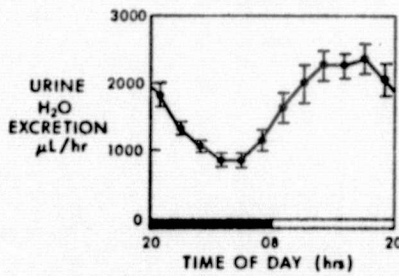
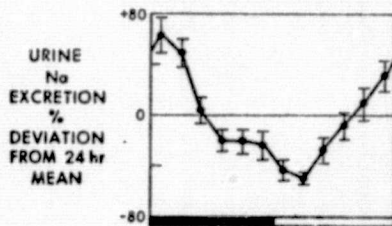
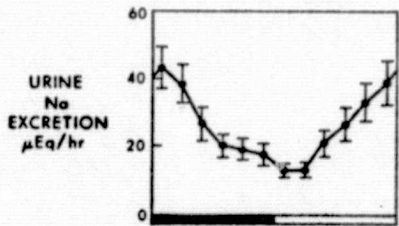
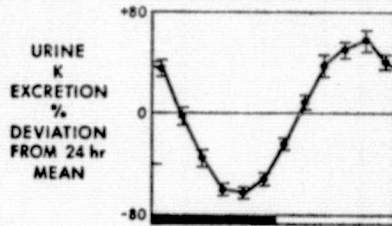
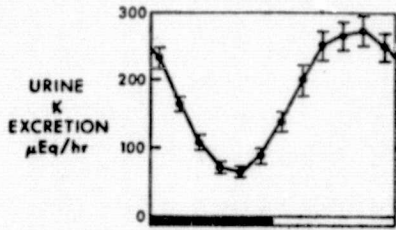
Figure 7. The circadian rhythms of urinary potassium, sodium and water excretion during one control day of ad lib feeding and drinking, 36 hours of water deprivation and 36 hours with the water supply returned to the monkey. Despite water excretion rates which ranged from 0.6 ml/hr during water deprivation to 12 ml/hr on the day after the water supply was returned, the circadian rhythm of urinary potassium excretion remained unchanged. In contrast, the circadian rhythm of sodium excretion varied markedly in amplitude and a natriuresis accompanied the high urine flow rates on Day 3.

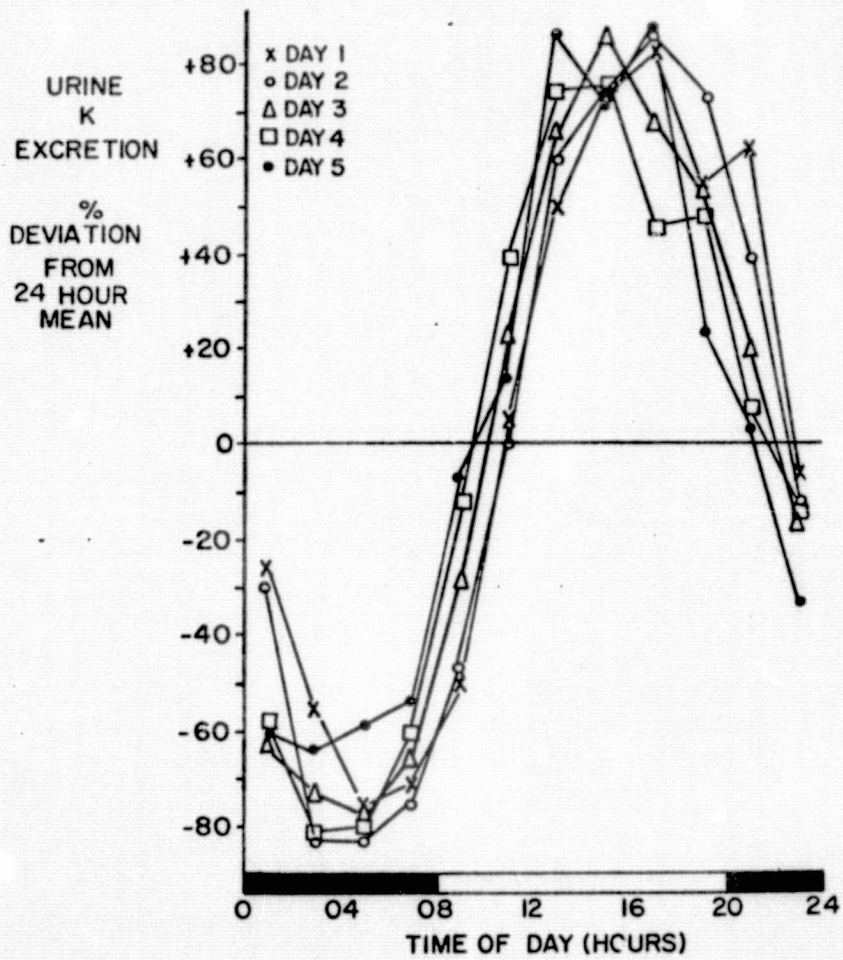
Figure 8. Urinary excretory rhythms during ad lib and two-hourly (q2h) feeding. The patterns of urinary potassium, sodium and water excretion are shown during ad lib feeding (shaded pattern) and in the same four monkeys during a two-hourly (q2h) feeding regimen. The rhythm of urinary potassium excretion was uninfluenced by the change in feeding pattern, but the circadian oscillations of urinary sodium and water excretion had increased amplitudes.

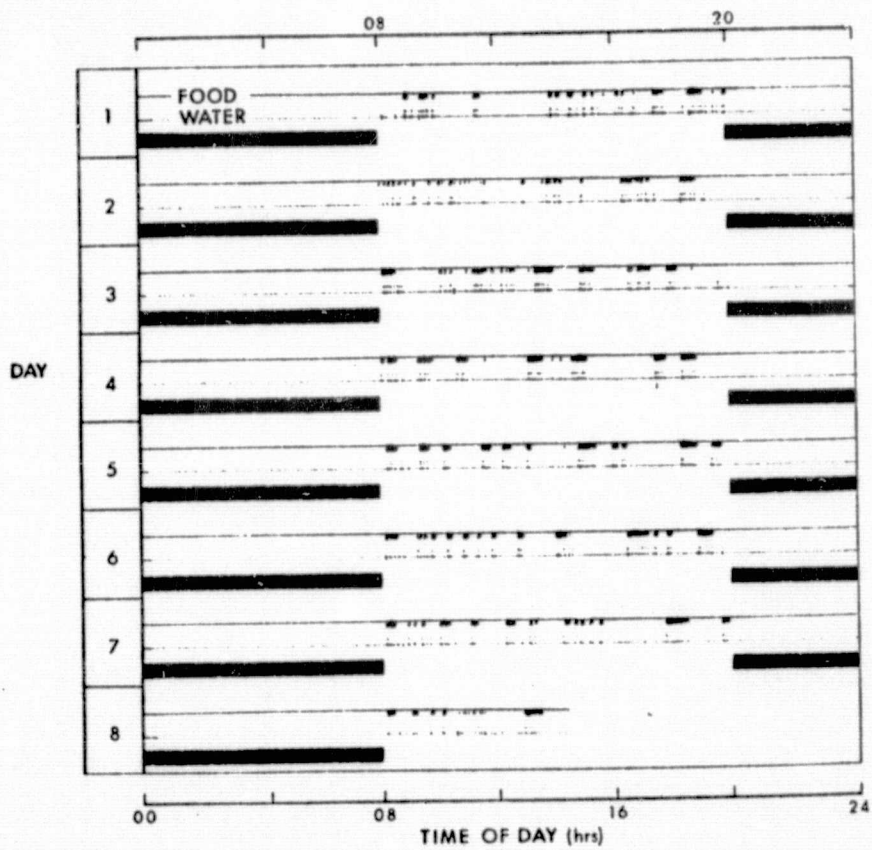
Table 1. Average Daily Intakes for Five Monkeys During Control Ad Lib Feeding Schedules

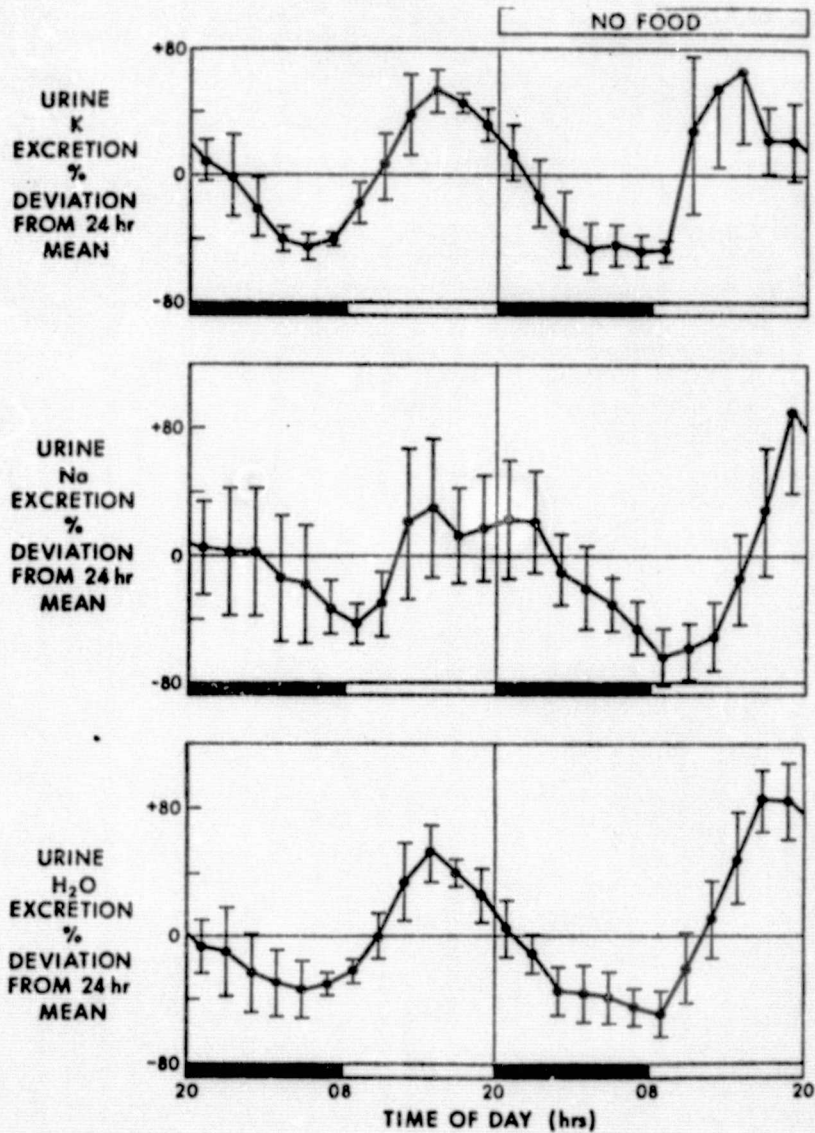
<u>Monkey</u>	<u>Pellets*</u>	<u>Potassium</u>	<u>Sodium</u>	<u>Calcium</u>	<u>Magnesium</u>	<u>Nitrogen</u>
	#/day	mEq/day	mEq/day	mEq/day	mEq/day	g/day
A	127	4.78	1.79	6.36	1.73	0.98
B	172	6.47	2.43	8.62	2.34	1.33
C	200	7.52	2.82	10.02	2.72	1.54
D	114	4.29	1.61	5.71	1.55	0.88
E	157	5.90	2.21	7.87	2.14	1.21
Mean	154	5.79	2.17	7.72	2.09	1.19
+ SD	+ 35	+ 1.30	+0.49	+1.73	+0.47	+0.27

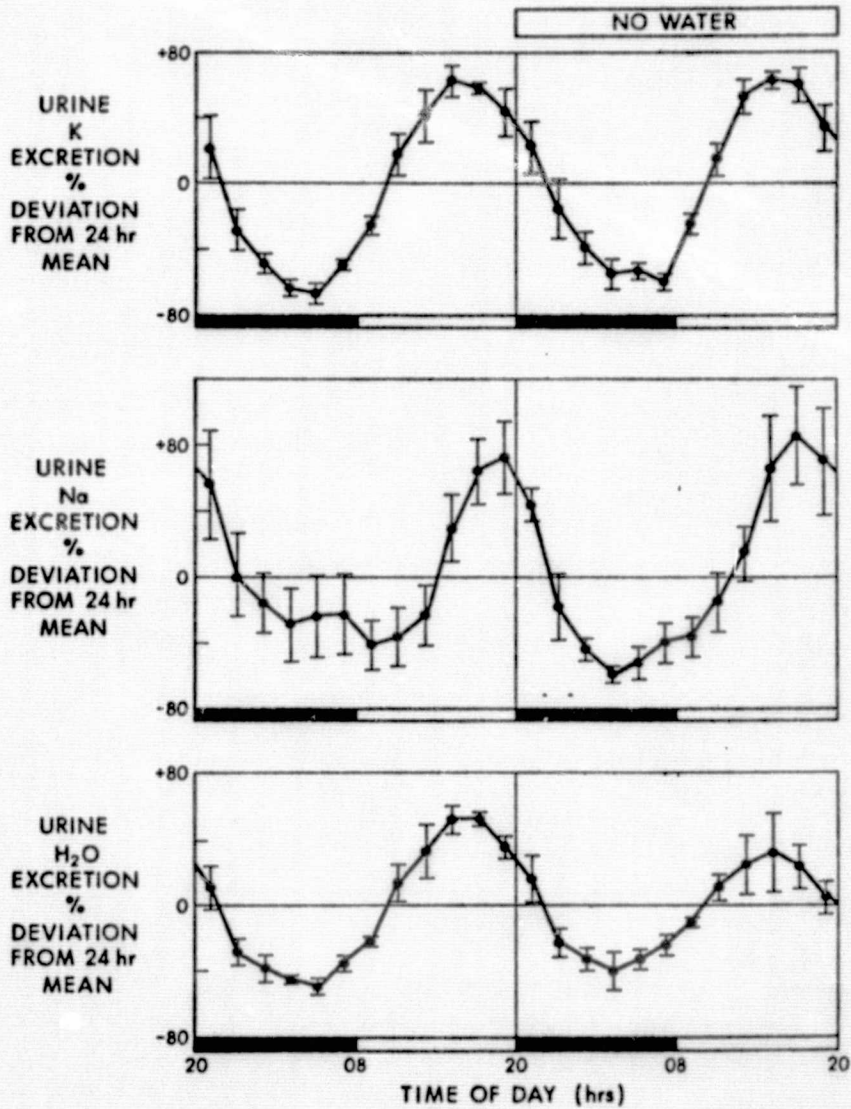
*Each pellet contained 37.6 μ Eq potassium, 14.1 μ Eq sodium, 50.1 μ Eq calcium, 13.6 μ Eq magnesium, and 7.7 mg nitrogen.



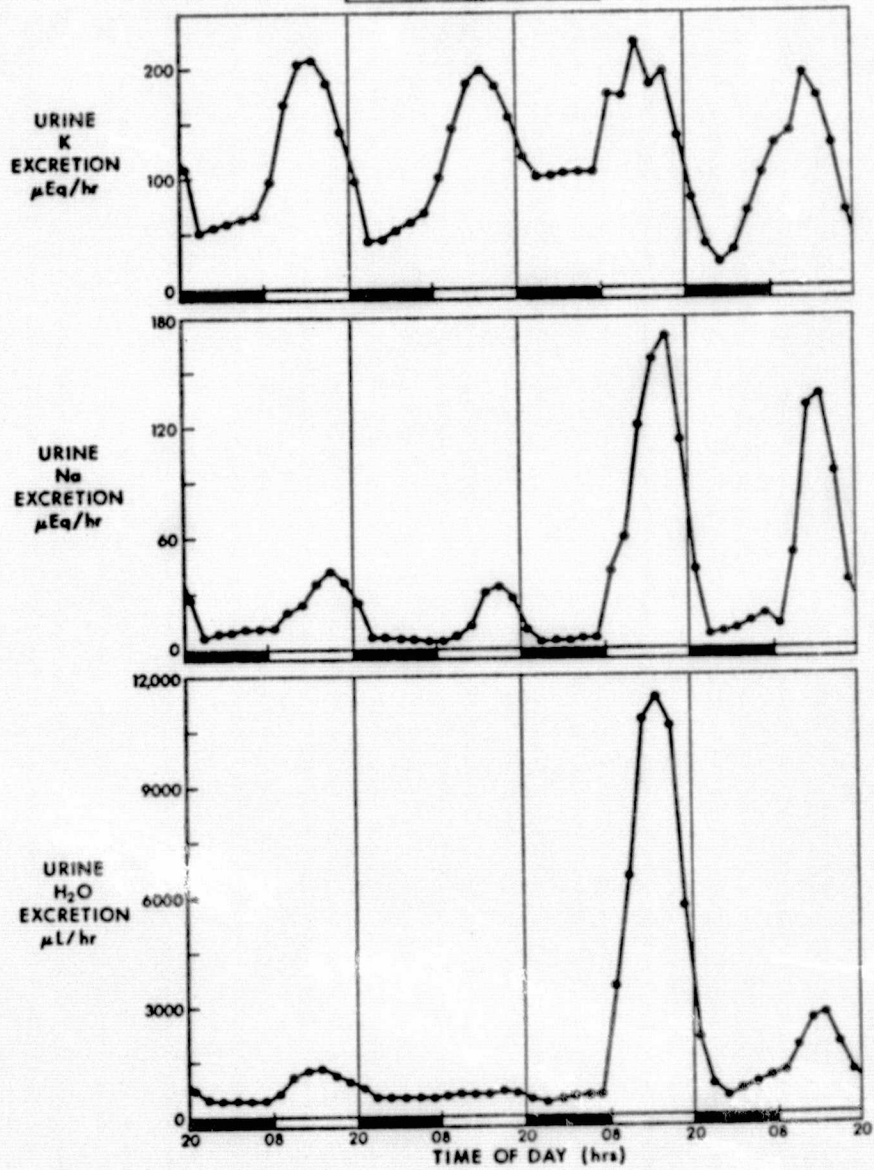


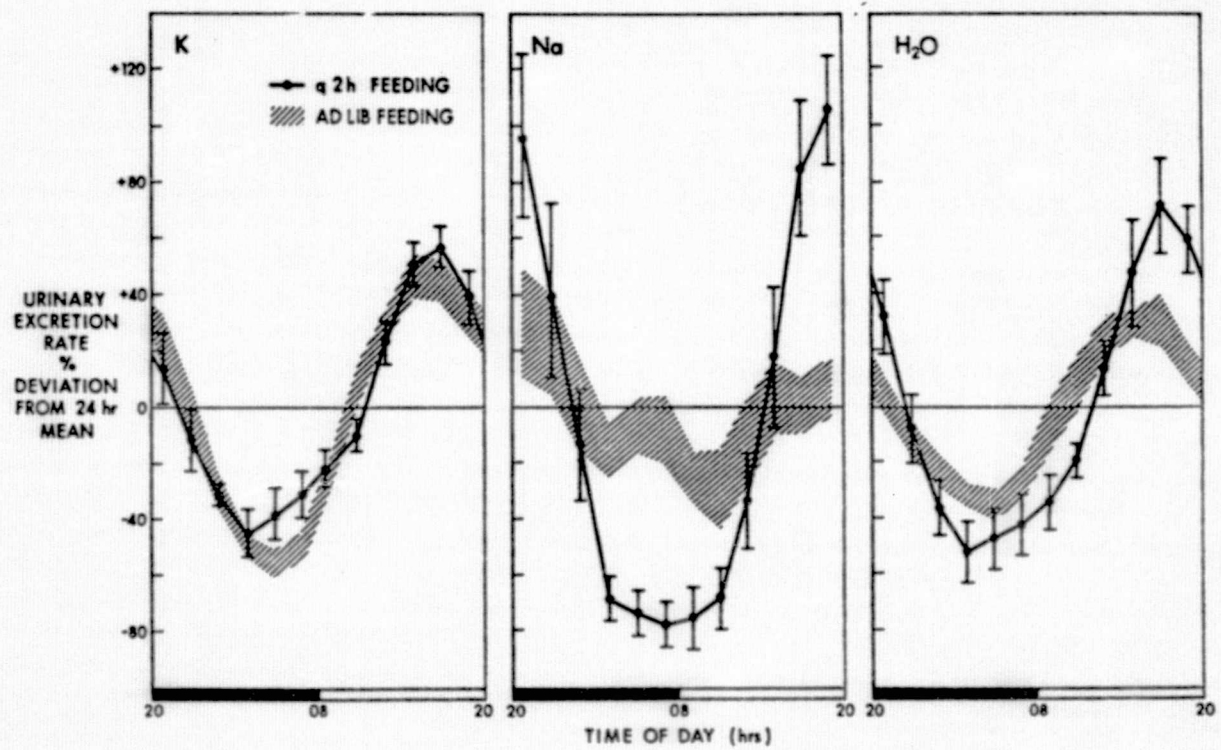






WATER DEPRIVATION





EPISODIC 24-HOUR CORTISOL SECRETORY PATTERNS
IN PATIENTS AWAITING ELECTIVE CARDIAC SURGERY

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Running Title: Preoperative 24-Hour Cortisol Patterns

This work was supported in part by National Aeronautics and Space Administration Contract NAS9-14249, by National Institutes of Health Grant HL-13872-16, by the Block Fund of the University of Chicago and by the Fisher Endocrine Research Fund.

ABSTRACT

The 24-hour pattern of plasma cortisol concentration in four patients on the day before major elective surgery was compared with that of five similarly hospitalized control subjects to study the effect of the expectation of surgery on the secretion pattern. Using an indwelling venous catheter which extended outside the patient's room to collect blood samples every 20 minutes for 24 hours, it was found that cortisol was secreted episodically in both control subjects and presurgical patients. The nycthemeral patterns of plasma cortisol concentration in the two groups were indistinguishable for most of the day despite the occurrence of intermittent events which appeared to cause anxiety in the presurgical patients. However, between 9 P.M. and 11 P.M., while each presurgical patient was being preoperatively prepared (body shaving, wash and enema), a major pulse of cortisol secretion occurred, raising the plasma cortisol concentration to between 6.9-10.5 standard deviations above the control subject mean for that time of day.

