CIRCADIAN MANIFESTATIONS OF BARBITURATE HABITUATION, ADDICTION AND WITHDRAWAL IN THE RAT

by

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INTRODUCTION

When administered in a punctate fashion to the rat, pentobarbital is a *chronobiotic*, i.e. it phase-shifts chronotypically the circadian rhythm (Ehret, et al., [2]). Chronic administration of dietary phenobarbital (0.3%) in the absence of other temporal cues results in dyschronism, i.e., the loss of circadian synchrony, within the first 5 or 6 days of the start of the habit (Ehret, C. F., and K. W. Dobra[3]. The present study extends these observations to include diverse habituation and drug ingestion circumstances, including a comparison of the effect of pre-entrainment cycles of programmed feeding and illumination (FS 7:17, DL 17:7 vs FS 12:12, DL 12:12) and subsequent conditions of exposure (FF, DL 12:12 vs FF, DD and FF, DL 17:7) of duration sufficient to permit a "steady-state" in the appearance of circadian patterns derived from core-temperature telemetry and from automated food-consumption measurements. Finally, measurements were permitted to continue long enough after the drug was withdrawn from the diet to allow characterization of an *abstinence syndrome*, indicative of addiction.

MATERIALS AND METHODS

Male rats of the Charles River /CD strain 40 days of age were housed individually in thirty-six cylindrical glass cages, suspended on stainless steel wire grids, and visually isolated from one another by a foil wrapped barrier that also served as a radio-frequency shield. Intraperitoneal temperatures of each animal were monitored by implanted miniature radio-telemeters, and temperature measures were recorded by a data acquisition system at 15 mi intervals. Programmed feeding of the animals was accomplished by stainless steel food hoppers, two per cage, which can

enter or leave the cage on clock command by a motor switching system. This arrangement permits the onset of administration of phenobarbital in the diet to be programmed to occur automatically and without room entry. The food hoppers are suspended from force transducers that permit the automatic monitoring of food consumption, recorded at prescribed intervals by the data acquisition system. Distilled water was continuously available for drinking.

Illumination during entrainment was provided by 15 W daylight fluorescent lamps that contributed about 100 lux within the cage during light (L) phases of dark-light (DL) entrainment cycles; the latter consisted of 12 h of dim light (D) and 12 h of L daily in one series (DL 12:12), and 17 h of D and 7 h of L (DL 17:7) in another. During D phases of LD cycles, as well as during free-run (DD), cages were dimly illuminated at about 0.1 lux from 15 W incandescent lamps operated constantly at 70% full power. Animals were housed in controlled environment rooms maintained at 20°C and 50% R.H. The entire system for programmed feeding and data acquisition is described in detail elsewhere (Meinert et al.,[6]). Control and experimental diets were nutritionally complete and pelleted, and contained 30% casein (Teklad TD 68483); the experimental diets also contained 0.25% phenobarbital.

Statistical time-series analysis of temperature data was accomplished by following the cosinor method (Halberg et at.,[4], Rummel et al.,[8], Halberg and Katinas,[5]).

RESULTS

Series 1--DL 12:12, FS 12:12

The relationship between intraperitoneal temperature and time in days since the beginning of entrainment (DL 12:12, FS 12:12) is shown for 8 rats (Figures 1-3). In this series, program feeding ended with the onset of D phase on day 11, and proceeded *ad libitum* (FF) until day 47 (Figure 3), when program feeding (FS 7:17) resumed; from the end of L phase, day 35

until day 44 (Figures 2 and 3) animals remained in free-run (DDFF). Within a day or two of entry of phenobarbital into the diet (dashed vertical line, day 14, Figure 1) the neatly symmetrical individual chronograms are significantly perturbed and at one time or another, selected individuals are markedly or marginally dyschronic for one or more days. Following this interval of habituation, characterized as a transient of several days of highly irregular wave form, a new and regularly symmetrical circadian oscillation develops and becomes established (days 19-22, Figure 1, and Figure 2, days 23-35) during LD, and persists even during a week of free-run (DD, days 34-43, Figure 3). The new wave form for the dietary phenobarbital group is characterized by a marked increase in the amplitude of the sharply defined circadian oscillation, and by a displacement of the thermal peak to the left. This shift of the peak to early in the presence of the LD zeitgeber represents a phase-angle difference--or psi-shift (Aschoff et al.[1])--between the oscillation and the zeitgeber of about 2.1 h, equivalent to an advance in thermal acrophase (Ψ-advance) relative to controls of about 32 degrees (days 34,35, Figure 2, and cosinors IC and IIIC, Figure 4). It is important to note that this Ψ-shift had already been established by the fourth day on phenobarbital (cosinor IIIB, Figure 4), and that controls retained their characteristic phase throughout days 14-35 (cosinor IB, and IC, Figure 4). From days 35-44 (Figures 2 and 3), LD entrainment ceased and all animals remained on free-run (DDFF). The control group showed the expected phase drift to late (see Table I), equivalent to a free-running period, τ, of about 24 h 24 mi $\pm$ 9 mi (cosinor IID, Figure 4), equivalent to a free running τ of about

24 h 22 mi + 4 mi. On the 44th day, the regimen of dietary phenobarbital was discontinued in the phenobarbital group, and all animals were restored to the control diet. A gap in the data occurs at this point, but beyond the gap it is evident that a striking change in the wave form has occurred: every animal is markedly dyschronic on at least one or more days for at least four days following removal of phenobarbital from the diet (Figure 3 and Figure 4, cosinor IVE, for abstinence days two and three). By the fifth day (day 49) on the control diet and in the presence of entrainment (DL 17:7, FS 7:17) circadian rhythmicity begins to resume (Figure 3 and Figure 4, cosinor IVF, for abstinence days four and five). After eight or nine days of abstinence from phenobarbital, a normal circadian oscillation appears to be fairly well reestablished (Figure 3, days 52, 53 and Figure 4, cosinor IVG). Throughout this interval, controls have retained circadian synchrony, with minor phase adjustments associated with shifts from DDFF to DLFS regimens (Figure 3, D-G, and Figure 4, cosinors IID-IIG).

