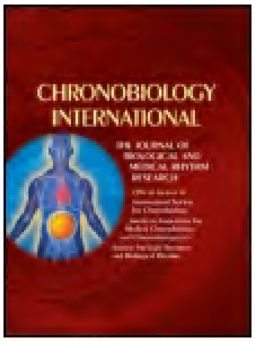

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THE EFFECTS OF VARIOUS LIGHTING SCHEDULES UPON THE CIRCADIAN RHYTHMS OF 23 LIVER OR BRAIN ENZYMES OF C57BL/6J MICE

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Abstract—The activities of 23 brain or liver enzymes were studied in 5–6 week old C57BL/6JNctr male and female mice that had been fed *ad libitum* and standardized for 2 weeks to either (1) 12 hr of light (0600–1800) alternating with 12 hr of darkness (1800–0600) (LD 12 : 12), (2) staggered sequences of 12 hr of light and 12 hr of dark (SLD 12 : 12) or (3) continuous illumination (LL 12 : 12) for 2 weeks. Mice in the LD 12 : 12 and LL 12 : 12 experiments were killed at 4 hr intervals along a 24-hr span in order to sample at six different circadian stages. Lighting schedules for mice in the SLD 12 : 12 experiment were organized such that six different circadian stages were sampled when all mice were killed at one time of day.

All 23 enzymes demonstrated a prominent circadian rhythm in at least one of the experiments. Moreover, about two-thirds of the enzymes in LD and SLD 12 : 12 had a statistically significant fit to a 24-hr cosine curve, while only one-third of the enzymes in LL 12 : 12 had significant fits to cosine curves. Peak activities of enzymes from mice in LD 12 : 12 were clustered at the time of transition from light to dark. This was also the trend for the activities of enzymes from mice in SLD 12 : 12, but resynchronization did not appear completed within the 2-week span. This, along with the observation that mesors (mean 24-hr activity) were reduced and amplitudes altered, indicated that the 2-week standardization period was not sufficient for some enzymes. Times of peak activities, mesors and amplitudes were affected for most enzymes from mice in the LL 12 : 12 environment. This suggests that individual mice became desynchronized from one another with respect to the original light–dark schedule and that rhythms were altered or lost because individual mice were free running with frequencies different from 24 hr.

Introduction

Metabolic functions mediated by enzymes are subject to rhythmic fluctuation across a 24-hr span; several enzymes have been reported to undergo pronounced daily fluctuation in activity (1–4). Such changes have been most often correlated to alteration in the rest–activity cycle, influenced in rodents by the environmental light–dark schedule and to meal timing (1, 5–8). In most laboratory animal studies only one or a few enzymes were measured. Recently we reported that 19 enzymes assayed from liver or brain of male mice of the (BALB/cNCox × DBA/2NCox) F_1 hybrid (CDF_1) demonstrated prominent circadian rhythms when standardized to either 12 hr light, followed by 12 hr dark (LD 12 : 12) or to a reversed light–dark cycle (DL 12 : 12) (9–11).

We recently developed a biochemical specific locus assay which identifies transmissible germ cell mutations by monitoring a battery of enzymes in progeny of chemical mutagen treated C57BL/6JNctr male mice (12–14). This assay detects the mutant as the heterozygote and is dependent upon measurement of relatively small changes in enzyme activity. Because circadian fluctuation might obscure changes caused by

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mutations, it was important to determine whether rhythms similar to those measured in CDF_1 mice existed for C57BL/6J Nctr mice and if there was a sex influence since animals of both sexes were used. Moreover, from a practical point it was important to obtain information about the alteration of enzyme rhythms following manipulation of the light-dark schedules or by subjecting animals to continuous illumination (LL).

Materials and Methods

Two hundred and ninety-four C57BL/6JNctr male and female weanling (3–4 weeks of age) mice were used in this study. The mice were randomized across cages. They were placed seven to a cage, and groups of two cages (one with seven males; one with seven females) were placed in separate isolation chambers. Each chamber was sound attenuated to equalize noise levels in each chamber, had its own ventilation system, and the lights were automatically regulated. Mice were standardized to these conditions for two weeks. Since there were three different experiments in this study, all done on the same day, they are described as Experiments 1, 2 and 3.

Experiment 1. In this experiment, seven chambers were illuminated from 0600 to 1800 and darkened from 1800 to 0600 (LD 12 : 12) [see light–dark schedule in chamber 1 (Figure 1)]. Thus to sample 'round the clock' the investigator had to remain awake 24 hr.

Experiment 2. Six chambers were illuminated with 12 hr of light alternating with 12 hr of dark (LD). The LD schedules were staggered (S) so that in one chamber the light went on and off at 0600 and 1800, in another at 1000 and 2200, in another at 1400 and 0200, in another at 1800 and 0600, in another from 2200 and 1000, and in the sixth chamber the lights went on at 0200 and off at 1400. We refer to such a schedule where the lights were turned on and off in sequence in each chamber as a staggered light-dark cycle (SLD 12 : 12). It should be noted that in chamber 1, where the lights went on at 0600 and off at 1800 there were two cages of males and two cages of females. This was done in order to have a group that was killed at the beginning and a group from the same light–dark cycle killed at the end of the sampling period, thus providing a balanced sampling of circadian stages relative to Experiments 1 and 3. (See Figure 1.) Use of the SLD conditions made it possible to sample all animals in Experiment 2 within a short span (0815–0900) and presumably equivalent to sampling at six different mouse circadian stages.

Experiment 3. Similar to Experiment 1 there were seven chambers; however, in this case the chambers were continuously illuminated (LL).

All mice received Purina Laboratory Chow No. 5008 ($\geqslant$ 23% protein, $\geqslant$ 6.5% fat, $\leqslant$ 4% fiber, $\leqslant$ 8% ash and $\leqslant$ 2.5% added minerals) and water *ad libitum*. They remained under these standardized conditions for 2 weeks prior to sampling and were not intentionally disturbed (except for the perturbation caused by replacing cages once a week and the replenishing of food and water). If it was necessary to open a chamber containing a group of animals subjected to dark, a dim red light was used.