We conclude that 1) expectation of a major surgical procedure for several weeks does not result in chronic activation of the pituitary-adrenocortical axis, 2) many discrete anxiety-provoking events do not evoke cortisol secretory episodes, 3) most episodes of cortisol secretion are part of an endogenous cyclical pattern with a circadian distribution and are not a direct result of environmental stimuli, and 4) preoperative preparation evokes a major cortisol secretory response in patients awaiting surgery. Whether that release of cortisol is a response to the physical manipulations or psychological implications of that stimulus is presently unknown.

Anxiety, and particularly the apprehension of personal injury, is generally considered to be a potent stimulus to ACTH and cortisol secretion (1-5). Yet attempts to correlate elevations in either plasma cortisol concentration or urinary 17-hydroxycorticosteroid excretion with psychologically stressful situations (both contrived and real) have often failed to demonstrate a consistent relationship in man (6-13). Such variability in the cortisol secretory response to a given situation among and within individuals has been explained in several ways. When falling plasma cortisol concentrations have been observed in the face of apparent stress some investigators have concluded that the psychoendocrine response was being masked by a concurrent diurnal fall of plasma cortisol concentration (12,13). Other authors have suggested that personality type was the overriding factor in cortisol output (14-16) or that increased cortisol secretion was only seen when an individual's psychological defenses were inadequate to cope with a situation (7,8,10,17).

While such explanations may yet prove to be valid, another reason for the lack of a consistent correlation between anxiety-provoking situations and elevated plasma cortisol concentrations has become apparent from the demonstration by Weitzman and colleagues that cortisol is secreted episodically in man (18,19). Their use of frequent (20 minute) plasma sampling has demonstrated that cortisol secretion is limited to short pulses with no obvious secretion between those pulses (20,21), although the average nycthemeral pattern of cortisol concentration still demonstrates the previously reported circadian variation (22-24). This episodic 24-hour pattern of cortisol secretion explains why attempts to show a precise correlation between infrequently monitored plasma cortisol concentrations and psychologically stressful situations have had little success. Accurate definition of any psychoendocrine response which is superimposed upon a complex, pulsatile

secretory cycle is simply not possible when only a few blood samples are taken on each day of study.

One naturally occurring situation which is associated with anxiety is the prospect of major surgery(25). Patients awaiting elective cardiac surgery were chosen for this study since most such patients feel that the operation poses a significant threat which they consent to undertake in the hope of a successful relief of their symptoms. Consequently, such patients might be expected to demonstrate a major adrenocortical response (1,2). This paper reports a study which compared the 24-hour patterns of plasma cortisol concentration measured at 20 minute intervals in four patients during the 24-hour period just prior to undergoing open-heart surgery with the 24-hour cortisol patterns of five control subjects who were similarly hospitalized.

MATERIALS AND METHODS

Control Subjects. Five healthy, normal male subjects (A,B,C,D & E), 21-43 years of age (mean=26.3 years), were studied in the Clinical Research Center of the Peter Bent Brigham Hospital. Each subject was a personal acquaintance of one of the authors (C.C.). Normality was established by clinical history, physical examination and routine clinical biochemical screening. Signed informed consent was obtained from each subject.

Each subject received several hours of instruction prior to the investigation in order to minimize the possible effects of uncertainty about the experimental procedures (11). For at least one week prior to the study, each subject kept a daily record of their estimated times of sleep onset and waking. A clinical psychiatrist (Q.R.), interviewed each subject for between one to three hours. Without prior knowledge of the endocrine data, he ranked each subject according to manifest display of emotional responses on a scale from the overt expression of emotionality to a tendency towards inhibition of emotional responses. This was done in

conformity with studies using similar clinical methods (15,26). On that scale (overt expression to inhibition) the subjects ranked in the following order: A,D,E,B,C.

The subjects were admitted to the Clinical Research Center on the day prior to the study in order to foster adjustment to the hospital environment. They were provided a normal diet containing 100 mEq potassium and 150 mEq sodium per 24 hours. The subjects were restricted to light activity or bedrest during the adaptation and experimental days. Lights were switched out at 11 P.M. and switched on at 7 A.M. daily (ID 16:8).

Venipuncture was performed on each of the control subjects three days prior to the study in order to reacquaint them with that procedure (27). In order that the reported adrenocortical secretory response to intravenous catheterization (28) would not confound the results of the control studies, such catheterization was performed at least 12 hours before the 24-hour blood sampling procedure was begun. A sterile teflon catheter was inserted into a forearm vein and connected to a 12 foot long section of polyethylene tubing (1.14 mm ID) which extended out into the hall adjacent to the subject's room. The tubing was insulated with larger diameter translucent tubing to prevent the subject from sensing temperature changes as blood was drawn through the line. This intravenous line was kept patent with a microdrip infusion of heparinized saline (500 U sodium heparin and 0.45 g NaCl per 100 ml) at a rate of 12 ml/hr. Frequent blood samples could thus be obtained from outside the subject's room without his being aware of the procedure (19). Blood samples (1.5 ml) were withdrawn from the extended indwelling catheter every 20 minutes, starting at 7 A.M. on the day after admission to the hospital and continuing for the subsequent 25 hour period. The subjects reported that they slept normally throughout the period of darkness (11 P.M.- 7 A.M.).

The degree of anxiety or apprehension was subjectively assessed using a 1-5 rating scale (13,29), at twenty minute intervals, throughout the period of the experiment. In addition, a detailed log was kept of events which occurred during the day of the experiment.

Presurgical Patients. Four patients age 36-59 (mean=44 years) were studied during the 24-hour period immediately prior to elective coronary artery bypass graft surgery. Three patients were men (W,Y & Z) and one was a woman (X). They had slept 3 to 8 nights in the hospital just prior to their studies. Except for a previous history of myocardial infarction in X,Y and Z, and significant occlusion of one or more coronary arteries as determined by coronary angiography in all cases, each patient had no other medical abnormalities. No patient had any endocrine or metabolic disorder, and specifically there was no evidence of congestive heart failure, hypertension, hyper- or hypothyroidism, Cushing's or Addison's disease, or recurrent angina pectoris although all reported the experience of angina pain on strenuous physical exertion. No episodes of angina occurred at any time during these studies. One of the patients (Y) had undergone the same operation one year before. None of the patients were receiving any medication with the following exceptions: all were given sodium methicillin (Staphcillin, 1 g) prophylactically at midnight; Y received isosorbide dinitrate (Isordil Tembids, 80 mg/day); W, X and Y consented to forego the usual preoperative sleep medication, but Z received glutethimide (Doriden, 0.5 g), a non-barbiturate hypnotic, at midnight.

All of the patients before cardiac surgery in this study were intellectually aware of the risks of major cardiac surgery. They each talked about their fears of the operation repeatedly during the day of study and described themselves as anxious.

Blood sampling from outside of the patient's room was accomplished at

20 minute intervals through an indwelling catheter as described above for the control subjects, from 6 A.M. on the day before surgery until 7 A.M. on the day of surgery. For the patient's comfort, the catheter was not placed until just prior to sampling. During the day of the study the patients engaged in light activity or bedrest similar to that of the normal volunteers. The degree of anxiety and apprehension was estimated as described for the normal subjects, and a log was kept with particular attention paid to the timing of potentially stressful events during the day--such as diagnostic procedures, venipuncture, etc.

Plasma Cortisol Assay. After each blood sample had been collected in a heparinized tube it was centrifuged and the plasma aliquot frozen for subsequent biochemical assay. The cortisol concentration in each of the 670 plasma samples drawn in the study was assayed in duplicate using the competitive protein binding technique in a modification of the method of Murphy (30) after Rosenfield et al. (31). The interassay coefficient of variation was 7%.

Presentation of Data. An average time of sleep onset for each subject was calculated from his record of the seven nights prior to the study. This was used as the zero point of the time scale for plotting his cortisol data. This time of reported mean sleep onset (MSO) for the previous week was chosen as a common reference point rather than the actual time of "lights out" on the night of the experiment because the circadian cortisol secretory pattern has been shown to persist with unaltered phase for several days after a phaseshift of the light-dark or sleep-wake cycles (32,33). The actual clock times of sleep onset and waking on the experimental days are shown in Figures 1 and 3 by downward and upward arrows respectively.

For purposes of statistical analysis, the 24-hour sleep-wake cycle was divided into the following 4 phases: Phase I=4 hours before until 2 hours after MSO, Phase II=2 to 4 hours after MSO, Phase III=4 to 9 hours after

MSO and Phase IV=15 to 4 hours before MSO. These divisions are similar to those outlined by Weitzman et al. (19), as were the criteria for defining secretory episodes. Comparisons of the cortisol data between the two experimental groups were made using Student's t test, and linear regression analysis was used to test for correlations between anxiety ratings and cortisol concentration.

RESULTS

Control Subjects. The patterns of plasma cortisol concentration for the 24-hour study period in the five normal subjects are plotted in Figure 1. The mean plasma cortisol concentration, range of values, number of secretory episodes and the longest period when the cortisol concentration did not rise above the mean are presented in Table 1. In each subject the concentration of cortisol fluctuated widely during the day in a manner suggesting discrete episodes of cortisol secretion. In the period from 4 hours before to 2 hours after mean sleep onset (MSO) there were no secretory pulses which rose above the 24-hour mean level in the normal subjects. This dormant period regularly ended between 2 and 3 hours after MSO, with the initiation of a series of major secretory pulses which continued throughout the remainder of the sleep period. Maximum concentrations were reached about 7 hours after MSO. A fall in plasma cortisol concentration was then seen in 4 out of the 5 subjects during the morning (16 to 13 hours before MSO). During the middle of the day, several secretory pulses were observed in each subject; none of these reached the values observed during the late sleep period. Day-time ratings of anxiety in these control subjects rarely rose above 2 on a scale of 1 low to 5 high. No incidents occurred which produced significant affective response; even mild apprehension about events in the environment was rare. The plasma concentration of cortisol was not significantly correlated with anxiety ratings in the twenty minutes immediately before blood sampling in the control subjects ($p > .05$). There was also no significant rank-order correlation between the mean plasma cortisol concentration (Table 1) and the psychiatric rating of these subjects on the scale of manifest display of emotional responses.

Figure 2 shows the mean pattern of plasma cortisol concentration in the 5 normal subjects. The circadian variation is readily apparent (at the expense of obscuring the pulsatile nature of the secretion) with a maximum plasma cortisol concentration of $16.9 \pm 3.9 \mu\text{g}/100 \text{ ml}$ (mean \pm SD) at 7 hours after MSO and a minimum level of $1.0 \pm 1.8 \mu\text{g}/100 \text{ ml}$ at 1 hour after MSO. Thus, although cortisol was secreted episodically, the average pattern of this group of individuals demonstrated a circadian rhythm.

Presurgical Patients. The patterns of plasma cortisol concentration in the four preoperative cardiac surgery patients are shown in Figure 3. Superimposed on each individual pattern is the mean pattern (\pm SD) of the normal subjects (from Figure 2). For most of the day the patterns of plasma cortisol concentration in the preoperative patients were very similar to those seen in the normal subjects, with a similar number of secretory episodes (Table 1). However, in Phase I, coincident with preoperative preparation (consisting of a complete chest, abdomen and leg shaving, antiseptic wash and enema--indicated in Figure 3 by a black bar with an "E" at the time of the enema) each patient had a major episode of cortisol secretion. Plasma cortisol concentration reached values that were between 6.9 and 10.5 standard deviations above the mean level for the control subjects at the corresponding time of day. The mean concentration for the presurgical patients during Phase I ($7.1 \mu\text{g}/100 \text{ ml}$) was 3.7 times higher ($p < .001$) than that of the normal subjects ($1.9 \mu\text{g}/100 \text{ ml}$) (Table 2). The difference in Phase I maximum concentrations (16.4 vs. $4.0 \mu\text{g}/100 \text{ ml}$) was also highly significant ($p < .001$), as was the difference between the two groups in the length of the dormant period ($p < .001$) (Table 1).

The elevations in plasma cortisol concentration coincident with preoperative preparation appeared to represent a discrete pulse of cortisol

secretion followed by a period of several hours with no further cortisol secretory pulses while the plasma concentration fell. The secretory pulse associated with preoperative preparation occurred during the period of the day when cortisol secretion was at a minimum in the control subjects.

It is difficult to separate out the influence of the different components of the presurgical preparation. For example, in one patient (W), the cortisol secretory episode associated with preoperative preparation preceded the enema, whereas in the other patients the enema either just preceded, or was coincident with the pulse of secretion. While a secretory episode always began during the period of preparation, no single component of that preoperative preparation (shaving, enema or antiseptic wash) showed a consistent temporal relationship with the timing of the secretory response.

Another major pulse of cortisol secretion appeared to be associated in patient X with the preoperative teaching procedure (shown by a "T" in Figure 3) in which the patient was instructed about the intensive care situation in which she would awaken after the operation. However, patients W and Z also experienced a similar preoperative teaching procedure and no major pulse of cortisol secretion immediately followed in either case, although patient W, who had that experience earlier in the day, did have a minor pulse afterward. Patient Y received no preoperative teaching because he had previously undergone the same operation a year earlier. It is interesting to note that he had the smallest peak of cortisol secretion in response to the preoperative shaving procedure.

Other pulses of cortisol secretion which occurred during the day could not be related consistently to potentially stressful events. For example, the insertion or reinsertion of an intravenous catheter (for this study or for laboratory tests which were performed on the patients--indicated in the Figures by the letters "IV") was occasionally, but not consistently,

followed by a pulse of cortisol secretion, but such pulses were unremarkable since cortisol concentration in those cases never rose above 2 standard deviations from the control subject mean. Similarly, times of high anxiety ratings could sometimes, but not consistently, be related to secretory episodes and no significant correlation could be detected between anxiety rating and plasma cortisol concentration ($p > .05$). Secretory episodes of similar magnitude and timing often occurred in both the control subjects and presurgical patients with no apparent psychogenic stimulus. In fact, inspection of the patterns either visually (Figures 1 and 3) or by phase statistics (Table 2) shows that the patterns of the presurgical patients were indistinguishable from those of the controls at all times except during preoperative preparation.

DISCUSSION

The twenty minute sampling procedure revealed an episodic 24-hour cortisol secretory pattern in both the control subjects and presurgical patients. The 24-hour patterns in the control subjects were consistent with previously published patterns of frequent plasma cortisol measurements in normal subjects (18-20,34,35).

The pattern of plasma cortisol concentration in the presurgical patients remained within the limits established for the normal controls for most of the preoperative day. This was in spite of (1) the mean age differences which existed between the two groups; (2) the fact that those in the control group were personal acquaintances of one of the authors whereas the presurgical patients were not; (3) differences in the time of catheter placement between the two groups; (4) the few medications noted in the methods section that were taken by the presurgical patients, but not the control subjects; and (5) most importantly, the many incidents during the presurgical studies which provoked overt and often verbal expressions of apprehension and anxiety relating to the patient's upcoming surgery. Furthermore, these patients who

had been facing the threat of the operation for some weeks did not have the kind of psychoendocrine activation of the adrenocortical axis which Sachar et al. have demonstrated in depressed patients (29). The presurgical patients neither had markedly elevated plasma cortisol concentrations throughout the day nor an increased number of daily secretory episodes (Table 1). This suggests that neither the discrete emotional stresses associated with the immediate presurgical situation nor the longer term anticipation of upcoming surgery resulted in hyperactivation of the hypothalamo-pituitary-adrenocortical axis. Most episodes of secretion that were observed in both the normal subjects and the presurgical patients could not be reliably correlated with environmental stimuli. These findings thus support the concept that even during the waking period, the episodic pattern of plasma cortisol concentration is part of an endogenous cyclical functioning of the pituitary-adrenocortical axis rather than a series of responses to intermittent environmental stimuli (19).

In contrast, there was a single event during the late evening that was consistently related to a major pulse of cortisol secretion in the presurgical patients. Preoperative preparation, which consisted of complete chest, abdomen and leg shaving, antiseptic wash, and an enema, induced a major pulse of cortisol secretion which raised the cortisol concentration between 6.9 and 10.5 standard deviations above the control subject mean values for that time of day. This response of the pituitary-adrenocortical axis occurred at whatever time in the evening each patient was preoperatively prepared. It therefore appears to have been induced by either the psychological or physiological components of that complex stimulus, since none of the differences between the two subject groups which were noted in the preceding paragraph could account for such a temporally related change in cortisol secretion.

It is possible that on the evening before open-heart surgery, body shaving

could provoke the acute focusing of diffuse and unconscious anxieties about an approaching surgical procedure, thus seriously challenging and perhaps temporarily overwhelming a patient's psychological defenses by confronting him with the reality and the immediacy of his forthcoming operation. This explanation would be consistent with previous observations on the parents of fatally-ill children during an acute challenge to psychological defenses (8). Alternatively, the preoperative preparation could act as a physiological stimulus since considerable non-specific sensory stimulation was involved although previous work has suggested that other late evening sensory stimuli do not always result in an increase in cortisol secretion (36). In either event, it would appear that while the normal pattern of episodic cortisol secretion is generated by an endogenous mechanism, additional secretory episodes can be specifically induced by episodes of stress.

The present study has shown that secretory episodes induced by environmental events during periods of normally minimal cortisol secretion can result in considerable disruption of the normal circadian distribution of plasma cortisol pulses. This finding may provide an explanation for the occurrence of circadian rhythm internal desynchronization in monkeys subjected to various stressors (37), and human subjects with a high neuroticism index who are placed in isolation (38). Moore Ede (39) has recently demonstrated that the circadian rhythm of plasma cortisol concentration plays an essential role in synchronizing circadian rhythms of electrolyte metabolism with the circadian rest-activity cycle. When the plasma cortisol circadian rhythm is eliminated by the continuous infusion of replacement corticosteroids in adrenalectomized human or animal subjects, circadian rhythms such as renal potassium excretion become desynchronized from the rest-activity cycle, and oscillate with their own free-running period. Environmentally-induced stresses which cause the circadian distribution of secretory episodes to approach a continuous series of secretory episodes

thus might cause the loss of the synchronizing cue normally provided by the plasma cortisol circadian rhythm. In this situation, those circadian oscillations in physiological variables which are normally synchronized by the plasma cortisol rhythm would begin to free-run, while other variables with circadian rhythms which are not dependent on the plasma cortisol rhythm would remain normally synchronized with environmental time cues. This postulated mechanism for the initiation of circadian internal desynchronization clearly requires further experimental test, but it is possible that this may be an important process in the pathophysiology of stress.

Another important conclusion from the present study is that frequent blood sampling for at least 24 hours must be used to define the influence of environmental variables on the pituitary-adrenal axis. The late evening pulses of secretion demonstrated herein would probably have been overlooked if samples had been taken infrequently or over limited periods of time. This explains why earlier studies of plasma cortisol concentration measured once or twice daily in presurgical patients (40-42) yielded inconsistent results. In the present study, the 8 AM mean cortisol concentration in our four preoperative patients was $11.6 \mu\text{g}/100 \text{ ml}$ (range 8.4 to 16.2), whereas the 8 AM mean of the normal volunteers was $14.0 \mu\text{g}/100 \text{ ml}$ (range 10.5 to 18.0)--statistics which fail to reflect the consistent differences which did exist between the two groups at a later time of day. Similarly, the 24-hour mean plasma cortisol levels did not indicate the differences between the two groups. Furthermore, it is clear that adequate analysis of the results of such frequent blood sampling must include statistical comparisons with a true control group at corresponding times of day; failure to do so adequately in an earlier study of multiply-sampled presurgical patients by Wise *et al.* led them to overlook the consistent and significant changes demonstrated in our study, which also, in retrospect, appear to have occurred in their patients before surgery (43).

In summary, in this study it has been possible to clarify the influence of environmental stimuli on the 24-hour secretion pattern of plasma cortisol by using the multiple frequent blood sampling technique. We have shown that the circadian pattern of plasma cortisol concentration consists of a sequence of episodic pulses which are normally unrelated to specific environmental stimuli, even in a situation in which there were many anxiety provoking events. However, major secretory pulses can reliably be superimposed on the endogenous cyclical pattern by certain acute environmental stimuli, such as preoperative surgical preparation.

Table 1. Analysis of plasma cortisol patterns over a full 24-hour period.

Subjects	Mean cortisol conc (μ g/100 ml)	Minimum cortisol conc (μ g/100 ml)	Maximum cortisol conc (μ g/100 ml)	# Secretory episodes	Length of quiescent period (hours)
A	6.1 \pm 4.2	0.2	17.3	7	9.0
B	6.3 \pm 4.9	0.0	13.9	7	8.9
C	8.3 \pm 4.9	0.4	21.5	5	9.3
D	7.2 \pm 4.8	0.0	18.6	7	9.0
E	8.1 \pm 5.8	0.0	18.0	7	9.7
Mean	7.2 \pm 1.0	0.1 \pm 0.2	17.9 \pm 2.7	6.6 \pm 0.9	9.2 \pm 0.3
W	9.6 \pm 4.7	2.1	20.5	7	7.3
X	9.8 \pm 4.4	4.6	27.2	7	4.7
Y*	7.9	3.1	16.0	7	4.7
Z	7.9 \pm 3.4	0.0	16.8	8	3.3
Mean	8.8 \pm 1.0	2.5 \pm 2.3	20.1 \pm 5.1	7.3 \pm 0.5	5.0 \pm 1.7
p value	NS	<.05	NS	NS	<.001

*Samples could not be collected for three hours (from 3 to 6 hours after MSO in subject Y; his values in this table are therefore based on the remaining 61 determinations.

NS-not significant ($p > .05$)

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Control

Presurgical

Table 2. Analysis of cortisol concentration ($\mu\text{g}/100 \text{ ml}$) during each phase of the 24-hour cycle.

