A CIRCADIAN SUSCEPTIBILITY-RESISTANCE CYCLE TO FLUOTHANE IN MALE B_1 MICE*

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BECAUSE THE POTENCY and margin of safety of Fluothane are topics of current interest, we are reinvestigating the toxicity of Fluothane using the mouse and the method of indirect periodicity analysis [1] This paper presents pertinent experimental results

Several earlier studies of Fluothane toxicity in the mouse constitute available background information In his first study, Raventós[2] found the LD_{50} of Fluothane vapour in oxygen to be 2 8 per cent ($\pm$0 34 per cent) during 30 minutes of exposure In a later article, Raventós and Dee[3] reported the value to be 3 16 per cent Krantz, Park, Truitt, and Ling[4] found a value of 3 1 per cent using 5 minutes of exposure and different vaporization techniques Study of Mørch and Jobgen's data[5] from tests with 10 minutes of exposure suggests a higher LD_{50} of between 3 5 and 4 0 per cent On the other hand, Jones, Margolis, and Steven[6] observed no deaths after the intraoesophageal instillation of 100 per cent Fluothane Because these lines of work were reported without reference to temporal aspects of tests conditions (discussed elsewhere[7]), it seemed desirable for us to employ techniques of indirect periodicity analysis for the bio-assay of Fluothane toxicity

The reliability of bio-assay techniques depends upon many factors [8] It increases with the extent to which one pays attention to variables such as the genetic background, sex, and past history of an animal population, as well as to test conditions Pertinent factors stem from the physical environment to which animals are exposed before and during testing, including still other sources of variation ranging from diet to infection Some of these parameters of bio-assay have temporal facets The age of test animals is the most obvious time factor Less well known is the role in bio-assay of another temporal parameter, the stage of physiologic rhythms with periods of about one day These so-called circadian (*circa, dies*)[7 9] rhythms can critically determine the outcome of a mammal's exposure to a variety of agents [1 10-16]

Studies of drug effects done at different times of the day that do not further specify conditions of observation may seem pertinent, although they are not identical in approach [17-19] Actually, an indication of an hour of the clock for increased or decreased susceptibility is meaningful only under standardized

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conditions [1] Those unfamiliar with physiologic periodicity analysis should note that by manipulating the lighting regimen to which certain experimental animals are exposed, and by several added precautions,[7] one may shift the peak and trough of a susceptibility rhythm to *any* clock hour of one's choice [20] Without reference to the lighting regimen and other factors,[7] studies of drug effects solely at different clock hours, therefore, are of restricted, if any, value They may, at times, confuse the issues on hand, and as long as conditions of observations are not standardized, the results may be quite variable

When temporal and other factors in bio-assay are taken into account to a certain extent, as in the present study, the results are surprising the mortality of mice after exposure to 3 5 per cent Fluothane for 7 to 10 minutes demonstrates a circadian susceptibility-resistance cycle The mortality from a given dose of Fluothane may be as low as 5 per cent or as high as 76 per cent, depending upon the organism's circadian system phase

Materials and Methods

Three experiments were done on male inbred mice of the C_{57} Black, subline 1 stock (briefly, B_1) This strain has been maintained by brother-to-sister mating for more than 10 years, in the Department of Pathology at the University of Minnestota Medical School The experimental mice were kept multiply-housed, with Purina Fox Chow and tap water freely available from weaning and throughout the study

One week prior to a given experiment, animals were transferred to disposable cages measuring $40 \times 32 \times 9$ cm —6 or 7 mice to a cage The cages were placed in a room shielded from natural light and sound, maintained at $24 \pm 1°$ C A clock-controlled switch turned the lights in the room on at 0600 and off at 1800 each day

On the day of study, separate groups of animals were tested at four-hourly intervals, starting at 0800 of one day and ending at either 0400 or at 0800 of the next day A group was composed of 14 to 21 mice The mice constituting different groups in a given experiment were comparable not only in terms of genetic background and sex, but also in terms of age

The anaesthetic concentration was prepared using a Heidbrink 660X Kinet o-Meter* to provide an oxygen flow rate of 4 litres/minute into a Mark II Fluotec vaporizer,† set at the 3 5 per cent marking A model 10 Fluothane Monitor‡ of the infrared type served to check the accuracy of the vaporizer

The anaesthetic chamber consisted of an 8 litre desiccator with a wire mesh platform resting across the glass ledge near the bottom Several minutes before the start of a test 3 5 per cent Fluothane in oxygen was introduced at 4 litres/minute into the bottom of the chamber, by rubber tubing ending below the platform

Mice from a given cage were then transferred into the chamber Anaesthetic vapour continued to flow into the bottom of the chamber during transfer and

*Ohio Chemical and Surgical Equipment Co , Madison, Wisconsin
†Fraser Sweatman, Inc , Buffalo, New York
‡Analytical Systems Company, Pasadena, California

throughout the next 10 minutes Excess vapour escaped through a hole in the lid After 10 minutes of exposure the wire platform holding the mice was lifted out and placed on a table in room air