Series 2--DL 17:7, FS 7:17

In this series, program feeding ended on day 11 at the end of F phase, and was followed by two days of starvation (SS) after which on day 14 *ad libitum* feeding (FF) began at the end of that day's L phase. Animals then remained for 9 days in free run (DDFF). Administration of dietary phenobarbital thus began in the phenobarbital group of animals on day 14, with the commencement of FF following SS (Ehret and Dobra[3]). As in the first series, disturbances in the normal wave form are evident for several days (Figure 5, days 15-17, and Figure 8, cosinor VIIB). In this case in the absence of a reference

zeitgeber one cannot speak of Ψ shift; but if any motion of the acrophase relative to control values has occurred, it is in the direction of *late* rather than early (cosinor VIIB compared with cosinor VB). The large error in phase and amplitude associated with free run is reduced during days 24-35, when the animals were returned to DL 17:7 entrainment, with FF continuing. Now in the presence of a zeitgeber one can speak in terms of Ψ shifts as before in Series 1, but this time there is none to see: the phase of the oscillation is securely established in the phenobarbital group, but is not significantly different from that of the controls (Figure 6C, and Figure 8, cosinors VC and VIIC). The amplitude of the oscillation is also enhanced, but not nearly so much nor so significantly as in series 1 on the DL 12:12 regimen. Upon return to DDFF, many of the animals in the phenobarbital group again became marginally or totally dyschronic on one or another day (Figure 7, days 40-43), although the group mean remains significantly in phase with controls (Figure 8, cosinors VIIID and VID). Upon withdrawal of phenobarbital from the diet, a wide range of responses are evident (Figure 7, days 45-53), including a transient of dyschronism (Figure 8, cosinor 8E) and recovery in most animals by the fourth and fifth days of abstinence (Figure 7, F, and Figure 8, cosinor VIIIF), and in all animals by the eight and ninth days (Figure 7, G, and Figure 8, cosinor VIIIG). During the second free-running interval in series 2(DDFF, days 35-44) the control group showed a free-running period of about 24 h 27 mi ± 9 mi (calculated from Table II, control group, C and D) and the phenobarbital group showed a nearly equivalent value for τ of about 24 h 28 mi ± 8 mi (calculated from Table II, phenobarbital group, C and D).

DISCUSSION

The present study shows that the dyschronogenic effect of dietary phenobarbital (Ehret and Dobra[3]) is a habituation transient that lasts for several days at the dosages administered (0.25%, Figs. 1 and 5), and is then followed by an indefinitely long tolerance interval. The latter is characterized by circadian oscillations with well-defined thermal acrophases, and moderately or considerably enhanced amplitudes (Figs. 2 and 6, Tables 1 and 2). Conditions of free-run (DDFF) tend to prolong habituation (Fig. 5) and to increase the probability of dyschronism even during tolerance (Figs. 3 and 7); animals previously entrained on DL 17:7 are more susceptible to DD dyschronism in the presence of phenobarbital than are animals previously entrained on DL 12:12 (Figs. 7 and 3). Finally, in each series, withdrawal of the drug from the diet results in an abstinence syndrome indicative of addiction and characterized by distorted wave forms and circadian dyschronism for 3-6 d even during DL entrainment, and before normal circadian patterns are restored (Figs. 3 and 7).

One of the most interesting points to emerge from the comparison of series 1 with series 2 animals, is the dramatic difference in response to phenobarbital during tolerance (Figs. 2 and 6), when animals are exposed to different illumination regimens, that compare DL phase ratios that are 1:1 (DL 12:12) with those that are $\sim$ 2.4:1 (DL 17:7). In series 1, during DL 12:12, the circadian wave form was dramatically distorted by an increase in amplitude (from 0.7°C to 1.2°C) and by a phase angle difference (Ψ-advance) of about 32 degrees, equivalent to an advance of 2.1 hours in the thermal acrophase (Table I and Figs. 2 and 4). In sharp contrast, in series 2, during DL 17:7, the amplitude of the oscillation was only slightly increased above the normal, and no significant Ψ-shift was seen (Table II, and Figs. 6 and 8). These results appear to fit remarkably well with the predictions of Wever[9] for circumstances analogous to these. To paraphrase Wever, in dark-active organisms, the phase of the biological oscillation advances relative to the zeitgeber (DL) if the DL phase-ratio increases. This is

precisely what is seen when one compares the initial state of control animals in series 1 with those in series 2: the latter DL 17:7 animals show acrophases earlier than middle of D phase (i.e., "advances"); whereas series 1 animals show acrophases later than middle of D phase (cf, Tables I and II, and cosinors IA and IB, Fig. 4, vs. cosinors ^{VA} and ^{VB}, Fig. 8). In the presence of phenobarbital series 2 animals appear as if already "Ψ-shifted" towards the limits of entrainment (Wever[9]), and show no further displacement; they are indistinguishable in phase from controls during entrainment (cosinor VC, Fig. 8). However in the presence of phenobarbital in series 1 animals (DL 12:12), a dramatic phase-angle difference (Ψ -advance) between the zeitgeber and the biological oscillation is seen (cosinor IIIC, Fig. 4). This Ψ-shift of 32 degrees during drug addiction appears as if in the presence of the drug the duration of the L phase were reduced and the DL phase-ratio were increased. These differences may also be associated with the different free-running periods of rats entrained on the two regimens: controls in series 1 (DL 12:12) had a τ of 24 h 24 mi, and addicted animals a τ of 24 h 22 mi; in series 2 (DL 17:7) controls and addicted animals had longer periods, with τ's of 24 h 27 mi and 24 h 28 mi respectively. Thus, free-running periods vary only slightly as a function of the values of the entrainment phase ratios (DL) that precede the measures; but the durations of these periods are not significantly altered during phenobarbital addiction (Tables I and II).

In each series, food consumption was monitored continually, and was nearly the same in controls and in drug-addicted animals; however FF animals on the control diet eat round the clock, albeit at a greater rate during dark than during light phase, whereas rats on phenobarbital (DL 12:12) start to eat earlier and confine their eating almost exclusively to early dark phase. These feeding data are analyzed in detail elsewhere, in a paper that focuses upon the role of dietary phenobarbital as a cocarcinogen (Peraino et al.[7]).

SUMMARY

The influence of dietary phenobarbital (0.25%) upon the circadian rhythm of intraperitoneal temperature in the Charles River male rat was measured in long time series. A complete barbiturate habituation, addition, and withdrawl sequence is observable from *circadian* criteria alone: 1) the early habituation effect, characterized by transients of circadian dyschronism, 2) the "steady-state" tolerance or addiction effect, with remarkably stable amplitude enhancements and Ψ-shifts dependent in magnitude upon phase ratio of the zeitgeber, and 3) an abstinence syndrome characterized by severe dyschronism when the drug is withdrawn.

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TABLE I

CONFIDENCE REGIONS FOR PHASE AND AMPLITUDE AS A FUNCTION OF TIME IN THE PHENOBARBITAL HABITUATION, ADDICTION AND WITHDRAWAL SEQUENCE.* FIRST SERIES, DL 12:12

		Days of Experiment	Days ON/OFF Phenobarbital	N	ϕ (h)	99% CA (± mi)	A (°C)	99% CI (± °C)	P
CONTROL GROUP	A	9,10	0	10	1212	40	.68	.11	.0001
	B	16,17	0	10	1312	64	.64	.16	.0001
	C	34,35	0	9	1328	40	.66	.11	.0001
	D	42,43	0	9	1616	90	.59	.20	.0001
	E	46,47	-	9	1636	40	.74	.13	.0001
	F	48,49	-	9	1736	32	.81	.11	.0001
	G	52,53	-	10	1720	32	.87	.11	.0001
PHENOBARBITAL GROUP	A	9,10	0	10	1224	32	.67	.09	.0001
	B	16,17	3,4	10	1040	60	.82	.22	.0001
	C	34,35	21,22	9	1044	24	.98	.11	.0001
	D	42,43	29,30	9	1300	32	.81	.11	.0001
	E	46,47	-2,-3	9	1532	164	.29	.13	.0001
	F	48,49	-4,-5	9	1704	96	.30	.12	.0001
	G	52,53	-8,-9	10	1656	36	.62	.08	.0001

*From cosine curves fitted to data from the two-day spectral windows indicated at the left (A-G in each group), acrophases (ϕ), and amplitudes (A) were calculated, along with their respective confidence arcs (CA) and confidence intervals (CI). N is the number of animals in a group, and P is the significance level as derived from the F-test (Halberg and Katinas[5]).