Subject	Phase I (-4 to 2)*			Phase II (2 to 4)*			Phase III (4 to 9)*			Phase IV (-15 to -4)*		
	Mean \pm SD	Min	Max	Mean \pm SD	Min	Max	Mean \pm SD	Min	Max	Mean \pm SD	Min	Max
A	1.2 \pm 0.6	0.2	2.5	5.6 \pm 5.2	1.0	12.3	11.9 \pm 2.6	8.5	17.3	6.1 \pm 1.5	2.6	9.0
B	0.3 \pm 0.6	0.0	2.1	6.6 \pm 5.0	0.0	12.7	11.6 \pm 1.6	8.4	13.3	7.0 \pm 3.6	0.3	13.9
C	3.5 \pm 1.0	0.4	5.3	9.5 \pm 6.0	3.2	17.4	12.4 \pm 6.6	4.5	21.5	8.9 \pm 2.7	4.7	14.6
D	2.7 \pm 2.3	0.0	7.2	9.1 \pm 4.5	4.7	15.4	13.6 \pm 2.8	9.1	18.6	6.4 \pm 3.1	0.4	13.7
E	1.6 \pm 1.1	0.0	3.1	5.0 \pm 3.1	0.8	13.3	16.4 \pm 1.4	13.3	18.0	8.5 \pm 3.6	3.2	15.5
Mean	1.9 \pm 1.3	0.1	4.0	7.2 \pm 2.0	1.9	14.2	13.2 \pm 2.0	8.8	17.7	7.4 \pm 1.3	2.2	13.3
W	8.1 \pm 5.6	2.1	20.5	7.1 \pm 5.7	2.1	16.8	15.0 \pm 3.2	9.3	19.0	8.3 \pm 2.0	4.5	13.1
X	10.3 \pm 3.3	6.2	17.6	6.4 \pm 1.1	5.2	7.9	13.9 \pm 6.3	4.6	27.2	8.2 \pm 2.5	5.9	16.6
Y+	4.5 \pm 1.9	3.1	11.3	(7.7 \pm)			(11.5)			8.1 \pm 2.3	4.3	14.1
Z	5.4 \pm 3.9	0.0	16.8	10.5 \pm 3.8	6.6	16.1	9.9 \pm 3.2	5.4	15.4	7.9 \pm 2.1	2.6	12.7
Mean	7.1 \pm 2.3	2.9	16.6	7.9 \pm 1.8	4.6	13.6	12.6 \pm 2.3	6.4	20.5	8.1 \pm 0.2	4.3	14.1
p value	<.001	<.05	<.001	NS	NS	NS	NS	NS	NS	NS	NS	NS

*Hours before and after mean sleep onset (MSO).

NS=not significant ($p > .05$)

+ See footnote about missing samples for "Y" in Table 1.

FIGURE LEGENDS

- Figure 1. 24-hour plasma cortisol concentration patterns in five control subjects. Time of mean sleep onset during the preceding week is shown as zero hours on the time scale. The actual time of lights out and lights on of the experimental day are shown by downward and upward arrows, respectively, along with the clock (E.D.T.) time at those points.
- Figure 2. Mean and standard deviation of plasma cortisol concentration at 20 minute intervals for a 24-hour period in five control subjects.
- Figure 3. 24-hour plasma cortisol concentration patterns in four patients on the day prior to elective coronary artery bypass graft surgery superimposed over the mean pattern (\pm SD) from the control subjects (of Figure 2). Time of mean sleep onset is used as the common time reference, as in Figure 1; the times of lights out and waking are also similarly indicated. Symbols beneath each graph indicate the time when certain events occurred: "IV"-insertion of an intravenous catheter; "T"-preoperative teaching, which involved instruction designed to acquaint patients with what they should expect after surgery. The time of presurgical preparation is indicated by a horizontal bar, with the letter "E" specifically denoting the time of the preoperative enema.

enclosed separately

24 HOUR PLASMA CORTISOL PATTERNS IN CONTROL SUBJECTS

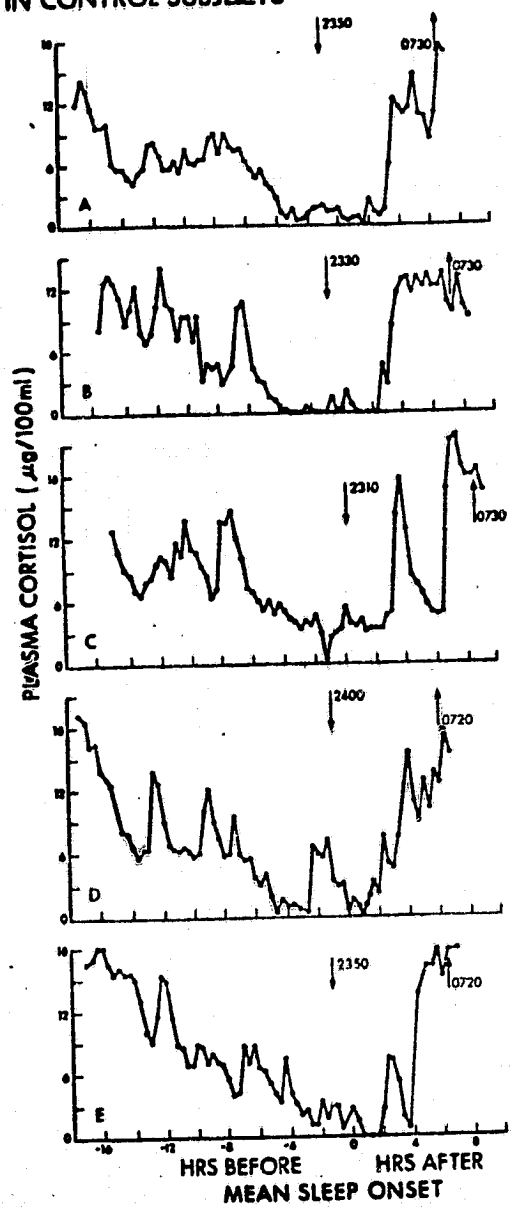


Figure 1

1954
Control
52

MEAN 24 HOUR PATTERN OF PLASMA CORTISOL CONCENTRATION IN 5 CONTROL SUBJECTS

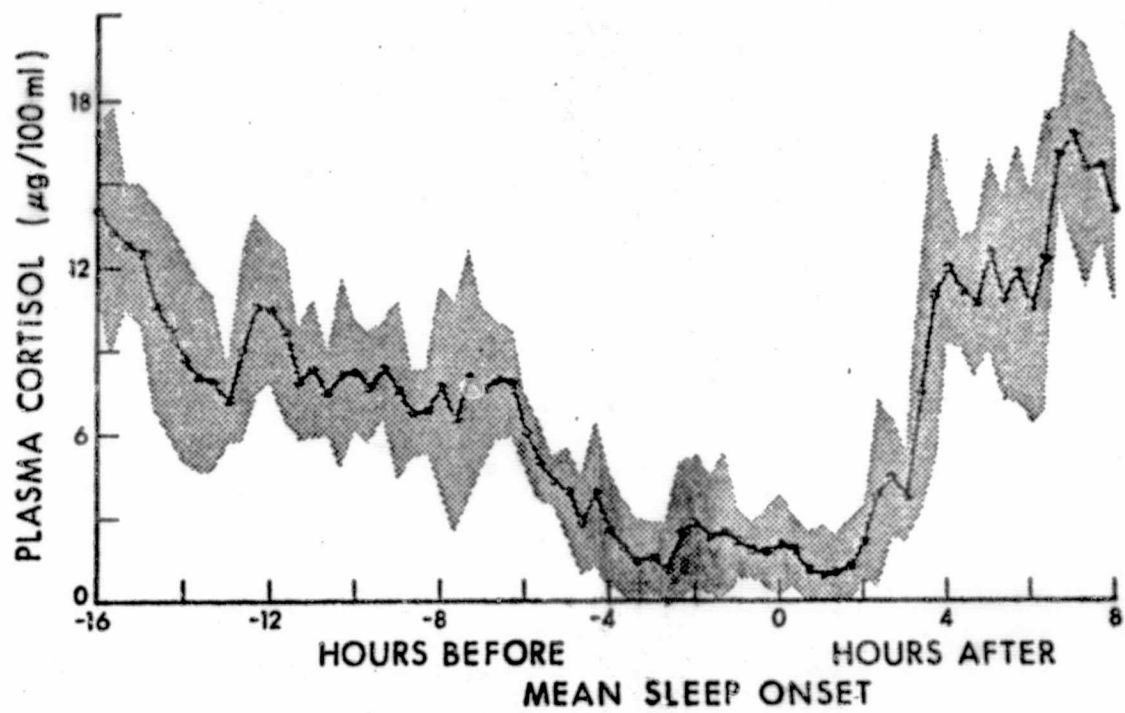
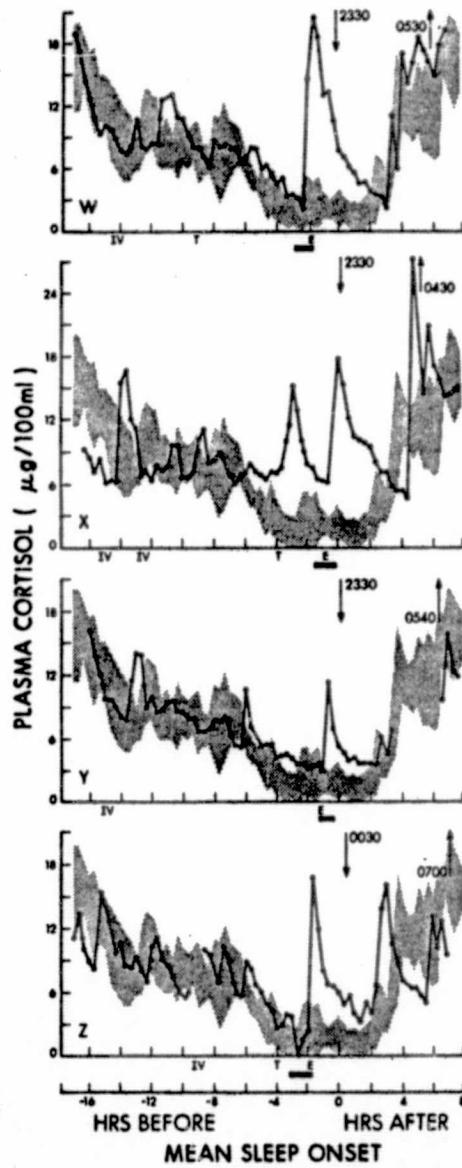


Figure 2

910554 p.m.
enclosed separately

24 HOUR PLASMA CORTISOL PATTERNS IN PREOPERATIVE PATIENTS



L-2

Figure 3

ACKNOWLEDGEMENTS

The authors wish to thank Doctors James C. Orr, Norman I. Gold, Gordon H. Williams and Francis D. Moore for their support and encouragement of this work. We are grateful to Doctors Toma Hoeksema and Luke Pascale for their permission and aid in the study of their patients. We also wish to express special thanks to Ms. Margaret R. Ball and Ms. Farida Siddiqui for their technical advice and help in the laboratory and Ms. Susan Ruane for her assistance in compiling the manuscript. We are indebted to the subjects who volunteered for these studies, and to the staffs of the Saint Francis Hospital in Chicago and the Peter Bent Brigham Hospital in Boston for their consistent cooperation.

1. Hamburg, D.A., Res Publ Ass Res Nerv Ment Dis 40:406, 1962.
2. Mason, J.W., Psychosom Med 30:576, 1968.
3. Williams, R.H., Textbook of Endocrinology, ed. 5, W.B. Saunders Co., Philadelphia, 1974, p. 240.
4. Donovan, B.T., Mammalian Neuroendocrinology, McGraw-Hill, London, 1970, p.65.
5. Ganong, W.F., Review of Medical Physiology, ed. 6, Lange Medical Publications, Los Altos, California, 1973, p. 282.
6. Bourne, P.G., R.M. Rose, and J.W. Mason, Arch Gen Psychiat 17:104, 1967.
7. Bourne, P.G., R.M. Rose, and J.W. Mason, Arch Gen Psychiat 19:135, 1968.
8. Friedman, S.B., J.W. Mason, and D.A. Hamburg, Psychosom Med 25:364, 1963.
9. Mason, J.W., E.J. Sachar, J.R. Fishman, D.A. Hamburg, and J.H. Handlon, Arch Gen Psychiat 13:1, 1965.
10. Katz, J.L., H. Weiner, T.F. Gallagher, And L. Hellman, Arch Gen Psychiat 23:131, 1970.
11. Wadeson, R.W., J.W. Mason, D.A. Hamburg, and J.H. Handlon, Arch Gen Psychiat 9:146, 1963.
12. Bliss, E.L., C.J. Migeon, C.H.H. Branch, and L.T. Samuels, Psychosom Med 18:56, 1956.
13. Persky H., R.R. Grinker, D.A. Hamburg, M.A. Sabshin, S.J. Korchin, H. Basowitz, and J.A. Chevalier, Arch Neurol Psychiat 76:549, 1956.
14. Fiorica, V., and S. Muehl, Psychosom Med 24:596, 1962.
15. Fox, H.M., S. Gifford, A.F. Valenstein, and B.J. Murawski, J Psychosom Res 14:71, 1970.
16. Bloch, S., and C. J. Brackenridge, J Psychosom Res 16:25, 1972.
17. Wolff, C.T., S.B. Friedman, M.A. Hofer, and J.W.Mason, Psychosom Med 26:576, 1964.
18. Weitzman, E.D., H. Schaumburg, and W. Fishbein, J Clin Endocrinol Metab 26:121, 1966.
19. Weitzman, E.D., D. Fukushima, C. Nogeire, H. Roffwarg, T.F. Gallagher, and L. Hellman, J.Clin Endocrinol Metab 33:14, 1971.
20. Hellman, L., F. Nakada, J. Curti, E.D. Weitzman, J. Kream, H. Roffwarg, S. Ellman, D.K. Fukushima, and T.F. Gallagher, J Clin Endocrinol Metab 30:411, 1970.

21. Gallagher, T.F., K. Yoshida, H.D. Roffwarg, D.K. Fukushima, E.D. Weitzman, and L. Hellman, J Clin Endocrinol Metab 36:1058, 1973.
22. Mills, J.N., Physiol Rev 46:128, 1966.
23. Nichols, C.T., and F.H. Tyler, Annu Rev Med 18:313, 1967.
24. Perkoff, G.T., K. Eik-Nes, C.A. Nugent, H.L. Fred, R.A. Nimer, L. Rush, L.T. Samuels, and F.H. Tyler, J Clin Endocrinol Metab 19:432, 1959.
25. Bodley, P.O., H.V.R. Jones and M.D. Mather, J Neurol Neurosurg Psychiat 37:230, 1974.
26. Fox, H.M., B.J. Murawski, A.F. Bartholomay, and S. Gifford, Psychosom Med 23:33, 1961.
27. Davis, J., R. Morrill, J. Fawcett, V. Upton, P.K. Bondy, and H. M. Spiro, J Psychosom Res 6:83, 1962.
28. Rose, R.M., and M.W. Hurst, J Human Stress 1:22, 1975.
29. Sachar, E.J., L. Hellman, H.P. Roffwarg, F.S. Halpern, D.K. Fukushima, and T.F. Gallagher, Arch Gen Psychiat 28:19, 1973.
30. Murphy, B.E.P., J Clin Endocrinol Metab 27:973, 1967.
31. Rosenfield, R.L., W.R. Eberlein, and A. M. Bongiovanni, J Clin Endocrinol Metab 29:854, 1969.
32. Orth, D.N., D.P. Island, and G.W. Liddle, J Clin Endocrinol Metab 27:549, 1967.
33. Weitzman, E.D., D.G. Kripke, J. Kream, P. MacGregor, and L. Hellman, Psychophysiology 7:307, 1970.
34. deLacerda, L., A Kowarski, and C.J. Migeon, J Clin Endocrinol Metab 36:227, 1973.
35. Krieger, D.T., W. Allen, F. Rizzo, and H. P. Krieger, J Clin Endocrinol Metab 32:266, 1971.
36. Ismail, A.A.A., D.W. Davidson, J.A. Loraine, and C.A. Fox, Nature 237:288, 1972.
37. Stroebel, C.F., Bibl. Primat 9:91, 1969.
38. Lund, R., Psychosom Med 36:224, 1974.
39. Moore Ede, M.C., Control of Circadian Oscillations in Renal Potassium Excretion in the Squirrel Monkey, Ph.D. Thesis, Harvard University, 1974.
40. Franksson, C., and C.A. Gemzell, J Clin Endocrinol Metab 15:1069, 1955.
41. Price, D.B., M. Thaler, and J.W. Mason, Arch Neurol Psychiat 77:646, 1957.
42. Bursten, B., and J.J. Russ, Psychosom Med 27:309, 1965.
43. Wise, L., H.W. Margraf, and W.F. Ballinger, Surgery 72:290, 1972.

**INTERNAL ORGANIZATION OF THE CIRCADIAN
TIMING SYSTEM IN MULTICELLULAR ANIMALS**

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ABSTRACT

Three models of the organization of the circadian timing system in multicellular animals are presented. Each can account for the observed internal synchronization of the various circadian rhythms within the organism and each is also compatible with the known responses of circadian systems to manipulations of environmental time cues. One is a single oscillator system (Model I) while the other two are multioscillator systems arranged in a hierarchical (Model II) or non-hierarchical (Model III) manner. Experiments which test the predictions of the different models are reviewed. These indicate that the circadian timing system in mammals is organized as a multioscillator system with oscillating concentrations of chemical mediators (nervous or endocrine) internally synchronizing the various potentially-independent oscillators by an entrainment mechanism. However, as yet, there is insufficient evidence to indicate whether the oscillators are arranged with a predominantly hierarchical (Model II) or non-hierarchical (Model III) organization.

Key Words: Circadian rhythms, internal synchronization, models, biological oscillators, nervous and endocrine mediators

Circadian rhythms in biological variables are one outward manifestation of an important evolutionary adaptation to life on a rotating planet: the ability to measure time. This capability enables organisms to predict the major changes in environmental conditions, and the consequent alterations in food supply and predator activity, which occur with a 24 hour periodicity because of the earth's rotation (9). Thus, for example, adaptive physiological and behavioral responses which may take several hours to be activated can be initiated in advance of the predicted environmental challenge, or events where timing may be critical for survival such as emergence in flies, can be timed to occur at the point of maximum environmental advantage (23).

There is now considerable evidence to indicate that such circadian time measurement is the product of an oscillating system within the organism (25,15). The responses of this oscillating system to manipulations in environmental time cues are now well established (24,3), but current knowledge of the anatomical and physiological organization of the circadian timing system within advanced multicellular organisms such as mammals is still very limited.

In most studies of the responses of the circadian timing system to environmental periodicities the assumption has been made that the system acts as a single self-sustained oscillator and that an endogenous circadian rhythm in any single physiological variable may be used as a marker for the response of the system. Thus, the response of only one variable, such as the rest-activity rhythm (3), has often been used in attempts to define the properties of the oscillating system.

This assumption has usually been accepted because in most steady-state situations within an individual subject the various oscillating physiological variables with a circadian period are "internally synchronized" with one another. This means that they demonstrate identical periods and stable phase relationships as if they were generated by a single oscillator.

For example, in Figure 1 the phase-relationships of the circadian rhythms of urinary potassium excretion and body temperature are plotted for one human subject who was entrained by a normal 24-hour environmental regimen (26) and for another subject who was demonstrating free-running rhythms in the absence of environmental time cues (5). In both subjects the various circadian rhythms which were monitored under a given set of environmental conditions demonstrated identical periods and stable phase relationships.

(Fig 1)

Mills has suggested (17,18) that the phenomenon of internal synchronization implies that the circadian timing system consists of a single self-sustained oscillator and that circadian rhythms represent passive responses of physiological systems to an oscillating driving force transmitted from the driving oscillator, or "circadian clock". However, it is not necessary to conclude that there is only one oscillator. A multioscillator system would also be compatible with the observed properties of the circadian system provided that the various oscillators were coupled with one another so that internal synchronization was maintained. It is the purpose of this paper to review three alternative models of the circadian timing system which can account for the phenomenon of internal synchronization while at the same time being compatible with the known responses of circadian systems in multicellular animals to manipulations of environmental time cues.

ALTERNATIVE MODELS OF THE CIRCADIAN TIMING SYSTEM

Three models of the circadian timing system are presented in Figure 2. Minor variants of these models, or combinations of their features are also possible, but the models presented here emphasize the contrasts between certain possible organizations of the circadian system.

(Fig 2)

Model I, which has been proposed by Mills (17,18) but has been assumed in many other investigations of the circadian timing system, consists of a network of cellular systems (A,B,C,...,etc.) which passively oscillate as

a forced response to a single self-sustained driving oscillator (D.O.). Where these cellular units are non-contiguous in a multicellular animal, the model requires that oscillating levels of physical or chemical mediators be postulated (a,b,c,...,etc.), with the period of D.O. but not necessarily the same phase. These mediating systems, which would presumably be nervous (neurotransmitter release) or endocrine (hormonal concentration), would transmit the forced oscillating response to D.O. to the various passively responding cellular units. The entire circadian system would be entrained by environmental time cues via exteroceptive sensory inputs to the driving oscillator.

Model II describes a network of cellular units which are each themselves self-sustained oscillators, able to maintain oscillations with an independent period in the absence of periodic inputs. One oscillator (D.O.) acts as a pacemaker and is entrained by exteroceptive sensory inputs from environmental time cues. As in Model I it is necessary to postulate oscillating nervous or endocrine mediators which maintain synchronization within the animal. However, the mediators in this model actively entrain the self-sustained cellular oscillators in a manner similar to the entrainment of the organism's circadian system by cycles of environmental illumination (3).

Model III also describes a multioscillator model but in this case no one oscillator consistently acts as a pacemaker. Instead the various exteroceptive sensory inputs entrain different oscillators. Internal synchronization within the system is maintained by the positive and negative feedback action of mediators (a,b,c,...,etc.) on the separate oscillating units (A,B,C,...,etc.). As in Model II, the mediators synchronize the oscillators by active entrainment.

SINGLE OR MULTIOSCILLATOR SYSTEM?

There are several ways in which it is possible to differentiate between the single oscillator system (Model I) and a multiple oscillator system (Models II or III). The evidence is strongly suggestive that the circadian timing function is a product of a multioscillator system.

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A. Spontaneous Internal Desynchronization. There are two main situations in which internal desynchronization between different circadian rhythms in the same animal have been observed. Firstly, in both experimental animals and man, rhythms in certain physiological variables take longer to resynchronize after a phaseshift of the environmental light-dark cycle than do others (10,12,14), so that the various monitored circadian rhythms demonstrate different transient periods and altered phase relationships during resynchronization and hence are temporarily internally desynchronized. Secondly, Aschoff and his colleagues (5) have demonstrated that while internal synchronization is normally observed between a wide variety of circadian rhythmic functions in men studied under constant isolation conditions with no time cues, 15% of their subjects demonstrate spontaneous internal desynchronization with the different rhythmic variables oscillating with independent free-running periods within the same subject. An example of this phenomenon is illustrated in Figure 3. In this subject the circadian rhythms of rest-activity and urinary calcium excretion spontaneously began to oscillate with a period of 32.6 hours while the rhythm of body temperature, urinary potassium and water excretion continued to oscillate with a period of 24.7 hours. Thus, the circadian rhythms in these two groups of variables became spontaneously desynchronized from one another. These demonstrations of internal desynchronization between the circadian rhythms within an organism are incompatible with a single oscillator model but would be predicted by a model, such as II or III in Figure 2, to occur whenever the coupling information between individual oscillators was lost.