The same procedure for anaesthesia was repeated for subgroups of mice from consecutive cages, at each time-point chosen for study Total test time required for mice from a group of cages (at least two, but usually three cages) did not exceed 45 minutes The animals were under continuous observation in the chamber and thereafter, until death or recovery from anaesthesia Recovery was judged by return of the righting reflex The time of death or recovery was followed with a stopwatch, started when the mice were transferred into the desiccator Results were recorded for consecutive one minute intervals

Results

Figure 1 shows the results of the three experiments Test-time, given in clock hours on the abscissa, is roughly the mid-point of the total period elapsed from start to end of testing a group of mice The lighting regimen on which mice were kept for a week prior to study and up to a minute prior to testing also is indicated above the abscissa, black areas denoting darkness During anaesthesia and thereafter the mice were kept in light at all times The percentage of deaths from anaesthesia at each test-time is shown on the ordinate

In the experiment recorded on the left 137 mice were used, 147 in that shown in the middle, and 125 animals comprise the right-hand graph The mean age in days (and standard error) of these three sets of mice was, in this order, 83 (±1), 116 (±1), and 283 (±2) days, respectively Mice summarized in the right-hand plot were thus much older than those described on the left or in the middle

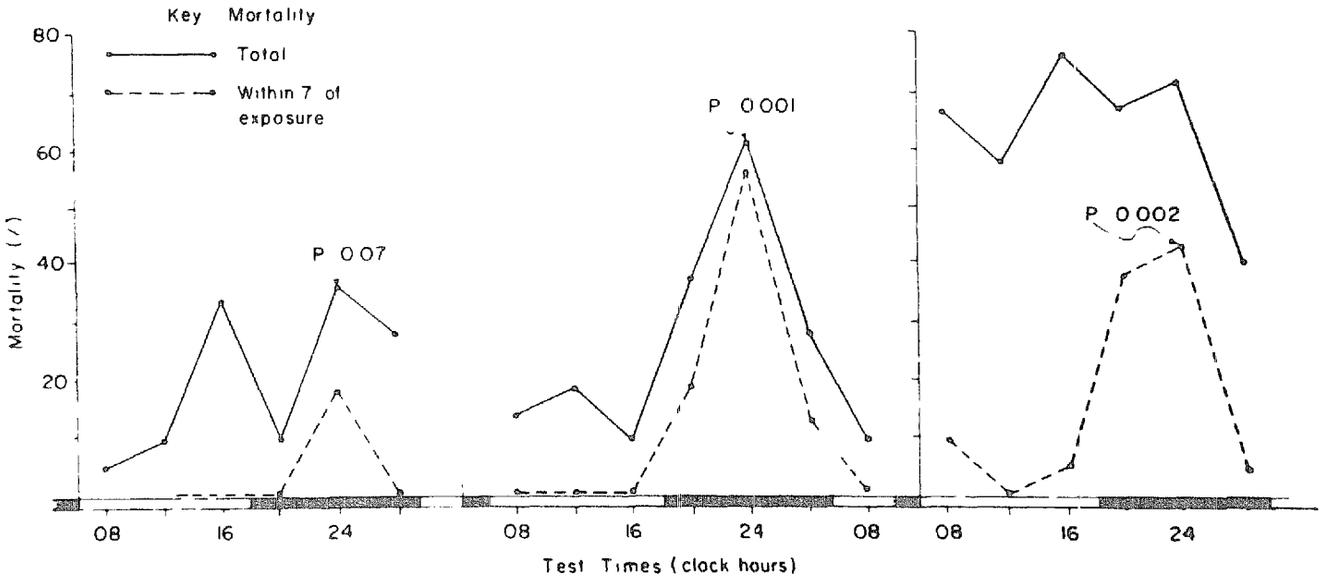

Figure 1 Dependence of Fluothane toxicity upon circadian system-phase Three experiments on male B_1 mice of three age groups Experiment on youngest mice on the left, those on older animals on the right (see text) In each experiment the 10-minute exposure of separate groups of comparable animals to 3 5 per cent Fluothane was associated with differences in mortality as a function of exposure time *P* values derived according to procedure for analysing the statistical significance of crests in physiologic time series [21]

The curves with solid lines show that total mortality, expressed as per cent of total tested, varied from 5 to 76 per cent, according to test time and age The greatest mortality was among older mice—the plot on the right Since the three experiments were done on different days, the age effect awaits further intra-experimental validation

Plots of mortality in a given experiment, in their turn, suggest differences related to test time These were analysed by a statistical procedure[21] described and proposed earlier for indirect periodicity analysis[1] From this test of the statistical significance of the crest in mortality occurring at 2400 in the experiment recorded on the left side of Figure 1, a P value of 0 07 was obtained For the data in the middle of the figure the corresponding P value was less than 0 001

An evaluation of the crest in total mortality was not done on the data of the third experiment At the time of the first exposure (0800) it was observed that the 3 5 per cent dose of Fluothane was too great for mice of this age Since a high percentage of mice of this first group died within 10 minutes, it was decided *at the outset*, i e , at the first test-time, that the experiment be continued with

