these fruits apparently still contain small amounts of hypoglycin (*14*). As is shown in our studies, hypoglycin stays in the body as long as 24 hours. It also interacts with isovaleryl CoA dehydrogenase (*4*), glutaryl CoA dehydrogenase (*13*), and CoA and (−)-carnitine (*5*). Therefore, one has to raise the likelihood that small amounts of hypoglycin A ingested over a long period of time may cause cellular injury and possibly liver damage. A number of chronic liver diseases of unknown etiology and of an endemic nature (such as veno-occlusive disease) occur in Jamaica. From our findings, it would seem to us important to explore the possible role of hypoglycin and deranged isovaleric acid metabolism as factors in the pathogenesis of these forms of liver disease.

KAY TANAKA
KURT J. ISSELBACHER
Department of Medicine, Massachusetts General Hospital (Gastrointestinal Unit), and Harvard Medical School, Boston 02114
VIVIAN SHIH
Department of Neurology, Harvard Medical School, and Joseph P. Kennedy Laboratories, Massachusetts General Hospital, Boston 02114

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Circadian Rhythm in Uptake of Tritiated Thymidine by Kidney, Parotid, and Duodenum of Isoproterenol-Treated Mice

Abstract. *The total uptake of [^{3}H]thymidine by the mouse parotid gland, kidney, and duodenum exhibits a circadian rhythm. A single injection of isoproterenol changes the phasing and amplitude of these rhythms. Depending on the organ, there are certain points in the circadian time structure when isoproterenol stimulates or inhibits the uptake of thymidine; at other time points there is no difference between the responses to isoproterenol and to saline.*

The stimulation of increased DNA biosynthesis in the parotid gland and kidney of mice by a single intraperitoneal injection of isoproterenol (IPR) is of current research importance (*1–3*). Our study demonstrates that the total uptake of [^{3}H]thymidine (TdR) by parotid gland, kidney, and duodenum, as well as the effect that IPR has on this uptake, is dependent on the mouse circadian time structure.

Seventy-two inbred male BDF_1 mice (18 to 24 g) were maintained under rigidly standardized conditions consisting of freely available food and water and a controlled light-dark cycle (light from 0600 to 1800 hours C.S.T.) for 2 weeks before the initiation of the experiment. Beginning at 0900 hours and continuing at 4-hour intervals (1300, 1700, 2100, 0100, and 0500 hours) two subgroups of six mice each were injected intraperitoneally at each time point with either 0.75 ml of saline or an equal volume of distilled water containing 7.5 mg of freshly dissolved isoproterenol hydrochloride. All mice were killed exactly 28 hours after injection of either saline or IPR. Thirty minutes before being killed by cervical dislocation each mouse received a subcutaneous injection of 10 μc of [^{3}H]TdR (22 c/mmole) (*4*). Samples of parotid gland, kidney, and duodenum were removed and fixed in 10 percent buffered formalin. Subsequently each piece was transferred to 70 percent ethyl alcohol for several days, dried overnight in a desiccator, weighed to the nearest 0.001 mg, and digested with 2 ml of hyamine hydroxide at 55°C. The radioactivity was measured in a Nuclear-Chicago Mark I liquid scintillation spectrometer. In one experiment, in addition to the protocol above, pieces of kidney were weighed, homogenized in 1 percent sodium lauryl sarcosinate, and centrifuged at 1000*g*. The supernatant was precipitated with 10 percent trichloroacetic acid (5 percent final concentration) and centrifuged. The precipitate was dissolved in 0.1*N* KOH, spotted on filter paper disks (*5*), and counted.

The uptake of TdR into the parotid gland is increased by IPR (Fig. 1) when compared to the saline controls ($P <$ .001 at all points except 0100 hours where it was $<$ .02). There is a circadian rhythm in the uptake of TdR into the parotid gland in both the saline- and IPR-treated animals. In the IPR-treated animals there is a 92 percent variation between the lowest (at 0100 hours) and highest (at 1300 hours) means recorded. The difference between these means is significant ($P < .05$). The highest (at 2100 hours) and lowest (at 1700 hours) means for the controls differ by 215 percent ($P < .05$). The occurrence of the peak in TdR uptake advanced from 2100 hours in the controls to 1300 hours in the IPR group.