Fig 3

B. Mode of Synchronization by Chemical Mediators. Both the single oscillator system and the multiple oscillator systems require the postulation that there are oscillating concentrations of chemical mediators which synchronize the potentially-independent oscillators in non-contiguous tissues. However, the characteristics of the mediating process in Model I differs considerably from that which would be predicted in Models II and III,

and this offers a critical test between the models.

Model I as defined by Mills (17,18) predicts that the oscillating chemical output "e" of cell unit "E", which is forced by the oscillation "b" will have the following characteristics: 1) that a change in the level of "b" must induce an equivalent change in "e" at any time in the 24-hour day; 2) that "e" must cease to oscillate if "b" is maintained at a constant level; 3) phaseshifting "b" will produce an equal and immediate phaseshift in "e"; 4) the normal variations in "b" over the course of the 24 hour day must be appropriately large to induce the circadian variation in "e".

In contrast, Models II or III would predict that: 1) changes in the level of "b" will have variable effects on "E" (and, hence, "e") depending on the circadian phase of the change (i.e., there will be a characteristic phase-response function); 2) if "b" is maintained at a constant level, oscillator "E" will yield a free-running rhythm in "e" which is no longer synchronize to other circadian rhythms; 3) a phaseshift in "b" will result in a phaseshift in "e" but only after a transient response; 4) the normal daily variations in "b" will not necessarily be of a size sufficient to passively induce variations in the level of "e".

To examine these predictions of the single oscillator and multioscillator models, we have studied the process by which the circadian rhythm in plasma cortisol concentration synchronizes the circadian rhythm of urinary potassium excretion in the squirrel monkey (Saimiri sciureus) (20,21). In adrenalectomized, chair-acclimatized squirrel monkeys maintained in isolation it was demonstrated that a phaseshift in the time of administration of physiological doses of cortisol resulted in a phaseshift of the rhythm of urinary potassium excretion (Figure 4). Thus, the plasma cortisol rhythm appeared to be the dominant synchronizer of the urinary potassium rhythm in this animal. However, it can be seen that the phase of the urinary potassium rhythm did not immediately reset to the new phase of the plasma cortisol

Fig 4

0

rhythm. Instead, there was a transient period while the urinary potassium rhythm resynchronized slowly over approximately 5 days. This finding suggests that the rhythm in urinary potassium excretion does not passively follow the oscillations in plasma cortisol concentration, but instead is generated by a system which is capable of oscillating spontaneously, although normally actively entrained by the cortisol circadian rhythm.

To examine this question further a bolus of 15 mg cortisol was given between 20.00 and 23.00 hr to squirrel monkeys with intact adrenal glands (Figure 5). This dose, which was sufficient to raise the plasma cortisol concentration to a level comparable or higher than the normal morning maximum of plasma cortisol concentration, did not induce a second elevation in urinary potassium excretion on the day of treatment. This again would indicate that the oscillation in urinary potassium excretion does not passively respond to the daily fluctuations in plasma cortisol concentration.

The final critical test was to eliminate all circadian oscillations in the mediator. In Figure 6 the circadian rhythms of urinary potassium excretion for three adrenalectomized monkeys are plotted during two control days of cortisol and aldosterone treatment at 08.00 hr daily and then for up to 7 days of infusion of the same 24 hour dose of cortisol and aldosterone, continuously administered throughout day and night. According to Model I this should result in the elimination of the urinary potassium rhythm, but according to Models II and III this would result in the appearance of a free-running oscillation in urinary potassium excretion. In each of these animals urinary potassium excretion continued to oscillate with an independent period which was clearly different from 24 hours. Fourier analysis has demonstrated this free-running period is always shorter than 24 hours; for example, in monkey E this period was approximately 14 hours.

These studies indicated that the circadian rhythm of urinary potassium excretion appears to be generated by a potentially-independent self-sustained oscillator which is synchronized with the light-dark cycle and with other oscillators within the animal through entrainment by the circadian rhythm in plasma cortisol concentration. These results were not compatible with a single oscillator system proposed in Model I and were instead suggestive of a multiple oscillator system as outlined in Models II and III.

C. Isolation of Circadian Oscillators in Vitro. If there are circadian oscillators in peripheral tissues which are organized in the manner suggested in Models II or III, then it should be possible to demonstrate free-running circadian oscillations in tissues maintained in vitro. This has now been achieved by several investigators. For example, free-running rhythms have been demonstrated in vitro in isolated adrenal glands (1,29), cardiac muscle (31) and liver (27). In Figure 7, the circadian rhythm of corticosteroid secretion by isolated hamster adrenal glands in vitro maintained under constant levels of ACTH is shown. In the lower two panels of Figure 7 it can be seen that the rhythm can be phaseshifted by a pulse of ACTH when it is applied at certain phases of the circadian cycle but not when applied at others. This is suggestive of a phase-response relationship of the adrenal cortex to ACTH administration. This would be predicted as a general feature of the entrainment of any self-sustained oscillator and has been demonstrated for the response of circadian oscillatory systems to manipulations of environmental lighting regimens (3).

Fig 7

HIERARCHICAL OR NON-HIERARCHICAL SYSTEM?

The evidence presented in the previous section indicates that the circadian timing system is a multioscillator system, as depicted in Models II or III, rather than a single oscillator system as described in Model I. Unfortunately, only limited evidence is available which can help us differentiate between Models II and III.

The essential difference between Model II and Model III is that the former is a hierarchical system with a single oscillator, or synchronous group of oscillators in a single anatomical location, acting as a pacemaker to the system, whereas the latter has no such single pacemaker and instead relies on the mutual synchronization of a number of self-sustained oscillating systems, in various anatomical locations, which determine the timing of the circadian system within the organism.

One testable prediction, which would differentiate between Models II and III, is that the hierarchical model (II) requires that all exteroceptive sensory inputs from environmental time cues be routed through the driving oscillator. The period and phase of the driving oscillator, which would thus be influenced by external time cues, would then determine the period and phase of the other spontaneous oscillators within the animal. In Model III, however, the various exteroceptive inputs would entrain separate oscillators within the organism. Thus, it should be possible, if Model III were correct, to entrain different oscillators, by separate time cues, such as the environmental rhythms in illumination (24), temperature (28), sound (16), social cues (4), or food availability (22). If this were so, then the individual oscillators might be manipulated separately by controlling the phase and period of the different environmental cues. The effectiveness of this approach would depend on the strength of the coupling between environmental cycles and internal oscillators, versus the strength of the mutual coupling between the various internal oscillators.

There is yet little evidence to indicate whether separate oscillators within an organism can be entrained by different time cues. Experiments where the coupling influences of more than one environmental time cue (such as environmental temperature and lighting cycles) have been examined have usually only monitored one rhythmic variable within the organism (8). Where rhythms in several variables have been monitored simultaneously there is

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some evidence that the phase of certain circadian rhythms are synchronized by one zeitgeber (such as the rhythm of corneal mitosis by the light-dark cycle) while other circadian rhythms within the same animal are synchronized by another (such as the circadian rhythm of serum corticosterone by the feeding regimen)(22). However, in such studies it has not been demonstrated how much of the "synchronization" is a passive response and how much is due to entrainment by an exogenous zeitgeber. It will be necessary to demonstrate that the new phase-relationships of the separately-synchronized variables persist for at least several cycles after the periodicities in the individual zeitgebers are eliminated.

Another approach to the differentiation between Models II and III is to attempt to identify the driving oscillator (D.O.), or "pacemaker" which Model II requires. Several investigators have tried to locate central nervous system oscillators which control an animal's circadian rhythms (6, 7, 11, 13, 19, 30). However, usually only one or two rhythmic variables have been monitored simultaneously and attempts have not been made to investigate whether internal desynchronization occurs between the various oscillating systems in the animal after lesions are placed. It is possible that studies, such as those of Stephan and Zucker (30), which demonstrated that supra-chiasmatic lesions in rats cause the loss of the rest-activity rhythm, may have located the site of the oscillators which control only the circadian rhythms in rest and activity. More elaborate studies will have to be done before the presence or absence of a driving oscillator can be asserted with any certainty.

In conclusion, although a single oscillator system may be ruled out, it is not possible with currently available evidence to differentiate between the alternate multioscillator systems described in Models II and III. Direct studies must be designed to answer this question. In addition, the localization and characterization of the individual oscillators within the organism must be accomplished before the morphology and physiology of

the intact circadian timing system can be understood in any detail.

REFERENCES.

1. ANDREWS, R.V. Temporal secretory response of cultured hamster adrenals. Comp Biochem Physiol 26: 179, 1968.
2. ASCHOFF, J. Circadian rhythms in man. Science 148: 1427, 1965.
3. ASCHOFF, J. Response curves in circadian periodicity. In: Circadian Clocks, edited by J. Aschoff, Amsterdam, North Holland Publishing Co., 1965, p. 95.
4. ASCHOFF, J. Desynchronization and resynchronization of human circadian rhythms. Aerosp Med 40: 844, 1969.
5. ASCHOFF, J., U. GERECKE, and R. WEVER. Desynchronization of human circadian rhythms. Jpn J Physiol 17: 450, 1967.
6. BALAGURA, S., R.H. WILCOX, and D.V. COSCINA. The effect of diencephalic lesions on food intake and motor activity. Physiol Behav 4: 629, 1969.
7. BLACK, I.B., and D.J. REIS. Central neural regulation by adrenergic nerves of the daily rhythm in hepatic tyrosine transaminase activity. J Physiol (London) 219: 267, 1971.
8. BRUCE, V.G. Environmental entrainment of circadian rhythms. Cold Spring Harbor Symp Quant Biol 25: 29, 1960.
9. CLOUDSLEY-THOMPSON, J.L. Rhythmic Activity in Animal Physiology and Behavior. New York, Academic Press, 1961, p. 38.
10. ELLIOTT, A.L., J.N. MILLS, D.S. MINORS, and J.M. WATERHOUSE. The effect of real and simulated time-zone shifts upon the circadian rhythms of body temperature, plasma 11-hydroxycorticosteroids and renal excretion in human subjects. J Physiol (London) 221: 227, 1972.
11. HALASZ, B., M.A. SLUSHER, and R.A. GORSKI. Adrenocorticotrophic hormone secretion in rats after partial or total deafferentation of the medial basal hypothalamus. Neuroendocrinology 2: 43, 1967.
12. HAUS, E., and F. HALBERG. Phase-shifting of circadian rhythms in rectal temperature serum corticosterone and liver glycogen of the male C-mouse. Russ Neurol Veg 23: 83, 1969.
13. KAKOLWESKI, J.W., E. DEAUX, J. CHRISTENSEN, and B. CASE. Diurnal patterns in water and food intake and body weight changes in rats with hypothalamic lesions. Am J Physiol 221: 711, 1971.
14. MARTEL, P.J., G.W.G. SHARP, S.A. SLORACH, and H.J. VIPOND. A study of the roles of adrenocortical steroids and glomerular filtration rate in the mechanism of the diurnal rhythm of water and electrolyte excretion. J Endocrinol 24: 159, 1962.
15. MENAKER, M. Aspects of the physiology of circadian rhythmicity in the vertebrate central nervous system. In: The Neurosciences, edited by F.O. Schmidt and F.G. Worden, Cambridge, M.I.T. Press, 1974, p. 479-489.

16. MENAKER, M. and A. ESKIN. Entrainment of circadian rhythms by sound in Passer domesticus. Science 154: 1579, 1966.
17. MILLS, J.N. Human circadian rhythms. Physiol Rev 46: 128, 1966.
18. MILLS, J.N. Transmission processes between clock and manifestations. In: Biological Aspects of Circadian Rhythms, edited by J.N. Mills, New York, Plenum Press, 1973, p. 27.
19. MOORE, R.Y., and H.B. QAVI. Circadian rhythm in adrenal adeny cyclase and corticosterone abolished by medial forebrain bundle transection in the rat. Experientia 27: 249, 1971.
20. MOORE EDE, M.C. Control of Circadian Oscillations in Renal Potassium Excretion in the Squirrel Monkey (Saimiri sciureus), Harvard University, Ph.D. Thesis, 1974.
21. MOORE EDE, M.C., W.S. SCHMELZER, D.A. KASS, and J.A. HERD. Circadian timing system: organization of multiple oscillators synchronized by chemical mediators. Submitted to Science, 1975.
22. NELSON, W., L. SCHEVING, and F. HALBERG. Circadian rhythms in mice fed a single daily meal at different stages of lighting regimen. J Nutr 105: 171, 1975.
23. PITTENDRIGH, C.S. On temperature independence in the clock system controlling emergence time in Drosophila. Proc Natl Acad Sci 40: 1018, 1954.
24. PITTENDRIGH, C.S. On the mechanism of the entrainment of a circadian rhythm by light cycles. In: Circadian Clocks, edited by J. Aschoff, Amsterdam, North Holland Publishing Co., 1965, p. 277.
25. PITTENDRIGH, C.S. Circadian oscillations in cells and the circadian organization of multicellular systems. In: The Neurosciences, edited by F.O. Schmidt and F.G. Worden, Cambridge, M.I.T. Press, 1974, p. 437.
26. REINBERG, A. Biological rhythms of potassium metabolism. In: Potassium in Biochemistry and Physiology, Berne, International Potash Institute, 1971, p. 160.
27. RINTOUL, D. Enzyme oscillations in cultured rat liver cells. Presented at Symposium of Physiological and Biochemical Aspects of Circadian Rhythms, 59th Annual Meeting of Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1975.
28. ROBERTS, S.K. Circadian activity rhythms in cockroaches. 2. Entrainment and phase-shifting. J Cell Comp Physiol 59: 175, 1962.
29. SHIOTSUKA, R., J. JOVONOVICH and J.A. JOVONOVICH. In vitro data on drug sensitivity: circadian and ultradian corticosterone rhythms in adrenal organ cultures. In: Chronobiological Aspects of Endocrinology, edited by J. Aschoff, F. Ceresa and F. Halberg, New York, Schaffauer-Verlag, 1974, p. 255.
30. STEPHAN, F., and I. ZUCKER. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. Proc Natl Acad Sci USA 69: 1583, 1972.

31. THARP, G.D. and GE. FOLK, JR. Rhythmic changes in rate of the mammalian heart and heart cells during prolonged isolation. Comp Biochem Physiol 14: 255, 1964.

FIGURE LEGENDS

Figure 1 The cumulative phaseshifts of the circadian rhythms in body temperature and urinary potassium excretion in a) a normal human subject demonstrating free-running circadian rhythms (●, ▲) while isolated from environmental time cues, and b) a normal subject studied while synchronized to a 24-hour environmental cycle (○, △). Replotted data from Aschoff (5) and Reinberg (26) with permission.






Figure 2 Three alternative models of the mammalian circadian timing system. The symbol  represents an active cellular unit capable of maintaining a self-sustained oscillation with its own independent period;  represents a cellular unit that responds passively to an oscillating driving force;  indicates the oscillating concentration of a chemical mediator;  indicates the entrainment of a self-sustained oscillator by a phase-response mechanism; and  is the direction of flow of passive responses to an oscillating driving force. Model I is therefore a single oscillator system whereas the other models are multioscillator systems arranged in a hierarchical (Model II) or non-hierarchical (Model III) manner.

Figure 3 Internal desynchronization of the circadian rhythms of a human subject studied in the absence of environmental time cues. From Day 3 the timing of the activity rhythm (black bar) and the maxima of the rhythm of urinary calcium excretion (△) demonstrated a spontaneously free-running period of 32.6 hours, whereas the maxima of the rhythms of body temperature (●), urinary potassium excretion (○) and urinary water excretion (x) demonstrated a 24.7 hour period. From Aschoff (2) with permission; copyright 1965 by the American Association for the Advancement of Science.

Figure 4 Phaseshifts of the rhythms of urinary potassium excretion (solid line) and feeding (interrupted line) in response to an eight-hour phase-delay of the time of cortisol administration in adrenalectomized squirrel monkeys. The light-dark cycle phase was kept unchanged throughout the experiment. All

animals continued to feed with a rhythm synchronized to the light-dark cycle, but the rhythm of urinary potassium excretion resynchronized with the new phase of cortisol administration.

Figure 5 Mean \pm SEM response of urinary potassium excretion in four intact squirrel monkeys to the administration of 15 mg cortisol between 20.00 and 23.00 hrs on Day 2. No second peak in urinary potassium excretion was seen which was comparable to the normal circadian maximum between 13.00 and 17.00 hrs.

Figure 6 Rhythms of urinary potassium in three adrenalectomized monkeys which received 5 mg cortisol and 0.001 mg aldosterone administered intravenously each day. Initially, the cortisol and aldosterone were administered in a single injection at 08.00 hr and then from Day 3 onward were given throughout the whole day and night in a continuous intravenous infusion. In each monkey urinary potassium excretion began to oscillate with a period of less than 24 hours when cortisol and aldosterone were given continuously.

Figure 7 Persisting rhythms of corticosteroid secretion by isolated hamster adrenal glands studied in vitro. The top panel displays the rhythm in untreated control glands whereas the lower two panels present the data from glands treated with a single pulse of 1.0 i.u. ACTH at the times indicated by arrows. Note that a phaseshift of the corticosteroid rhythm was only obtained when ACTH was administered at a certain phase of the adrenal cycle, just before the maximum of adrenocortical secretion. From Menaker (15) with permission (M.I.T. Press) after Andrews.

