high rates of lung cancer among female residents in the PIC raise the possibility of a pollution hazard spreading beyond the workplace.

The findings of this survey suggest the need for industry-wide epidemiologic studies to clarify the risk of cancer among various groups of petroleum workers and to evaluate the possible effects of petrochemical emissions released into neighboring communities.

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9. For lung cancer, the rates (deaths per year per 100,000 population) increased from 30.0 to 35.7 to 45.5 among the three urbanization groups of the PIC as opposed to 27.2 to 31.1 to 39.0 for the control counties. The rates for nasal cancer varied little with urbanization, remaining at about 0.6 in the PIC and 0.4 in the control counties. Skin cancer rates declined with urbanization (4.2, 3.3, and 3.3 in the PIC compared to 3.4, 3.2, and 3.0 in the control counties).
10. The PIC/control counties mortality ratios for white females were (total and three urbanization categories of Table 3, respectively): lung cancer, 1.06, 1.21, 1.07, 1.05; nasal cancer, 0.77, 0.70, 0.58, 0.76; skin cancer, 1.01, 1.00, 1.05, 0.99; all cancers combined, 1.01, 0.98, 1.02, 1.02.
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12. The mortality rate for nasal cancer for the 11 PIC combined was 0.8 (about double the control and national rates) and exceeded 0.7 in eight of the 11 counties. The rate for the 28 PIC without heavy chemical industrial involvement was 0.5.
13. The mortality rates for each of nasal, lung, and skin cancers were reported to be higher by 10 percent in chemical-industry counties (*11*). The mortality rates for lung and skin cancers in the PIC where chemical industries were heavily concentrated were roughly 10 percent higher than in the PIC without a heavy concentration of chemical industries, which were in turn higher (12 percent for lung cancer and 7 percent for skin cancer) than the control rates. However, rates for nasal cancer in the PIC with chemical industries were over 50 percent higher than rates in the remaining PIC.
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Bifunctional Intercalators: Relationship of Antitumor Activity of Diacridines to the Cell Membrane

Abstract. *The in vivo antitumor effectiveness [as measured by the percentage increase in life-span (ILS%)] of 28 diacridine bis-intercalators of nucleic acids shows a highly significant correlation with their effect on phenomena associated with plasma membrane as well as a high degree of structural specificity. In contrast, the ILS% does not correlate with the uptake of these diacridines by cells, nor with the inhibition of RNA synthesis or of DNA synthesis or with the inhibition of growth of cells in culture. The possibility that the antitumor effectiveness of actinomycin D, another DNA intercalator, is associated with sites of action other than the hibition of RNA synthesis is discussed.*

In an attempt to develop new antitumor compounds, we have enhanced the well-known ability of the acridine ring to intercalate with DNA by synthesizing diacridines (*1–3*). These compounds consist of two acridine rings connected through their 9-amino position by hydrocarbon chains of various lengths. In addition, various substitutions have been made on the acridine rings; only symmetrical compounds have been synthesized, the two acridine rings of each diacridine are identical (see Table 1). It was anticipated that such compounds would intercalate more strongly with DNA. In order for the DNA to be free of the diacridine, both rings would have to deintercalate simultaneously; a delay of one ring would permit the other ring to reintercalate. Work done in our laboratory (*1, 2*), as well as in others, with a limited number of diacridines (*4*) has shown them to bind more strongly than monoacridines to DNA and RNA.

In the biological studies described below, the diacridines in which the connecting paraffinic chain has six or more methylene groups ($n \geq 6$ in Table 1) have proved more effective than when $n \leq 4$. This result seems to find an explanation both in model building (*1*) and in studies on PM 2 DNA with Wakelin *et al.* (*5*). These studies (*1, 5*) indicate that when $n \geq 6$ the diacrines can effectively act as bifunctional intercalators with PM 2 DNA while they act only as monofunctional intercalators when $n \leq 4$; when $n = 5$, intermediate results are obtained.