TABLE II

CONFIDENCE REGIONS FOR PHASE AND AMPLITUDE AS A FUNCTION OF TIME IN THE PHENOBARBITAL HABITUATION, ADDICTION AND WITHDRAWAL SEQUENCE. SECOND SERIES, DL 17:7

		Days of Experiment	Days ON/OFF Phenobarbital	N	ϕ (h)	99% CA (± mi)	A (°C)	99% CI (± °C)	P
CONTROL GROUP	A	9,10	0	7	1608	44	.71	.13	.0001
	B	16,17	0	7	1628	116	.25	.11	.0001
	C	34,35	0	7	1700	76	.46	.14	.0001
	D	42,43	0	7	2008	60	.56	.14	.0001
	E	46,47	-	7	1828	60	.65	.16	.0001
	F	48,49	-	7	1800	56	.54	.13	.0001
	G	52,53	-	7	1652	44	.71	.13	.0001
PHENOBARBITAL GROUP	A	9,10	0	7	1552	44	.64	.13	.0001
	B	16,17	3,4	7	1912	64	.41	.12	.0001
	C	34,35	21,22	7	1728	40	.67	.11	.0001
	D	42,43	29,30	7	2048	76	.31	.09	.0001
	E	46,47	-2,-3	7	0048	356	.12	.12	.0013
	F	48,49	-4,-5	7	1512	84	.28	.09	.0001
	G	52,53	-8,-9	7	1432	40	.72	.11	.0001

Figure 1. Intraperitoneal temperature measured every 15 minutes for 8 animals over 14 days. The regimens for lighting and feeding (DL 12:12) are given at the top, L = light phase from 1800-0600 h, F = food available, S = food withdrawn. The top animal is a control and the 7 remaining animals received a diet containing 0.25% phenobarbital *ad libitum* (FF) commencing at the dashed line (0600 on day 14). Bracketed areas at bottom (A and B) indicate that statistical analyses were performed on the data on these days.

Figure 2. Intraperitoneal temperature measured every 15 minutes for 8 animals over 14 days. The regimens for lighting (L) with the light phase from 1800-0600 h (DL 12:12), and feeding (ad libitum, FF) are given at the top. The upper 2 animals are controls and the remaining 6 animals were given phenobarbital in the diet at a concentration of 0.25%. Bracketed area at bottom (C) indicates that statistical analyses were performed on the data on these days.

Figure 3. Intraperitoneal temperature measured every 15 minutes for 8 animals over 14 days. The regimens for lighting and feeding are given at the top L = light phase from 0200-0900 h, F = food available, S = food withdrawn. The top animal is a control and the remaining 7 animals were exposed to phenobarbital in the diet at a concentration of 0.25%. The phenobarbital diet was removed at the dashed line and feeding on the control diet began. The gap in the data (day 44-45) was due to a malfunction in the data acquisition equipment. Bracketed areas at bottom (D, E, F, and G) indicate that statistical analyses were performed on the data on these days.

Figure 4. Statistical analyses (cosinors) of specific day groups shown in Figures 1, 2, and 3 (see A through G). In all cases the lines from the center of any cosinor diagram point to the time of day (clock face) at which the circadian deep body temperature oscillation peaks (acrophase). The length of these lines defines the amplitude (°C) of the oscillation and can be read from the scale given in the right half of the cosinor (0.2-0.6). That portion of the line which lies within the small circle at the tip of each line defines the error in amplitude and the circle itself defines the 99% confidence interval around the mean value for the acrophase. The shaded areas on the perimeter of the cosinors indicate the particular LD-cycle which was in effect. I and III, DL 12:12 L-phase 1800-0600 h all groups A, B, and C; II and IV DD (continuous darkness, outerperimeter) group D only, DL 17:7 L-phase 0200-0900 h (inner perimeter), groups E, F, and G. Cosinors I and II pertain to the control group and III and IV pertain to the phenobarbital group.

Figure 5. Intraperitoneal temperature measured every 15 minutes for 8 animals over 14 days. The regimens for lighting and feeding (DL 17:7) are given at the top, L = light phase from 0200-0900 h, F = food available, S = food withdrawn. The top animal is a control and the remaining 7 animals received a diet containing 0.25% phenobarbital ad libitum (FF) commencing at the dashed line (0900 on day 14). Bracketed areas at bottom (A and B) indicate that statistical analyses were performed on the data on these days.

Figure 6. Intraperitoneal temperature measured every 15 minutes for 8 animals over 14 days. The regimens for lighting and feeding are given at the top: L = light phase from 0200-0900 h (DL 17:7), FF = ad libitum feeding. The upper 2 animals were controls and the remaining 6 animals were given a diet containing 0.25% phenobarbital. Bracketed area at bottom (C) indicates that statistical analyses were performed on the data on these days.

Figure 7. Intraperitoneal temperature measured every 15 minutes for 8 animals over 14 days. The regimens for lighting and feeding are given at the top: L = light phase from 0200-0900 h, F = food available, S = food withdrawn. The top animal is a control and the remaining 7 animals were given a diet containing 0.25% phenobarbital prior to the dashed line (0900 h, day 44). At the dashed line the phenobarbital diet was replaced with the control diet. The gap in the data (day 44-45) was due to a malfunction in the data acquisition equipment. Bracketed areas at bottom (D, E, F, and G) indicate that statistical analyses were performed on the data on these days.

Figure 8. Statistical analyses (cosinors) of specific day groups shown in Figures 5, 6, and 7 (see A through G). In all cases the lines from the center of any cosinor diagram point to the time of day (clock face) at which the circadian deep body temperature oscillation peaks (acrophase). The length of these lines defines the amplitude (°C) of the oscillation and can be read from the scale given in the right half of the cosinor (0.2-0.6). That portion of the line which lies within the small circle at the tip of each line defines the error in amplitude and the circle itself defines the 99% confidence interval around the mean value for the acrophase. The shaded areas on the perimeter of the cosinors indicate the particular LD-cycle which was in effect. V and VII: DD (continuous darkness, outer perimeter) group B only; DL 17:7 L-phase 0200-0900 h, inner perimeter, group A and C. VI and VIII: DD (continuous darkness, outer perimeter) group D only; DL 17:7 L-phase 0200-0900 h, inner perimenter, group E, F, and G. Cosinors V and VI pertain to the control group, and III and IV pertain to the phenobarbital group.