Figure 1

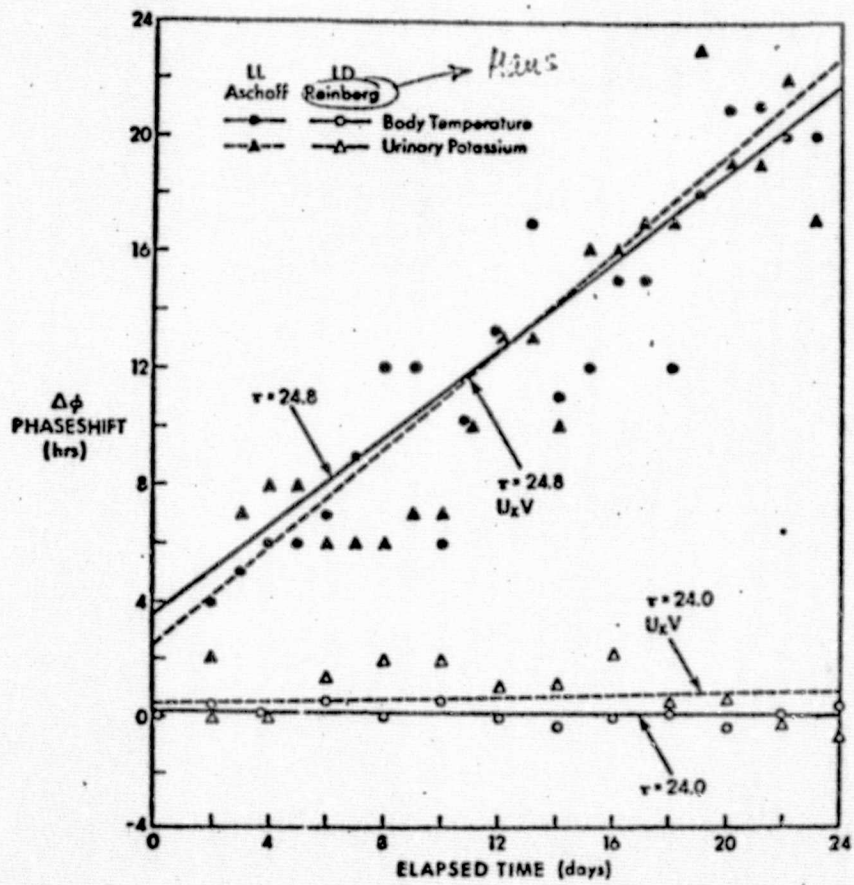


Figure 2

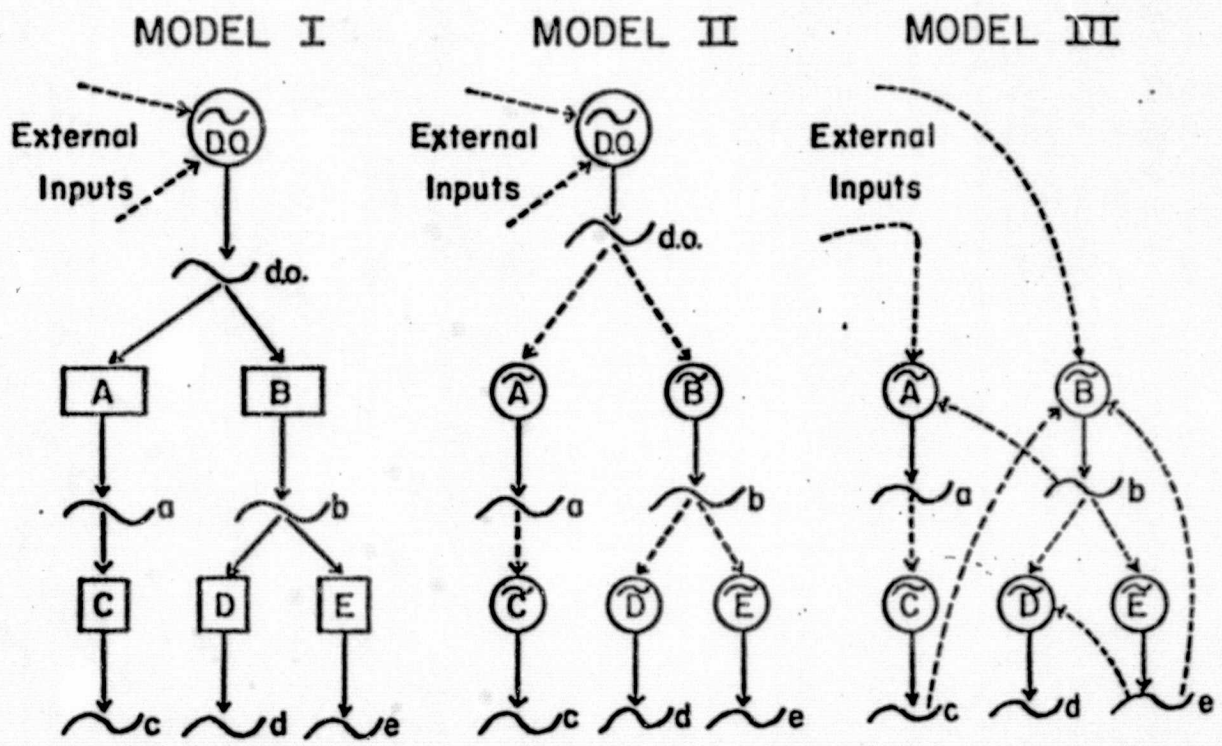


Figure 3

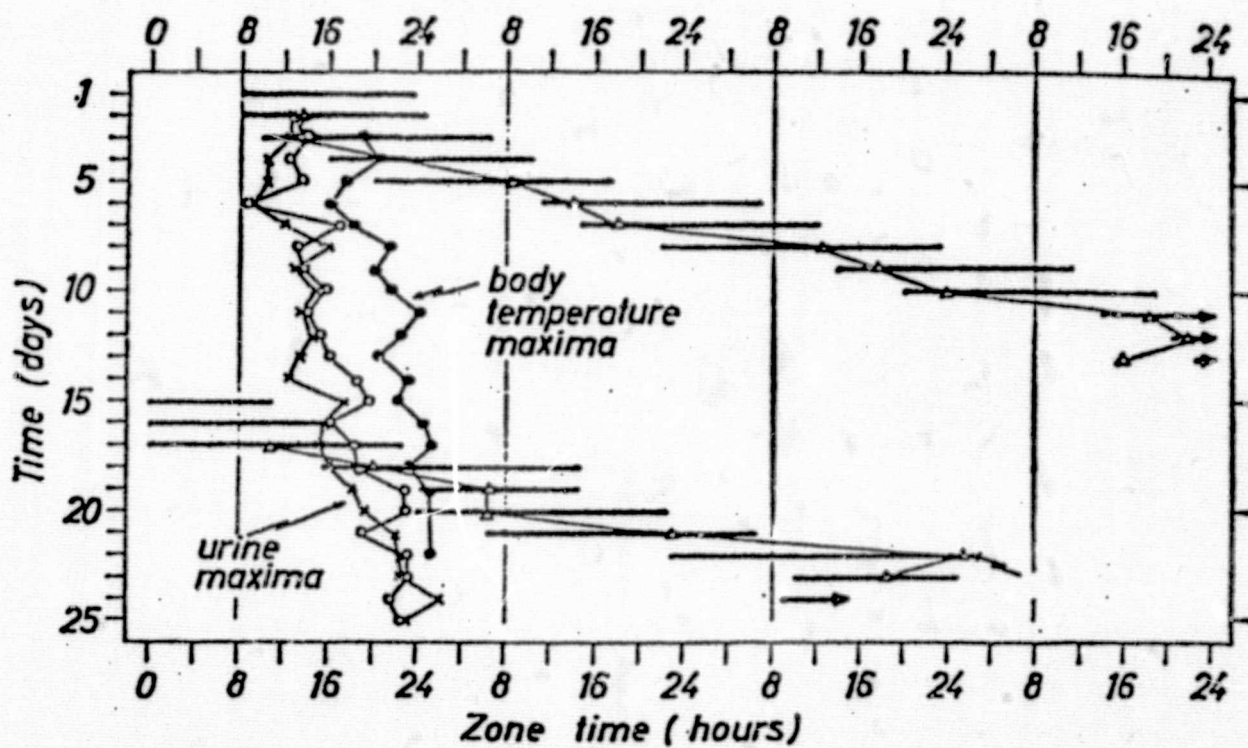


Figure 4

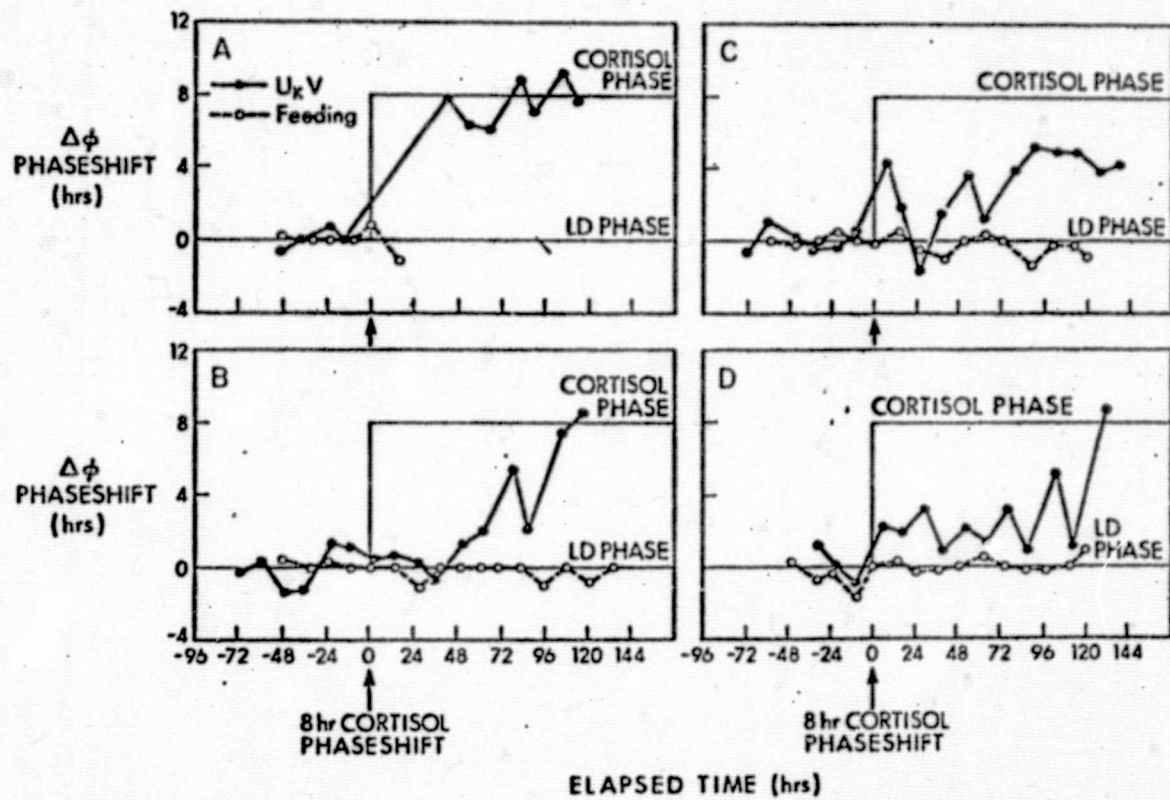


Figure 5

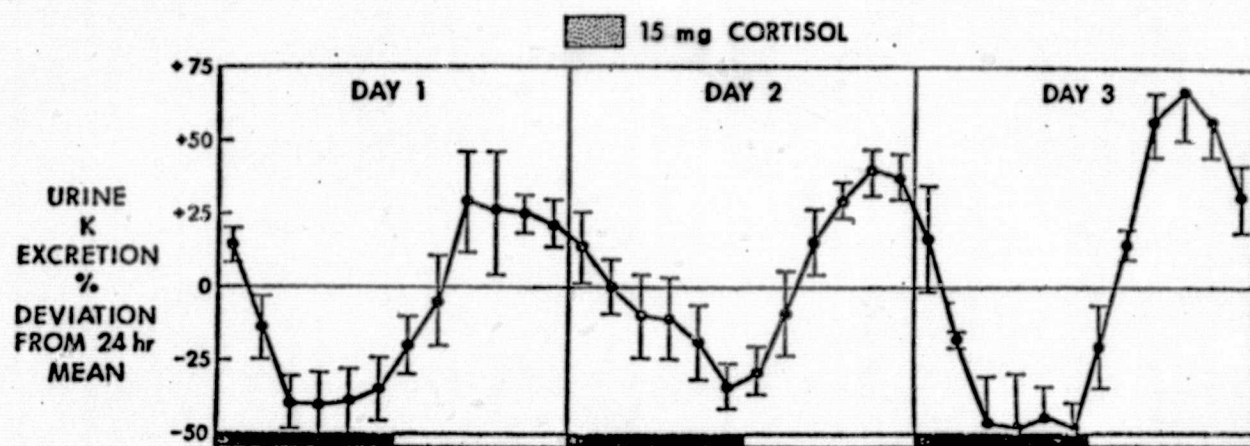
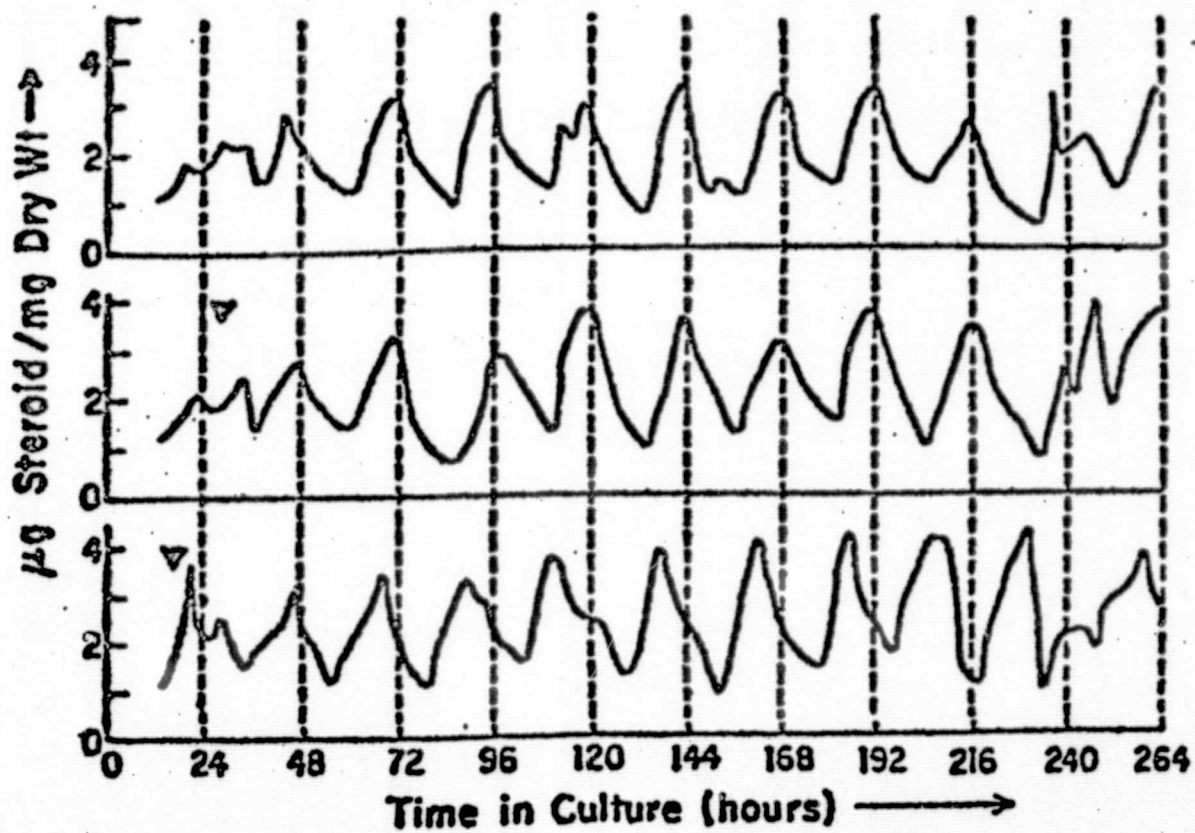


Figure 7



CIRCADIAN TIMING SYSTEM: ORGANIZATION OF MULTIPLE OSCILLATORS

SYNCHRONIZED BY CHEMICAL MEDIATORS

Abstract

The circadian rhythm of renal potassium excretion in the squirrel monkey (Saimiri sciureus) is normally synchronized with the light-dark cycle and with other circadian rhythms in the animal via the circadian rhythm in the plasma concentration of a hormonal mediator, cortisol. In the absence of circadian oscillations in the mediating hormone, renal potassium excretion demonstrates independent free-running oscillations significantly different from 24 hours. These findings suggest that the circadian timing system in primates consists of an organization of multiple, potentially independent oscillators synchronized by hormonal, and possibly nervous, mediators.

Circadian (≈ 24 hour) rhythms in many physiological variables are generated within the organism by a self-sustained oscillating system (1) which serves a variety of essential timing functions (2). The responses of this circadian timing system to manipulations of environmental time cues have received considerable attention (3) but the determination of the anatomical and physiological organization of the oscillating system within the organism has lagged behind. This report is concerned with the question of whether the circadian timing system in advanced multicellular animals is composed of one or of several spontaneously oscillating units, or "oscillators", which will demonstrate an independent period in the absence of periodic inputs. Evidence will be presented from studies in a non-human primate which suggests that the circadian timing system consists of an organization of multiple potentially independent oscillators in various tissues which are synchronized with one another by hormonal and possibly nervous mediators.

Investigations of the response of the circadian timing system to manipulations of environmental time cues have usually treated endogenous circadian rhythms as the product of a single self-sustained oscillator within the organism (4). This approach has been adopted because circadian rhythms in diverse physiological functions in an individual animal are usually found to have identical periods and stable phase-relationships (5) whether the animal is synchronized with environmental time cues, or has its rhythms "free-running" with a period significantly different from 24 hours (6). This phenomenon of "internal synchronization" does not necessarily imply, however, that circadian rhythms in all physiological variables are passive responses to a single, self-sustained driving oscillator. It is also possible that circadian rhythms are generated by several potentially independent oscillators provided that these are coupled in such a way that internal synchronization is normally maintained.

Figure 1 presents models of the organization of single (Model I) and

multiple (Model II) oscillator systems. These can account for the phenomenon of internal synchronization in multicellular animals and are at the same time compatible with the known responses of the circadian timing system to environmental time cues. Variants of these models, combinations of their features, or a non-hierarchical version of Model II are also possible, but the two models in Figure 1 were selected because they emphasize the contrasts between single and multiple oscillator systems.

In both single (Model I) and multiple (Model II) oscillator systems it is necessary to postulate oscillating intermediates (a,b,c,...,etc.) which would presumably be endocrine (hormone concentration) or nervous (neuro-transmitter release) in nature. The characteristics of the cellular systems (A,B,C,...,etc.) which mark the difference between Models I and II will also be manifested in the properties of the chemical intermediates. These can be more easily monitored and can be manipulated in order to perform critical tests between the models.

Model I, which was proposed by Mills (7), predicts that the oscillating chemical output "e" of cell unit "E", which is forced by the oscillation in "b" will have the following characteristics: 1) that a change in the level of "b" must induce an equivalent change in "e" at any time in the 24-hour day; 2) that "e" must cease to oscillate if "b" is maintained at a constant level; 3) phaseshifting "b" will produce an equal and immediate phaseshift in "e"; 4) the normal variations in "b" over the course of the 24 hour day must be appropriately large to induce the observed circadian variations in "e".

In contrast, the second model would suggest that: 1) changes in the level of "b" will have variable effects on "E" (and, hence, "e") depending on the circadian phase of the change, (i.e., there will be a characteristic phase-response function); 2) if "b" is maintained at a constant level, oscillator "E" will yield a free-running rhythm in "e" no longer synchronized to other circadian rhythms; 3) a phaseshift in "b" will result in a

phaseshift in "e" but only after a transient response; 4) the normal daily variations in "b" will not normally be of a size sufficient to passively induce the circadian variations in the level of "e".

To test between a single oscillator system (Model I), on the one hand, and a multiple oscillator network (Model II) on the other, an effort was made to identify a mediator which synchronized a circadian rhythm in a physiological variable and which could be continuously monitored for many successive cycles in an individual animal. When it was found that the phase of the circadian rhythm of urinary potassium excretion was determined by the phase of administration of physiological doses of cortisol in adrenalectomized squirrel monkeys (Saimiri sciureus) it was decided to use this system to differentiate between the single oscillator (I) and multiple oscillator (II) models.

Adult male monkeys weighing 600-900 g were prepared at least two weeks prior to the studies with chronically implanted arterial and venous catheters. They were conditioned to sit in a special metabolism chair, restrained at the waist. Urine was collected with a funnel between the monkey's legs. The urine passed to a fraction collector which aliquoted samples into 2-hourly collections. The monkey had a lever which it was trained to operate to gain food pellets and drinking water was also continuously available. Studies were conducted within an isolation chamber with the arterial and venous catheters led outside via extension tubing. Blood sampling and hormonal infusions could thereby be conducted from outside the chamber without disturbing the monkey. Within the isolation chamber temperature was maintained at $25^{\circ} \pm 1^{\circ}\text{C}$., external auditory stimuli were muffled by noise sources (91 dB, RE: $20 \mu\text{N/m}^2$) within the chamber and illumination was provided at 600 lux from 08.00-20.00 hr and < 1 lux from 20.00-08.00 hr (LD 12:12) daily. The monkeys rapidly became accustomed to sitting in the metabolism chair and repeated studies of up to three weeks in length were well tolerated with no ill effects (8).

Four adrenalectomized monkeys when infused with 5 mg cortisol and 0.001 mg aldosterone between 08.00 and 09.00 hr daily demonstrated circadian rhythms of urinary potassium and 17-hydroxycorticosteroid excretion which were similar in mean, phase and amplitude to those of intact control animals (8). Similarly the adrenalectomized animals in this regimen demonstrated circadian rhythms of feeding, drinking and activity which were identical to those in intact controls. When the time of cortisol administration was phaseshifted by 8 hours (so that it was infused between 16.00 and 17.00 hr daily) the circadian rhythm of urinary potassium excretion phaseshifted by 5 to 8 hours although the phase of the light-dark cycle was unchanged (Figure 2a). The circadian rhythm of feeding, however, did not phaseshift and remained synchronized with the light-dark cycle. Although the phase of cortisol administration determined the phase of the circadian rhythm of urinary potassium excretion it was apparent that the synchronization was not achieved by the passive dependence of the rhythm in urinary potassium excretion on the rhythm in plasma cortisol concentration. Instead, the urinary potassium rhythm took approximately five days to resynchronize with the new phase of cortisol administration, a response predicted for a rhythm generated by an actively oscillating unit as described in Model II but not compatible with the passive cellular units of Model I.

The conclusion that cortisol synchronized the urinary potassium rhythm by an active entrainment process was confirmed by two further studies. Firstly, a bolus of 15 mg cortisol was infused into four intact monkeys between 20.00 and 23.00 hr, the time of the circadian minimum of plasma cortisol concentration, thus inducing a second maximum in plasma cortisol concentration in a single 24 hour day. This was found to have little influence on the circadian rhythm of urinary potassium excretion and produced no second daily maximum of potassium excretion which was comparable to the normal daily maximum between 13.00 and 17.00 hr. Thus, the rate of urinary potassium excretion was shown not to be passively dependent on plasma cortisol concentration. In the second, and critical study, 5 mg

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cortisol and 0.001 mg aldosterone per 24 hours were continuously infused into adrenalectomized monkeys for up to 7 days (Figure 2b). As predicted by Model II but not by Model I, urinary potassium excretion continued to oscillate with free-running periods which were demonstrated by period analysis (9) to be significantly different from 24 hours. There was some evidence of an initial 24.0 hour component but this faded out within approximately 3 days.

These results suggest that the circadian rhythm of urinary potassium excretion is generated by a potentially independent peripheral oscillator or synchronous group of oscillators. These are synchronized with the light-dark cycle and with other oscillators in the animal by the circadian rhythm in plasma cortisol concentration through an active entrainment process (10). The most probable site for this oscillator, or synchronous group of oscillators, would be the distal tubular cells of the kidney, since these are known to determine renal potassium excretion largely independently of plasma potassium concentration, glomerular filtration rate or proximal tubular function (11). This conclusion, however, will require verification by further direct studies of renal distal tubular function.

It is noteworthy that cortisol rather than the more potent mineralocorticosteroid aldosterone appears to act as the hormonal mediator synchronizing the circadian rhythm of renal potassium excretion with the light-dark cycle. When the 8 hour phaseshift experiment (Figure 2a) was repeated with the time of both cortisol and aldosterone administration phaseshifted together, the resynchronization of the urinary potassium rhythm with the new phase of adrenal steroid administration was no more rapid or more complete than when cortisol was phaseshifted alone. That aldosterone administration at doses equal to the normal 24 hour adrenal secretion rate does not appear to play an essential role in synchronizing the urinary potassium rhythm may be because the high plasma cortisol concentrations normally seen in the squirrel monkey (10) and reproduced in these experiments, were sufficient to induce a maximal "mineralocorticoid" effect (11). However, Vagnucci *et al.*, (12)

have shown in man that the circadian rhythm of urinary potassium excretion is well correlated with the rhythm of urinary 17-hydroxycorticosteroid excretion, but is poorly correlated with rhythms in the urinary excretion of aldosterone. Thus, while aldosterone may act as a potent mineralocorticoid under the usual test situations, the slower time-course changes in distal tubular function such as are involved in circadian rhythm synchronization may be induced by the glucocorticoid effects of cortisol (10).

The circadian oscillation in plasma cortisol concentration, however, may not be the only mediator synchronizing the circadian rhythm of urinary potassium excretion. A 24.0 hour component persisted for 3 days in the oscillations of urinary potassium excretion when adrenal steroids were infused at a constant rate throughout day and night, and the phase-delay of cortisol administration failed to induce an equally complete phase-delay in the urinary potassium rhythm in all monkeys (Figure 2a). These findings both suggest that some other minor synchronizing mediator which continued to influence urinary potassium excretion may be operating to a variable extent. The nature of this alternate synchronizing pathway is at the moment a matter for speculation.

There are several further pieces of evidence which support our conclusion that the circadian timing system in advanced multicellular animals, such as mammals, is organized as a multiple oscillator system (such as in Model II) rather than as a system with only a single independent oscillator (Model I). Firstly, Aschoff and his colleagues (15) have demonstrated that although internal synchronization was normally observed between a wide variety of circadian rhythmic functions in men studied under isolation conditions, 15% of their subjects demonstrated internal desynchronization, with various monitored rhythmic variables oscillating with independent free-running periods within the same subject. This observation is incompatible with Model I, but is readily predicted from Model II, since this could occur whenever there was a loss of the circadian rhythm of a synchronizing mediator.