Unlike the parotid gland which responds maximally 28 hours after injection of IPR, the kidney does not re-

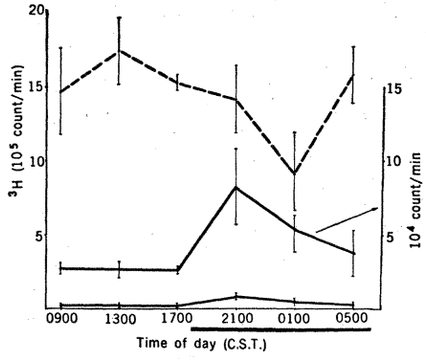

Fig. 1. Circadian pattern in the uptake of [^{3}H]thymidine (TdR) into the mouse parotid gland (solid line) and the effect of a single intraperitoneal injection of isoproterenol (dashed line) administered 28 hours previously. For example, mice injected at 0900 hours were killed at 1300 hours, or 28 hours later. On the abscissa is the time of day with darkness from 1800 to 0600 hours C.S.T. The time of killing is plotted against the uptake of [^{3}H]-TdR (10^5 count/min per gram of dry weight) for the top dashed line and the bottom solid line. (The middle solid line has an expanded scale for the saline-treated animals, the unit being 10^4 count/min per gram.) All animals received TdR 30 minutes prior to killing. Each point is the mean for six animals ± the standard error of the mean.

spond maximally until 34 hours after a single injection of IPR (*3*). Our data for the kidney (Fig. 2) were obtained 28 hours after each injection. There are only three time points along the 24-hour time scale where the uptake of TdR in the kidneys of IPR-treated animals is significantly different from the uptake in the controls. One such point occurs at 0900 hours. At this time the kidneys from IPR-treated animals have more radioactivity than controls ($P < .05$). The two other points where there is a significant difference between IPR and control groups are 0100 and 0500 hours ($P < .05$ and $< .01$, respectively). However, at these points the highest amount of radioactivity occurs in the kidneys of the controls, not in the IPR group. The effect of IPR on the kidney, like that on the parotid gland, varies significantly over the 24-hour period. The maximum mean value occurs at 0900 hours and is 113 percent different ($P < .01$) from the minimum at 0100 hours. There is a rhythm also in the uptake of TdR into the kidneys of the controls (27 percent fluctuation). Because the standard error of the highest recorded mean (0100 hours) of the saline-treated animals is so large, we could not obtain statistical significance between it and the lowest recorded mean (0900 hours); however, the mean for 0500 hours is significantly different from the 0900-hour mean ($P < .05$). The occurrence of the peak in TdR uptake advanced from 0100 hours in the controls to 0900 hours under the influence of IPR. In the kidneys subjected to the homogenization procedure (not illustrated) the overall radioactivity was greatly reduced. In this experiment the differences between the highest and lowest means recorded for the IPR- and saline-treated animals are significant ($P < .05$). The phasing of the circadian rhythms in both groups is essentially identical to that described for the total TdR uptake rhythm in kidney.

There is a rhythm in the uptake of TdR into the duodenum in both IPR- and saline-treated animals (Fig. 3). In the IPR animals, the maximum uptake occurs at 1300 hours and is different ($P < .02$) from the minimum at 0100 hours (60 percent fluctuation). The peak uptake into the duodenum of the controls occurs at 0100 and is different ($P < .01$) from the trough at 2100 hours (134 percent fluctuation). There is a significantly greater amount of radioactivity in the duodenums of IPR-treated animals than in the saline-treated group at 1300 hours ($P < .01$), 1700

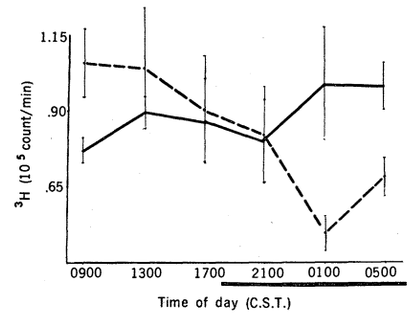

Fig. 2. Circadian pattern in the uptake of TdR into the mouse kidney (solid line) and the effect of a single intraperitoneal injection of isoproterenol (dashed line) when administered 28 hours previously. On the abscissa is the time of day with darkness from 1800 to 0600 hours C.S.T. The time of killing is plotted against the uptake of [^{3}H]TdR (10^5 count/min per gram of dry weight). All animals received TdR 30 minutes before killing. Each point is the mean of six animals ± the standard error of the mean.