The diacridines are potent inhibitors of the growth of P-388, L-1210, and HeLa cells in culture (*6*). More detailed studies with these cells indicated that RNA synthesis was the primary site of action; DNA and protein synthesis were affected to a lesser extent (*6, 7*). Additional studies showed that the synthesis of 45*S* ribosomal RNA was specifically inhibited as well as the further processing of this RNA to 28*S* and 18*S* RNA (*8, 9*) and the methylation of transfer RNA (tRNA) (*9*). A study of the synthesis of well-defined messenger RNA's (mRNA) in vitro (T7 DNA-dependent RNA polymerase transcribing the late cistrons of T7 DNA in vitro) showed that the diacridines inhibited the initiation of RNA synthesis in contrast to actinomycin D, which inhibited elongation (*10*). In addition, we have found the diacridines to be extensively taken up by cells in culture (*8*) and by tumor cells in vivo (R.M.F., unpublished observations). The diacridines also have considerable in vivo antitumor effect when assayed in BDF/1 mice bearing P-388 leukemia tumors in ascites form (*1*).

It was therefore of interest to establish whether the in vivo antitumor effect of these diacridines, as measured by the percentage increase in life-span (ILS%), would correlate with the well-defined inhibition of RNA or of DNA synthesis or with their cellular uptake. Using all the diacridines for which we have in vivo antitumor data (ILS% values), we related the ILS% with the ability of the diacridines to inhibit RNA synthesis (Fig. 1a) and DNA synthesis (Fig. 1b), as well as their uptake by P-388 cells in culture (Fig. 1c). These results indicate that there is no significant correlation between any of these three parameters and the antitumor activity of the diacridines as represented by ILS%. A corresponding study relating ILS% to the inhibition of growth of P-388 cells in culture also showed no significant correlation (*3*).

However, we also evaluated the cell membrane as a possible site of action of

these diacridines—their high lipophilicity as expressed by log P (*11, 12*) makes the membrane a very probable site of action (P is the partition coefficient between octanol and water). It has been shown by Hwang *et al.* (*13*) and by Murphree *et al.* (*14*) that adriamycin, an antitumor agent (*15*) that intercalates with DNA (*15*), reacts with the membrane of S-180 cells in a concanavalin A–mediated agglutination assay (*13, 14*). Our results with this assay emphasize that a highly significant inverse correlation exists between the ILS% and the ability of the diacridines to enhance the rate of agglutination of S-180 cells (Fig. 1d). The monofunctional intercalators 1 and 2, where $n = 2$ and 4, respectively, do not fit this correlation.

These studies indicate that the calculated log P cannot be the only determinant because the calculated log P of compound 14 and that of compound 15 are very similar (*11, 12*), yet their antitumor effects vary widely. In addition, the structure of the diacridines, in the face of identical calculated log P's must be very determinant because the ILS% results obtained with compound 14 and compounds 15 or 16 are widely divergent. Taken together, these considerations indicate that the antitumor effectiveness of these diacridines is associated with very specific membrane sites that may be primarily characteristic of tumor cells. This association is expressed, through tumor and host cell interactions, in an ILS% that correlates well with the agglutination assay, which in turn is an indicator of membrane-related interactions. However, the uptake of diacridines, their inhibition of cell growth and of syntheses of RNA and DNA may also be required for the full expression of their antitumor activity. It is likely that the doses re-