Beginning at 0815, 5/23/80, all mice from the SLD schedule of Experiment 2 were killed by rapid cervical dislocation. This was done by successively removing two cages from each of the six chambers and killing all mice in the two cages within a minute to avoid full activation of the pituitary adrenal axis which might affect enzyme

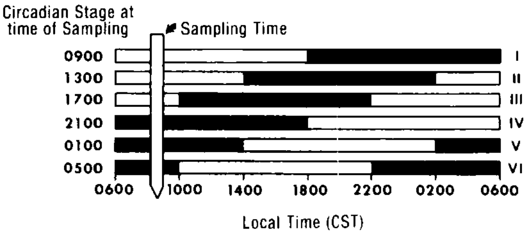

Figure 1. The abscissa relates to local time and permits a comparison of the time when the lights went 'on and off' in each of the six chambers. The vertical bar is the 45-min time span during which sampling took place for all six chambers. The times on the ordinate are the comparable mouse circadian times for animals subjected to SLD 12 : 12, assuming that phase shifting was completed. The Roman numerals are the chamber numbers.

activity. This procedure was repeated for both Experiments 1 and 3 at 0900; however, only the mice in two cages (seven males; seven females) from each of the two experiments were killed at each circadian stage. The procedures of both Experiments 1 and 3 were repeated at 4-hr intervals throughout the day and night with the 0900 sampling repeated on the second day.

The livers and brains were quickly removed after killing. The tissues were wrapped in prelabeled aluminium foil squares and immediately frozen in liquid nitrogen. Sampling of each circadian stage was completed in less than 7 min. The tissues were subsequently stored at -70°C to await preparation and analysis.

Materials, tissue preparation and enzyme analysis have been previously described (9, 15). Homogenates from tissues were assayed for a total of 23 enzymes; 17 from the liver and six from the brain. Livers were assayed for isocitrate dehydrogenase (ICD, EC 1.1.1.42), glutamate dehydrogenase (GlDH, EC 1.4.1.3), lactate dehydrogenase (LDH, EC 1.1.1.27), alcohol dehydrogenase (ADH, EC 1.1.1.1), glutathione reductase (GR, EC 1.6.4.2) glyoxalate reductase (GlyR, EC 1.1.1.26), L-alanine aminotransferase (GPT, EC 2.6.1.2), glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1), pyruvate decarboxylase (PDC, EC 4.1.1.1), fructose-1-phosphate aldolase (F1P, Ald, EC 4.1.2.7), fructose diphosphate aldolase (FDP, Ald, EC 4.1.2.13), fructose diphosphatase, (FDPase, EC 3.1.3.11), fatty acid synthetase (FAS, EC 2.3.1.38), amino acid oxidase (AAO, EC 1.4.3.3), citrate cleavage enzyme (CCE, EC 4.1.3.8), cytochrome c reductase (CCR, EC 1.6.2.5) and sorbitol dehydrogenase (SbDH, EC 1.1.1.14). Brains were assayed for phosophoglucose iso-

Table 1. Rhythmometric summary of C57BL/6NJctr mice standardized to LD12 : 12

	Male							Female						
Enzyme	Cosine fit (*P*)	Mesor *M* ±	S.E.	Amplitude *A* ±	S.E.	Acrophase in: DEG±	S.E.	Cosine fit (*P*)	Mesor *M* ±	S.E.	Amplitude *A* ±	S.E.	Acrophase in: DEG±	S.E.
Brain														
AK	0.11	2606	20	58	29	-312	28	0.52	2464	31	53	43	-229	47
ATPase	0.08	705	10	31	14	-117	26	0.04	629	20	72	28	-168	22
CPK	0.01	2795	4	19	6	-238	18	0.26	2775	6	13	8	-270	35
MDH	0.05	32671	326	1235	463	-220	21	<0.01	31250	346	1930	487	-196	14
PGI	<0.01	4667	12	87	16	-249	11	<0.01	4617	15	83	21	-240	14
PK	<0.01	7186	12	359	89	-239	14	0.19	6932	62	171	88	-215	29
Liver														
AAO	<0.01	228	8	71	11	-251	9	<0.01	261	9	107	12	-260	6
ADH	0.21	186	3	7	4	-319	34	<0.01	213	4	19	5	-38	15
CCE	<0.01	189	7	64	10	-262	9	<0.01	237	9	93	13	-263	8
CCR	0.17	848	25	65	35	-348	31	<0.01	1380	22	221	31	-239	8
FAS	<0.01	352	5	41	7	-264	10	<0.01	331	6	29	9	-339	17
FDP, Ald	0.02	420	8	30	11	-308	20	0.03	389	10	43	15	-48	20
FDPase	0.15	169	3	10	5	-271	29	0.04	181	3	13	5	-6	21
FIP, Ald	0.01	411	7	28	9	-292	20	0.33	383	9	21	13	-65	37
GlDH	0.02	1871	35	135	50	-286	21	<0.01	2075	34	184	48	-271	15
GlyR	<0.01	19564	81	71	12	-231	9	<0.01	18977	10	70	14	-253	11
GOT	0.21	9216	169	403	239	-301	34	0.05	8482	142	513	201	-238	22
GPT	<0.01	2117	29	224	41	-256	11	<0.01	2454	36	403	51	-249	7
GR	<0.01	505	4	41	6	-248	8	<0.01	475	4	25	5	-261	12
ICD	0.23	3082	45	107	64	-283	34	0.27	3339	34	83	48	-249	33
LDH	<0.01	22467	369	2038	520	-286	15	0.03	19044	504	2032	712	-16	20
PDC	0.03	6018	43	159	61	-309	22	0.26	5600	73	177	103	-14	33
SBDH	<0.01	896	10	63	15	-242	13	0.55	924	15	22	21	-313	56

Activities (Mesor and amplitude) = μ moles NAD$^+$/hr/g tissue.