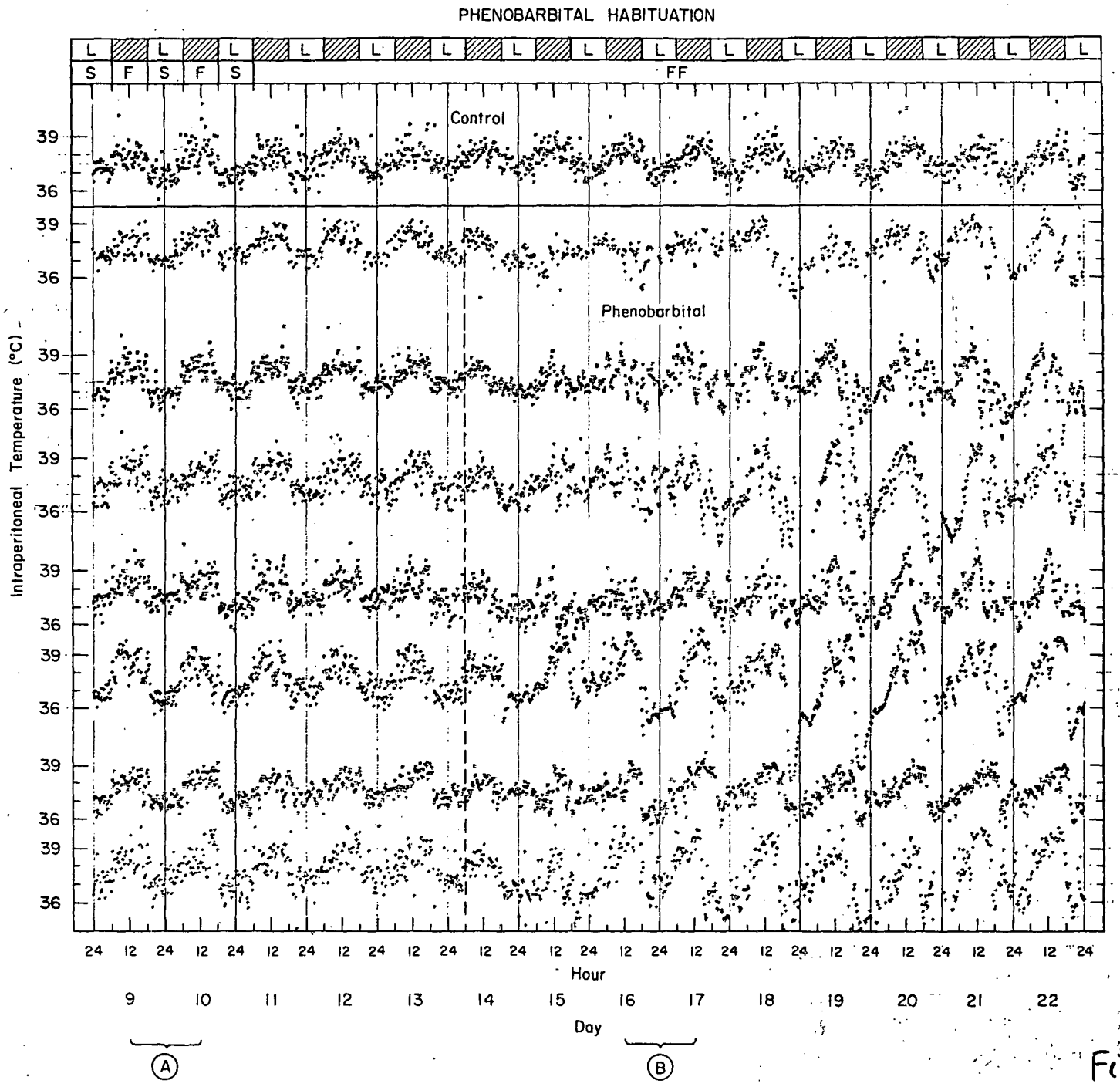
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Fig 1

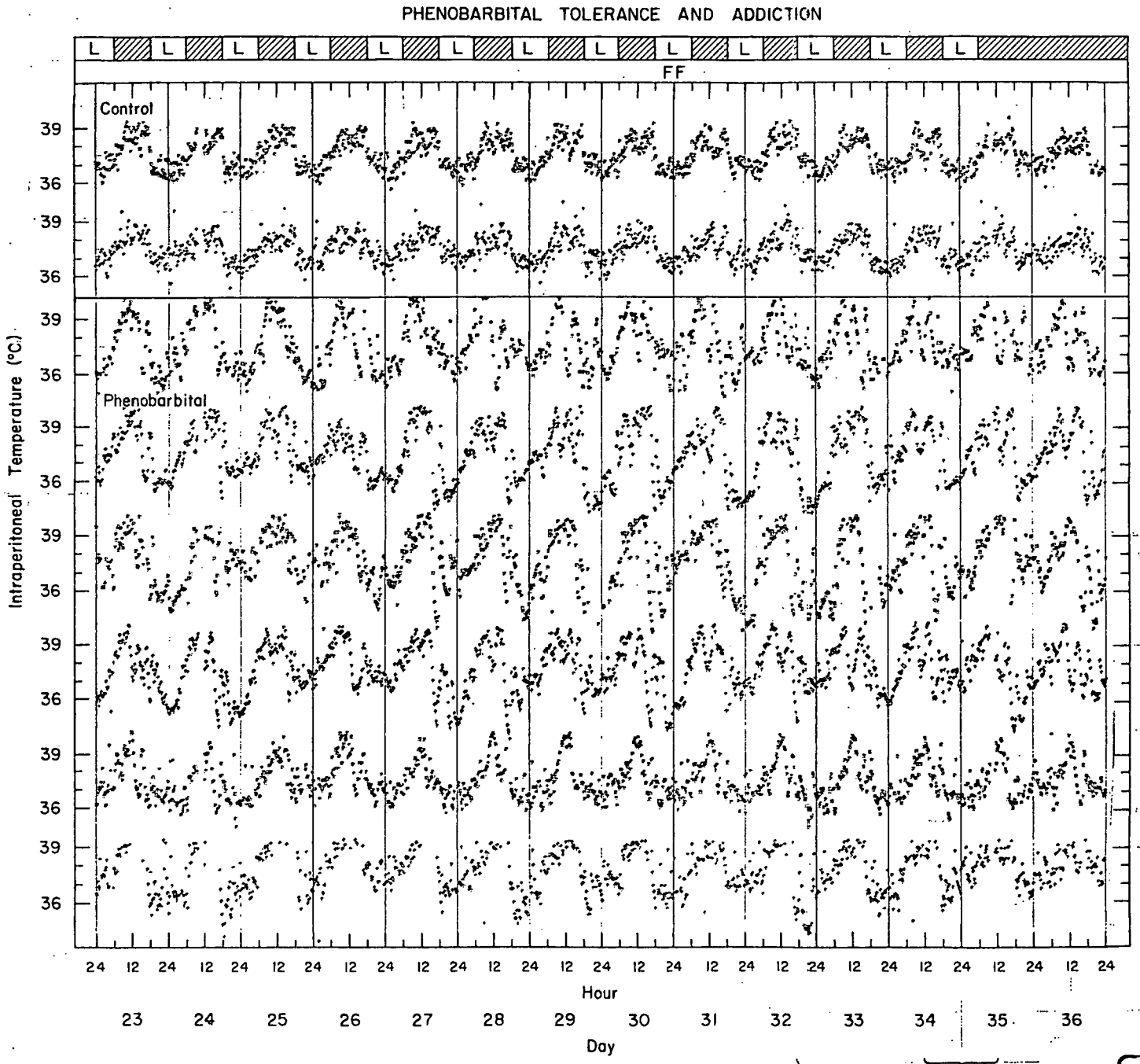
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Fig 2

