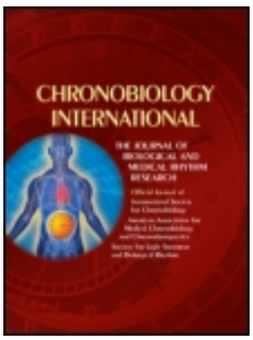

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CIRCADIAN DEPENDENT EFFECT OF EPIDERMAL GROWTH FACTOR, INSULIN AND GLUCAGON ON HEPATIC PYRUVATE KINASE AND MALIC ENZYME OF MICE

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Abstract—The influence of epidermal growth factor (EGF), 0.75 μg g^{-1}; insulin, 1.5 μg g^{-1}; glucagon, 1.25 μg g^{-1} and their combinations on the activities of hepatic pyruvate kinase (PK) and malic enzymes (ME) was monitored. Male CD2F$_1$ mice were treated toward the end of the light or dark periods, 9 or 23 *h*ours *a*fter *l*ights *o*n (9 or 23 HALO), and subgroups of six mice were killed at 4, 8 or 12 hr post-treatment. PK and ME activities from control mice were well characterized by cosine curves. The PK activity was maximal when ME activity was minimal at the transition from light to dark (9 HALO plus 4 hr) and PK was at a minimum when ME was highest (23 HALO plus 4 hr). Both enzymes were influenced by at least one peptide hormone, and the effects were strongly circadian-stage dependent. The only effect attributed to EGF was an increase of PK activity (23%) 12 hr after injection at 23 HALO. PK activity was increased by insulin (23%) at 23 HALO (4 hr after injection), but not at 9 HALO, and decreased (17%) by glucagon 12 hr after injection at 9 HALO. Several reductions in PK activity in response to various combinations of peptides were observed, and appeared to be caused by glucagon but influenced by insulin. The activity of ME was decreased (33%) in response to insulin 4 hr after injection at 23 HALO but not at 9 HALO and increased (60–70%) by glucagon alone or in combinations with insulin or EGF, or both, at 4 hr after injection at 9 HALO but not at 23 HALO. In general, when ME activity was altered by either insulin or glucagon, PK activity was also altered in the opposite direction, and the effects of glucagon were opposed by insulin.

Key words—Pyruvate kinase, malic enzyme, liver, brain, epidermal growth factor, insulin, glucagon, circadian.

Introduction

Insulin, glucagon, and epidermal growth factor (EGF) have each been shown to potently affect various aspects of liver physiology. Each of these hormones stimulate DNA synthesis in the normal mouse liver (1–4) and may play a role in hepatic regeneration (5–7). Moreover, insulin and glucagon have a proven role in hepatic glucose metabolism, and have been shown to alter a variety of intra-cellular enzymes (8). For example, insulin stimulates while glucagon inhibits pyruvate kinase (PK) activity in hepatocytes through a phosphorylation–dephosphorylation mechanism (10). However, their roles in the regulation of other enzymes, such as malic enzyme (ME), have not been established (11–13). EGF, which has abundant hepatocellular cell membrane receptors (14, 15), induced hepatic lipid accumulation in chronically injected adult female mice (16) and inhibited the stimulatory effect of glucagon on hepatocellular amino acid uptake *in vitro* (17). Studies concerning the *in vivo* effects of EGF on hepatic enzymes of glucose metabolism of the mouse have not been undertaken.

We have previously demonstrated that the enzymatic activities of PK and malic enzyme exhibited circadian variation (18). These changes could be related, in part, to circadian changes in the portal and arterial concentrations of various peptides, including insulin, glucagon and EGF. In this study, we examined the individual and combined effects of these three peptides on the activities of hepatic malic enzyme and pyruvate kinase. The hormones were administered at two different stages of the enzyme's circadian rhythms to determine if there was any evidence that the responses to them might show circadian dependence.

Materials and Methods

Two hundred and eighty-eight $CD2F_1$ treatment group male mice, 6 weeks of age, (Simmonsen Lab, Inc., Gilroy, CA) were assigned randomly to groups of six per cage, and four cages were placed in each of 12 sound-attenuated, temperature-regulated isolation chambers. The lighting was automatically regulated such that six of the chambers were illuminated from 1900 to 0700 hr (DL), whereas the other six chambers were illuminated from 0700 to 1900 hr (LD) daily. Food and water were available *ad libitum*. Thirty-six additional mice were included as untreated controls. The mice remained standardized in this manner for 5 weeks before the experiment and were disturbed weekly to clean cages and replenish food and water. Under these lighting conditions, 2 weeks was a sufficient standardization period for ME and PK activity (18). The two lighting schedules made it possible to inject all of the mice within a short span of time within the working day.