P is probability, anything less than 0.05 indicates a statistically significant 24-hr rhythm detection.

DEG is acrophase assigned in degrees (e.g. 1200 CST = 180°) with reference to 0000.

merase (PGI, EC 5.3.1.9), adenosine triphosphatase (ATPase, EC 3.6.1.3), creatine phosphokinase (CPK, EC 2.7.3.2), pyruvate kinase (PK, EC 2.7.1.40), adenylate kinase (AK, EC 2.7.4.3) and malate dehydrogenase (MDH, EC 1.1.1.37) activities. All values are reported as μM of nicotinamide adenine dinucleotide produced/hr/g of tissue.

The data for each enzyme were analyzed by a factorial analysis of variance (ANOVA) on sex, circadian stage and their interaction. This analysis determined whether significant changes in enzyme activity occurred among the six different circadian stages or sexes within each of the three experiments. The interaction sex vs the circadian stage provided a measure of whether or not the pattern of enzyme activities across the 24-hr span was similar in the two sexes.

In addition, the data for each enzyme and sex were analyzed by the 'cosinor' method (16, 17) to determine: (1) the significance of the approximation of the data by cosine curve with a period equaling 24 hr. Essentially, this is a test that the amplitude of the rhythm is not equal to zero, that is, a straight line would not better be an approximate characterization of the data (17); and (2) three circadian rhythmic parameters (and their dispersions in the case of rhythm detection): the acrophase (ϕ), mesor (M) and amplitude (A).

The acrophase represents the crest of the approximating cosine curve in relation to some arbitrarily selected reference point along the 24-hr time scale. Usually ϕ corresponds to the time when the actual values were, on average, highest; however, it should be noted that ϕ may not necessarily be the time when the peak value was recorded. The reference point chosen in this study was local midnight. Frequently, ϕ is expressed in degrees rather than hr. If 360° = 24 hr, then 15° = 1 hr. The minus sign preceding

Table 2. Rhythmometric summary of C57BL/6JNctr mice standardized to SLD 12 : 12

	Male							Female						
Enzyme	Cosine fit (*P*)	Mesor *M* ±	S.E.	Amplitude *A* ±	S.E.	Acrophase in: DEG±	S.E.	Cosine fit (*P*)	Mesor *M* ±	S.E.	Amplitude *A* ±	S.E.	Acrophase in: DEG±	S.E.
Brain														
AK	<0.01	4233	112	519	159	-350	18	0.04	4206	115	4185	164	-355	22
ATPase	0.22	460	12	32	17	-250	31	0.10	436	12	40	18	-243	25
CPK	<0.01	1808	11	63	16	-345	15	<0.01	1787	9	68	13	-14	11
MDH	0.23	40132	346	917	491	-41	31	0.09	38403	524	1710	748	-72	25
PGI	<0.01	4962	17	139	25	-35	10	<0.01	4931	27	137	38	-50	16
PK	<0.01	5945	79	394	113	-346	16	<0.01	5944	88	433	125	-6	16
Liver														
AAO	<0.01	137	6	34	8	-208	13	0.14	160	8	23	11	-245	27
ADH	<0.01	118	3	18	5	-175	15	0.15	106	4	12	6	-60	27
CCE	0.02	129	5	20	6	-217	18	<0.01	129	5	7	7	-255	13
CCR	0.14	645	13	38	18	-234	27	<0.01	843	15	116	21	-273	10
FAS	<0.01	389	6	42	9	-194	12	0.98	328	7	2	10	-275	319
FDP, Ald	0.27	228	8	17	11	-116	37	<0.01	208	7	42	10	-39	14
FDPase	0.02	123	2	10	3	-232	18	0.65	119	3	4	4	-342	64
FIP, Ald	0.05	245	7	21	9	-131	25	<0.01	217	7	36	9	-45	15
GIDH	<0.01	2309	20	144	29	-206	11	<0.01	2486	39	202	55	-272	16
GlyR	<0.01	17164	177	1015	251	-208	14	<0.01	14973	186	959	266	-240	16
GOT	<0.01	7290	101	648	144	-177	13	<0.01	8354	148	786	212	-213	15
GPT	<0.01	1621	24	151	33	-232	13	0.10	1852	35	107	50	-281	27
GR	<0.01	473	6	44	9	-205	12	<0.01	421	7	38	9	-234	14
ICD	0.03	1805	71	299	100	-43	19	0.02	1790	64	262	92	-154	20
LDH	0.10	16473	418	1321	597	-190	26	0.21	13199	438	1181	625	-35	30
PDC	<0.01	8117	149	712	212	-173	17	0.12	6576	180	558	257	-77	26
SBDH	<0.01	675	11	70	15	-225	12	0.12	642	15	43	22	-316	29

Activities (Mesor and amplitude) = μmoles NAD$^+$/hr/g tissue.

P is probability, anything less than 0.05 indicates a statistically significant 24-hr rhythm detection.

DEG is acrophase assigned in degrees (e.g. 1200 CST = 180°) with reference to 0000.

the ϕ indicates the acrophase is given as a delay from the selected reference.

The mesor is the cosinor-determined overall 24-hr mean and is equivalent to the 24-hr arithmetical mean only if the data were equidistant as in this study. The amplitude is defined as one-half of the total cosine excursion best approximating the rhythm. It represents the distance between the mesor and the crest of the approximating function. Both A and M are expressed in actual units of enzyme activity (μmol NAD$^+$/hr/g tissue).