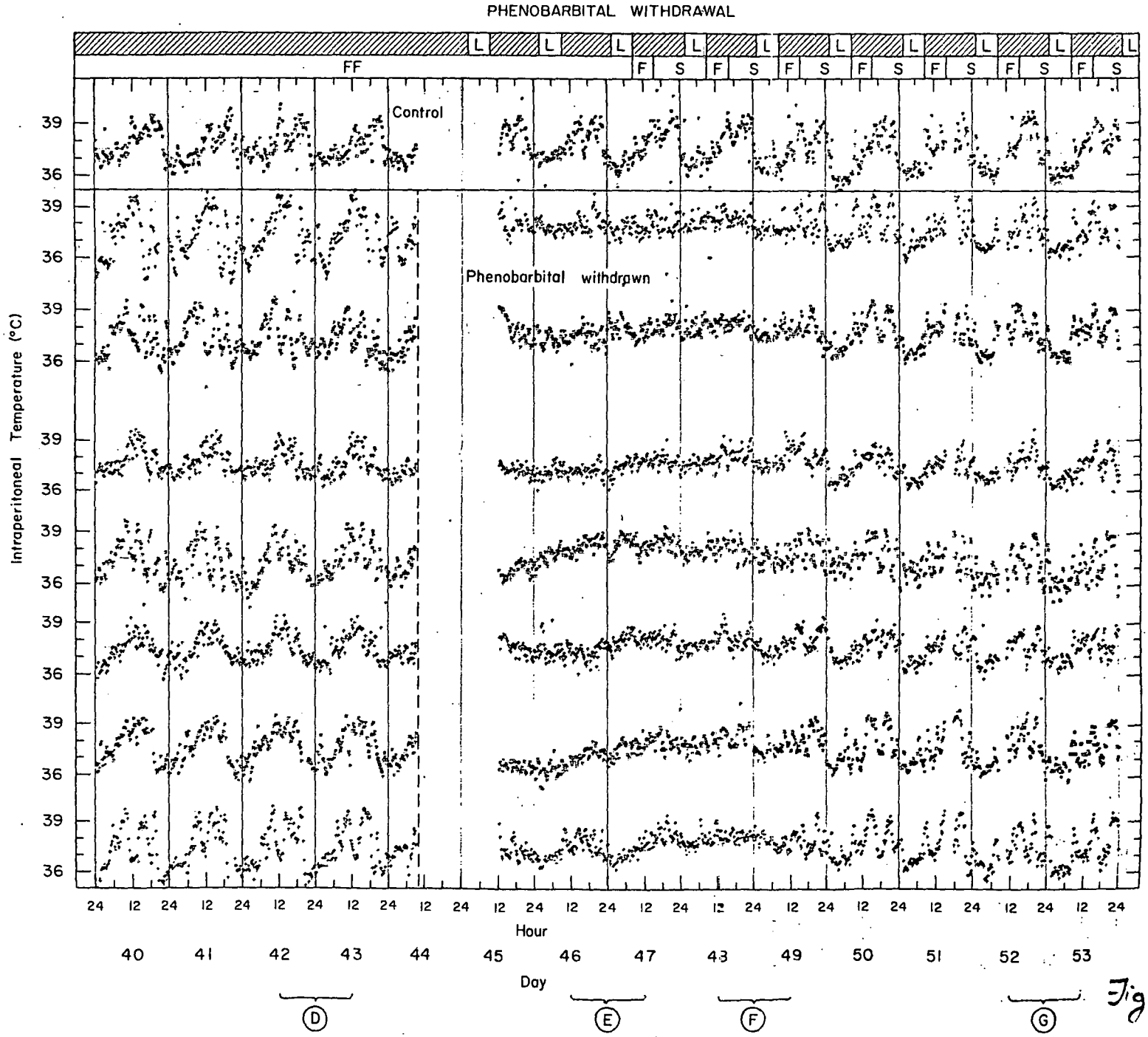
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Fig 3