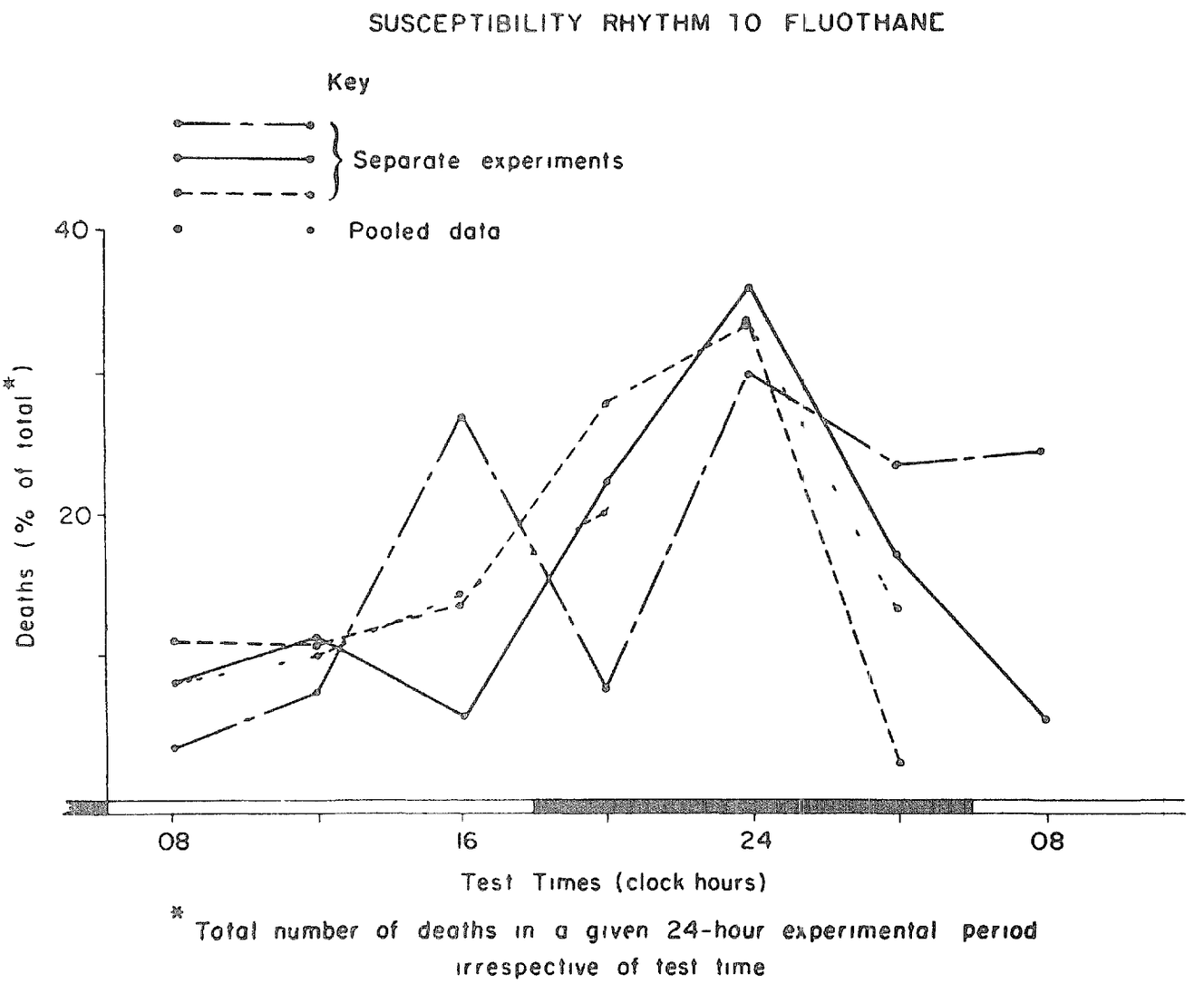

FIGURE 2 Data from Figure 1 re-expressed as percentage of total mortality in a given experiment on mice of a given age Transformation reduces effect of differences in level of susceptibility or resistance to Fluothane as a function of age The temporal change in susceptibility or resistance as a function of circadian system-phase, rather than age, can now be seen from the pool of data from all three experiments (see text)

10-minute exposures, as originally planned, but that results be evaluated according to the mortality occurring within the first 7 minutes, rather than during the entire 10 minutes of exposure Analysed on this basis, i e , in terms of the data summarized by the dashed line, the crest at 2400 in the right-hand graph is statistically significant, with a P of 0 002

For comparison, the deaths occurring within 7 minutes of exposure in the other two experiments also are shown by dashed lines Viewing the seven-minute data as well, it can be seen that mortality at 2400 is much higher than at other times Note, for instance, that in the experiment plotted on the left, no deaths had occurred prior to the seventh minute at any time except at 2400

Figure 2 shows mortality expressed in percentage of total mortality in a given experiment For the first two experiments all deaths were included in this computation Deaths occurring after the seventh minute of exposure in the last experiment were excluded from calculation, for the reasons stated above The three curves are roughly congruent, their mean is shown as a dotted line