The 24 cages within DL chambers were randomly divided into eight treatment groups of three cages each. At 0400 hr [9 HALO (*h*ours *a*fter *l*ights *o*n) for the DL illumination period], mice within the individual treatment groups were injected intraperitoneally with one of eight separate treatments; saline, 0.75 $\mu g\ g^{-1}$ EGF (Bethesda Res. Lab.), 1.5 $\mu g\ g^{-1}$ of insulin (Sigma), 1.25 $\mu g\ g^{-1}$ of glucagon (Eli Lily), 0.75 $\mu g\ g^{-1}$ EGF plus 1.25 $\mu g\ g^{-1}$ glucagon, 0.75 $\mu g\ g^{-1}$ EGF plus 1.5 $\mu g\ g^{-1}$ insulin, 1.5 $\mu g\ g^{-1}$ insulin plus 1.25 $\mu g\ g^{-1}$ glucagon and 0.75 $\mu g\ g^{-1}$ EGF, 1.5 $\mu g\ g^{-1}$ insulin plus 1.25 $\mu g\ g^{-1}$ glucagon. All concentrations were adjusted in saline so that volumes of approximately 0.2ml were delivered. At 0600hr (23 HALO for the LD illumination period) the process was repeated for mice within the LD chambers. At 4, 8 and 12 hr after the 9 and 23 HALO injection times, mice were killed, livers taken and frozen in liquid nitrogen. They were subsequently prepared and analysed for PK and ME using standard procedures routinely used in our laboratory (19). This method of standardization of light schedules, treatment and sampling has previously been reported in detail (20).

A cosine regression (21, 22) was calculated for data from saline treated mice. The PK and ME activities were regressed against the relative circadian hour where the relative circadian hour was defined by the light schedule and sacrifice time. The relative circadian hour assigned to mice on the LD schedule was their actual time of killing and the relative circadian hour assigned to the mice on the DL schedule was their actual time of sacrifice plus 12 hr. The cosinor analysis determined estimates of mesor (mean activity of the rhythm), acrophase (crest of the fitted cosine curve), and amplitude (one-half the total excursion of the fitted curve). Both amplitude and mesor were expressed in enzyme activity units (μmoles product $h^{-1}g$ $tissue^{-1}$) and acrophases were reported in hours. A lack of fit test (23) was also performed to determine if the rhythm displayed any departure from the cosine model. A statistically significant cosine regression and a non-significant ($P>0.05$) sum of squares attributed to the lack of fit indicated not only that the activities were cyclic, but that a cosine curve was an accurate description of the rhythm. Treatment effects were judged by a factorial analysis of variance.

Results

Pyruvate Kinase

The data obtained for pyruvate kinase activity was well characterized by a cosine wave with a period equal to 24 hr (Figure 1). This was indicated by the significant cosine regression of PK activity from saline treated mice (untreated mice had rhythms that were statistically similar) against relative circadian hour ($P<0.0001$) with a nonsignificant sum of squares attributed to lack of fit ($P = 0.49$). The mesor was 106.3 μmoles pyruvate $h^{-1}g$ $tissue^{-1} \times 10^2$ in which the 95% confidence interval was 104.7–107.9. The amplitude was calculated to be 14 μmoles pyruvate $h^{-1}g$ $tissue^{-1} \times 10^2$ with a 95% confidence interval from 11.7 to 16.2. Thus, the calculated percentage change in activity from trough (bathyphase) to acrophase was 30%. The cosinor-determined acrophase was 2127 hr, which corresponds to 9 HALO plus 5:27 hr, with a 95% confidence interval from 2050 to 2204 hr. The highest observed activity was 122 μmole

