circadian periods for some time and (ii) the two components occasionally rejoin with a new phase relationship. Both phenomena, which are also typical for some of the mammalian data, are difficult to reconcile with a one-oscillator model (*3, 6, 7*). Hence, the present results suggest that the coupling between circadian oscillators controlling the diurnal pattern of locomotor activity is effected by hormones.

3) The phenomena of "splitting," increased activity time, and continuous locomotor activity are apparently expressions of the same physiological processes. This is suggested by the facts that (i) testicular growth or injections of testosterone may cause any of these three phenomena in different individuals and (ii) increasing activity time or developing continuous activity are often the result of or preceded by "splitting." Therefore, the present data suggest that the development of continuous activity is at least partly a consequence of the dissociation of two or more circadian oscillators.

Continuous and apparent arhythmic activity patterns have also been observed in various animal species kept under high intensities of continuous illumination and in birds in which the pineal gland had been surgically removed (*11, 12*). It has been suggested by several authors that continuous activity under these conditions results from the uncoupling of two or more circadian oscillators controlling locomotor activity (*3, 7, 11*). The present data support this hypothesis.

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10. Such a differential testicular response to continuous light was expected on the basis of other photoperiodic studies on starlings and other Temperate Zone birds. It reflects the seasonal change from photoresponsiveness during winter and spring to photorefractoriness in summer and fall [D. S. Farner and R. A. Lewis, in *Photophysiology*, A. C. Giese, Ed. (Academic Press, New York, 1971), vol. 6, p. 325].
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Localization of Sister Chromatid Exchanges in Human Chromosomes

Abstract. *The bromodeoxyuridine sensitivity of 33258 Hoechst fluorescence allows microfluorometric analysis of sister chromatid exchanges in human metaphase chromosomes. The frequency of sister chromatid exchanges among chromosomes correlates with chromosome length. Exchanges appear to occur predominantly in interband regions, as defined by quinacrine fluorescence, or very near band-interband junctions. A few regions are involved unusually frequently.*

Metaphase chromosomes of a number of organisms exhibit distinctive quinacrine and modified Giemsa (*1*) banding patterns. The bands, which exhibit bright fluorescence with quinacrine and stain intensely with Giemsa, appear to consist predominantly of heterochromatin (*1, 2*). Heterochromatic regions have long been considered sensitive to damage by a variety of agents (*3*), although recent studies suggest that high energy radiation breaks human chromosomes principally in the interband regions (*4*). Chromosome breaks, however, may represent only that small fraction of damaged regions that have not undergone repair.

Sister chromatid exchanges are events that involve breaks in both chromatids at coincident locations with subsequent

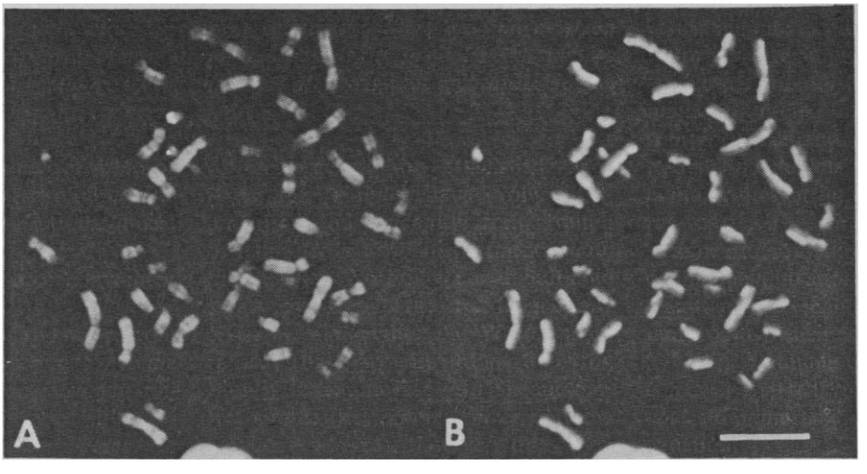

Fig. 1. Quinacrine and 33258 Hoechst fluorescence of human chromosomes from cells grown two divisions in medium containing 5-bromodeoxyuridine (BrdU). Chromosomes were prepared from peripheral leukocytes of a normal human male (46,XY) which were grown 72 hours in medium containing 0.02 m*M* BrdU. (A) A metaphase was stained first with quinacrine and photographed, and (B) destained, then stained with 33258 Hoechst, and photographed. The former (A) displays a characteristic quinacrine banded fluorescence pattern while the latter (B) exhibits the 33258 Hoechst fluorescence pattern expected after two cell divisions in medium containing BrdU. The bar in (B) represents 10 μm.