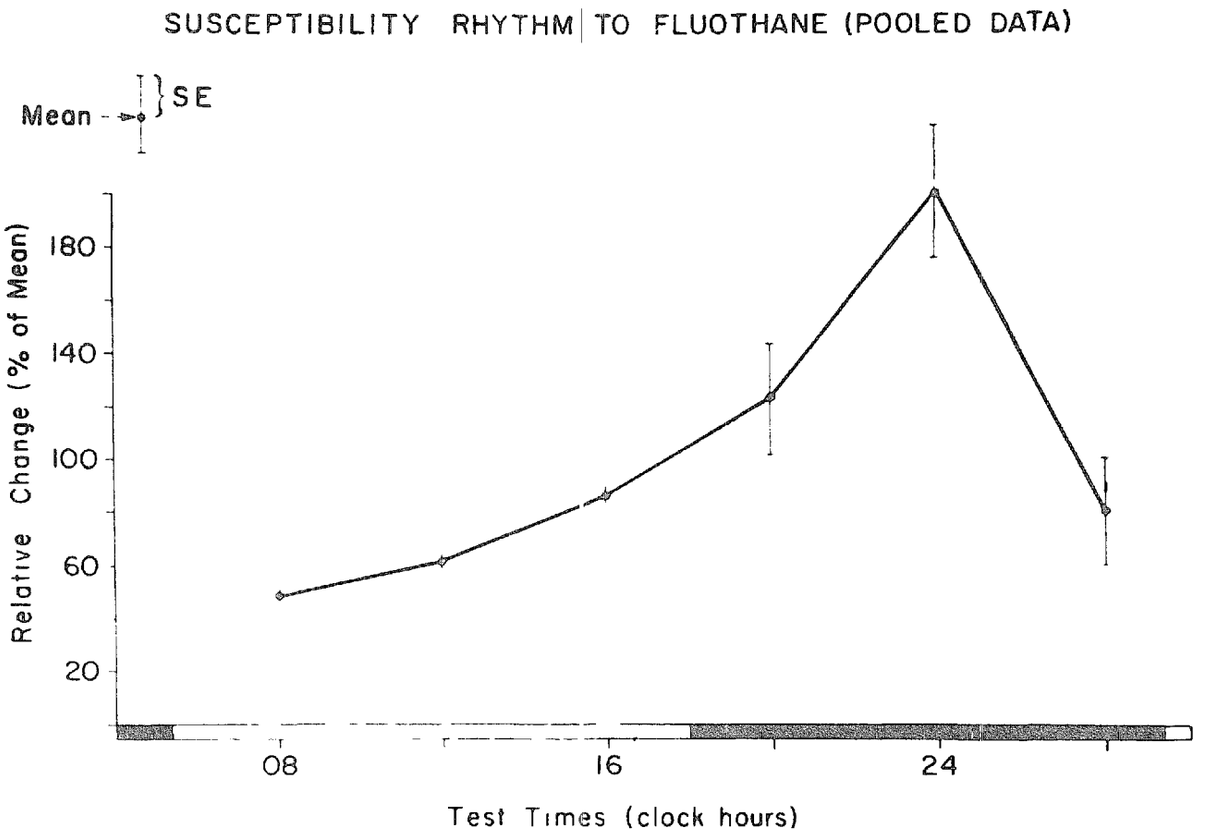

FIGURE 3 Conversion of the pooled data in Figure 2 into "relative changes"[22]—with estimates of variability also provided for results from each test-time

The crest in mortality at 2400 is apparent from this summary presentation as well as from the solid line in Figure 3, which shows the so-called "relative change"[22] in susceptibility to Fluothane To obtain the curve in Figure 3, mortality at a given test-time was expressed as a percentage of the average mortality in a given experiment, with the latter mean equated to 100 per cent Thereafter, these relative values for each test-time were averaged to obtain the mean relative change, plotted in Figure 3

Discussion

These experiments involving a 7 or 10 minute exposure to 3 5 per cent Fluothane in oxygen demonstrate, first, a circadian susceptibility-resistance cycle to this agent Secondly, the data also suggest, but do not prove, a difference in susceptibility among different age groups The location of the crest in susceptibility at 2400 and the influence of differences in age were anticipated on the basis of previous studies using another agent affecting the CNS—Librium (Fig 4) [15,16]

Along the same line, it is interesting that the susceptibility of mice to ethanol[14] and audiogenic convulsions,[10 11 20] tested under comparable conditions, is highest at about 2000 (Figs 5 and 6) [23] The location of the crests of these susceptibility rhythms shows relatively small, if any, differences in phase Pertinent also are circadian susceptibility rhythms with a similar timing described by Davis for pentobarbital[24] and by Davis and Webb for hexafluorodiethyl ether,[25] as well as a most recent study on Nembutal by Emlen and Kem [26] The response of rodents to all of these agents affecting the CNS thus appears to depend upon a similar circadian system phase, awaiting further definition Susceptibility rhythms to