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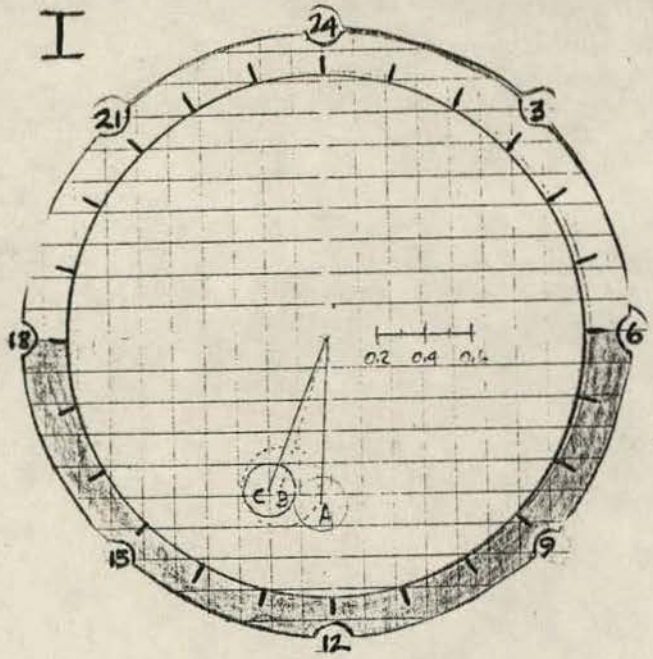
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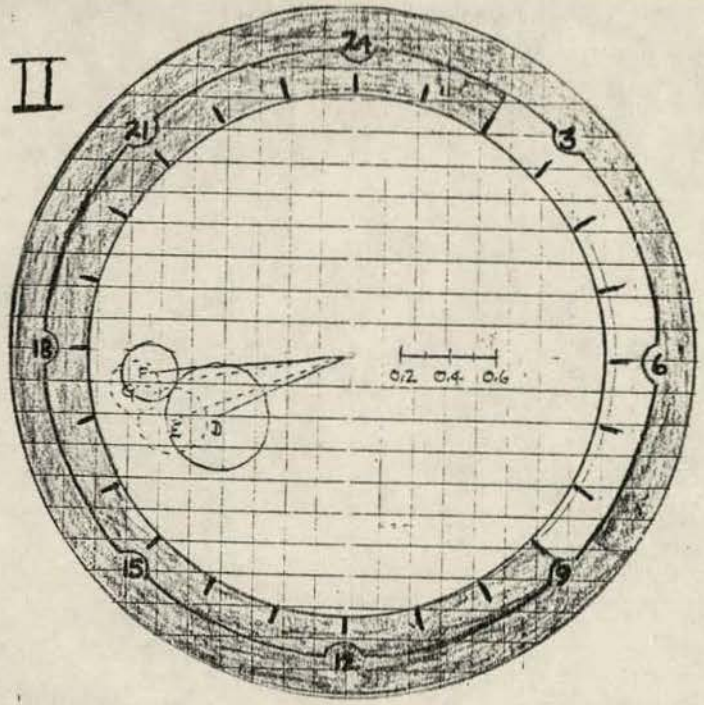
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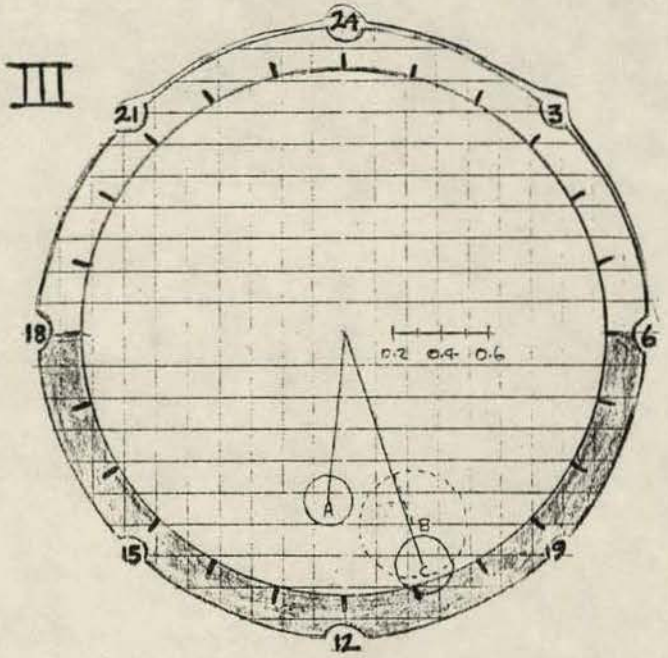
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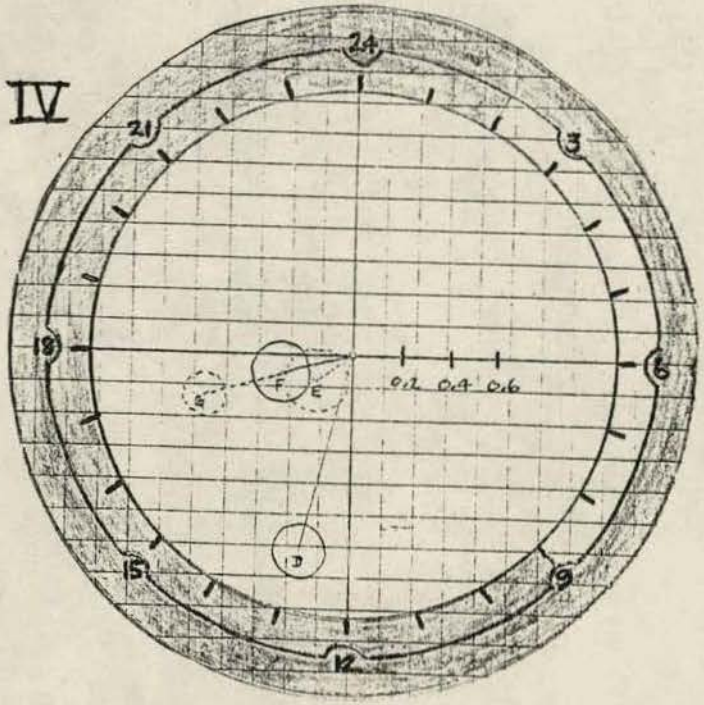
C

