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A Light-Responsive and Periodic NADH Oxidase Activity of the Cell Surface of *Tetrahymena* and of Human Buffy Coat Cells

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ABSTRACT

Oxidation of external NADH (NADH is an impermeant substrate) by cells of *Tetrahymena pyriformis* oscillated with a period of 24–26 min. The period length in darkness (25.6 min) appeared to be slightly longer than the period in light (~24 min). When *Tetrahymena* were placed in darkness for 30–50 min and then returned to light, a new maximum in the rate of NADH oxidation was observed 36–38 min (13 + 24) min after the beginning of the light treatment. The cell-surface NADH oxidase of human buffy coats (a mixture of white cells and platelets) also was periodic and light responsive. Antiox. Redox Signal. 2, 289–300.

INTRODUCTION

CIRCADIAN AND OTHER FORMS of periodic activities have been studied widely (Edmunds, 1988; Wilsbacher and Takahashi, 1998), and circadian oscillators based upon transcription/translation feedback loops have been postulated (Dunlap, 1996; Hall, 1996; Barinaga, 1997; Crosthwaite *et al.*, 1997). However, no biochemical entities have been reported with the potential to conform to the restraints of temperature compensation and entrainment that characterize the circadian clock (Edmunds, 1998).

One such activity recently described (Morré, 1998; Morré and Morré, 1998) is represented by a family of novel cell surface hydroquinone (NADH) oxidases with protein disulfide-thiol interchange activities (designated NOX proteins) (Morré *et al.*, 1997, 1998; Kishi *et al.*, 1999; Morré, 1998; Morré and Morré, 1998). The NOX proteins are located at the external cell surface and have proven suitable for assay and identification using whole cells and organisms (Hicks and Morré, 1998).

The principal defining characteristic of the NOX proteins is a regular periodic oscillation of activity (Morré, 1998). They exhibit both hydroquinone (Kishi *et al.*, 1999) or NADH (Morré and Morré, 1998) oxidase activity as well as a protein disulfide-thiol interchange (Morré *et al.*, 1997, 1998).

The oxidase and interchange activities alternate at 12 min intervals giving an ~24-min period (Morré, 1998; Morré and Morré, 1998). The periodic oscillations were observed with purified proteins (Wang *et al.*, 1997; Morré, 1998; Morré and Morré, 1998) and by the cloned protein from HeLa cells expressed in *Escherichia coli* (Morré, 1998). The periodic oscillations of

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the activities of the proteins serve to distinguish the NOX proteins from other cellular oxidative and protein disulfide isomerase-like activities. The potential function of the NOX protein as a terminal oxidase of plasma membrane electron transport adds to the importance of the observations

Assay of the oscillatory nature of the activity is facilitated by a high degree of synchrony among individual NOX molecules. In plants, one way of achieving synchrony among NOX molecules is by light entrainment (Morré *et al.*, 1999). These experiments were to determine if the cell-surface NOX activities of non-plant origins were subject to a similar synchronization by light.

MATERIALS AND METHODS

Growth of Tetrahymena

Tetrahymena pyriformis were maintained at room temperature in darkness on soybean broth. Assays were with 3 ml of culture to which NADH was added directly.

Isolation of pooled buffy coats

Pooled buffy coats were isolated from blood provided by St. Elizabeth Medical Center laboratory (Lafayette, IN) from routinely collected blood samples. The samples were maintained at 4°C prior to collection and assay.

