Progress in the developing science of chronobiology has closely paralleled the emergence of new and improved research methodologies. Methodological requirements for biological rhythm study have been reviewed previously (Halberg et al. 1972, 1977; Reinberg 1971, 1974; Smolensky et al. 1974). This chapter outlines a minimum set of conditions and procedures necessary for conducting sound chronobiologic investigations. The recommendations which are put forward should be regarded as proposals and/or suggestions rather than rules or criteria for judging the quality of experimentation. It is obvious that each study dictates a specific methodology depending upon, among other things, the goals of the investigation and the state of knowledge. The contents of this chapter provide necessary information for designing chronobiologic research protocols and for minimizing the occurrence of those types of mistakes typically experienced in earlier chronobiologic investigations.

**Types of Synchronizers and the Synchronization of Biological Rhythms**

Common to all biological research, particulars related to species, sex, age, weight, height, food intake, state of health or disease, etc., must be stated. These general requirements are mentioned here as a reminder, only, despite the fact they are critical.

With respect to chronobiologic methods, when experimenting on laboratory animals, it is mandatory that the timing of the natural or artificial light (L)–dark (D) cycle be monitored, recorded, and reported since the LD cycle is recognized as a primary synchronizer of circadian and possibly other rhythms. The LD cycle influences the period length and peak time of rhythms of many species, such as birds, rodents, and monkeys.* Other potential synchronizers,

* The term synchronizer refers to an environmental periodicity capable of determining the temporal staging, with respect to clock hour or calendar date, of a given endogenous rhythmicity.
such as cyclic changes in temperature, noise, odors, humidity, and food availability, should be maintained at more or less constant levels. In so doing, only one (known) synchronizer is operational, whether or not it is manipulated. The concurrent influence of several synchronizers may lead to a complex situation which may be difficult to analyze. Even with one synchronizer, such as the LD alternation, the duration, intensity, and quality (wavelength) of the light as well as the abruptness of change from L to D can influence the findings (Aschoff 1960; Boissin and Assenmacher 1971; Halberg et al. 1959). Therefore each must be well defined.

Many authors prefer using the LD:12/12 lighting regimen in which 12 hr of light alternate with 12 hr of darkness.* With regard to animal models involving typically nocturnally active rodents (rats and mice), some authors (Halberg 1973; von Mayersbach 1978) recommend a LD:8/16 schedule, since it seems to better simulate human synchronization, i.e., 16 hr of activity alternating with 8 hr of rest.

Selection of appropriate LD schedules constitutes one of the most fundamentally important steps in conducting animal research, whether or not one is involved in chronobiologic studies, for many reasons as discussed below. Some experimenters are unaware of, or choose to ignore, the significance of the LD schedule as a synchronizer of animal rhythms. Oftentimes, animals are maintained under constant illumination. The assumption made in housing rodents and other species under such conditions is that the absence of alternating LD cyclicity attenuates or obliterates biological rhythmicity. Some investigators, even if a LD schedule is provided, attempt to “control” for rhythm effects by restricting research procedures to one or two particular clock hours. The experimenter by adhering to this type of sampling schedule does a disservice to his research and to science for several reasons. With respect to research on rodents, a vast number of chronobiologic investigations conducted during the past two to three decades utilizing comparable LD synchronizer schedules (usually LD:12/12) have enabled the “mapping” of a multitude of circadian rhythmicities. The term mapping refers to aspects of the rhythm’s form over time, for example, the peak and trough times, with respect to the stated LD schedule. When the LD schedule is known, it is possible to predict rather precisely when either high, low, or average levels of a constituent of tissue, blood, urine, etc., will occur. Thus, the maximum of the circadian rhythm in serum corticosterone (the major adrenal corticosteroid hormone in mice) is expected to coincide with the timing of the transition of light to darkness in the animal room. It is also predictable that reduced levels of corticosterone will occur approximately 12 hr earlier or later, around the transition of darkness to light (Fig. 1) (Smolensky et al. 1978). In other words, different—early, middle, or late—stages of the light or dark span are coincident with particular features, such as the peak values (also referred to as the acrophase, discussed later in this chapter) of various circadian rhythmicities (Fig. 2). Relating the LD schedule to local time enables the determination of the clock hour of the aforementioned rhythmic features, since animals synchronized to fixed LD cycles display periodicities of almost precisely 24.0 hr. Under such highly standardized research conditions, clock hour thus is representative of circadian stage.

When animals are housed under constant light or darkness, i.e., without a LD synchronizer, the phenomenon of “free-running” often results—the occurrence of differing non-24-hr circadian cycles which vary in periodicity (τ) between biological functions in the same animal and/or be-

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* Chronobiologists rely upon certain abbreviations to convey pertinent information about synchronizer type and schedule. LD:12/12 designates the synchronizer to be the Light (L)-dark (D) cycle with L and D each having a 12-hr duration. LD:8/16 conveys that the synchronizer is the light–dark cycle with the duration of the former being 8 hr and the latter 16 hr. Time or clock hour is expressed using the international designation, e.g., 1:00 p.m. is referred to as 1300.