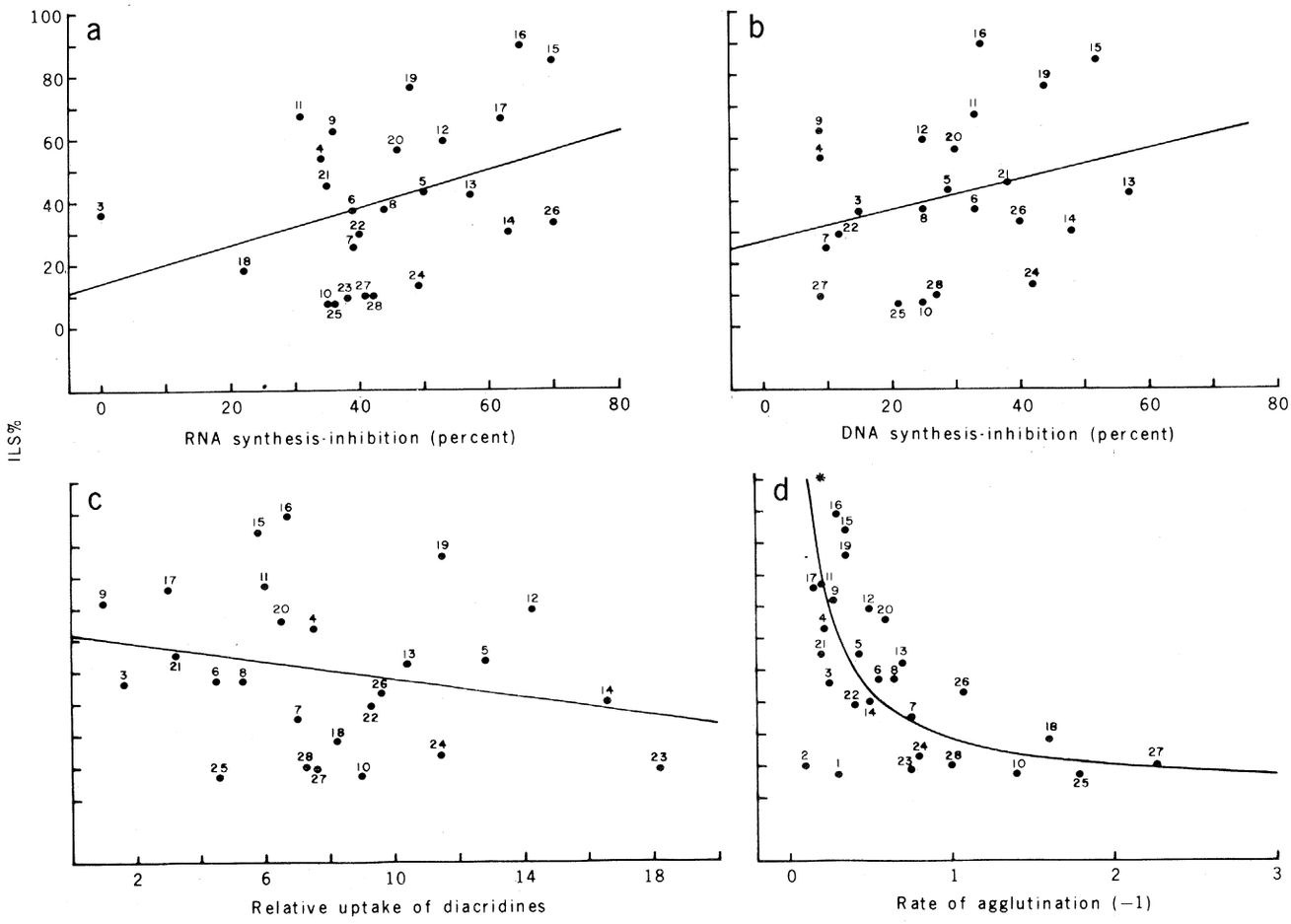


Fig. 1. Least-squares regression analysis of (a) ILS% and inhibition of RNA synthesis, (b) ILS% and inhibition of DNA synthesis, (c) ILS% and uptake of diacridines, and (d) ILS% and rate of agglutination of S-180 cells. The ILS%: to individual BDF/1 mice 1×10^6 P-388 cells from DBA donor mice were administered intraperitoneally on day zero. The drug was administered intraperitoneally once daily for 9 days beginning at day 1. The results were obtained from six BDF/1 mice per group; the drug dosage extended from toxic to ineffective.

$$\text{ILS\%} = \frac{\text{Survival (days) of treated} \times 100}{\text{Survival (days) of control}} - 100$$

The highest value for each compound is used; these have been normalized to the value for compound 10 = 160 percent, which was assayed as a control, in all the antitumor tests. (a) Logarithmically growing P-388 cells were exposed to [^{3}H]uridine for 30 minutes in presence of diacridine (0.5 μg/ml) (*6*). The values are expressed as inhibition of incorporation into RNA per 2×10^5 cells. Incorporation was linear during this time. Control: 10,000 count/min per 2×10^5. (b) Same as (a), except that [^{3}H]thymidine was used (*6*). The values are expressed as inhibition of incorporation into DNA per 2×10^5 cells. Incorporation was linear during this time. Control: 5000 count/min per 2×10^5 cells. (c) P-388 cells exposed to diacridines for 30 minutes. Uptake was the ratio of final intracellular to initial extracellular molar concentration of diacridine. All diacridines were tested at 6 μg/ml except for compounds 1 and 2 (18 μg/ml) and compound 3 (12 μg/ml). For most diacridines these are plateau values. All values are normalized relative to compound 9 = 1.0 (internal : external = 26). (d) S-180 cells incubated for 10 minutes at 37°C with each individual $10^{-4}M$ diacridine, washed, and treated with concanavalin A. The rate of agglutination was measured (*14, 15*). The values are expressed as (experimental/control) − 1. Similar results were obtained with selected diacridines at $1.25 \times 10^{-5}M$. The asterisk indicates $1 \times 10^{-4}M$ actinomycin D. *Statistics*: equations used to obtain correlation coefficients (r) and appropriate curves. (a) $y = 13.8 + 0.60x$, $r = 0.37$; (b) $y = 27.02 + 0.49x$, $r = 0.28$; (c) $y = 51.32 - 1.39x$, $r = 0.25$; and (d) $y = 18.25/x^{0.87}$, $r = 0.80$.