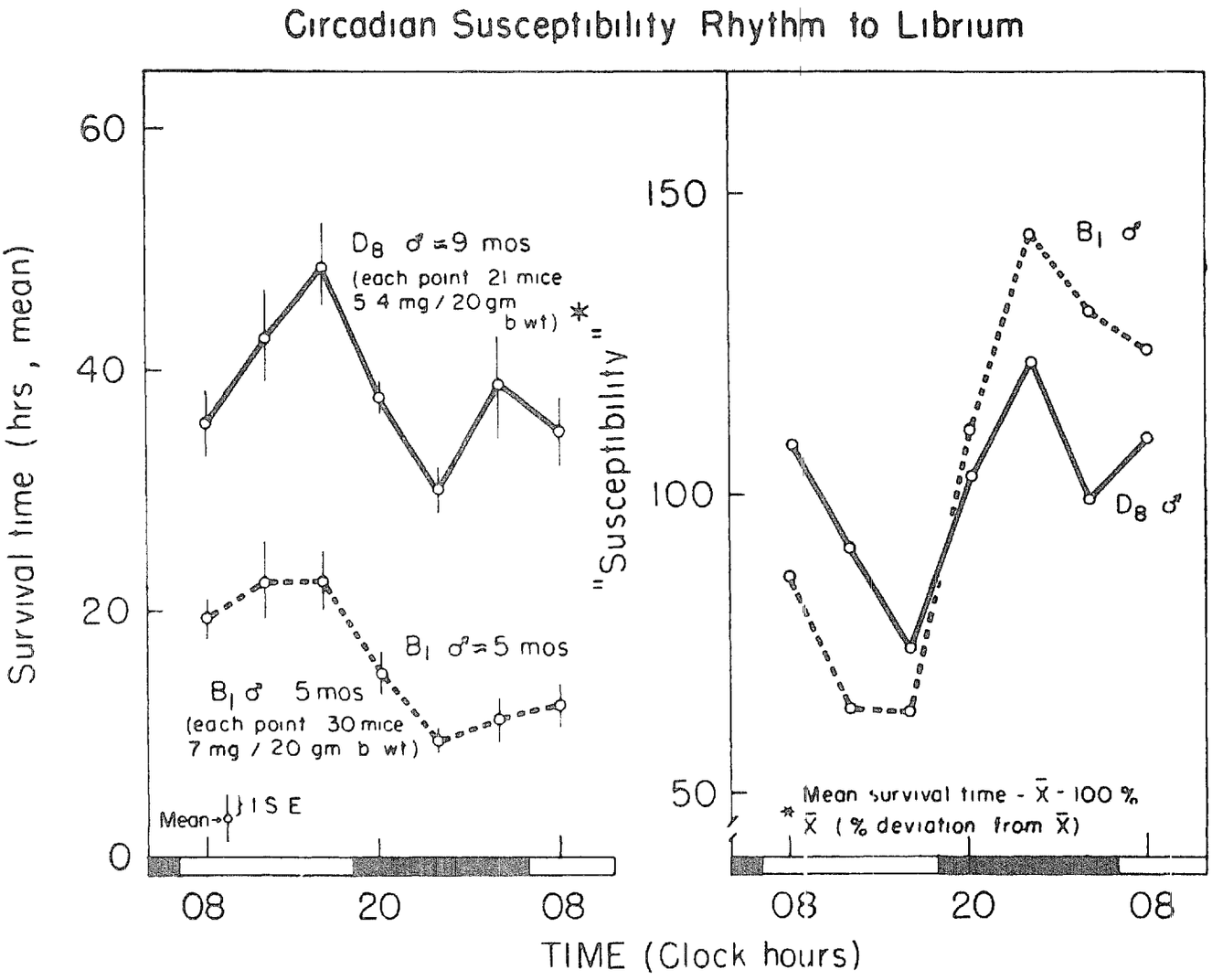

Figure 4 Circadian susceptibility-resistance cycle to Librium By contrast to Figures 1–3, data in the left half of this figure are expressed in terms of survival time rather than as per cent deaths Transformed data on the right of figure [10 16]