($P < .01$), and 2100 hours ($P < .02$). The opposite effect is found at 0100 hours, where there is a significant decrease ($P < .02$) in radioactivity in the IPR-treated animals. At 0500 and 0900 hours there is no significant difference in the levels of radioactivity in the duodenums of the two groups. The occurrence of the peak in TdR uptake advanced from 0100 to 1300 hours in the IPR-treated animals.

There is a circadian rhythm in the response of all three organs to a single injection of IPR administered 28 hours prior to killing. The profile and phasing

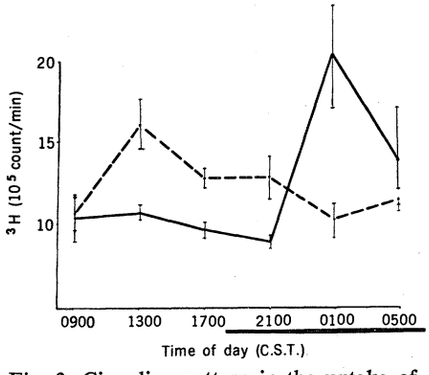

Fig. 3. Circadian pattern in the uptake of TdR into the mouse duodenum (solid line) and the effect of single intraperitoneal injection of isoproterenol (dashed line) administered 28 hours previously. On the abscissa is the time of day with darkness from 1800 to 0600 hours C.S.T. The time of killing is plotted against the uptake of [^{3}H]TdR (10^5 count/min per gram of dry weight). All animals received TdR 30 minutes prior to killing. Each point is the mean of six animals ± the standard error of the mean.

of the curves of these three organs is essentially identical. All organs demonstrate a trough at 0100 hours. The uptake of TdR reaches a peak at 1300 hours in the parotid gland and duodenum, whereas the peak in the kidney is at 0900 hours. The control mice demonstrate a circadian rhythm in the uptake of TdR, with the peak occurring at 2100 hours in the parotid gland and at 0100 hours in the kidney and duodenum. The minimum amount of radioactivity occurs at 0900 hours in the kidney, at 2100 hours in the duodenum, and 1700 hours in the parotid gland.

Because the response of the parotid gland to a single injection of IPR in standardized mice is significantly greater at all time points along the 24-hour scale, there would be little likelihood that an investigator, using the customary single time point sampling technique, would encounter any seriously conflicting results due to circadian variation. According to our data there is a more statistically significant response at the time of peak response (1300 hours) of the mouse circadian time structure than at the time of minimum response (0100 hours).

The response in the kidney and duodenum to IPR is much more complicated. In both organs there are times in the mouse biological system when IPR has no statistically significant effect, when the response is significantly stimulatory, or when the same drug causes a significant inhibition of the uptake of TdR. In short, three different results can be obtained depending on the time of the circadian time structure the drug was applied. Such differences in response to drugs or the concept of "hours of changing resistance" has been demonstrated and discussed many times (*6*).

It has been stated that the parotid gland and kidney are the only organs that respond to a single injection of IPR with cellular proliferation (*2*). We have demonstrated that this also may be the case, at certain times in the circadian time structure, in the duodenum. The response in the kidney, measured 28 hours after a single injection of IPR, also is time dependent.

We can offer no explanation to account for the timing of these rhythms. The results do emphasize the need for precise knowledge and control of the circadian time structure. To ignore this fundamental temporal organization in an organism can lead one into pitfalls. The common practice of sampling at

one time of the day, to avoid or minimize the effect of rhythms, without knowledge of the circadian time structure, is not acceptable.

E. ROBERT BURNS
LAWRENCE E. SCHEVING
TIEN-HU TSAI
Department of Anatomy,
University of Arkansas Medical Center,
Little Rock 72201

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Cyclic Adenosine Monophosphate Stimulation of Axonal Elongation

Abstract. *Elevated concentrations of adenosine 3',5'-monophosphate induce a variety of cell movements. The role of adenosine 3',5'-monophosphate in promoting those movements associated with growth prompted our study of in vitro microtubule-dependent axonal elongation. Ganglia treated with adenosine 5'-monophosphate show no enhancement over controls; treatment with adenosine 3',5'-monophosphate or its dibutyryl derivative significantly enhances elongation, as measured by increases in both axonal numbers and length. Our study suggests that adenosine 3',5'-monophosphate promotes elongation by stimulation of microtubule assembly.*

It has been suggested that many cellular events influenced by adenosine 3',5'-monophosphate (cyclic AMP) depend on intact microtubules; that is, aggregation (*1*), contact inhibition (*2*), growth (*2*), pigment migration (*3*), and secretion (*4*). In order to examine the possible relation between intracellular concentrations of cyclic AMP and microtubule assembly, a system with an easily measurable morphological feature dependent on microtubules is essential. Since cultured dorsal root ganglia undergo colchicine-sensitive axonal elongation that is dependent on microtubules (*5*), they provide an ideal system for this study. Test agents can be incorporated into the media, and the degree of resultant axonal elongation can be measured readily and can be compared to appropriate controls.