Results and Discussion

For each experiment and each enzyme, a factorial analysis of variance (ANOVA) was computed to test the significance of the activity differences attributed to circadian stage, sex or their interactions. By ANOVA, statistically significant differences in the activity of most enzymes along the 24-hr time scale in all three experiments were documented (95% Experiment 1, 83% Experiment 2 and 93% Experiment 3), thus suggesting rhythmic variation. The exceptions were FDPase and F1P, Ald in the LD 12 : 12 experiment, FAS, FDPase and the LDH in the SLD 12 : 12 experiment and MDH, PGI and PK in the LL 12 : 12 experiment (P values > 0.05). The enzymes PDC and SbDH in the LD 12 : 12 experiment exhibited borderline statistical significance ($P = 0.05$). However, four of these enzymes, F1P,Ald and MDH, PGI and PK also showed a statistically significant sex circadian stage interaction ($P < 0.05$). This meant the temporal patterns for these four enzymes differed between males and females. ANOVA showed statistically significant differences in enzyme activity between males and females in these same four enzymes.

Some of the enzymes showed no sex differences (P values > 0.05) and the circadian stage vs sex comparison also was not significant (SbDH in Experiment 1, CPK, FDPase and PGI in Experiment 2 and CPK and FAS in Experiment 3) indicating that the sexes exhibited similar temporal patterns for the activity of these enzymes across the 24-hr span. For the other enzymes which did not show activity differences between sexes (AK, CCE, ICD and PK in Experiment 2 and AAO, AK, ATPase, FDP,Ald, LDH, MDH, PK and SbDH in Experiment 3), there was a significant effect which was attributed to an interaction between sex and circadian stage.

More detailed analysis of these variations in activity across the 24-hr span was made using the 'cosinor' technique. Seventy per cent of the enzymes from LD males and females were characterized by a statistically significant fit of a cosine curve ($P \leqslant 0.05$), almost 50% were significant below the 0.01 level. Sixty-seven per cent of the enzymes from SLD male and female demonstrated statistically significant circadian rhythmicity at the 0.05 level; however, only 35% of the LL male and female enzymes exhibited statistical significance at the 0.05 level. Those enzymes which were assigned acrophases, without confidence intervals were rhythmic according to the ANOVA test but not by cosinor (Tables 1, 2 and 3, Figures 2–7).

The data from the conventionally conducted LD 12 : 12 experiment showed that the ϕ of both liver and brain enzymes were generally clustered around the time of transition from light to dark (Table 1, Figure 2). This was not surprising since in rodents the onset of feeding, which has been associated with stimulation of some enzyme activities in mice (1), usually occurs at the time of transition from light to dark. In the case of brain enzymes, this was in contrast to findings in a previous study (27 June 1978) using CDF$_1$ mice when it was found the ϕ clustered near the end of the dark span (9). Moreover, in that study, the phasing of the brain enzyme rhythms had shifted within a 2-week span after the LD 12 : 12 schedule had simply been inverted by 180° (DL 12 : 12).

Staggered light–dark schedule

From a practical point it was important to know whether the staging of the various enzymes of animals subjected to the SLD 12 : 12 schedule was different from the staging of the rhythms of enzymes in the animals subjected to the conventional LD schedule. If the rhythmic parameters of an enzyme rhythm from mice subjected to the SLD 12 : 12 were similar to those of mice

Table 3. Rhythmometric summary of C57BL/6JNctr mice standardized to LL 12 : 12

	Male							Female						
Enzyme	Cosine fit (P)	Mesor M ±	S.E.	Amplitude A ±	S.E.	Acrophase in: DEG ±	S.E.	Cosine fit (P)	Mesor M ±	S.E.	Amplitude A ±	S.E.	Acrophase in: DEG±	S.E.
Brain														
AK	0.10	1858	12	85	17	-111	11	0.30	1872	15	80	21	-56	15
ATPase	0.01	709	8	38	12	-201	18	<0.01	727	13	63	18	-222	16
CPK	0.06	2674	5	17	7	-165	24	0.78	2666	9	8	12	-139	87
MDH	<0.01	28064	147	929	208	-200	13	0.98	28322	234	75	332	-202	255
PGI	0.88	5319	14	10	20	-139	122	0.07	5369	15	51	21	-1	24
PK	0.44	7720	54	94	77	-115	47	0.32	7875	85	186	120	-13	37
Liver														
AAO	0.11	280	10	32	14	-21	26	0.65	266	12	17	17	-43	58
ADH	<0.01	183	4	26	5	-330	11	<0.01	194	5	38	7	-24	10
CCE	0.65	221	8	10	11	-335	65	0.67	238	8	9	11	-122	67
CCR	0.52	355	12	18	17	-167	52	0.07	440	5	18	8	-104	25
FAS	0.23	274	5	25	7	-347	16	0.06	268	7	39	10	-334	14
FDP, Ald	0.06	370	10	55	14	-316	14	0.06	355	11	63	16	-342	14
FDPase	0.28	145	3	17	5	-292	17	0.06	158	6	35	8	-331	13
FIP, Ald	0.47	350	8	13	11	-333	49	0.08	334	8	26	12	-357	25
GIDH	0.02	1800	27	109	39	-332	21	0.16	1909	43	124	61	-44	28
GlyR	<0.01	16169	138	961	195	-299	12	<0.01	15722	173	1447	246	-310	10
GOT	0.05	10032	158	615	224	-263	21	0.06	11406	251	949	355	-29	21
GPT	0.14	2503	48	142	68	-15	28	<0.01	2633	45	268	63	-49	14
GR	<0.01	417	2	16	4	-69	14	0.51	401	4	7	6	-12	48
ICD	0.54	3113	37	56	52	-354	53	<0.01	3403	41	271	59	-27	12
LDH	0.12	16328	373	1114	529	-1	27	<0.01	15633	473	3076	671	-334	13
PDC	0.01	3091	29	127	41	-11	18	<0.01	2982	46	363	65	-3	10
SBDH	0.12	809	10	28	14	-167	28	0.01	822	14	65	20	-39	17

Activities (Mesor and amplitude) = μmoles NAD+/hr/g tissue.
P is probability, anything less than 0.05 indicates a statistically significant 24-hr rhythm detection.
DEG is acrophase assigned in degrees (e.g. 1200 CST = 180°) with reference to 0000.