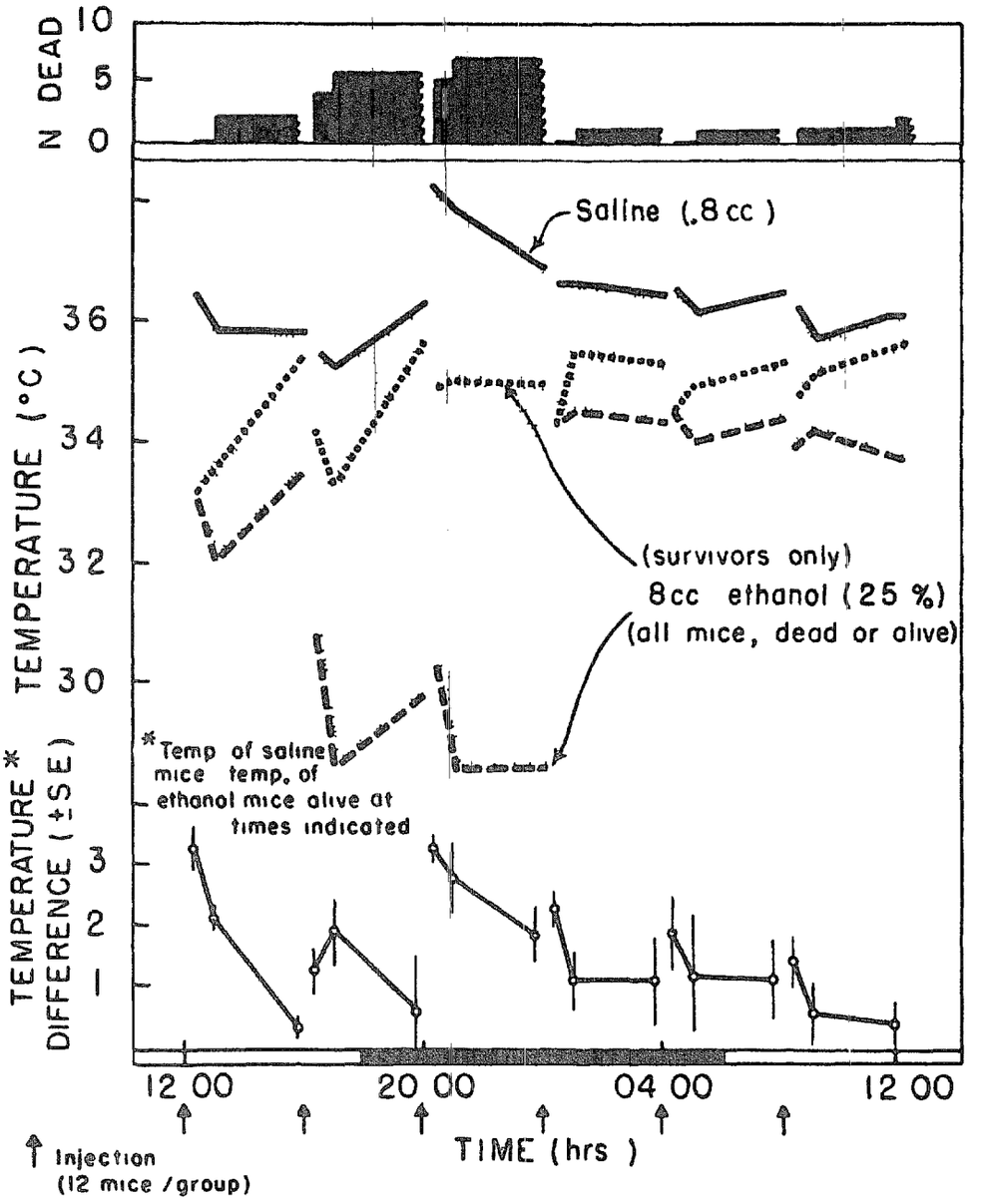

FIGURE 5 Circadian susceptibility-resistance cycle to ethanol Black columns on top of figure denote mortality of comparable male C mice, 4 months ± 2 weeks of age, injected i p with 0 8 c c of a 25 per cent solution of ethanol in saline Ethanol-induced hypothermia as well as mortality from ethanol depends upon circadian system-phase at injection time Details of this experiment and of confirmatory ones are in References 1 and 14

other agents, however, peak at quite different times, as shown in Figure 7, summarizing the so-called "hours of changing resistance"[1]

These predictable physiologic changes in susceptibility of mammals to drugs would be important as models for clinical studies if toxic-therapeutic ratios also can be found to vary depending upon an organism's circadian system phase For example, anaesthetics with undesirable cardiovascular side-effects, such as Fluothane, might have a relatively small margin of safety if at a given time the organism exhibits an increased susceptibility to cardiovascular disturbances and concomitantly a decreased susceptibility to cortical depression In other words, the same amount of drug might produce the desired effect and little undesirable side reactions at one time (i e, circadian system phase) and at another time considerable side reactions with little desirable effect