quired to affect the membrane-related interactions adversely affect the metabolism of the host cells as well. As a result, such doses probably supress factors like host resistance and host modulation of metastasis and may even promote selection of resistant tumors. These various adverse effects on the host would minimize the primary membrane-related effects. Consequently, a lack of correlation between ILS% and these individual effects on tumor cells in vitro is seen, because these are derivative and not primary in vivo antitumor effects.

The agglutination assay used with the S-180 cells cannot be used with murine ascites P-388 cells; such P-388 cells are not agglutinated by concanavalin A (up to 300 μg/ml). However, if wheat germ agglutinin (WGA) is used in place of concanavalin A, it will agglutinate P-388 cells; this agglutination is affected by the diacridines and correlates well to ILS% (r = 0.80) (Fig. 2). In addition, there is substantial agreement between the results with P-388 and S-180 cells, both in the inverse correlation and the relative position of most of the compounds. However, there are two disadvantages with the P-388 assay. (i) Although the results within one experiment are reproducible, there is a high degree of variability between experiments, which is not seen with the S-180 cells; and (ii) P-388 cells in the absence of growth media are subject to lysis.

At this time, we cannot attribute any biological significance to the inverse correlation between ILS% and the lectin-mediated agglutination assay. This does not mean that the active diacridines with high ILS% do not react with the membranes, because results of other experiments have shown that all these diacridines bind to the membranes (R.M.F., unpublished observations). The interaction with the membrane is also evident in the P-388 assay, where many of the diacridines with high ILS% inhibit the rate of agglutination.

In order to compare the diacridines with another known intercalator, we have assayed actinomycin D in the agglutination assay and related this effect to its antitumor effect ILS%; this relation is shown by an asterisk in Fig. 1d and fits the data presented so far for the diacridines.

It is interesting to examine and interpret, within the context of our experiments, the data of Paphadjopoulos *et al.* (*16*) with actinomycin D. In their experiments the decreased membrane-mediated transport of actinomycin D by resistant cells is bypassed by introducing the drug into these cells within lipid vesicles.

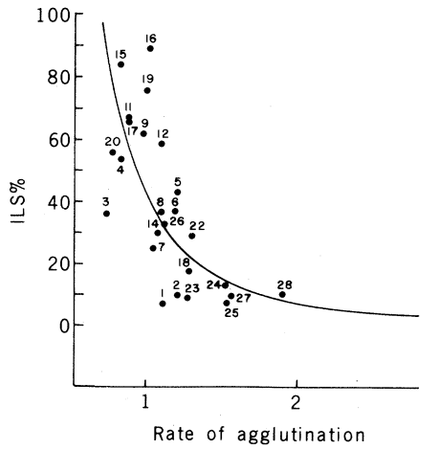
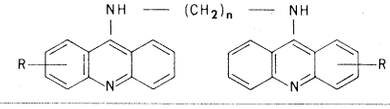


Fig. 2. Least-squares regression analysis of ILS% and rate of agglutination of P-388 cells. The P-388 cells (2×10^7 cell/ml) were incubated for 10 minutes at 37°C with $10^{-4}M$ individual diacridines, washed, and treated with wheat germ agglutinin. The rate of agglutination was measured as described in Fig. 1d. Some of the diacridines inhibit the rate of agglutination; consequently the control ratio could not be adjusted to zero. Therefore the values are expressed as the ratio of the experimental to the control. *Statistics*: the equation used to obtain correlation coefficient (r) was $y = 41.93/x^{2.58}$ and $r = 0.80$. For this correlation the following compounds were not included: compound 10 because it causes lysis of P-388 cells under the assay conditions; compound 13 because it gave highly variable effects; and compound 21 because our present supply was exhausted.