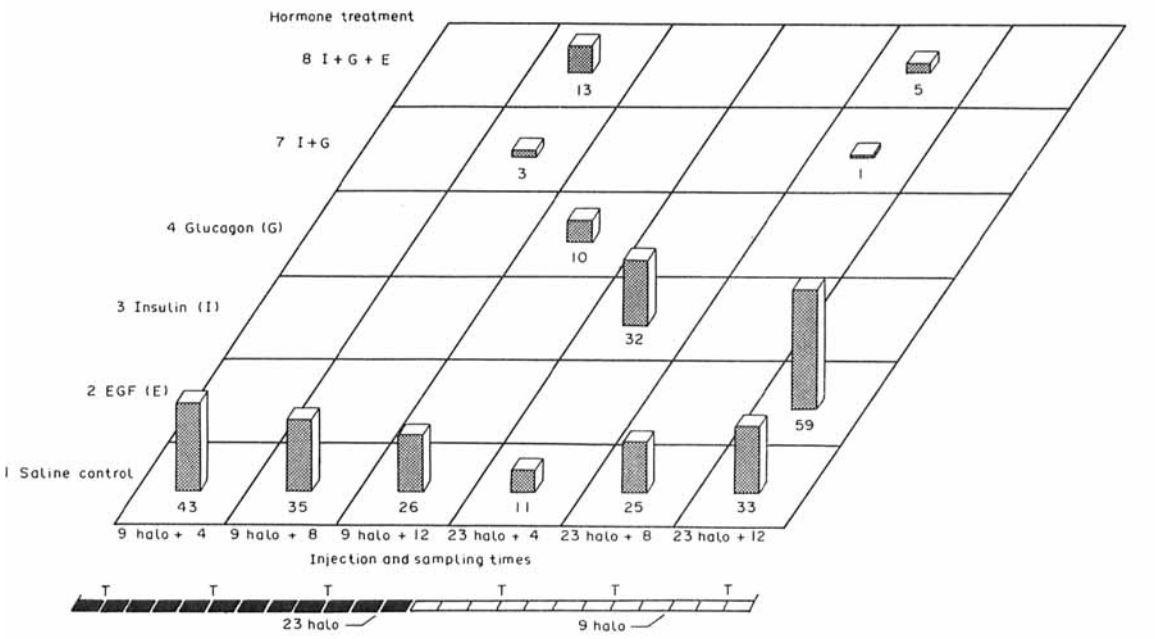

Figure 1. Effect of peptide hormones on pyruvate kinase. Only values statistically different from controls are shown. All values are expressed as μmoles pyruvate h^{-1} tissue^{-1} $\times$ 10^2, and all values were reduced by 79. HALO = *h*ours *a*fter *l*ights *o*n, arrows indicate treatment times and T's represent kill times.

pyruvate h^{-1}g tissue^{-1} $\times$ 10^2 at the 9 HALO plus 4 hr stage, which corresponds very closely to the calculated acrophase (Table 1). The lowest observed activity was 90 μmole pyruvate h^{-1}mg tissue^{-1} at 23 HALO plus 4 hr (1000 hr) which corresponds well with an anticipated lowest activity at 0927 hr (acrophase plus 12 hr).

Pyruvate kinase activity was altered by several hormone treatments (Table 1). Under the conditions of this study, EGF stimulated PK activity only at 12 hr after injection at 23 HALO (1800 hr). The increase observed was 23% above the control value. Insulin did not cause any statistically significant increases in PK activity when injections were made at 9 HALO and samplings were in the dark again. The dark span represents the time during which PK activity reached its acrophase in saline treated mice. However, PK activity was significantly increased when insulin was administered at 23 HALO and

Table 1. Hepatic pyruvate kinase circadian variation and the circadian effects of epidermal growth factor (EGF), insulin (I) and glucagon (G)

	Injection at 9 HALO			Injection at 23 HALO		
Treatment	4 hr	8 hr	12 hr	4 hr	8 hr	12 hr
Control	*122 ± 4	114 ± 3	107 ± 3	90 ± 3	104 ± 4	112 ± 6
EGF						†23
(I)				23		
(G)			-17			
EGF + G						
EGF + I						
I + G		-28			-23	
I + G + EGF		-19			-19	

*μmoles pyruvate h^{-1}g tissue^{-1} $\times$ 10^2 ± S.E.
†Percentage change listed only when statistically different from controls.

sampling was 4 hr post-treatment (time of minimal PK activity in saline treated mice). Glucagon caused a decrease in PK activity when given at 9 HALO and sampled at 12 hr post-treatment. Of the combinations of peptides tested, only the insulin plus glucagon and insulin plus glucagon plus EGF yielded statistically significant PK activity changes. Insulin plus glucagon decreased PK activity at the 9 and 23 HALO plus 8 hr stages. Addition of EGF to this mixture did not alter the result at either injection time.