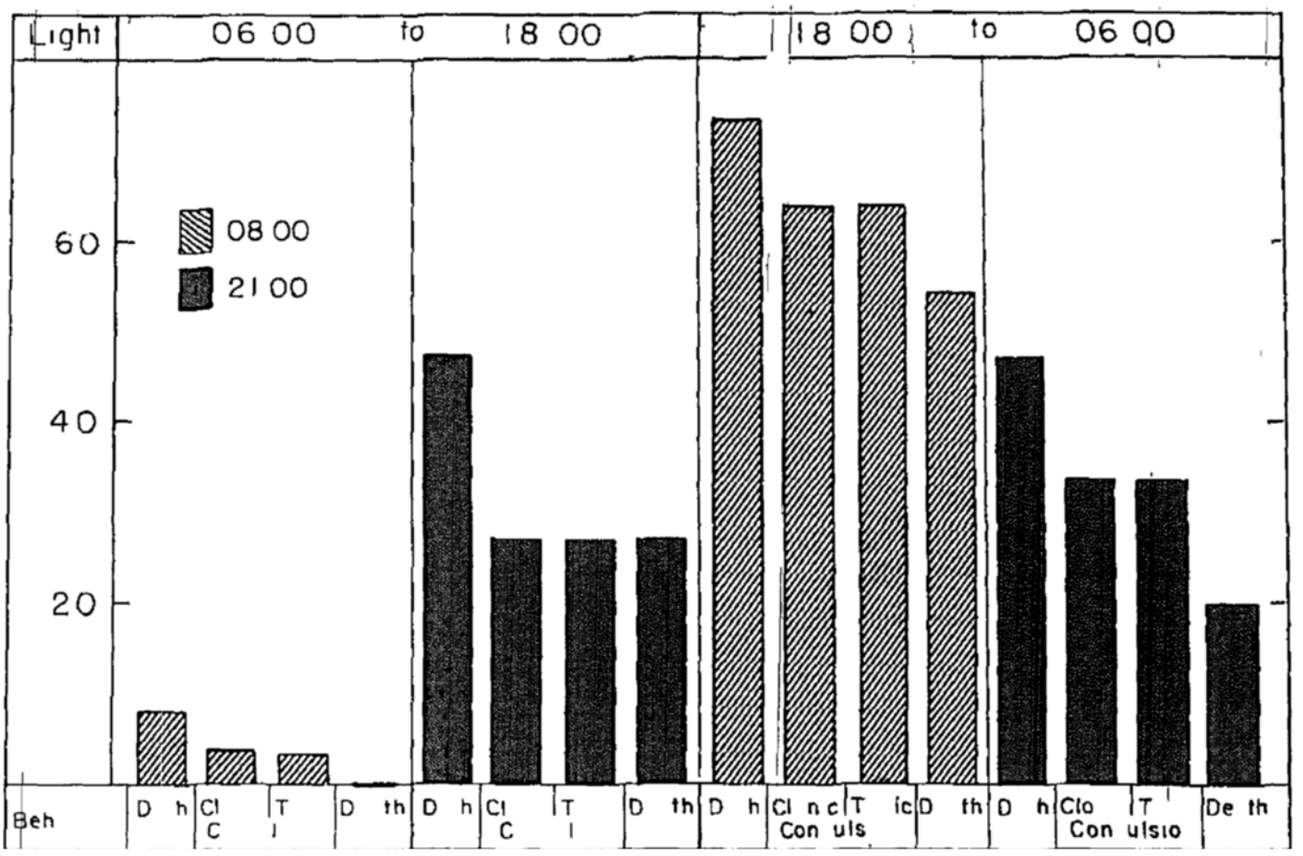

FIGURE 6 Abnormal audiogenic resonses in *D* mice on two schedules of light and darkness alternating at 12-hour intervals Incidence of several types of noise-induced abnormality differs at 0800 as compared with 2100 in mice exposed to light from 1800 to 0600 as compared with that in mice exposed to light from 0600 to 1800 Note feasibility of phase-shifting of a circadian susceptibility resistance cycle Total tested 120 mice about five weeks of age of both sexes [20]

We must qualify these initial experimental data on Fluothane in several ways First, in speaking of certain phases of susceptibility rhythms such as a crest we mean to refer to circadian system phase rather than to clock hour We do so since a given phase of circadian rhythm can be shifted to any clock hour of one's choice by manipulating the environment [0 27] We also must refer to a number of technical problems One of these revolves around the accuracy of the Fluotec vaporizer This question was resolved for the time being only through verification of the vapour concentration provided by infrared analysis and through consistent use of the same flow rate and vaporizer settings Subsequent experiments have necessitated a more accurate system and one which permits the study of other anaesthetic vapours and gases

A second technical problem resulted from a small water leak in the periodicity room but not into the cages containing the mice represented by the left hand graph of Figure 1 The effect of the noise from dripping water remains unevaluated

In subsequent studies we are extending the design not only to replicate the circadian susceptibility rhythms but also to test within various experiments the influences of age sex strain variations in dosage of Fluothane premedication and other common anaesthetic factors More accurate observation of induction