As a result, an identical inhibition of RNA synthesis is incurred in the resistant and in the sensitive cells for the same concentrations of actinomycin D. However, this identical inhibition of RNA synthesis in the two cell lines is not reflected in a similar inhibition of growth of the resistant and of the sensitive cells. The resistant cells still require a 15-fold higher concentration of actinomycin D than do the sensitive cells for a comparable inhibition of growth. We suggest that their data, in association with our data on the ILS% value of actinomycin D and on its effect on the agglutination of cells, indicate that actinomycin D exerts a growth-inhibitory effect on tumor cells in vivo that may be related to sites other than the inhibition of RNA synthesis. It will be of interest to relate the other cellular sites that react with actinomycin D to those that react with the diacridines.

Table 1. Structures of diacridines evaluated in these studies. The drug code numbers correspond to those in Figs. 1 and 2.



Drug code No.	CH_2 groups n	R
1	2	
2	4	
3	5	
4	6	
5	6	4-Ethyl-
6	6	3-Nitro-
7	6	2-Methoxy
8	6	3-Propoxy
9	6	4-Methoxy
10	6	4-Butoxy
11	7	
12	8	
13	8	4-Ethyl
14	8	2-Methoxy
15	8	3-Methoxy
16	8	4-Methoxy
17	8	3,5-Dimethoxy
18	8	3-Methoxy-7-chloro
19	8	4-Ethoxy
20	8	3-Propoxy
21	8	3-Nitro
22	8	3-Chloro
23	8	3-Bromo
24	8	4-Methylthio
25	8	4-Butoxy
26	10	
27	12	
28	12	4-Aza

The emphasis in chemotherapy to date has been to develop agents that differentially interfere with cellular metabolism. We originally developed the diacridines on this basis; however, the cumulative results presented above on this family of compounds suggest that a viable alternative is to develop compounds that primarily affect membrane-related intercellular interactions with minimum metabolic repercussions. Such compounds may then be combined with specific metabolic inhibitors, in amounts that can be adjusted to provide the correct amount of metabolic inhibition.

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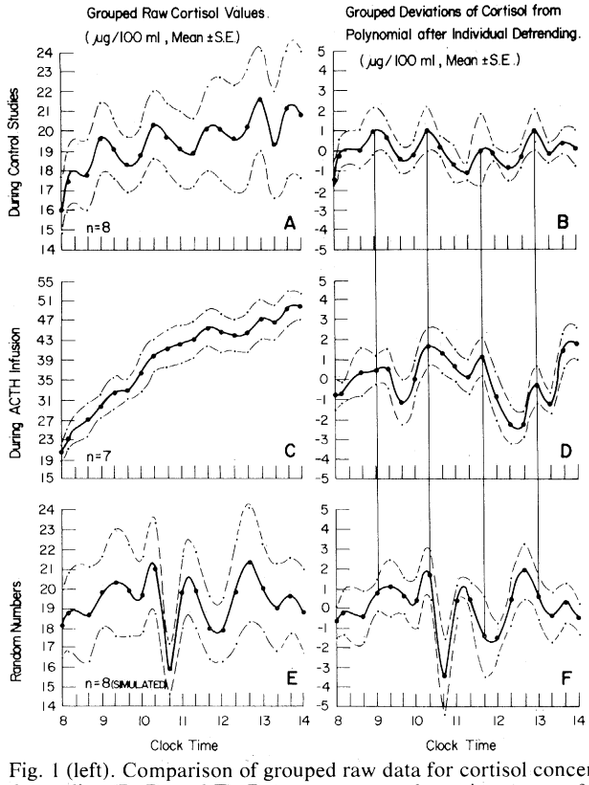
Synchronized Ultradian Cortisol Rhythms in Monkeys: Persistence During Corticotropin Infusion