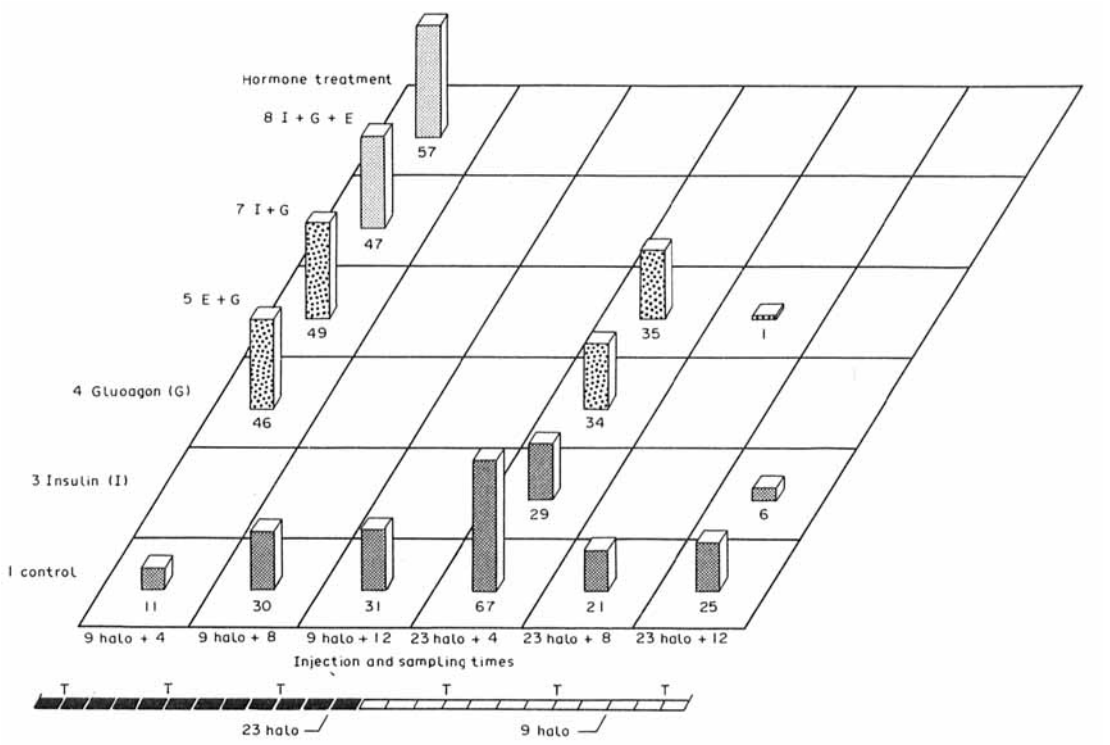

Figure 2. Effect of peptide hormones on malic enzyme. Only values statistically different from controls are shown. All values are expressed as μmoles NADPH $h^{-1}g$ $tissue^{-1}$, and all values were reduced by 49. HALO = *h*ours *a*fter *l*ights *o*n, arrows indicate treatment times and T's represent kill times.

Table 2. Hepatic malic enzyme circadian variation and the circadian dependent effects of epidermal growth factor (EGF), insulin (I) and glucagon (G)

	Injection at 9 HALO			Injection at 23 HALO		
Treatment	4 hr	8 hr	12 hr	4 hr	8 hr	12 hr
Control	*59 ± 6	79 ± 9	80 ± 6	115 ± 11	70 ± 6	73 ± 8
EGF						
(I)				-33		-26
(G)	†60			-29		
EGF + G	65			-28	-30	
EGF + I						
I + G	61					
I + G + EGF	77					

*μmoles NADPH $h^{-1}g$ $tissue^{-1}$ ± S.E.
†Percent change listed only when statistically different from controls.