subjected to the LD schedule, this would imply that the rhythm had completely phase-shifted to the six different light–dark schedules of the chambers within a span of 2 weeks. If this had been the case, such a schedule could be employed to achieve within a short span of the day the equivalent of sampling animals at fixed intervals 'round the clock', thereby eliminating the inconvenience of having to remain awake during both the day and the night to sample. In the past we have found that some rhythmic variables, such as temperature, the mitotic index in some tissues and other variables had completely phase-shifted to such a SLD cycle well within 2 weeks and this was the rationale for selecting the 2-week waiting period before studying the enzymes. Unfortunately, in the case of at least some enzymes, the 2-week span was not a sufficient time for the SLD schedule to bring about a completed shift in circadian staging. This becomes evident simply by comparing the ϕs of the enzymes of Figures 2 and 3. In Figure 2, the typical phasing of enzymes was seen with most ϕs clustering around the beginning of the dark span. It was obvious that such a state had not yet been achieved for some enzymes in the SLD animals (Figure 3). However, it was clear that the process of resynchronizing to the new light–dark schedule within the chambers was occurring as shown in Figures 5–7 for glutathione reductase, amino acid oxidase and cytochrome reductase. These three enzymes were arbitrarily selected as typical examples. From previous experience we predict that approximately one more week would have been sufficient to completely phase-shift most, if not all, rhythms but this remains to be proven.

It is of practical interest that our previous study (9) of CDF_1 mice showed that two weeks was sufficient time to phase-shift all the enzyme

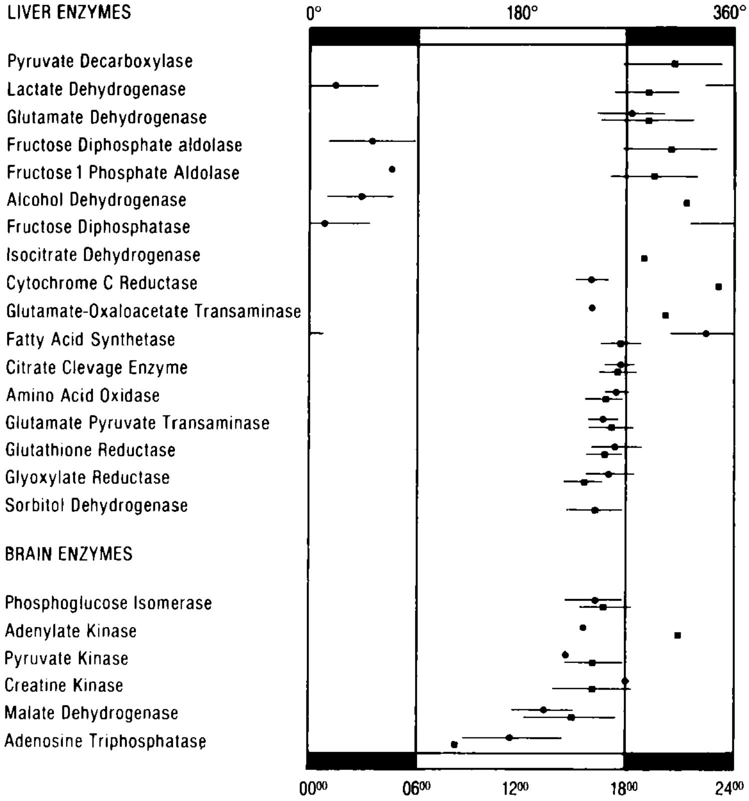

Figure 2. Acrophase map for enzymes from male (square) and female (circle) mice of Experiment 1 (LD 12 : 12). The acrophase approximates the crest of the circadian cycle and is shown in reference to the light-dark cycle. Those acrophases that did not show a statistically significant fit to a 24-hr cosine curve are not shown with confidence limits.

rhythms described herein by simply inverting the light-dark schedule by 180°. This would be the equivalent of having the two groups of animals standardized for 2 weeks in Chambers I and IV as shown in Figure 1. It is understandable from knowledge of phase shifting and the complexity of the SLD schedule why some rhythmic variables such as enzymes might take longer to completely phase shift when compared to simple 180° phase shift. From the practical point of view when working with these enzymes, one may simply standardize two groups of animals for two weeks; 1 group subjected to a LD 12 : 12 light-dark schedule, and the other to a DL 12 : 12 schedule. With such schedules, after 2 weeks, sampling can be performed at 4-hr intervals during a 12-hr span of the normal day and one can obtain the physiological equivalent of sampling three times during the light and three times during the dark; of course shorter sampling intervals could also be used.

The process of phase shifting in response to the SLD system involves re-attaining expected amplitude and relative activity (mesor) normally associated with the enzyme rhythm demonstrated in animals subjected to a conventional LD cycle. Thirteen enzymes from males showed statistical significance by cosinor for data gathered during both the LD 12 : 12 and SLD 12 : 12 experiments. Of these, amplitudes were significantly decreased for SLD 12 : 12 mice in two cases, increased in two cases, and unchanged or back to expected levels in the other nine cases. Seven enzymes from females exhibited statistical