Secondly, several investigators (16) have been able to demonstrate free-running rhythms in isolated tissues maintained in vitro under constant conditions; presumably they have therefore isolated tissue containing self-sustained oscillators as would be predicted from Model II. Thirdly, it has been repeatedly observed in advanced multicellular animals (17) that after the abrupt phaseshift of environmental time cues the various monitored circadian rhythms take different lengths of time to resynchronize with the new phase of environmental cues, so that temporary internal desynchronization occurs. Fourthly, activity rhythms in many different species have been observed to "split" under certain lighting conditions in a manner suggestive of a multiple oscillator system (18) and fifthly, the extensive data that has been accumulated on the rephasing of the *Drosophila* eclosion rhythm by pulses of light can only readily be explained by postulating that there is more than one independent circadian oscillator in this organism (19).

This report has shown that the circadian timing system in advanced multicellular animals, such as primates, appears to be organized as a set of multiple, potentially independent oscillators which are normally synchronized with one another through chemical mediators. The further localization and characterization of these oscillators within the animal will be necessary for the understanding of the function of this timing system, and will form an essential base for examining the physiological roles which the timing system performs.

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References and Notes

1. E. Bunning, The Physiological Clock (Springer-Verlag, New York, ed. 3, 1973); C.S. Pittendrigh, in The Neurosciences, F.O. Schmidt and F.G. Worden, eds, (M.I.T. Press, Cambridge, 1974), pp. 437-458; J Aschoff, in Biological Clocks, Cold Spring Harbor Symp Quant Biol 25, 11 (1960).
2. These include the timing of developmental sequences (D.S. Saunders, Proc Natl Acad Sci USA 69, 2738, 1972), the initiation of adaptive changes before the occurrence of a periodic environmental challenge (J.L. Cloudsley-Thompson, Rhythmic Activity in Animal Physiology and Behavior [Academic Press, New York, ed. 1, 1961], pp. 38-80) and the time compensation of sun orientation mechanisms (G. Kramer and U.V. Saint Paul, Naturwissenschaften 37, 526, 1950).
3. C.S. Pittendrigh, in Biological Clocks, Cold Spring Harbor Symp Quant Biol 25, 159 (1960); in Circadian Clocks, J. Aschoff, ed. (North Holland Publishing Co., Amsterdam, 1965), pp. 277-297; J Aschoff, ibid., pp. 95-111; S.K. Roberts, J Cell Comp Physiol 59, 175 (1962); M. Menaker and A.Eskin, Science 154, 1579 (1966); W. Nelson, L Scheving and F. Halberg, J Nutr 105, 171 (1975).
4. J Aschoff, in Biological Clocks, Cold Spring Harbor Symp Quant Biol 25, 11 (1960).
5. The phase-relationships between the circadian rhythms within an animal are not identical when it is free-running in constant conditions as compared to when it is synchronized to environmental 24 hour cycles (J. Aschoff in Chronobiological Aspects of Endocrinology, J. Aschoff, F. Ceresa and F. Halberg, eds., (Schattauer-Verlag, New York, 1974), pp. 250-253. However, after the initial transient internal phaseshifts the phase relationships of the various circadian rhythms in an individual animal are constant in both free-running and synchronized conditions.

6. L. McMurray and J.W. Hastings, *Science* 175, 1137 (1972); J. Aschoff and H. Pohl, *Fed Proc* 29, 1541 (1970); F.P. Gibbs and P. Van Brunt, *Fed Proc* 34, 301 (1975); J. Aschoff, *Science* 148, 1427 (1965); *Aerosp Med* 40, 844 (1969).
7. J.N. Mills, *Physiol Rev* 46, 128 (1966); J.N. Mills in Biological Aspects of Circadian Rhythms, J.N. Mills, ed. (Plenum Press, New York, 1973) pp. 27-84.
8. M.C. Moore Ede and J.A. Herd, submitted to *Am J Physiol*; M.C. Moore Ede, Control of Circadian Oscillations in Renal Potassium Excretion in the Squirrel Monkey (Harvard University, Ph.D. Thesis, 1974). In five intact animals studied under these conditions urinary potassium excretion demonstrated a regular circadian rhythm with a maximum of $274 \pm 23 \mu\text{Eq/hr}$ (mean \pm SEM) at 17.00 hr and a minimum of $64 \pm 6 \mu\text{Eq/hr}$ at 05.00 hr.
9. Periodicities in the data were analyzed by a combined linear-nonlinear least squares iterative period analysis which is based on the Marquardt algorithm (J. Rummel, J.K. Lee and F. Halberg in Biorhythms and Human Reproduction, M. Ferin, F. Halberg, R.M. Richart and R. L. Vandewiele, eds. (John Wiley and Sons, New York, 1974), pp. 53-84). We are grateful to Dr. J.A. Rummel for permission to use this program.
10. A phase-response relationship has been reported for phaseshifts of the circadian rhythm of urinary potassium excretion induced by the administration of identical doses of the synthetic glucocorticoid, prednisone, at different phases of the circadian cycle (K. Reindl, C. Falliers, F. Halberg, H. Chai, D. Hillman and W. Nelson, *Rass Neurol Veg* 23, 5 (1969). Such a phase-response relationship is indicative of the active entrainment of the urinary potassium circadian rhythm by glucocorticoid rhythms.

11. G. Giebisch in the Kidney, C. Rouiller and A.F. Muller, eds. (Academic Press, New York, 1971) vol 3, pp. 329-382; B.M. Brenner and R.W. Berliner in Handbook of Physiology, Section 8: Renal Physiology, J. Orloff and R.W. Berliner, eds. (American Physiological Society, Washington, DC, 1973) pp. 497-519.
12. G.M. Brown, L.J. Grotta, D.P. Penney and S. Reichlin, Endocrinology 86, 519 (1970); G.M. Brown, D.S. Schalch and S. Reichlin, Endocrinology 88, 956 (1971).
13. G.E. Swaneck, L.L.H. Chu and I.S. Edelman, J Biol Chem 245, 5382 (1970).
14. A. Vagnucci, A.P. Shapiro and R.H. McDonald, Jr., J Appl Physiol 26, 720 (1969).
15. J. Aschoff, U. Gerecke and R. Wever, Jap J Physiol 17, 450 (1967).
16. R.V. Andrews, Comp Biochem Physiol 26, 179 (1968); R. Shiotsuka, J. Jovonovich and J.A. Jovonovich, in Chronobiological Aspects of Endocrinology, J. Aschoff, F. Ceresa and F. Halberg, eds. (Schattauer-Verlag, New York, 1974) pp. 255-267; G.D. Tharp and G.E. Folk, Jr., Comp Biochem Physiol 14, 255 (1964); A. Eskin, J Comp Physiol 80, 353 (1972); J.W. Jacklett and J. Geronimo, Science, 174, 299 (1971).
17. P.J. Martel, G.W.G. Sharp, S.A. Slorach, and H.J. Vipond, J Endocrinol 24, 159 (1962); E.B. Flink and R.P. Doe, Proc Soc Expt Biol Med 100, 498 (1959); A.L. Elliot, J.N. Mills, D.S. Minors and J.M. Waterhouse, J Physiol (Lond) 221, 227 (1972); E. Haus and F. Halberg, Rass Neurol Veg 23, 83 (1969).
18. K. Hoffman, in Biochronometry, M. Menaker, ed. (National Academy of Sciences, Washington, D.C., 1971) pp. 134-148; H. Pohl, J Comp Physiol 78, 60 (1972); E. Gwinner, Science 185, 72 (1974).
19. A.T. Winfree, Nature 253, 315 (1975).
20. Supported by National Aeronautics and Space Administration Contract NAS9-14249, and by National Institutes of Health Grants HL-13872, HL-14150 and HL-09154.

Figure Legends

Figure 1: Models of single (Model I) and multiple (Model II) oscillator systems which could describe the circadian timing system in advanced multicellular animals. Both represent networks of cellular systems (A,B,C,...,etc.) which are either passively oscillating as a forced response to a single self-sustained driving oscillator (D.O.) (Model I), or are themselves potentially independent oscillators with their own individual characteristics which are normally entrained by a driving oscillator acting as a pacemaker (Model II). Where these cellular units are non-contiguous in a multicellular animal each model requires that oscillating levels of physical or chemical mediators with the period of the driving oscillator but not necessarily the same phase, be postulated (a,b,c,...,etc.). These mediators, which would presumably be nervous or hormonal, would either transmit the forced response from the driving oscillator to the various passively responding cellular units (Model I) or would actively entrain the self-sustained cellular oscillators to the driving oscillator by a mechanism similar to the entrainment of circadian rhythms by the light-dark cycle (Model II). Each circadian timing system would be entrained by environmental time cues via exteroceptive sensory inputs to the driving oscillator. It should be noted, however, that the multioscillator system (Model II) does not necessarily require a driving oscillator. The multiple oscillators could be arranged in a non-hierarchical manner with the different exteroceptive sensory inputs impinging on different oscillators, and internal synchronization being a product of mutual coupling between oscillators through positive and negative feedback. The predictions which are made of the behavior of the oscillating chemical mediators in such a system are similar to those made from Model II and therefore they will not be considered separately.

Figure 2 2a) The response of the circadian rhythms of urinary potassium

excretion and feeding to an 8 hour phase delay of the time of administration of cortisol in adrenalectomized monkeys. The rhythm of urinary potassium resynchronized with the new phase of cortisol administration over a 5 day period, but the feeding rhythm remained synchronized to the phase of the light-dark cycle.

2b) When circadian oscillations in cortisol and aldosterone administration were eliminated by infusing these steroids at a constant rate throughout day and night the urinary potassium excretion began to oscillate independently with free-running periods of greater or less than 24 hours in each animal studied. A least squares period analysis (9) revealed that the significant periods ($p < .05$) were for monkey D, 18.0, 16.0 and 12.4 hours; for monkey E, 25.8 and 18.2 hours; and for monkey F, 38.4, 24.6, 12.4 and 10.0 hours. In addition, in each animal there was a transiently persisting 24.0 hour component which faded to an insignificant amplitude after the first three days of continuous adrenal steroid infusion.

FIG 1

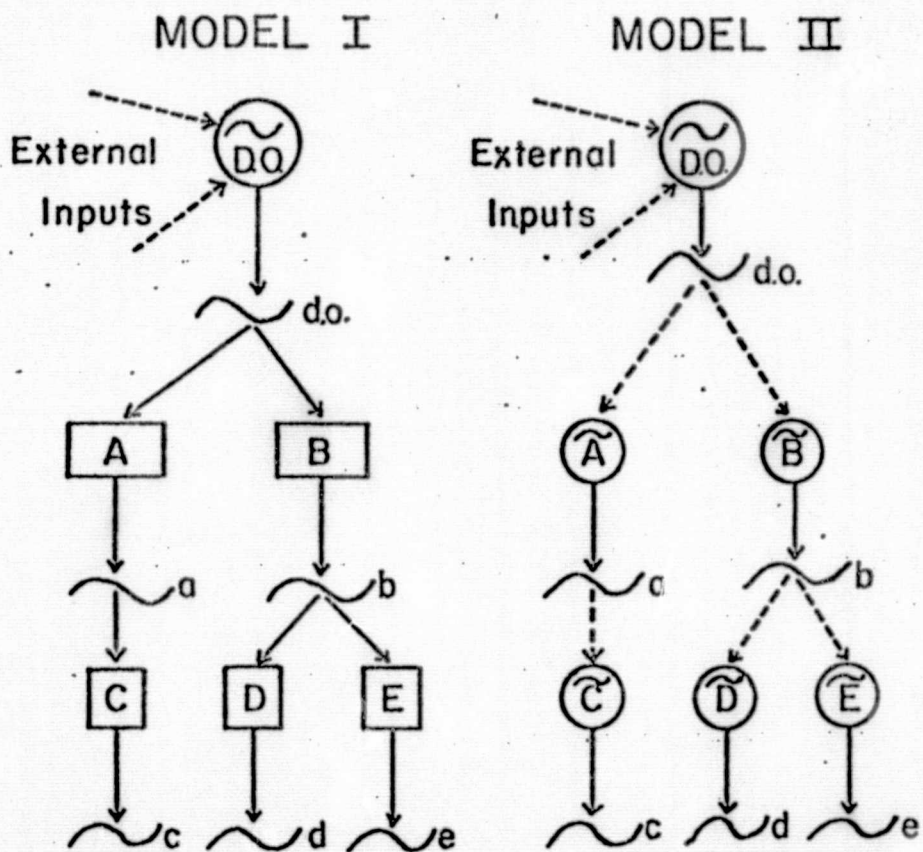
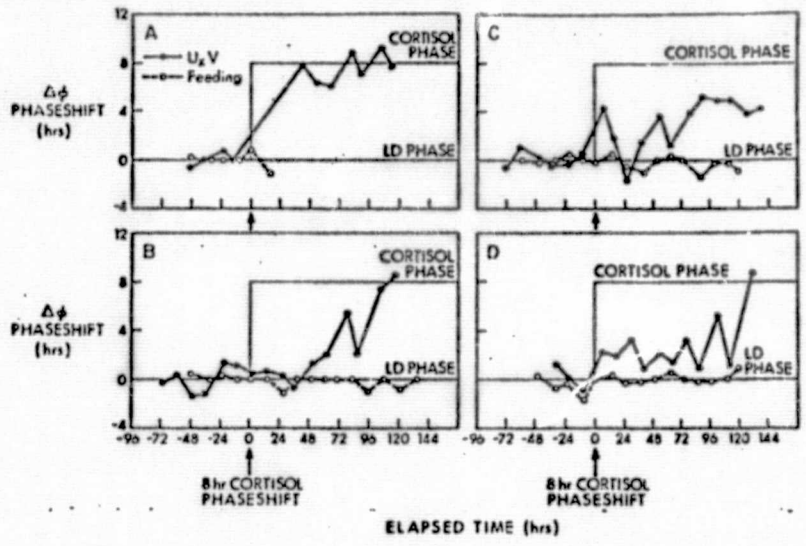
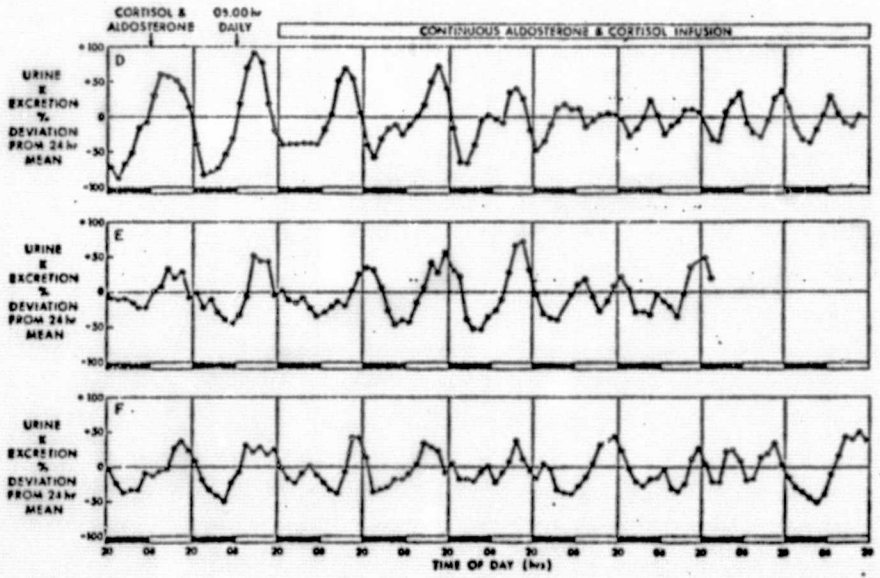


FIG 2

A.



B.



THE EFFECT OF PSYCHOLOGICAL STRESS IN THE
PRESURGICAL PERIOD ON THE EPISODIC 24-HOUR
PLASMA CORTISOL SECRETION PATTERN

Presented on Saturday, March 22, 1975 at
2:45 PM at the Annual Meeting of the American
Psychosomatic Society in New Orleans, Louisiana.

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94305

**AMERICAN PSYCHOSOMATIC
SOCIETY**

Program Insert

SATURDAY AFTERNOON, MARCH 22, 1975

EMERALD BALLROOM BRIEF COMMUNICATIONS

Chairman: SIDNEY COBB, M.D.

*Professor of Community Health and Psychiatry,
Division of Biological and Medical Sciences,
Brown University, Providence*

**2:45 p.m. THE EFFECT OF PSYCHOLOGICAL STRESS IN THE
PREOPERATIVE PERIOD IN THE EPISODIC 24-HOUR
PLASMA CORTISOL SECRETION PATTERN**

CHARLES A. CZEISLER
MARTIN C. MOORE EDE, M.B.
QUENTIN R. REGENSTEIN, M.D.
ELDAD KISCH, M.D.
VICTOR S. FANG, Ph.D.
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*Stanford Medical School, Stanford; Harvard
Medical School, Boston; University of Chicago
Medical School, Chicago*

**AGGRESSIVENESS AND TESTOSTERONE PRODUCTION
OF XYY SUBJECTS**

HEINO F. L. MEYER-BAHLBURG, Ph.D.
MINOTT SHARMA, Ph.D.
DONALD A. BOON, Ph.D.
W. ROY SLAUNWHITE, JR., Ph.D.
JOHN A. EDWARDS, M.D.

*State University of New York at Buffalo, School
of Medicine, Buffalo*

**EFFECT OF COGNITIVE ACTIVITY ON RESPIRATION
AND HEART RATE**

DONALD P. SPENCE, Ph.D.
DAVID BEYDA

Rutgers Medical School, Piscataway, N.J.

**PHYSIOLOGICAL CORRELATES OF THE
PSYCHOTHERAPEUTIC PROCESS**

PAUL C. MOHL, M.D.
REDFORD B. WILLIAMS, M.D.
LINDA C. WYRICK, Ph.D.
DAVID M. HAWKINS, M.D.
LINDA J. BURDETTE, B.A.

Duke University Medical Center, Durham

FIVE-YEAR FOLLOW-UP OF TREATMENT FOR OBESITY

SYDNOR B. PENICK, M.D.
ALBERT J. STUNKARD, M.D.

*Princeton Hospital, Princeton; Stanford Univer-
sity Medical Center, Stanford*

**PSYCHOPHYSIOLOGIC AROUSAL IN ACUTE
SCHIZOPHRENIA**

WILLIAM T. CARPENTER, JR., M.D.
THOMAS H. MCGLASHAN, M.D.
THEODORE P. ZAHN, Ph.D.

NIMH, Bethesda

**ANTINUCLEAR ANTIBODIES IN CHLORPROMAZINE-
TREATED PATIENTS WITH CHRONIC PSYCHOSES**

WILLIAM F. KIELY, M.D.
F. P. QUISMORIO, M.D.
E. L. DUBOIS, M.D.

*University of Southern California Medical Center,
Los Angeles*

Extensive evidence indicates that cortisol secretion increases in response to anxiety and particularly to the threat of personal injury. Yet investigators in the field have encountered a great deal of variability and apparent inconsistency in their results. On inspection of many earlier studies which are currently cited as evidence of the "highly sensitive response" of the hypothalamic-pituitary - adrenal axis to psychological stress, it becomes clear that although the evidence shows that the system is not unresponsive it fails to show predictably when or how the system does respond. Such variability in the cortisol secretory response to a given situation among and within individuals has been explained in several ways. Falling plasma cortisol concentration observed in the face of apparent stress have led some investigators to conclude that the psychoendocrine response was being masked by a concurrent diurnal fall of plasma cortisol concentration in the morning, when those studies were done. Others have concluded that personality type was the overriding factor in cortisol output or that increased cortisol secretion was only seen when an individual's psychological defenses ^{were} inadequate to cope with a situation.

While such explanations may yet prove to be valid, the demonstration by Weitzman and colleagues at the Montefiore Hospital in New York that cortisol is normally secreted episodically in man has made it obvious that infrequent plasma sampling is a wholly inadequate technique for such investigations. By taking blood samples every 20

minutes for an entire day, they demonstrated that cortisol secretion is limited to brief episodes which are distributed throughout the day. That episodic nature of the secretion explains why attempts to show precise correlation between infrequently monitored plasma cortisol levels (such as 1 or 2 per day) and psychologically stressful situations have yielded confusing results.

The purpose of the present study was to see how the apprehension of a major life threatening surgical procedure would affect the 24-hour secretion pattern of cortisol. In order to do so, four patients during the 24-hours just prior to coronary artery bypass graft surgery were compared with 5 similarly hospitalized normal control subjects in no apparent distress. Blood sampling was accomplished every 20 minutes for 24 hours in each subject by means of an indwelling intravenous catheter, inserted 16 hours before the study began in the normal control subjects. The catheter extended outside his room in order to allow the frequent blood sampling without the subject's awareness. To minimize uncertainty of the procedure I briefed each of the subjects (who were all personal acquaintances) for several hours about the procedures involved in the study.

A clinical psychiatrist interviewed each subject and without prior knowledge of the endocrine data ranked them on a scale of overt expression of emotional responses to see if there were any obvious correlations with the data in this necessarily small study population.

A detailed log was also kept of events which occurred during the course of each experiment. The 670 plasma samples were assayed in duplicate by the competitive protein binding method.

The following slides will illustrate the results first in the normal subjects and then in those awaiting surgery.

SLIDE I

In these and subsequent graphs plasma cortisol concentration is plotted on each vertical axis and the time of day - referenced to the time of mean sleep onset for the seven days prior to this study - is on the horizontal axis. As expected, cortisol was secreted in episodes in each of the five subjects. Those episodes appeared to be part of an endogenous rhythm of cortisol secretion since they could not be correlated with environmental stimuli. Furthermore, the mean daily cortisol level did not correlate with the psychiatric rating of the subjects on a scale of overt emotionality.

You will notice that the pulses of secretion have a circadian distribution, with most of them occurring in the latter half of sleep (3 a.m. to 9 a.m.) and very few during the late evening. Those trends are perhaps illustrated more clearly on the next slide, which shows the mean and standard deviation of the cortisol concentrations in each of the five normal subjects throughout the day. This slide shows the highly reproducible circadian rhythm in these subjects. Each of them had a 9 hour period here when the level never once rose above the daily mean and, in fact, consistently fell to a cortisol concentration at or near zero.