Malic Enzyme

The data obtained for malic enzyme activity also was well characterized by a 24 hr cosine wave (Figure 2). As with PK, this was indicated by the significant cosine regression of ME activity from saline treated mice against relative circadian hour (P=0.004) with a non-significant sum of squares attributed to lack of fit (P=0.06). The mesor was 82.7 μmoles NADPH h^{-1} g tissue^{-1} with a 95% confidence interval of 74.6–90.7, and the amplitude was 20.2 ± 5.6 μmoles NADPH h^{-1} g tissue^{-1}. The percentage change in activity from the trough (bathyphase) to acrophase was 65%. The cosinor-determined acrophase was 0818 hr, which corresponds to 23 HALO plus 2:18 hr, with a confidence interval of 0609 to 1028 hr. The highest observed activity was 115 μmoles NADPH h^{-1} g tissue^{-1} at 23 HALO plus 4 hr which corresponds closely to the calculated acrophase (Table 2). The lowest observed activity was 59 μmoles NADPH h^{-1} g tissue^{-1} at the 9 HALO plus 4 hr stage which corresponds well with an anticipated lowest activity at 2018 hr (acrophase plus 12 hr).

Malic enzyme activity was altered by nine of the various peptide treatments (Table 2). Under the conditions of this study, EGF had no affect on the activity of ME, while insulin treatment decreased ME activities at the 23 HALO plus 4 and 12 hr stages. Insulin decreased ME activity only at the time when ME was shown to be at its acrophase in saline treated mice. Glucagon increased ME activity at the 9 HALO plus 4 hr stage (the time of minimum activity in saline treated mice), while yielding a decrease at the 23 HALO plus 4 hr stage. Similarly, glucagon in combination with EGF, insulin or both EGF and insulin increased ME activity at the 9 HALO plus 4 sampling times. EGF plus glucagon decreased ME activity at the 23 HALO plus 4 and 8 stages, respectively.

Discussion

The activities of PK and ME from saline or untreated mice were well described by 24-hr cosine curves which were out of phase by approximately 12 hr (e.g. PK acrophase = 2127 hr and ME acrophase = 0818 hr). Pyruvate kinase activity was refractory to insulin but was

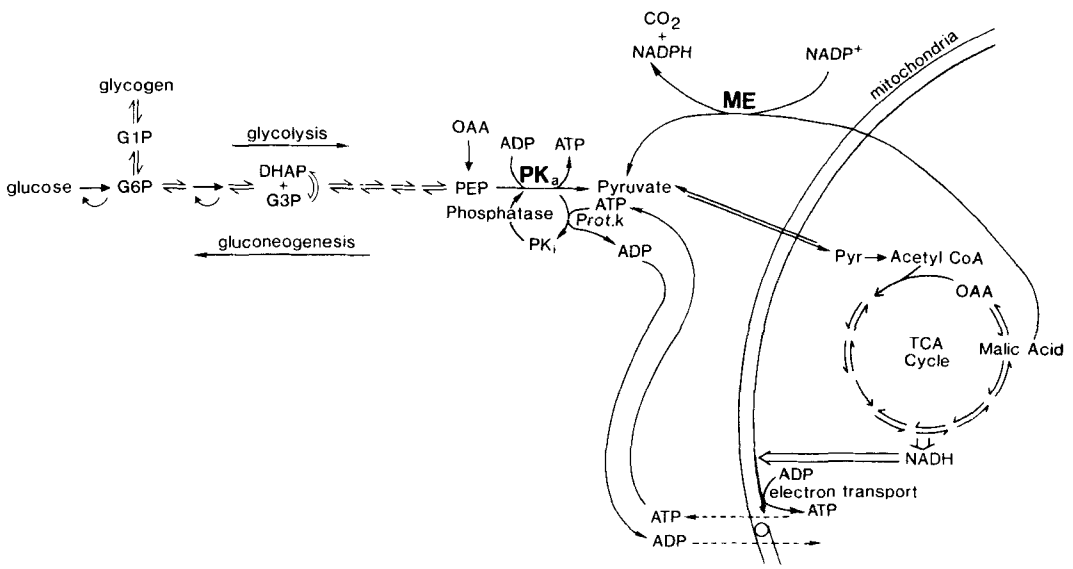

Figure 3. Relationship of pyruvate kinase and malic enzyme to cellular energy functions. Glucagon binds its receptor and stimulates adenylate cyclase which produces cAMP. Cyclic-AMP then stimulates a protein kinase which phosphorylates and inactivates PK. A phosphatase is activated upon insulin binding to its receptor which leads to the dephosphorylation of the phosphatase and reactivation of PK (Blair *et* 9, 10). Malic enzyme is probably allosterically regulated by fluctuating substrate levels (11).ME produces NADPH and shuttles pyruvate back out of the mitochondria in opposition to PK. In this context it would be appropriate for PK activity to be high when ME was low and vice versa.