IV


24
21
3
18
6
15
9
12
0.2 0.4 0.6
F
E
D

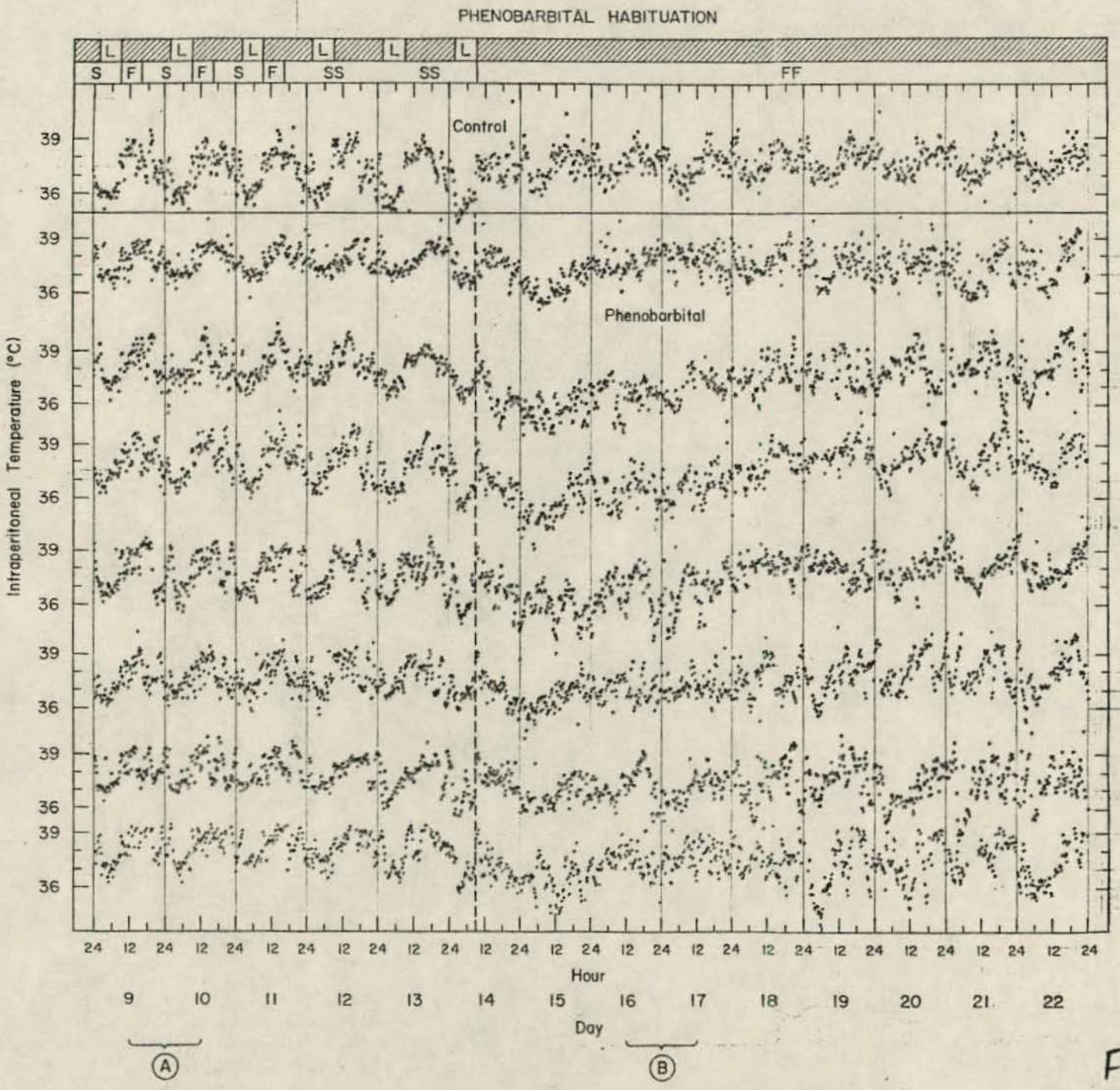

PHENOBARBITAL HABITUATION
L
S
F
SS
FF
Control
Phenobarbital
Intraperitoneal Temperature (°C)
39
36
24
12
Hour
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Day
A
B

Fig 5

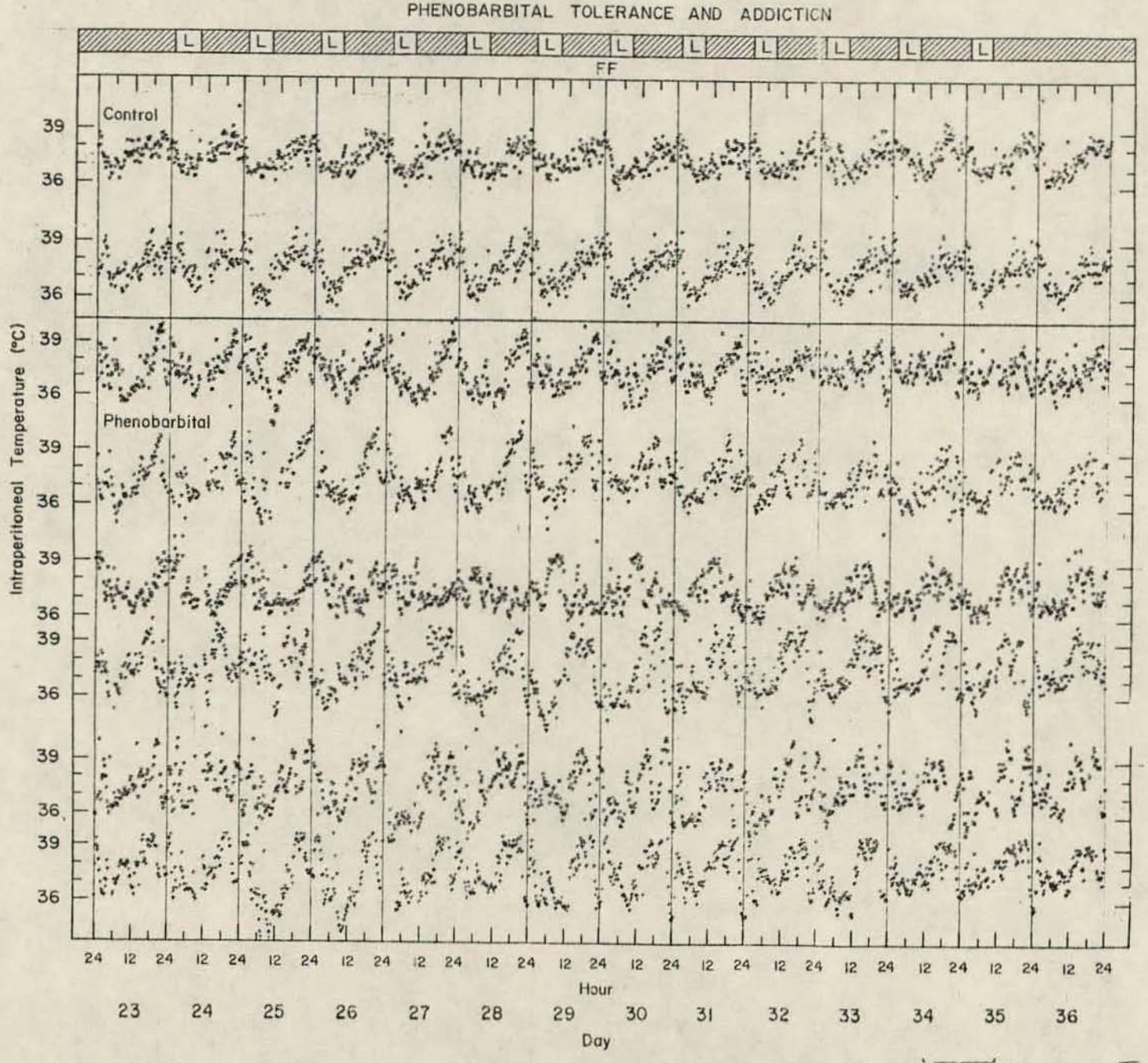

PHENOBARBITAL TOLERANCE AND ADDICTION
L
FF
Control
Phenobarbital
Intraperitoneal Temperature (°C)
39
36
24
12
Hour
23
24
25
26
27
28
29
30
31
32
33
34
35
36
Day