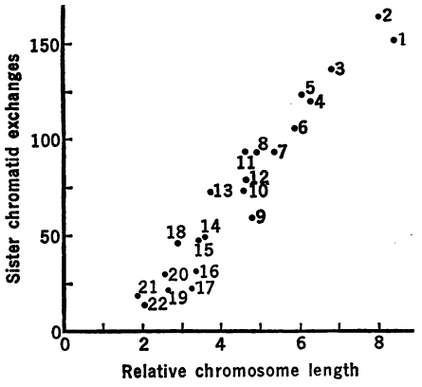

Fig. 2. The dependence of sister chromatid exchange frequency on metaphase chromosome length. A total of 126 human leukocytes in metaphase (48 from one female, 78 from two males) were examined (Fig. 1). The cells were grown two replication cycles in medium containing 0.01 or 0.02 m*M* BrdU and then harvested. Chromosomes were identified by their quinacrine fluorescence patterns, and sister chromatid exchanges were detected by 33258 Hoechst fluorescence. The total number of sister chromatid exchanges detected for each pair of autosomes is plotted against the relative metaphase chromosome length [as given in column D, table 5 of (*13*)]. The chromosome identification number is given beside each point. Not included in this plot are 13 sister chromatid exchanges in the Y chromosomes (relative chromosome length, 2.15) of 78 male cells and 85 sister chromatid exchanges in the X chromosomes (relative chromosome length, 5.12) of 48 female and 78 male cells.

interchange and repair. Such exchanges, first detected autoradiographically (*5*), are much more numerous than unhealed breaks, and their analysis should provide information about chromosome stability. However, the effective resolution of autoradiographic techniques is not sufficient to make practical a comparison between the locations of exchanges and those of quinacrine or Giemsa bands.

DNA synthesis in chromosomes can now be detected microfluorometrically, by a technique that is based on the sensitivity of the fluorescence of bisbenzimidazole dyes such as 33258 Hoechst to 5-bromodeoxyuridine (BrdU) (*6*). Sister chromatids that differ in the number of DNA chains substituted with BrdU can be easily distinguished, and sister chromatid exchanges can thereby be identified and located relative to the quinacrine banding patterns.

Human peripheral leukocytes were cultured 70 to 72 hours in Eagle's minimal essential medium supplemented with 20 percent fetal calf serum, 2 m*M* L-glutamine, an extract of red kidney beans containing phytohemagglutinin, 0.4 μM 5-fluorodeoxyuridine, 6 μM uridine, and 0.01 or 0.02 m*M* BrdU. Care was taken to protect all cultures from light. Slides were stained with quinacrine dihydrochloride, photographed, destained with a mixture of methanol and acetic acid (3 : 1), and restained with 33258 Hoechst (0.5 μg/ml) (*7*) dissolved in 0.15*M* NaCl, 0.03*M* KCl, 0.01*M* phosphate (*p*H 7). After being washed, the slides were mounted in 0.16*M* sodium phosphate–0.04*M* sodium citrate (*p*H 7), and photographed (*6*, *8*).