Dorsal root ganglia from 8½-day-old chick embryos (White Leghorn) were cultured on collagen-coated cover slips as double cover-slip preparations in classical Maximow culture chambers. The test agents were incorporated into basal medium 199 (Grand Island) and supplemented with 10 percent fetal calf serum (heat inactivated) (Colorado Serum). All cultures were incubated for 48 hours at 37°C, and then each culture was rated with regard to neurite length (measured with a micrometer to nearest 25 μm) and number (per quadrant). Representative cultures were then fixed overnight in Bouin's solution and photographed for permanent records. Various experimental groups were compared to controls with the aid of the *t*-test for comparison of two sample means. An IBM 1130 computer was used to calculate the mean and standard deviation of each group. Graphs were drawn to the Gaussian distribution by the IBM 1130 with the use of an IBM 1627 Calcomp plotter.

Explanted fragments of embryonic chick dorsal root ganglia exhibit an initial period of relative quiescence, usually 4 to 6 hours, followed by glial and connective tissue migration and then a period of axonal elongation. The time course of these events was variable and therefore large numbers of explants for statistical evaluation were used.

Table 1. The effects of test agents on axonal elongation.

Test condition	Axons per quadrant (No.)	Axonal length (μm)
Control	86 ± 50	178 ± 114
5'-AMP	50 ± 32	156 ± 112*
Cyclic AMP	120 ± 68	370 ± 264
Dibutyryl cyclic AMP	165 ± 68	686 ± 350

* Not significantly different from controls. All other values were significantly different when compared to controls, $P < .05$.

The effects of 48 hours of exposure to medium enriched with 5 m*M* concentrations of 5'-AMP, cyclic AMP, and dibutyryl cyclic AMP (all obtained from Schwarz Biological Research) on axonal length are shown in Fig. 1A. Treatment with 5'-AMP has no significant effect on axonal length, whereas treatment with either cyclic AMP or its dibutyryl derivative produces considerably longer axons (Table 1). Axons grown in media enriched with cyclic AMP or dibutyryl cyclic AMP are longer and are more numerous (see Fig. 1B). In contrast, explants exposed to 5'-AMP show a slight reduction in the total number of extended axons (Table 1).

Ganglia cultured in media containing 5'-AMP exhibit only slight differences from controls, whereas those ganglia treated with cyclic AMP or dibutyryl cyclic AMP show large increases in axonal numbers and lengths, two parameters that are a direct result of elongation. It is evident that cyclic AMP and dibutyryl cyclic AMP stimulate axonal elongation. The fact that dibutyryl cyclic AMP produces a higher degree of axonal elongation than cyclic AMP is not surprising, because the former is known to more readily penetrate the plasma membrane (*6*). Dibu-

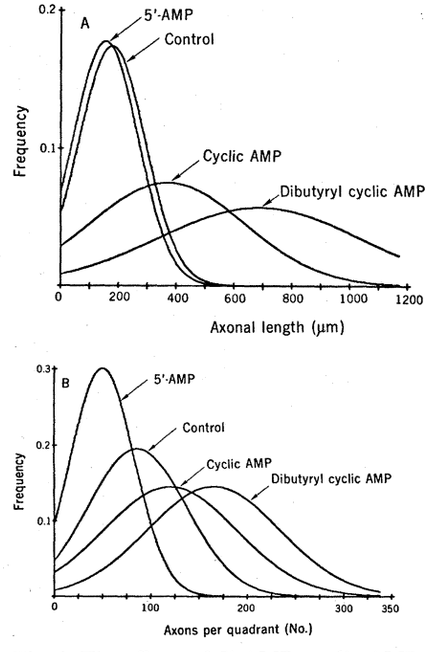

Fig. 1. The effects of 5'-AMP, cyclic AMP, and dibutyryl cyclic AMP on axonal elongation are summarized in these normalized frequency distribution histograms. (A) Axonal length. (B) Axonal number.