This stippled area of normal control mean \pm standard deviation was used as a background for comparing each of the presurgical patients in the next slide. (Slide 3, please). There are several things I would like to highlight here. 1) For most of the day (with the exception of the late evening) the patterns of concentration in these four presurgical patients fell closely within the normal range in spite of many incidents which provoked overt and often verbal expressions of apprehension and anxiety relating to the patient's upcoming surgery. 2) These patients, who had been facing the threat of operation for some weeks, did not have the kind of psychoendocrine activation of the adrenocortical axis which Sachar and others demonstrated in depressed patients. That is to say, these presurgical patients neither had markedly elevated plasma cortisol concentrations throughout the day nor an increased number of secretory episodes per day. This suggests that neither the discrete emotional stresses associated with the immediate presurgical situation nor the longer term anticipation of upcoming surgery resulted in a general hyperactivation of the hypothalmo-pituitary-adrenocortical axis. 3) While most episodes of secretion that were observed in the presurgical patients could again not be reliably correlated with environmental stimuli, there was, in contrast, a single event during the late evening that was consistently related to a major pulse of cortisol secretion in the presurgical patients:

In this normal period of secretory quiescence in the late evening each patient was preoperatively prepared, a procedure which

consisted of body shaving, antiseptic wash and enema and which is indicated in this figure by the black bar below the time axis. As you can see, each patient had a major episode of cortisol secretion. Plasma cortisol concentration reached values that were between 7 and 11 standard deviations above the mean level for the control subjects at the corresponding time of day. That highly unusual pulse of secretion resulted in a very significant drop in the average length of the quiescent period from 9 hours in the control subjects to only 5 hours in the presurgical patients.

(LIGHTS ON PLEASE)

The question then is: Was this response of the pituitary-adrenocortical axis induced by the psychological or physiological components of that complex stimulus. It is possible that on the evening before surgery body shaving could provoke the acute focusing of diffuse and unconscious anxieties about approaching surgery, thus seriously challenging and perhaps temporarily overwhelming a patient's psychological defenses by confronting him with the reality and the immediacy of his operation.

Alternatively, the preoperative preparation could act as a physiological stimulus since considerable non-specific sensory stimulation was involved.

The next slide illustrates very recent preliminary results from a study I am currently conducting at Stanford in collaboration with Dr. Elliott Weitzman to investigate that question. These are the results from a normal control subject who was not in anticipation of surgery but who nonetheless was subjected (at the same time of day)

to the same physical stimulus of presurgical preparation (that is, body shaving, antiseptic wash & enema). As you can see, our first results indicate that the physical stimulus alone (shown here at this black bar) was not enough to induce an episode of secretion. That would suggest that the episodes of secretion which were consistently observed in the presurgical patients when they were shaved were related to anxiety about the operation which the shaving procedure consistently prompted.

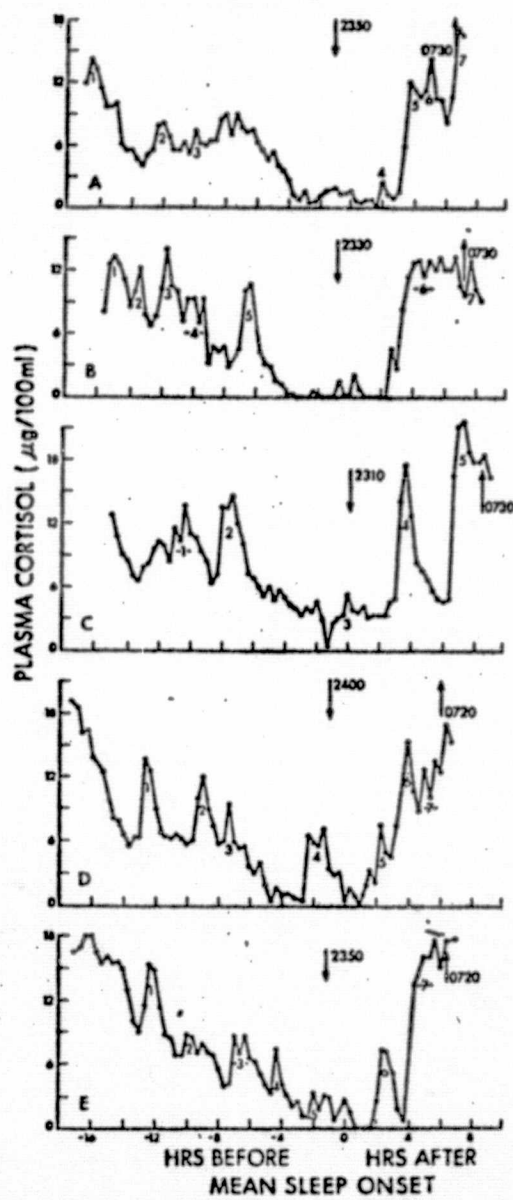
An important implication of the present study is that frequent blood sampling for at least 24 hours must be used to define the influence of environmental variables on the pituitary-adrenal axis. The late evening pulses of secretion demonstrated herein would probably have been overlooked if samples had been taken infrequently or over limited periods of time. This explains why earlier studies of plasma cortisol concentration measured once or twice daily in presurgical patients yielded inconsistent results.

Similarly, the 24 hour mean plasma cortisol levels were not significantly different between the two groups, so the significant differences in the secretion patterns demonstrated here would have been overlooked by measurement of 24 hour urinary excretion rates of cortisol metabolites.

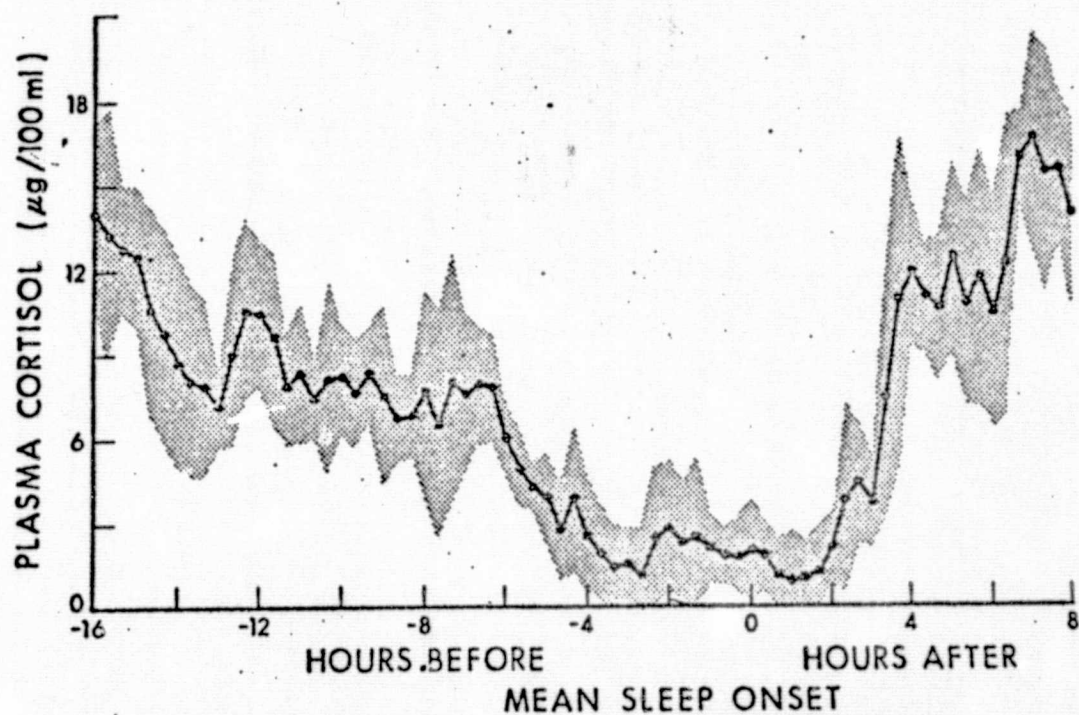
In summary, we conclude: 1) that the pituitary-adrenocortical axis is not chronically activated by a prolonged period of psychological stress, 2) that it was not significantly perturbed by most emotional stressors, 3) that spontaneous episodes of secretion were part of the

endogenous pattern and could not be reliably correlated with environmental stimuli, 4) that the adrenocortical axis responds actively and consistently to the stimulus of preoperative preparation in patients awaiting major surgery.

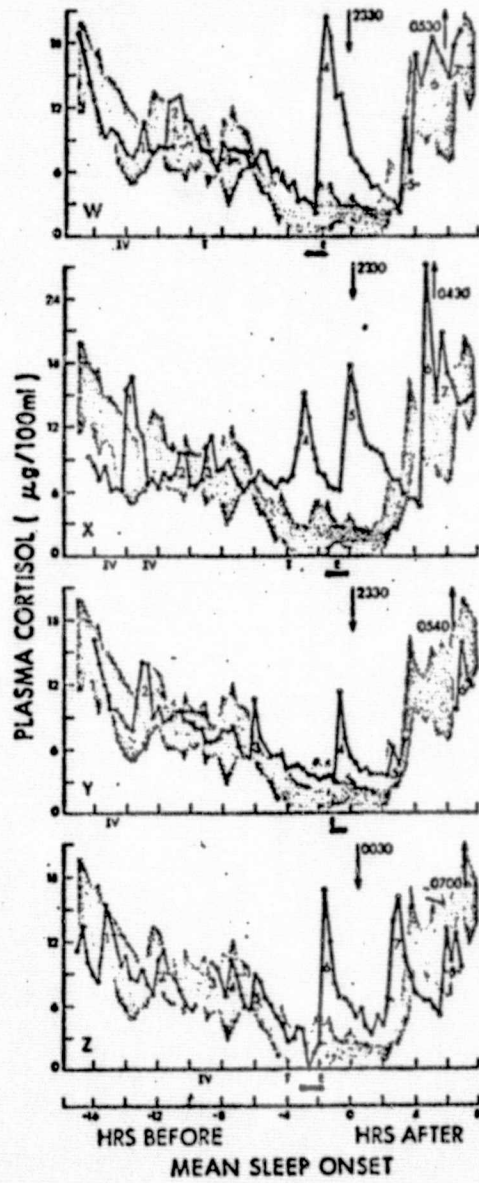
24 HOUR PLASMA CORTISOL PATTERNS
IN CONTROL SUBJECTS



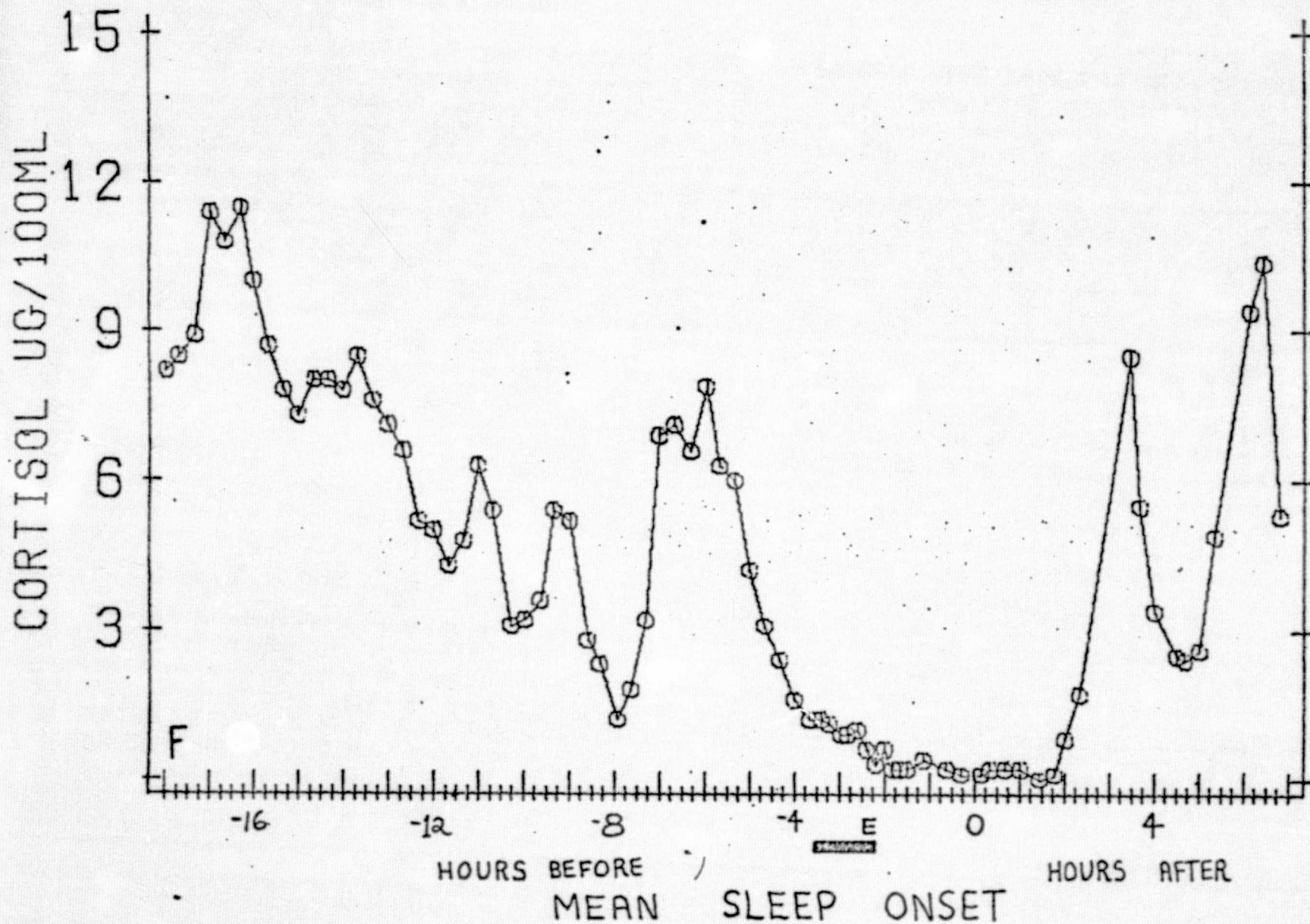
MEAN 24 HOUR PATTERN OF PLASMA CORTISOL
CONCENTRATION IN 5 CONTROL SUBJECTS



24 HOUR PLASMA CORTISOL PATTERNS
IN PREOPERATIVE PATIENTS



PREOPERATIVELY PREPARED CONTROL



**INTERNAL SYNCHRONIZATION OF SPONTANEOUS CIRCADIAN OSCILLATORS: THE
IDENTIFICATION OF THE HORMONAL MEDIATOR SYNCHRONIZING A RENAL OSCILLATOR**

Presented on Tuesday, April 15th at 2:10 P.M. in the Symposium on "Physiological and Biochemical Aspects of Circadian Rhythms" at the 59th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J.

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Federation of American Societies for Experimental Biology, 59th Annual Meeting

Subject..... Internal Synchronization of Spontaneous Circadian Oscillators: the Identification of the Hormonal Mediator Synchronizing a Renal Oscillator
Author..... Martin C. Moore Ede, M.B., B.S., Ph.D.
Address..... Department of Physiology, Harvard Medical School, Boston, Massachusetts. 02115
Time of Presentation.... 2:10 P.M., Tuesday, April 15, 1975
Place..... Convention Hall, Ballroom, Atlantic City, New Jersey
Program..... Symposium on Physiological and Biochemical Aspects of Circadian Rhythms
Convention Address..... Diplomat Hotel, 2800 Pacific Ave., Atlantic City, NJ 08401

RELEASE TIME*
April 15 - 2:10 P.M.

Today I would like to discuss some of our recent work on the control of circadian rhythms in primates. I will present evidence which suggests that circadian rhythms in such animals are generated by an internal timing system consisting of multiple tissue oscillators which are normally kept synchronized with one another through circadian oscillations in hormonal and nervous mediators. In particular, I will be discussing how the circadian rhythm of renal potassium excretion appears to be generated by an oscillator or synchronous group of oscillators within the kidney. Furthermore, I will show that this oscillator is normally synchronized with other circadian oscillators within the animal, as well as with the environmental time cues such as the light-dark cycle. This synchronization is mediated by the pituitary-adrenal axis via circadian oscillations in plasma cortisol concentration.

As research in this field progresses we are becoming more and more aware that rhythms in biological variables are but one outward manifestation of the internal timing systems that living organisms have evolved. These have many essential functions, ranging from the precise timing of the transcription of genetic information during development through the chronometry necessary for the effective functioning of physiological pumps such as the heart, to the control of seasonal adaptive processes such as hibernation.

The circadian rhythms with which we are particularly concerned in this

Symposium are an outward manifestation of an important evolutionary adaptation to life on a rotating planet. The time-span we know as 24 hours is an important unit of time for living organisms because of the major changes in environmental conditions that occur with this periodicity due to the earth's rotation. It appears that the ability to measure time, which is afforded by an internal circadian timing system, is important to living organisms because it enables them to predict when periodic environmental changes and dependent alterations in food supply and predator activity will occur. This temporal prediction capability may be essential for survival especially when the adaptive physiological or behavioral response takes several hours to be activated and thus must be initiated well in advance of the environmental challenge.

Dr. Menaker and Dr. Aschoff have already reviewed many of the properties of these circadian timing systems and the circadian rhythms in physiological variables which are generated by them. Work from many laboratories has demonstrated that living organisms, ranging from unicellular algae to man have endogenous circadian oscillators with well defined properties. Dr. Aschoff, for example, has already described his elegant studies of these self-sustained oscillators in man and has demonstrated their free-running characteristics in the absence of external time cues, and has shown how they are normally synchronized by certain environmental circadian periodicities.

There are many fascinating physiological questions which are raised by the work of Dr. Aschoff and his associates, but many of them unfortunately cannot be answered directly in man. Like students of the respiratory center and cardiac pacemaker, we would like to know 1) where the oscillators are located within the organism, 2) how they are synchronized with one another, 3) the physiological and biochemical mechanisms that generate the self-sustained circadian rhythms, and 4) how the oscillators drive the various timed physiological functions. Unlike students of the respiratory and cardiac oscillators, we also have to be concerned

with how the internal oscillators are synchronized with cyclical environmental variables.

While I do not plan to and obviously could not answer all these questions today, I will tell you something about how circadian oscillators are organized in primates and will particularly discuss the mechanisms that are responsible for internal synchronization. We have conducted our studies in squirrel monkeys because they have circadian rhythms with many similar properties to those in man, as well as having the advantage that physiological interventions are more readily performed.

But first, before discussing the evidence from studies in primates, I would like to consider one important clue as to the organization of circadian oscillators which comes from the work of Aschoff, Wever and associates in man. They have shown when circadian rhythms in several physiological variables such activity, body temperature and urinary potassium excretion are monitored simultaneously in an individual subject, the various rhythms are usually internally synchronized with identical periods and constant phase relationships whether the animal is synchronized with environmental time cues or has all its rhythms free-running in isolated constant conditions.

This is illustrated on the first slide.

FIRST SLIDE (#1--Internal synchronization of U_KV and temperature in man)

This slide shows the progressive changes in circadian rhythms phase in two human subjects over the course of 24 days of study. This is estimated by measuring on each cycle the time of day that a defined part on the cycle, such as maximum or minimum, occurs. One subject was studied by Drs. Aschoff and Wever in constant light and had all his monitored circadian rhythms free-running with a period of approximately 24.8 hours. Thus we can see the circadian rhythms of body temperature and urinary potassium excretion phaseshifted on average of 0.8 hours per day. The other,

with open symbols, was studied by Reinberg in a normal 24-hour light-dark cycle and was found to be synchronized to the 24 hour day. On this slide the phase of the rhythms of body temperature and urinary potassium excretion did not shift over the consecutive days of each experiment. You will note that whether the subject was free-running or synchronized to the 24 hour day, the two rhythms remained synchronized with each other with identical periods and constant phase-relationships. In this experiment many other variables were simultaneously monitored, such as rest-activity cycle and the urinary excretion of various other metabolites and ions. The regression of the phaseshifts of the circadian oscillations in all these variables could be superimposed upon the body temperature and urinary potassium phaseshifts plotted here. This phenomenon has been demonstrated in other species and is known as "internal synchronization".

NEXT SLIDE (#2-- Possible mechanisms of internal synchronization.)

There are several ways in which circadian oscillators could be organized to account for the phenomenon of internal synchronization. Firstly, some investigators such as Mills in England have proposed that there could be one central self-sustaining oscillator (labelled here as D.O. for driving oscillator) and that all circadian rhythms in various physiological variables are passively dependent upon that oscillator, presumably through sequences of oscillating variables with each oscillating variable passively dependent upon the previous oscillation in the sequence. Thus, for example, the circadian rhythm in plasma cortisol concentration would be passively dependent upon that of ACTH, which in turn would be passively dependent on the circadian rhythm of CRF release from the hypothalamus. Thus, the circadian oscillation in body temperature might be at the end of one sequence, and the rhythm of urinary potassium excretion as the end of another. Each would be synchronized with the driving oscillator by separate sequences of oscillating variables.

The second possible mechanism depicted here is that there are multiple

potentially independent circadian oscillators in various tissues depicted in the diagram as A,B,C,D & E. However, the central driving oscillator (D.O.) which normally acts as a pacemaker would synchronize the other oscillators in the body via circadian oscillations in chemical, hormonal or nervous mediators. These oscillators would be potentially-independent and if the synchronizing cue were insufficient the peripheral oscillator would be capable of free-running with an independent period. An analogous system to this is the pacemaker control of cardiac contractions, where ventricular muscle contraction can free-run with an independent period when the conducting pathway is damaged in heart block.

The third possibility represented on this diagram is that there might be multiple potentially independent oscillators but without any one oscillator consistently acting as a pacemaker. The oscillators would be mutually synchronized through circadian oscillations in nervous and hormonal mediators, but the system would be non-hierarchical as opposed to the hierarchical system described in alternative #2.

Our approach to the investigation of circadian oscillator organization in higher animals such as the primates was to identify the synchronizing mediators which it is necessary to postulate for each of the three models. By manipulating a synchronizing mediator once it is identified one can test the predictions of the three models. For example, the elimination of oscillations in a synchronizing mediator in alternative #1 would result in the immediate elimination of all the rhythms in physiological variables further down the synchronization pathway. However, in alternatives 2&3 the elimination of a rhythm in a synchronizing mediator would enable the normally synchronized but potentially-independent oscillator to free-run with respect to the other oscillators in the animal. In order to differentiate between alternatives 2&3 it would be necessary to identify several synchronizing mediators and their normally synchronized but potentially-independent oscillators and investigate the inter-relationships.