Most cells grown 3 days in medium containing BrdU have chromosomes that exhibit characteristic quinacrine fluorescence patterns (Fig. 1A), in accordance with the observation that the fluorescence intensity of quinacrine bound to $(dA\text{-}BrdU)_n$ (dA is deoxyadenylate) is little different from that bound to $(dA\text{-}dT)_n$ (dT is deoxythymidylate) (*6*). However, after the quinacrine is removed and the chromosomes are restained with 33258 Hoechst, a marked difference is apparent between the fluorescence of sister chromatids (Fig. 1B). Autoradiographic analysis (*6*) confirms that the dully fluorescing chromatids of such chromosomes contain approximately twice as much BrdU as do the brighter chromatids. The pattern shown is that expected after two replication cycles, at which time one chromatid contains DNA with one chain substituted with BrdU (the brightly fluorescent chromatid) and the other chromatid contains DNA with both chains substituted with BrdU. Cells which replicate three times during this period contain chromosomes with an average of only one-fourth of the total chromatid material fluorescing brightly, as expected.

Sharp reciprocal alterations in fluorescence intensity along chromatids stained with 33258 Hoechst identify sister chromatid exchanges in cells which replicated twice before harvest. An average of 14 sister chromatid exchanges are observed per metaphase. (The 126 metaphases examined contained 1737 sister chromatid exchanges, but only 7 chromosome or chromatid breaks.) A similar density of sister chromatid exchanges has been observed by others in studies in which cells were administered low doses of [^{3}H]dT and analyzed by autoradiography (*9*).

The frequency of sister chromatid exchanges per chromosome generally increases with chromosome length (Fig. 2). Additional data will be required to establish whether the exchange frequency in individual chromosomes or particular chromosome regions such as the secondary constrictions of chromosomes 1, 9, and 16 deviates significantly from this correlation. The smaller chromosomes as a group exhibit somewhat fewer exchanges than would be expected from their metaphase lengths, perhaps indicating an unusually low contribution from the centromeric region, which constitutes a larger portion of these chromosomes.

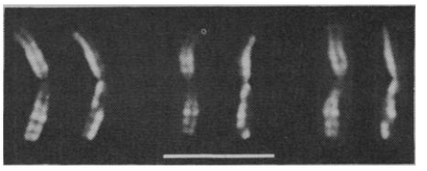

Fig. 3. Sister chromatid exchanges in human chromosome 1. Chromosomes were prepared from peripheral leukocytes obtained from normal human subjects (46, XY or 46,XX) and grown 70 to 72 hours in medium containing 0.01 or 0.02 m*M* BrdU. Chromosomes were stained with quinacrine (left-hand member of each pair), destained, and restained with 33258 Hoechst (right-hand member of each pair). Sister chromatid exchanges are evident as abrupt reciprocal alternations in 33258 Hoechst fluorescence along chromatids. The bar represents 10 μm.

Since quinacrine and 33258 Hoechst fluorescence patterns can be sequentially obtained for the same chromosome, the position of these sister chromatid exchanges relative to the quinacrine bands can be examined. Chromosome 1 is especially favorable for this analysis because the quinacrine bands, which account for somewhat more than half of the length of this chromosome, are very clearly demarcated. Furthermore, there is a slight unevenness in the 33258 Hoechst fluorescence along this chromosome, corresponding to the quinacrine bands, which aids in determining the location of sister chromatid exchanges (Fig. 3). The majority of the sister chromatid exchanges in chromosome 1 appear to be located either in the interband regions or very near the band-interband junctions. A few regions in chromosome 1 adjacent to very bright quinacrine bands are sites of unusually frequent sister chromatid exchange.

Precise location of sister chromatid exchanges in chromosomes with less distinct quinacrine banding patterns is less certain. However, the majority of sister chromatid exchanges observed in these chromosomes also appear to be in interband regions, and a few regions are

sites of unusually frequent sister chromatid exchange. These data are unavoidably subject to the influence of selective forces (*10*) operative during the two division cycles required for the analysis described here. The magnitude of this influence is unknown.

While the present method obviates the use of radioactivity for analysis of sister chromatid exchanges, it requires the incorporation of BrdU into chromosomal DNA. BrdU has been observed to cause breaks in some mammalian chromosomes (*11*), but it does not appear to contribute appreciably to them under the conditions employed here. Moreover, the BrdU-induced chromosome breaks reported (*11*) occurred in heterochromatic regions, which are thought to correspond to the quinacrine band regions (*1, 2*), while the observed sister chromatid exchanges occur predominantly in interband regions. This location of sister chromatid exchanges does not correlate with the expected distribution of BrdU along the chromatids, assuming that regions fluorescing brightly when stained with quinacrine are relatively A-T rich (*12*). Nonetheless, at this time, the possibility that BrdU may contribute to the exchange frequency cannot be ruled out.