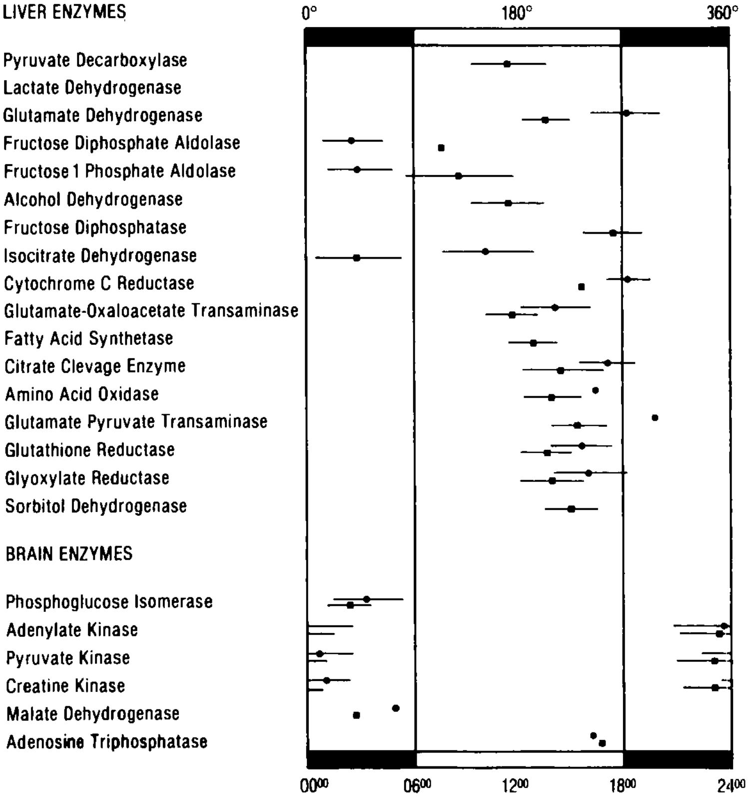

Figure 3. Acrophase map for enzymes from male (square) and female (circle) mice of Experiment 2 (SLD12 : 12). Enzymes which were not found to have statistically significant temporal variation by ANOVA were not considered in the cosinor analysis, therefore no acrophase could be assigned. The acrophase approximates the peak of the circadian cycle and is shown in reference to the light–dark cycle. The light–dark cycle represents local time so that the data of all three experiments can be compared. Those acrophases that did not show a statistically significant fit to a 24-hr cosine curve are not shown with confidence limits.

significance by cosinor for data gathered during both the LD 12 : 12 and SLD 12 : 12 experiments. Of these, amplitudes were significantly decreased for SLD 12 : 12 mice in three cases, increased in no cases and unchanged or back to expected levels in the other four cases. Even in the cases where the enzyme rhythms were not documented by cosinor in both experiments, the relative range of values over time were similar in SLD and LD mice. Statistical comparisons of SLD or LD mesors of both males and females for the 23 enzymes indicate that significant decreases were present in 16 cases, increases were present in five cases, while mesors of two male and female enzymes were unchanged or back to normal levels. This suggests that phase-shifting affects the level of enzyme activity and amplitude as a group phenomenon. The re-synchronization of amplitude occurs more rapidly than that for mesor for mice in the SLD 12 : 12 system. Taken together, these data indicate that phase-shifting of rhythms was, in many cases, completed while very nearly completed for several other enzymes (Tables 1 and 2; Figures 2, 3 and 5–7).

The chronograms shown in Figures 5–7 are interesting in that they illustrate the complexity of the phase shifting process. In the first case

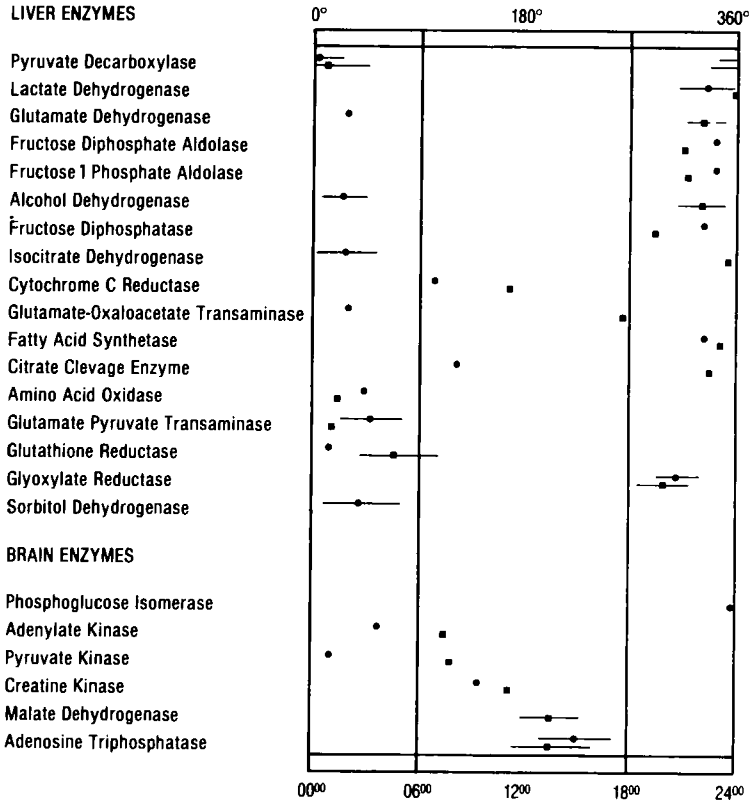

Figure 4. Acrophase map for enzymes from male (square) and female (circle) mice of Experiment 3 subjected to continuous illumination (LL 12 : 12). The vertical lines represent where the dark span began and ended prior to animals being placed in LL 12 : 12. Acrophases without confidence limits represent a lack of statistical significance for the fit of a 24-hr cosine curve to the data.

shown in Figure 5 (glutathione reductase) the rhythm in the SLD mice was almost completely phase shifted within 2 weeks but the mesor of the same rhythm in LL animals was dramatically reduced. In still another enzyme rhythm (amino acid oxidase), a primary acrophase was seen in the SLD animals at precisely the same time as the acrophase for the LD animals; however, a minor peak also persisted indicating in the case of this enzyme that it had not completely phase shifted in some animals. We predict that with time this enzyme would completely phase shift in all animals. In the case of this enzyme in animals subjected to LL the mesor remained about the same but the rhythm had become multiphasic. In the case of a third enzyme (cytochrome C reductase) the acrophase of the rhythm in SLD animals had completely phase shifted but the mesor was still dramatically reduced. This enzyme in animals subjected to LL completely flattened within 2 weeks.