decreased by glucagon at its acrophase while being refractory to glucagon but increased by insulin at its bathyphase. Malic enzyme activity was not increased by glucagon but was decreased by insulin at its acrophase, yet it was refractory to insulin, but increased by glucagon at its bathyphase. These observations indicate that the responses of these enzymes to the hormones were strongly influenced by the circadian stage at which the injection was made. The time dependencies described by these data are consistent with the notion that the circadian variations of these hormones may play prominent roles in modulation of the circadian rhythms of PK and ME. This argument can be further supported by the observations that the circadian rhythms of insulin and glucagon have been shown to be out of phase in rodents (24, 25) and insulin's rhythm approximates, but immediately precedes the phasing of PK, while the glucagon rhythm approximates that of ME.

Hormone receptor numbers have been found to vary in a circadian manner; adrenoreceptor sites from rat heart ventricles were 40% higher at the beginning of the dark than at the beginning of the light span (26), and down regulation of insulin receptors in hepatocytes diminishes glucogenic responses to insulin (27). Down regulation of insulin and glucagon receptors in response to high endogenous levels of the hormones could account for the refractory responses of PK to insulin and ME to glucagon at their respective acrophases. These refractory responses indicate there may be a maximum (and minimum) level past which PK and ME cannot be stimulated (or reduced) by insulin or glucagon. Each of these points, and the fact that the responses were at early times after treatment implicate regulation of existing PK and ME by insulin and glucagon, but not new synthesis and degradation, as the mechanism for modulation of their activities across the light and dark spans (Figure 3).

Under the conditions of this study, epidermal growth factor does not appear to be strongly involved in the modulation of the circadian rhythm of either PK or ME. Epidermal growth factor had no effect on ME at either treatment time. However, there was an increase in PK activity in response to EGF at only the latest sampling time subsequent to treatment just before the end of the dark span (23 HALO plus 12 hr stage). In a more recent study, EGF has been found to stimulate PK when sampling was at 16 hr after injection at the beginning of the light or dark span (data not shown). This later increase at 16 hr may indicate that new synthesis was required to increase PK activity in response to EGF.

The administration of the various combinations of peptides may bring about several outcomes. Glucagon increased ME activity, and in combination with any other peptide brought about statistically significant increases of the same magnitude. EGF plus insulin yielded no effect. We conclude that only glucagon affects ME activity at 9 HALO and it overrides any effect of either insulin or EGF. Unfortunately, the peptides given in combination produced complex interactions and no other clear interpretation of these observations can be made at this time.

Glucagon treatment at 9 HALO vs treatment at 23 HALO yielded opposite effects on ME. At 9 HALO, glucagon stimulated ME activity and at 23 HALO the peptide depressed ME activity. Therefore, a strong circadian-stage dependence was noted for the two selected time points. The paracrine effect of glucagon vs insulin may play a role since glucagon stimulates the release of insulin from beta cells of the pancreas, while insulin inhibits release of glucagon from the pancreas. Thus, the decreased ME activity observed at 23 HALO for glucagon may, in fact, be a response to insulin.

Insulin, glucagon, and EGF appear to be involved in the regulation of hepatic ME and PK activities. Further, insulin and glucagon, but not EGF, may be involved in the modulation of the circadian variations which have been shown to characterize these enzymes. It should be re-emphasized that the data presented represent testing for circadian-stage effect at only two time points and therefore does not fully resolve the temporal influence. However, the selection of the two time points was made against earlier denser data on untreated mice. Additionally, the present results may have been influenced by the

sampling times employed, and 4 hr may be too late for observation of maximal hormonal activation or inhibition of previously synthesized enzymes, while 12 hr may not be sufficient to observe activity changes due to altered rates of enzyme synthesis or degradation. To resolve more fully these influences of time, additional studies must be undertaken. Given the complexity of this experiment, it also is possible that an observation was statistically significant by chance. This is less likely where the data have reasonable and logical explanations, but may account for a portion of the other observations, especially those involving combinations of peptides. To those interested in enzyme regulation or intermediary metabolism, these data strongly suggest a sophisticated system for modulation of circadian rhythms of enzymes which may be fruitfully exploited.

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