Chromatid breakage prior to sister chromatid exchange may involve events similar to those of unhealed chromatid breaks, chromosome translocations, and meiotic crossing-over. The preferential location of sister chromatid exchanges in interband regions might then indicate that these other processes follow a similar nonrandom distribution. Since interband regions are enriched for less condensed, early replicating chromatin (*1, 2*), these regions might be the sites of actively expressed genetic loci. Translocations occurring in these interband regions might then subdivide some of these loci, perhaps accounting for some of the pathology observed in patients with apparently reciprocal translocations.

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Chloride Spike: A Third Type of Action Potential in Tissue-Cultured Skeletal Muscle Cells from the Chick

Abstract. *In addition to sodium and calcium spikes, tissue-cultured skeletal muscle cells from the chick can initiate spikes lasting tens of seconds. The peak membrane potential of the spike correlates with the chloride ion concentration, but not with the calcium or sodium ion concentration. The chloride spike is blocked by manganese ion but not by cobalt ion.*

Besides the Na and Ca spikes (*1*), skeletal muscle cells from the chick in tissue culture can generate a spike lasting tens of seconds (*2*). This late spike (LS) has been considered a Ca spike because it is blocked by Mn^{2+} but not by tetrodotoxin (*2*). If the LS were a Ca spike, its size should depend on the Ca^{2+} concentration and Co^{2+} should inhibit it (*3*). These expectations, however, were not verified by the experiments reported here. On the other hand, changes in Cl^- concentration significantly altered the peak potential of the LS. Therefore, the LS may be a Cl spike, similar to that found in certain plant cells (*4*) and in fish electroplaques (*5*).

Monocellular layers of skeletal muscle cells (6 to 30 days old), which were 20 to 50 μm thick and 0.5 to 5 mm long (in the myotubular and striated stage), were obtained by culturing myoblasts dissociated from pectoral muscles of chick embryos (10 to 12 days old) in modified Eagle minimum essential medium (*6, 7*). Fibroblasts were usually eliminated by adding $10^{-5}M$ D-arabinofuranosylcytosine (Ara-C) to the medium (*6*), but there was no essential difference in the results obtained with cells not treated with Ara-C.

Intracellular recordings were obtained in the conventional way by using glass microelectrodes (20 to 60 megohms) filled with either 3*M* KCl or 3*M* potassium acetate solution. Usually a cell was impaled by two microelectrodes with a tip separation of less than 50 μm; one microelectrode was used for passing current and the other for recording membrane potential. The standard extracellular solution contained 128.4 m*M* NaCl, 5.0 m*M* KCl, 5.0 m*M* $CaCl_2$, and 15.0 m*M* tris-Cl or Na-HEPES buffer of *p*H 7.4 (Na-HEPES is the sodium salt of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). The ionic species and concentrations were changed as follows: Na^+ was replaced by tris or tetramethylammonium ion, NaCl by sucrose or urea, Cl^- by acetate, and $CaCl_2$ by NaCl, so as to maintain a constant osmolarity. The extracellular solution was changed within 3 minutes while the microelectrodes remained in the cell (*8*). Solution changes sometimes caused a shift in the reference potential (*9*). After an experiment involving a solution change, therefore, the microelectrodes were always withdrawn from the cell and the new resting, holding, and LS peak potentials were calculated on the basis of the new reference potential.

The resting potential of the muscle cells varied from − 35 to − 80 mv and appeared to correlate with cell length (*7, 10*). When the membrane potential was held around − 80 mv with steady hyperpolarizing current, a depolarizing current pulse could elicit a Na spike and a LS (Fig. 1A). This Na spike was eliminated in Na-free solution or in solution containing $10^{-5}M$ tetrodotoxin (*1*), but the LS remained in these solutions. In Na-free sucrose solution a depolarizing pulse elicited a small