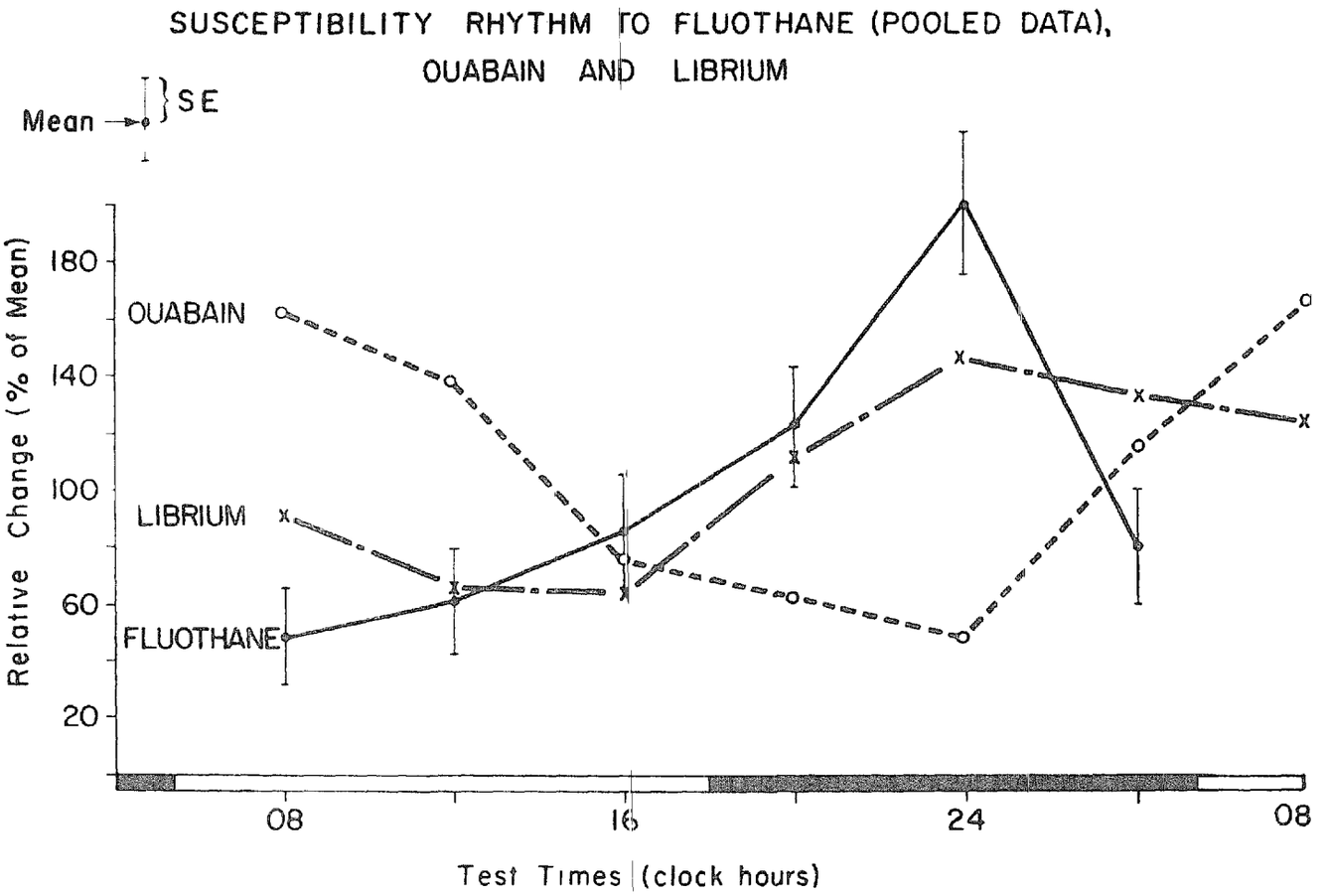

FIGURE 7 The concept of hours of changing resistance can be further documented by data on Fluothane (•————) The data of this paper are aligned with some obtained on other susceptibility-resistance cycles earlier This figure should convey the degree of generality of changes in response to toxic doses of several drugs as a function of circadian system-phase The same figure may be used further to suggest that crests and troughs in susceptibility to different agents do not all occur at the same time circadian synchronization occurs with differences in phase, some of which are positive or negative, as well as zero (*Cold Spring Harbor Symposia on Quantitative Biology*, vol 25, Long Island Biological Association, New York, 1960, p 524)

times, anaesthetic levels, and respiratory and cardiovascular responses may help to clarify mechanisms of death

The importance of these initial studies to anaesthesiologists may be the demonstration that circadian rhythms characterize the susceptibility of a mammal to Fluothane, and the suggestion that circadian periodicity analysis of therapeutic-toxic ratios may eventually contribute to a better understanding of the lethality and margin of safety of anaesthetics

SUMMARY

The mortality of mature, male B_1 mice upon exposure to 3 5 per cent Fluothane in oxygen for 10 minutes may be as low as 5 per cent or as high as 76 per cent, depending upon the organism's circadian system phase, Predictably periodic changes in susceptibility can be found in test animals of several age groups, apart from differences in susceptibility as a function of age Further consideration of the organism's time structure, as revealed by its rhythms, may lead to safer anaesthetic practices

Résumé

Nous faisons une étude de la toxicité de l'halothane, en nous servant de souris et de la méthode d'analyse de périodicité indirecte Ce travail rapporte des résultats expérimentaux sur une cycle circadien (*circa, dies*, un rhythme dont la période est d'environ 24 heures) dans la resistance d'un organisme vis-à-vis de l'halothane En tenant compte du facteur temps et de facteurs de technique biologique expérimentale, la mortalité de souris B_1 mâles—ayant respiré des vapeurs d'halothane à 3 5 pour cent dans l'oxygène durant 7 à 10 minutes—aurait varié de 5 à 76 pour cent selon la phase du système circadien, i e, la distribution des temps internes de l'organisme (fig 3) Ces résultats étaient à prévoir d'après les études antérieures sur le librium, sur l'éthanol, sur les convulsions audiogéniques, sur le pentobarbital et sur l'éther hexaflurodiéthylique La discussion de ces travaux soulève la question de l'amélioration possible de la pratique de l'anesthésie, si ces données s'appliquent au rapport toxicité/thérapeutique des agents anesthésiques

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