Abstract. *A highly synchronized ultradian cortisol rhythm with a predominant periodicity of 85 to 90 minutes was observed in eight isolated monkeys; this rhythm may be harmonically related to the circadian rhythm. The persistence of this synchronized rhythm during supramaximal infusions of adrenocorticotropin not only suggests that feedback is not causative but also challenges the classic concept that bursts of cortisol secretion are dependent upon an immediately preceding release of adrenocorticotropin.*

Biological ultradian rhythms are those with a frequency greater than one cycle in 20 hours (*1*). These rhythms are described by Halberg as being wobbling frequencies that are difficult to evaluate by the inspection of raw data (*1*). Such a description especially holds for rapid changes in adrenal corticosteroid concentrations over time. These have been reported to fluctuate variably in both man and monkeys (*2*), and time-series analyses of these fast-frequency adrenocortical fluctuations have uniformly shown broad variance spectra (*3*) consistent with this irregularity. Consequently, the pulsatile patterns of cortisol release have been referred to as "episodic" rather than rhythmic (*1–3*).

In recent work designed to minimize variance of cortisol concentrations both within and among subjects by rigorous environmental control, we reported rapid, cyclic fluctuations of cortisol concentrations in individual monkeys that are superimposed upon a circadian rhythm (*4, 5*). We now report (i) that these fluctuations over time do not disappear when data from individually isolated monkeys are grouped, indicating phasic consistency or synchrony of this ultradian rhythm among animals, (ii) that the variance spectra calculated from these raw data are narrow with a predominant periodicity of 85 to 90 minutes, and (iii) that this ultradian rhythm is not disrupted by the infusion of large doses of adrenocorticotropin (ACTH). Furthermore, evidence is presented that suggests that ultradian cortisol rhythms are harmonically related to the circadian rhythm.

Eight adult male monkeys (*Macaca mulatta*) were adapted to living in pri-



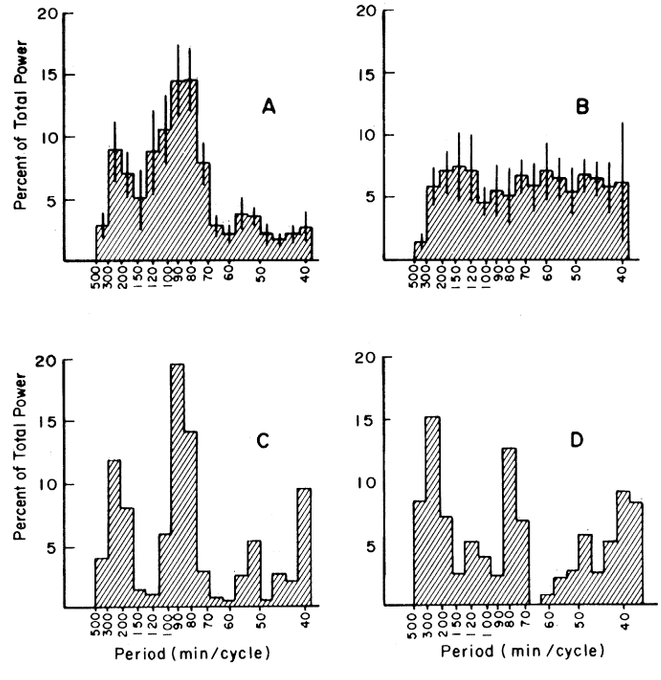


Fig. 1 (left). Comparison of grouped raw data for cortisol concentrations (A, C, and E) with the same data subsequent to individual polynomial detrending (B, D, and F). Data are expressed as micrograms of cortisol per 100 ml of plasma. Dashed lines indicate standard error of the mean computed at each 20-minute interval. Note the different scale on the ordinate in (C). Vertical lines (right) accentuate the exact synchrony between data for control and ACTH-stimulated animals. Random numbers are asynchronous. Fig. 2 (right). Power spectra depicting predominant periods of ultradian cortisol oscillations. (A) Composite of individually detrended control power spectra (mean ± standard error; $n = 8$). (B) Composite of individually detrended random number power spectra (mean ± standard error; $n = 8$). (C) Power spectrum of detrended, grouped control data ($n = 8$). (D) Power spectrum of detrended, grouped cortisol data during ACTH infusion ($n = 7$). The data in (D) reveal the same rhythmic components as seen in (A) and (C). Random number periodograms (B) are arrhythmic. These high-resolution power spectra were computed with essentially a 100 percent lag.