Fig 6

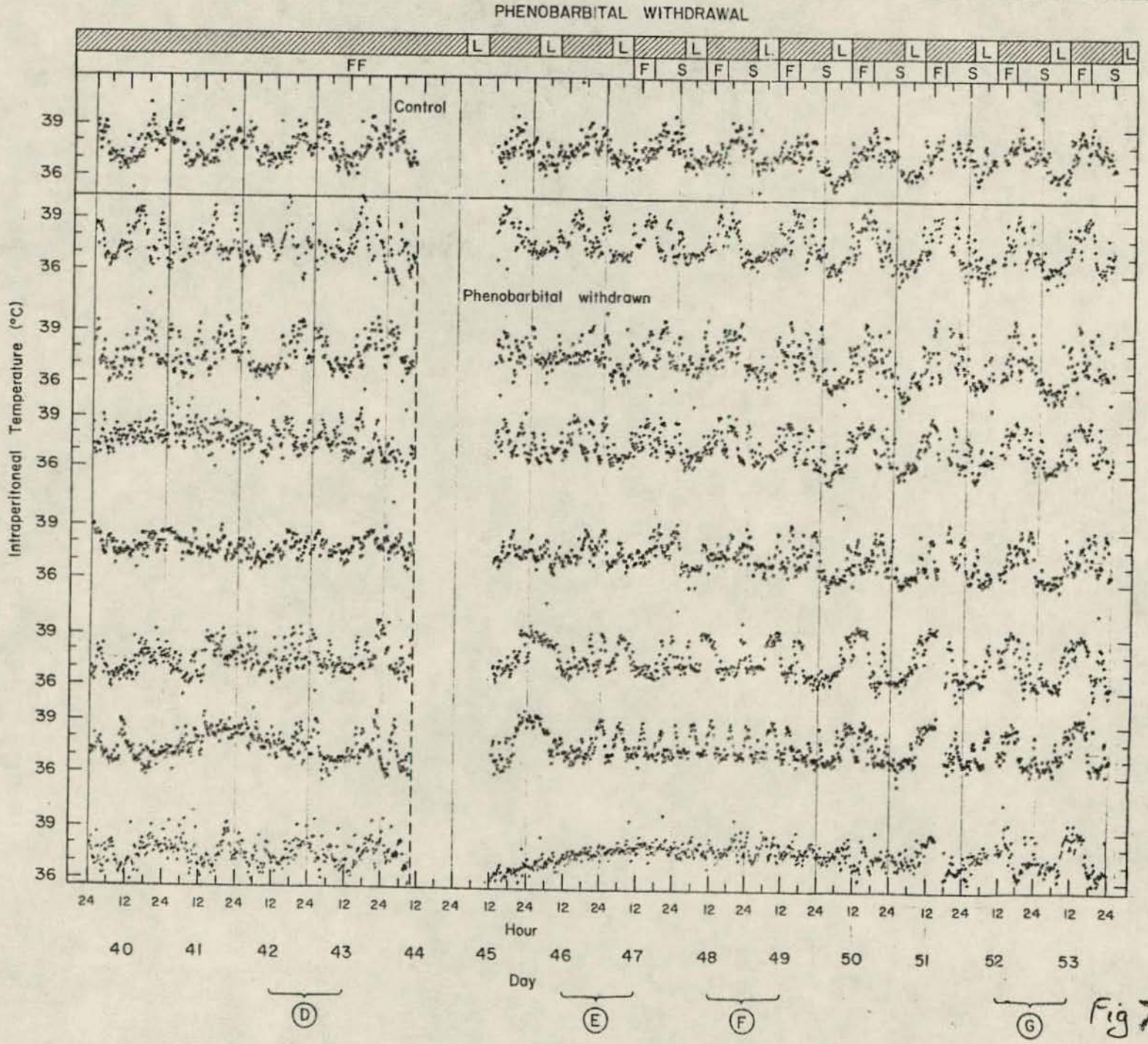

PHENOBARBITAL WITHDRAWAL
FF
L
F
S
Control
Phenobarbital withdrawn
Intraperitoneal Temperature (°C)
39
36
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24
Hour
40 41 42 43 44 45 46 47 48 49 50 51 52 53
Day
D
E
F
G

Fig 7

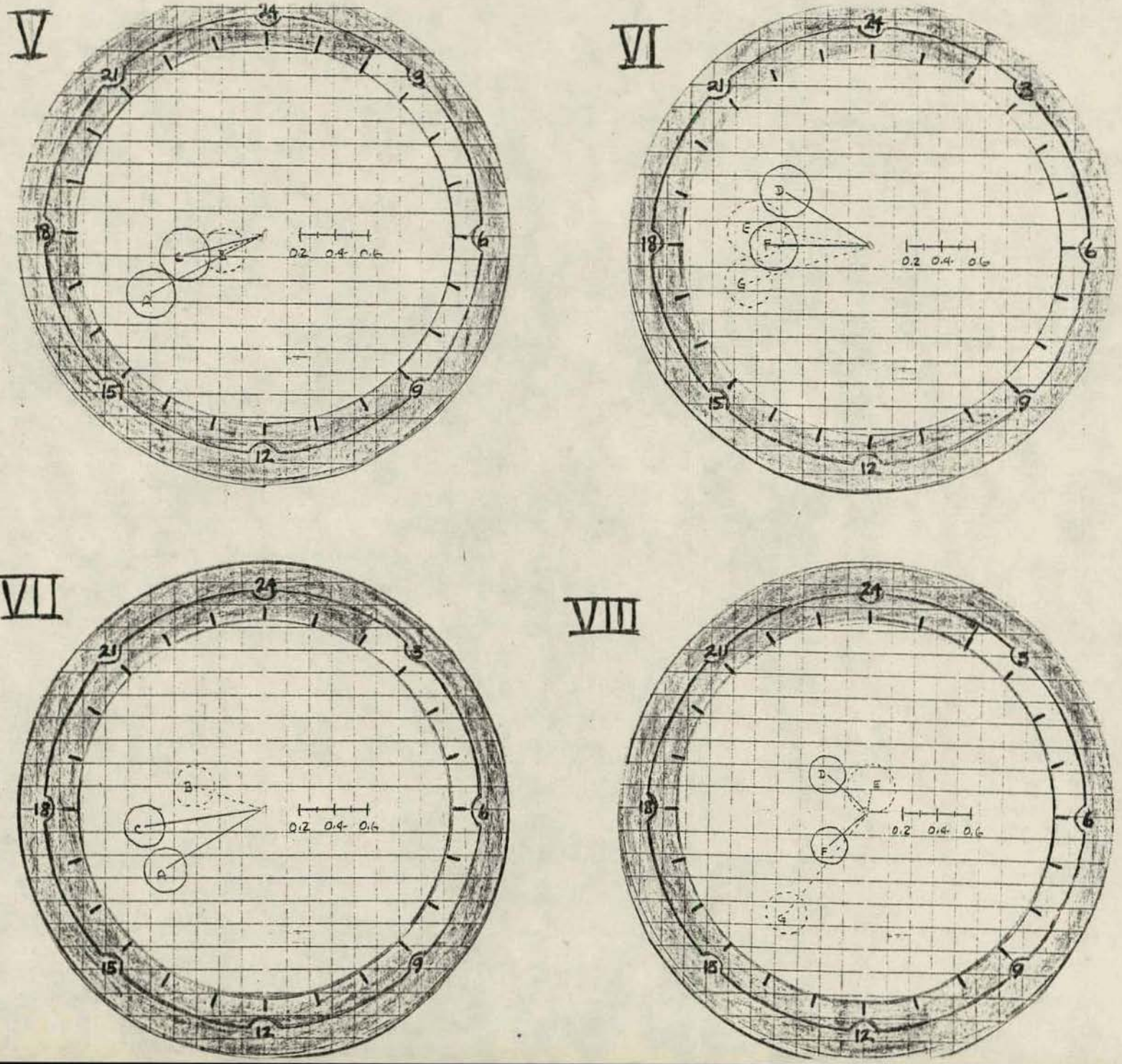


Fig 8