The different responses to the SLD can be explained at the molecular level since the regulation of gene expression contains the element of timing in relation to feeding, light schedule and/or other environmental factors. It is known that cyclic feeding might generate enzyme rhythms and be related to the availability and concentration of amino acids and other monomers which affect the synthesis of certain enzymes (1). The phase shift to the SLD schedule may be a function of the genome

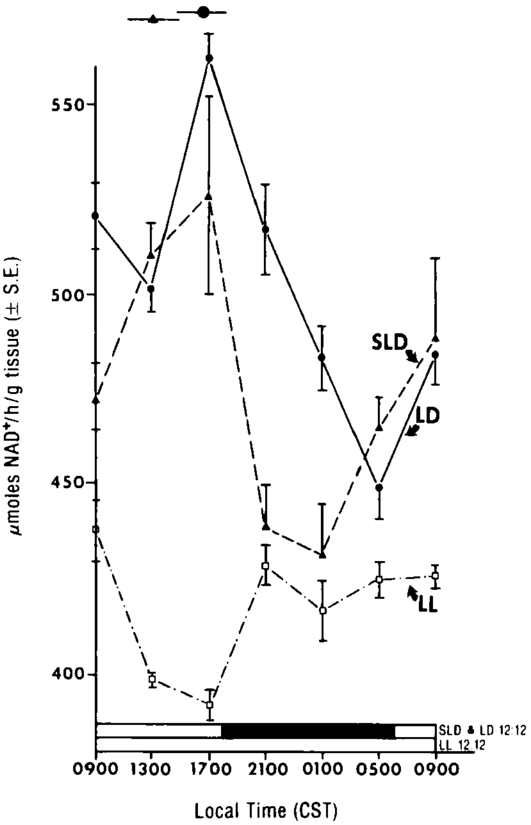

Figure 5. Chronograms illustrating daily variations in glutathione reductase activity in male mice standardized to either LD 12 : 12 in Experiment 1 (●————●), SLD 12 : 12 in Experiment 2 (▲— — — —▲) or LL 12 : 12 in Experiment 3 (□ —— — —— □). ———●——— and ———▲——— denote cosinor assigned acrophases and 95% confidence interval where cosinor P value was < 0.05. For this enzyme SLD mice had completed phase shifting. LL mice had a flattened response with a depressed mesor. For this enzyme the 2-week standardization span was sufficient for the SLD experiment.

differentially adjusting to the environmental signals. It is also known that hormone synthesis and release is rhythmic (18) and can affect permeability of enzyme substrates and synthesis of a variety of gene products. The phase shift to the SLD schedule could have a dramatic effect on the availability of various hormones and indirectly affect the synthesis of specific gene products. Additionally, translation of messenger RNA is very important relative to gene expression and has been shown to display daily and/or circadian rhythmicity (18). It is conceivable that translation of different messenger RNAs, as well as their stability, may be affected by the SLD phase shifting, accounting for the observed results.

Enzyme activity in animals subjected to LL

It has been generally accepted that when animals have been subjected to prolonged LL,

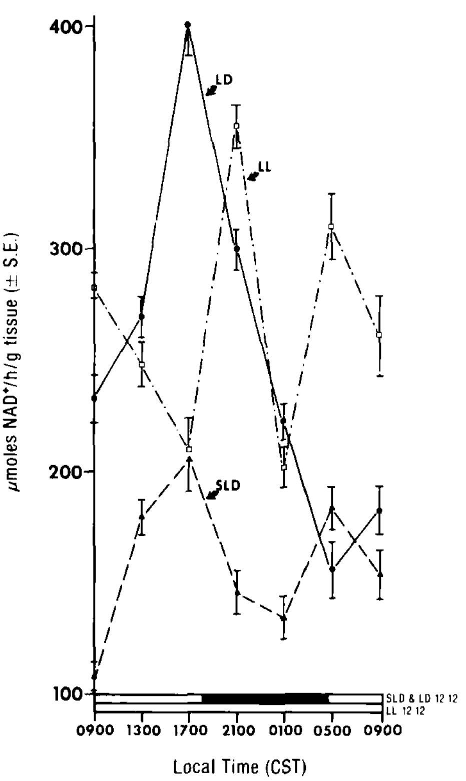

Figure 6. Chronograms illustrating daily variations in amino acid oxidase activity in female mice standardized to either LD 12 : 12 in Experiment 1 (●————●), SLD 12 : 12 in Experiment 2 (▲— — — —▲) or LL 12 : 12 in Experiment 3 (□————□). ————●———— denotes cosinor assigned acrophase with 95% confidence interval for LD mice. For SLD mice phase-shifting was nearing completion, but amplitude and mesor were reduced. Note the biphasic response to the SLD. It was speculated that with longer standardization times phase-shifting would go to completion with a single peak of activity and regained amplitude and mesor. This example suggests that enzymes phase shift at different rates (see Figure 5). LL mice were unchanged with respect to mesor and range of the data; however, the original monophasic rhythm was lost and the transient rhythm was multi-phasic. The initial loss of the monophasic rhythm was rapid. With time, further changes would be predicted; there may even be a flattening of the rhythm (Figure 5).

the typical population rhythm seen in LD gradually becomes modified in phasing and amplitude. The reproducibility of the rhythm no longer can be demonstrated from one study to another. A population rhythm in a variable being measured in LL animals may manifest itself in the following different ways: (1) it may remain with the unimodal wave form for a period of time, or it may become bi- or trimodal; (2) it may flatten or become completely obliterated and remain so or (3) it may reappear at a later date in a modified form. It is evident from a number of studies on rodents (19–24) that the general tendency over time is for the LD