Today I will describe how we have demonstrated that the circadian rhythm of plasma cortisol concentration acts as the internal mediator synchronizing the circadian rhythm of urinary potassium excretion in the squirrel monkey. Studies

of the mode of synchronization have suggested that the circadian rhythm of urinary potassium excretion is generated by potentially-independent intra-renal circadian oscillators, and that although these are synchronized by the plasma cortisol rhythm, the renal potassium rhythms is not passively dependent on the mediator.

NEXT SLIDE (#3--Monkey in metabolism chair)

These studies have been conducted using the squirrel monkey (Saimiri sciureus) a small South American primate weighing 600-900 g or approximately 1/100th the body weight of man. This is a strictly diurnal animal confining all of its activity and feeding to the daytime and resting at night. We have conditioned male monkeys to sit in a special metabolism chair restrained at the waist. Urine is collected with a funnel between the monkey's legs. The urine passes to a fraction collector containing test tubes which collect urine samples as two-hour fractions. The monkey has a lever which it was trained to operate to gain food pellets and also had drinking water available. An ultrasound activity monitor, above the animal, records activity.

NEXT SLIDE (#4--Full set up photo-chair and chamber and recorders)

The chair and monkey are placed in a temperature-controlled isolation chamber which enabled the control of environmental illumination, temperature and auditory stimuli. The lighting of the chamber provided 600 lux of illumination from 8 AM to 8 PM daily with less than one lux of illumination at night. The system was controlled by standard switching circuitry outside the chamber and continuous recording was made of food pellets gained, water drunk, activity recorded by the ultrasound motion detector and body temperature recorded from indwelling retroperitoneal thermistors. For some experiments, the monkeys were also prepared at least two weeks previously with chronically implanted arterial and venous catheters, which were protected by a nylon mesh jacket. During experiments, these catheters were connected to extension tubing leading outside the chamber. Blood sampling, and hormonal and electrolyte infusions could thereby

be undertaken without the monkey being disturbed. The animals rapidly became conditioned to sitting in the metabolism chair and the studies of up to three weeks in length were well tolerated.

NEXT SLIDE (#5 --Control data on U_{K^+} , $T^{\circ}C.$, etc.)

The squirrel monkey has highly reproducible circadian rhythms in many physiological variables. In order to examine internal synchronization mechanisms we chose to examine in particular the circadian rhythms in feeding, activity, body temperature and renal potassium excretion.

In this slide, I have shown the characteristic patterns of urinary potassium excretion, body temperature, feeding and activity in a monkey studied under a 24 hour light-dark cycle with lights on from 8 AM to 8 PM and off from 8 PM to 8 AM. Urinary potassium excretion rises to a maximum of approximately 270 $\mu\text{Eq/hr}$ during the last part of the lights-on period and then falls to a minimum of approximately 60 $\mu\text{Eq/hr}$ during the last part of the dark period daily. This is plotted on this slide as a percentage deviation from the 24 hour mean. Body temperature demonstrates a $2^{\circ}C.$ circadian variation beginning to rise before the time of lights-on and then reaching a plateau level during the lights-on period before falling during the dark to a second plateau level some $2^{\circ}C.$ lower. Feeding and activity were confined to the lights-on period of the 24 hours.

NEXT SLIDE (#6 --2h feeding experiment)

Since the squirrel monkey confines all its feeding and hence, all its dietary potassium intake, to the lights-on period of the 24 hours and the maximum of the urinary potassium excretion occurs at the end of that period of feeding, it was necessary to establish whether the urinary potassium rhythm was to any extent directly dependent on the pattern of feeding. To do this four monkeys were trained to operate the food pellet lever only when a low intensity green signal light came on. It was found that the monkeys could be trained to work for and consume an identical number of food pellets every two hours throughout day and

night. Thus during this regimen they evenly distributed their feeding, drinking and most of their activity throughout the 24 hours instead of confining it to the lights on period. However, when the patterns of urinary potassium excretion are compared during ad lib daytime feeding and during the 2-hourly feeding regimen the urinary potassium excretory rhythms are virtually identical. Thus, the pattern of potassium intake does not influence the circadian rhythm of potassium excretion.

NEXT SLIDE (#7--Richter slide of blinded monkey 3 years)

In the squirrel monkey, free-running circadian rhythms have been demonstrated to persist for considerable periods of time in monkeys deprived of time cues from the light-dark cycle. This may be achieved by placing the animal in constant light environments or by blinding the animal by optic nerve section as was done to the monkey whose results are shown on this slide. The activity pattern of a squirrel monkey in a light-dark cycle of twelve hours of light and twelve hours of dark is plotted. In June, 1964 this animal was blinded and then continued to be kept in the 24 hour light-dark cycle. Shortly after blinding, a free-running period was observed with the monkey beginning its activity on average 46 minutes later each day that when each 24 hours of record is placed under the previous 24 hours a strip pattern is observed. This free-running period continued throughout the length of this experiment right through until September 1967 more than 3 years later. The free-running period was so precise that one could practically predict what time the monkey was going to wake up the next Christmas morning. These experiments have been repeated several times. Some animals free-run with a period less than 24 hours and others with periods of greater than 24 hours.

NEXT SLIDE (#8--Internal synchronization between monkey rhythms)

This slide demonstrates the progressive phaseshifts of the rhythms of urinary potassium and water excretion and feeding of a monkey in constant light. As was seen before from Dr. Aschoff's data in man internal synchronization is observed between the rhythms whether they are free-running in constant light or are

NEXT SLIDE (#9 --8 hour LD shift raw data)

The appearance of a free-running period in constant light suggests the normal light-dark cycle is the dominant environmental synchronizer in this animal. We have confirmed this in our preparation by studying the response of monkeys to a light-dark cycle phaseshift. In this slide, the patterns of urinary potassium excretion, body temperature, feeding, activity and drinking are shown before and after light-dark cycle is phase-delayed by 8 hours. The rhythms of feeding, drinking and activity can be seen to resynchronize with the new light-dark cycle within approximately 2-3 days as does the rhythm of body temperature. The urinary potassium rhythm also can be seen to resynchronize but this occurs more slowly. To measure the rate of phaseshift, the movement of defined points of the rhythms was measured with respect to the 24 hour time scale. The defined points chosen in the studies described in the remainder of this talk will be the times of mean crossing upwards and downwards each cycle.

NEXT SLIDE (10 --Body temperature phaseshift plot)

This slide shows the phaseshift of the body temperature rhythm. Before the light-dark cycle phaseshift at time 0 the body temperature rhythm was closely synchronized with the light-dark cycle. Then after the abrupt 8 hour phase delay, plotted here as a +8 hour phaseshift, the body temperature rhythm resynchronized in 2-3 days, so that it finally resumed its original phase-relationship with the light-dark cycle. Similar rates of resynchronization were observed for feeding, drinking and activity rhythms.

NEXT SLIDE (#11--U_KV phaseshift plot)

In contrast, the urinary potassium rhythm took 7 days to resynchronize with the light-dark cycle. It did not start to resynchronize until approximately 24 hours after the light-dark cycle phaseshift and phase-delayed significantly more slowly until resynchronization was finally achieved.

Thus, there was a temporary desynchronization between the rapidly resynchronizing circadian rhythms like body temperature and the more slowly resynchronizing rhythms such as urinary potassium excretion. The time interval

when temporary internal desynchronization occurs between circadian rhythms such as these has been postulated to underly the deterioration in performance that has been demonstrated in man after rapid travel across time zones which is commonly known as "jet-lag".

NEXT SLIDE (#12--LD disruption)

The relative stability of the urinary potassium circadian rhythm can be demonstrated in another way. In this experiment, four monkeys were subjected to a disrupted light-dark cycle which consisted of 36 hours of continuous darkness followed by 36 hours of continuous light. The urinary potassium rhythm, shown in the top portion of this slide, demonstrated an unchanged amplitude and phase and was not disrupted by the acute manipulations of the light-dark cycle.

NEXT SLIDE (#13--Schematic representation of circadian oscillators in primate)

Thus, we have developed a physiological preparation in which we can examine the organization of circadian oscillators and the mechanisms by which they are internally synchronized in primates. This slide schematically represents the physiological system in which we have studied the effects of input oscillations in environmental illumination on several oscillating physiological variables. We have demonstrated that a phaseshift in the environmental illumination pattern can result in a phaseshift over a period of several days in the feeding, body temperature and urinary potassium rhythms although the urinary potassium rhythm will take considerably longer to resynchronize than the other two. Similarly, we have demonstrated that all the functions will oscillate with the same period and constant phase relationships, i.e., they will be internally synchronized when the animal is placed in a constant level of illumination. We know very little, however, about the internal physiological machinery that is responsible for the generation of these rhythms. Thus, next we turned our attention to the investigation of whether there is a single oscillator upon which all functions are passively dependent or whether there are multiple potentially-

independent oscillators, with or without a central pacemaker. Because it is the most easily isolatable function, our next series of studies were aimed at investigating how the circadian variation of renal potassium excretion was synchronized with the other circadian oscillations which we monitored in this animal. Our aim was firstly to identify which oscillating physiological functions were responsible for synchronizing a rhythm generated in the kidney.

NEXT SLIDE (#15--Possible routes of synchronization of kidney)

Clearly there are many potential influences on the kidney. This slide depicts the oscillating physiological variables which could act as synchronizing mediators in the control of the circadian rhythm of renal potassium excretion. These include plasma potassium concentration, body core temperature, plasma concentration of various hormones such as aldosterone, cortisol and ADH, the activity of the autonomic nervous innervation of the kidney, including both cholinergic and/or adrenergic components, hemodynamic variables such as blood pressure, renal blood flow and GFR and, in addition, many other blood constituents including glucose, hydrogen ion, protein, osmolarity and electrolytes.

We first examined the role of circadian rhythms in plasma concentration of the adrenal hormones aldosterone and cortisol. These hormones were chosen because each is known to influence the rate of potassium excretion and each hormone has a circadian oscillation of concentration in the plasma which reaches the maximum at a time prior to the urinary potassium rhythm that is suggestive of a causal relationship.

NEXT SLIDE (#16--Control 8 AM cortisol and aldosterone urinary rhythms)

Monkeys were adrenalectomized and maintained by cortisol and aldosterone administered intravenously between 08.00 and 09.00 hr daily. By using implanted catheters with extensions which led outside the isolation chamber it was possible to administer the steroids in any pattern we wished without the monkeys being aware of the time of administration. This slide shows the circadian rhythm of

urinary 17-hydroxycorticosteroid, potassium and water excretion in 3 monkeys before adrenalectomy (mean and SEM represented by shaded area) and again after adrenalectomy (represented by the black line) when cortisol and aldosterone were being provided intravenously between 08.00 and 09.00 hr daily. The doses chosen were equivalent to the normal daily adrenal output of these hormones in intact animals. As can be seen from this slide, the circadian rhythm of urinary potassium excretion and that of urinary water excretion were virtually indistinguishable in intact monkeys and in adrenalectomized monkeys on this adrenal steroid replacement regimen. The urinary rhythm of 17-hydroxycorticosteroid excretion indicates that a pattern similar to normal was achieved by the adrenal steroid administration.

NEXT SLIDE (#17 --Possible mechanisms of internal synchronization, same as #3)

Because we are able to reproduce the circadian rhythms of cortisol and aldosterone secretion by the artificial infusion of these hormones in adrenalectomized animals, we can test whether these hormones are involved in the synchronization of the circadian rhythm of renal potassium excretion.

If this rhythm marked "R" is renal potassium excretion then if cortisol or aldosterone is the rhythm marked "Q" we ought to be able to phaseshift "R" by phaseshifting Q. The manner in which it is phaseshifted will give further information on whether "R", the renal potassium rhythm, is passively dependent on "Q" or whether it is controlled by an independent oscillator (marked here as "E"). We will thus be able to test whether the mechanisms of alternative 1 or those of 2 and 3 are more applicable.

NEXT SLIDE (#1 --Responses of R to Phaseshift in Q)

Thus, if R is passively dependent on Q which directly controls it a phaseshift in Q will cause an immediate phaseshift in R while other rhythms that are synchronized as before. If Q plays no role in the synchronization of the rhythm in R, then the phase of R will remain unchanged when Q is phaseshifted.

However, if Q synchronizes R in the manner the light-dark cycle synchronizes the body temperature and other rhythms but does not actually control it, then we will probably see a more slow phaseshift of R over several days so that eventually resynchronizes with Q. This would suggest that renal potassium excretion is controlled by potentially-independent oscillators. Other possible situations might be envisaged, including one where there is more than one synchronizing pathway acting on R and, hence, the phaseshift of one pathway would result in only a partial phaseshift in the synchronized rhythm R. The extent of the phaseshift would depend on the relative strengths of the couplings of the two synchronization pathways.

NEXT SLIDE (#18--Cortisol 8 hour phaseshift)

Accordingly, four adrenalectomized monkeys were subjected to an 8 hour phaseshift of the time of cortisol administration so that cortisol was given at 16:00 hours after than 08:00 hr. The light-dark cycle was kept unchanged. As can be seen from this slide the urinary potassium rhythm resynchronized with the new phase of cortisol administration. This resynchronization was not quite complete in all animals and this suggests that there may be some other minor synchronizing influences which are competing with the plasma cortisol rhythm. On this slide is also shown the phase of the feeding rhythm throughout this experiment and it can be seen that the rhythm of feeding remained synchronized with the light-dark cycle while the urinary potassium rhythm was resynchronizing with the cortisol rhythm. Thus, we have induced an internal desynchronization of these circadian rhythms.

This same response was seen whether the monkeys were given cortisol and aldosterone and both were phaseshifted or whether the monkeys were only given cortisol and the cortisol was phaseshifted. This suggests that aldosterone does not play a essential role in the synchronization process.

One interesting feature of the resynchronization of the urinary potassium rhythm with the rhythm of cortisol administration was that it took several days

before resynchronization was complete. The urinary potassium phase did not immediately jump to the new phase of the cortisol rhythm, suggesting that although cortisol acted as a synchronizing agent the urinary potassium rhythm was not passively dependent on the plasma concentration of cortisol.

NEXT SLIDE (#19 --Evening infusion of cortisol)

To test whether urinary potassium excretion was passively dependent on plasma cortisol concentration in intact monkeys, animals were given a bolus of cortisol between 20.00 and 22.00 hrs at the time that plasma cortisol concentration is normally at a minimum. Thus, these monkeys had within one day, two successive maxima of plasma cortisol concentration. Despite the two peaks of plasma cortisol concentration approximately 12 hours apart, the urinary potassium rhythm was virtually unaffected and showed only a minor change in response to the cortisol infusion. This again confirms that plasma cortisol concentration does not directly control urinary potassium excretion although it is a potent synchronizing agent.

NEXT SLIDE (#20--Possible responses to elimination of oscillation)

There is one further test that can be performed in the examination of the role of plasma cortisol concentration as a synchronizing agent. Again, we are looking at the synchronization of a rhythm R by a hormonal mediator Q. If one can control Q, or plasma cortisol concentration in this case, then one can eliminate the oscillation in Q and test whether R continues with an independent oscillation or whether it is passively dependent on Q. The first possibility is that the elimination of Q results in the total elimination of R. This would occur if R is passively dependent on Q. The second possibility is that Q does not influence R and R continues oscillating with a 24 hour period which is synchronized with other circadian rhythms within the animal. The third possibility is that Q synchronizes R but R is not passively dependent on Q. In this situation, one would expect to find free-running oscillations in R.

This is the possibility that we particularly wish to examine since the other experiments are strongly suggestive of such a synchronizing mechanism. If there were an alternate synchronization pathway such as Y then one might see simply a phaseshift in the rhythm of R but then R would regain a 24 hour period now synchronized entirely by Y.

NEXT SLIDE (#21--Continuous cortisol plus aldo infusion)

Adrenalectomized squirrel monkeys were therefore given for four days their routine 08.00 hr cortisol and aldosterone infusions through remote catheters outside the isolation chamber. Then after the first 2 days shown on this slide, a continuous infusion of aldosterone and cortisol was given so that the same 24 hour dosage was administered but was evenly spread over the 24 hours with no circadian rhythm of administration. When this continuous infusion of aldosterone and cortisol infusion was given the urinary potassium rhythm did not damp out. Instead the oscillations persisted, but demonstrated free-running periods shorter than 24 hours. Thus, the circadian rhythm of renal potassium excretion lost its strict phase relationship with the light-dark cycle.

NEXT SLIDE (#22--Fourier analysis of S-557)

A Fourier analysis of the urinary potassium rhythm of the first monkey demonstrated a free-running rhythm with two period components--one of approximately 13 hours and the other of approximately 15 hours. It appeared that the second monkey had similar length periods although there was insufficient data for complete Fourier analysis, and the third monkey studied had a circadian rhythm of around 20 hours.

NEXT SLIDE (#23--Postulated major pathway)

We therefore wish to propose that the circadian rhythm of urinary potassium excretion is generated by potentially independent oscillators which are probably intrarenal. These are synchronized with the light-dark cycle and with other circadian rhythms within the animal via the circadian oscillation in the

plasma concentration of a hormonal mediator, cortisol. The release of this hormone is known to be under the control of ACTH which demonstrates a similar circadian rhythm and this, in turn, is under the release of corticotropin releasing factor which originates from the hypothalamus. Thus, it is possible to postulate that the pituitary-adrenal axis plays an essential role in synchronizing the circadian rhythm of renal potassium excretion. We are currently examining the control at other levels of this pathway. There is evidence that similar mechanisms of synchronization may apply at other levels.

LIGHTS PLEASE

Is this model of internal synchronization compatible with other evidence on the organization of circadian oscillators? The answer appears to be "yes". There are two main pieces of evidence which would suggest that our model describes the general mode of operation of these circadian timing systems in higher animals.

Firstly, Dr. Aschoff has already pointed out that internal desynchronization occurs in some 15% of his human subjects isolated under constant conditions. He found that some of the monitored physiological variables spontaneously began to oscillate with different frequencies so that in one example body temperature oscillated with a 25 hour period while the rest-activity cycle oscillated with a 33 hour period. This is strongly suggestive that there is more than one potentially independent oscillator in primates.

Secondly, several groups have shown that certain mammalian tissues maintained in vitro will demonstrate continued circadian rhythmicity, and will function although they are kept under constant conditions. For example, Andrews has kept hamster adrenal glands in culture for up to 10 days and has demonstrated persistent circadian rhythms in corticosteroid production in these isolated glands. David Rintoul later in this Symposium will report on work in isolated liver cells maintained in vitro which show a continued circadian rhythm of enzyme

activity. These lines of evidence suggest that individual tissues and maybe even individual cells, may contain separate potentially-independent circadian oscillators. Thus, the circadian timing system would appear to consist of a large number of potentially independent oscillators which are synchronized through oscillations in hormonal and perhaps nervous mediators.

THANK YOU.

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Circadian internal desynchronization: causation by circadian arrhythmias in hormonal mediators?

When circadian rhythms in several physiological variables are monitored simultaneously in an animal, internal synchronization is usually observed. The monitored circadian rhythms have constant phase relationships whether they are synchronized with environmental time cues or free-running under constant conditions. However, internal desynchronization may occasionally occur in animals and man, particularly when the subject is in isolation. Predisposing factors include environmentally-induced stresses, psychopathology and age. We have demonstrated that the circadian rhythm of plasma cortisol concentration acts as the mediator synchronizing the circadian rhythm of renal potassium excretion with other rhythms. Internal desynchronization of the renal potassium rhythm from the activity and feeding rhythms can be induced in adrenalectomized monkeys by either a) phase-shifting the time of cortisol administration or b) by giving a continuous infusion of cortisol so that the renal potassium rhythm becomes free-running. To investigate whether such alterations of the circadian rhythm of plasma cortisol concentration occur in stressful situations, eleven hospitalized human subjects were studied. Blood samples were taken at 20-min intervals for 24 hrs from an indwelling venous catheter from four patients anticipating cardiac surgery within 24 hrs, two patients 72 hrs after surgery and five similarly hospitalized control subjects. Plasma cortisol concentration demonstrated an episodic pulsatile pattern with a circadian distribution in the control subjects, with maximum pulse frequency and plasma concentration 1-h before waking. In contrast, the pre- and post-operative patients demonstrated additional pulses of secretion which tended to result in an even distribution of cortisol pulses throughout the 24 hrs. These findings lead us to propose that internal desynchronization of circadian rhythms in situations of stress and psychopathology may be the result of circadian arrhythmias in hormonal synchronizing mediators. Supported by NASA 14249 and NIH HL 13872.

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FORMULAR FÜR DIE ABFASSUNG DER KURZFASSUNG
ABSTRACT REPRODUCTION FORM
FEUILLET DESTINÉE À LA DACTYLOGRAPHIE DU
RESUME

1 N 2 2 0 1
PLASMA CORTISOL OSCILLATIONS SYNCHRONIZE THE CIR-
CADIAN RHYTHM OF RENAL POTASSIUM EXCRETION IN THE
SQUIRREL MONKEY

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The rate of urinary potassium excretion demon-
strates a circadian rhythm in the conscious chair-
acclimatized squirrel monkey (*Saimiri sciureus*)
maintained in isolation on ad lib feeding at 25°C.
In five monkeys studied in an LD 12:12 (600:41 lux)
light-dark cycle urinary potassium excretion oscil-
lated between a maximum of $274 \pm 23 \mu\text{Eq/hr}$ (mean
 \pm SEM) at 09.00 hr CT and a minimum of $64 \pm 6 \mu\text{Eq/}$
hr at 21.00 hr CT. This rhythm was synchronized
by the light-dark cycle since it a) free-ran in
constant light and b) responded to an 8 hour
phase-delay of the LD 12:12 light-dark cycle by
resynchronizing in 7 days with the new LD phase.

To investigate whether synchronization with
the LD cycle was mediated by the pituitary-adrenal
axis, adrenalectomized monkeys were studied. The
control urinary potassium rhythm could be repro-
duced by intravenously infusing 5 mg cortisol at
00.00 hr CT daily. When the time of cortisol ad-
ministration was phase-delayed by 8 hours the
urinary potassium rhythm responded with a 6-8 hour
phase delay within 3-6 days although the LD phase
remained unchanged. This finding, together with
the observation that free-running oscillations in
urinary potassium excretion appeared when cortisol
was given as a continuous infusion in adrenalecto-
mized monkeys suggests that the renal potassium
circadian rhythm is synchronized by, but not pas-
sively dependent on the circadian rhythm of plasma
cortisol concentration. Presented by M.C.
Moore Ede, Section II-4.