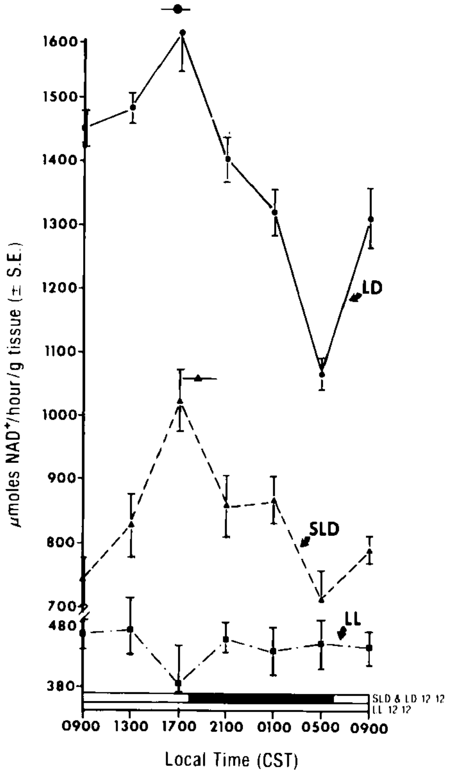

Figure 7. Chronograms illustrating daily variations in cytochrome c reductase activity in female mice standardized to either LD 12 : 12 in Experiment 1 (●————●, SLD 12 : 12 in Experiment 2 (▲— — — — ▲) or LL 12 : 12 in Experiment 3 (□— — — — □). ————●———— denotes cosinor assigned acrophase with 95% confidence interval for LD mice. For SLD mice phase-shifting was essentially completed and original amplitude was present, but mesor remained significantly depressed after the 2-week standardization period. This demonstrates that although 2 weeks was sufficient time to complete phase shifting, it was not enough time to re-obtain a normal mesor. The response of LL mice has become essentially flat and mesor is dramatically depressed. This was evidence that the original rhythm was lost within the 2-week standardization period for this enzyme.

population rhythm to flatten in LL; the flattening process may take considerable time to develop, and consequently, a relatively high amplitude rhythm may remain for some time after placing the animals in LL. The flattening of the population rhythm does not imply that the rhythms in the individual mouse have been obliterated. It is quite possible that the rhythm in each mouse persists and the individual mice were free-running on different circadian frequencies with the net result being a deterioration of the population rhythms. We have demonstrated such a phenomenon, in response to histamine (20). A classic description of this phenomenon of free-running, for the body temperature of single mice was that of Halberg (22–24). Of course the alternative explanation would be that a rhythm in a particular enzyme was lost in each mouse.

In this study only the activities of four of the enzymes in the LL environment were found to exhibit statistically significant circadian changes in both sexes (ADH, GR, and PDC from liver and ATPase from brain). Seven other enzymes exhibited statistically significant rhythms in one sex only; the rest of the enzyme activities were not circadian rhythmic by cosinor analysis; this implies that the waveform of the rhythm was dramatically altered in LL. Five enzymes from males were circadian rhythmic in both LD and LL environments. Of these, the amplitude of one was decreased and four were unchanged. Five enzymes from females were circadian rhythmic in both LD and LL. The amplitudes were increased in one enzyme, decreased in one enzyme and remained the same in three enzymes. The range in the time series data for most of the enzymes in LL not documented to evidence rhythmicity was unchanged or even decreased when compared to that of enzymes from mice of LD environments (data not shown). Mesors for males and females were significantly reduced for 12 enzymes, increased for four enzymes and unchanged for seven enzymes (see Tables 2 and 3, Figures 2, 4 and 5–7). In cases where there were increases in amplitude, bi- or trimodal waveforms were observed and mesors were usually not significantly changed, indicating that the desynchronization process was initiated. To summarize, most enzymes from mice in LL were found to exhibit statistically significant temporal differences by the ANOVA test parameters, but were not found to be circadian rhythmic by the cosinor technique. The amplitudes and range of the data were either reduced or unchanged while mesors also were either reduced or unchanged for the majority of the enzymes. Together, this suggests, but does not prove, the same phenomenon for enzymes that we have described previously for other rhythms in animals kept in LL (19–20). All such data demonstrate that exposure of animals to continuous illumination disrupts the circadian pattern of enzyme behavior in a complex manner.

From a practical point, we know that it is not uncommon to find laboratories which subject animals to LL (some claim they do this to avoid rhythms), whether it be by design or by accident. Such a practice is unwarranted not only because of the enzyme activity rhythm disruptions presented herein, but also because it has been shown (21) that by subjecting rats to LL and comparing them with blinded animals or animals subjected to LD that the LL has a deleterious effect on overall health. The fact that we have demonstrated herein such variation in enzyme rhythms suggests further the importance of not subjecting rodents to such an environment whether it be for a long or a short span of time.

In summary, the majority of the enzymes studied here were remarkably rhythmic in the LD and SLD animals and the activities from many show a statistically significant fit to a 24-hr cosine curve. It appears that in animals fed *ad libitum* that the phasing of the rhythms was related to the light–dark schedule; this does not imply that the time of food intake was not important in determining the circadian staging of these enzymes. However, the 2-week standardization period used in the SLD study was not as effective in terms of phase shifting as was the technique used in our earlier study (27 June 1978) of simply reversing the light–dark cycle 180°. In the SLD schedule it was not uncommon to see a bi-modal rhythm, this is brought about by the gradual phase shifting; most likely some animals from certain chambers phase shifted much faster than animals from other chambers. The data suggest that mice subjected to continuous illumination were desynchronized from one another as evidenced by the gradual loss of the typical population rhythm. This does not imply that the enzyme rhythms in individual mice disappeared (although this is not proven). From earlier work we know that rhythms in mice subjected to LL do persist; examples of such rhythmic variables are body temperature (22), motor activity (25) and response to histamine (20). A consideration of the organism's time structure, as revealed by its rhythms, may lead to a better understanding of the use of enzymes as endpoints in biochemical and toxicological studies. This becomes especially pertinent when it can be shown that a fixed dose of a potentially toxic agent may kill most of the animals to which it is given at one circadian stage, whereas in those treated at another stage, only a few animals or none may die (22, 26, 27). Finally, recognition of rhythms may account for much variation that once was uncritically described as 'random'.

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