Spectrophotometric assay

NADH oxidase activity was determined from the disappearance of NADH at 340 nm as reference using a Beckman DU 640 spectrophotometer with readings at 30-sec intervals averaged over 1 min. For assays of buffy coats, the reaction mixture contained the sample, 50 m*M* Tris-MES, pH 7.0, 2 m*M* KCN, 100 μM reduced glutathione (GSH), and 150 μ*M* NADH as substrate in a total volume of 2.5 ml. Hydrogen peroxide (H_2O_2) (2.5 μl of 30%) was

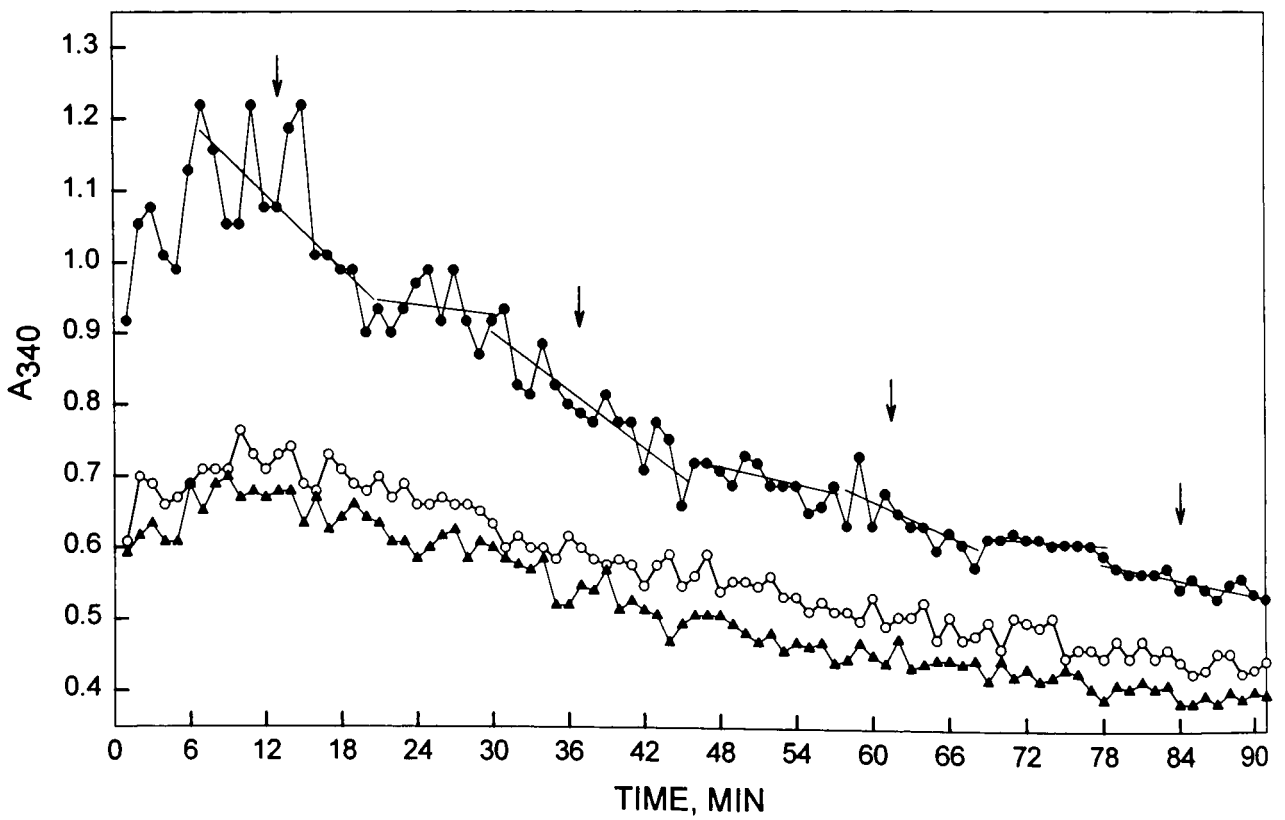

FIG. 1. NADH oxidation by *Tetrahymena pyriformis* (~1,000 cells/ml) in continuous light (●) compared to an autoclaved control (○) or a preparation in which the cells were removed by centrifugation (▲). The periodic response was observed only in the preparation with living cells. Approximate midpoints of periods of rapid NADH oxidation as determined by linear regression analysis are indicated by the arrows. The NADH concentration was 150 μ*M*.

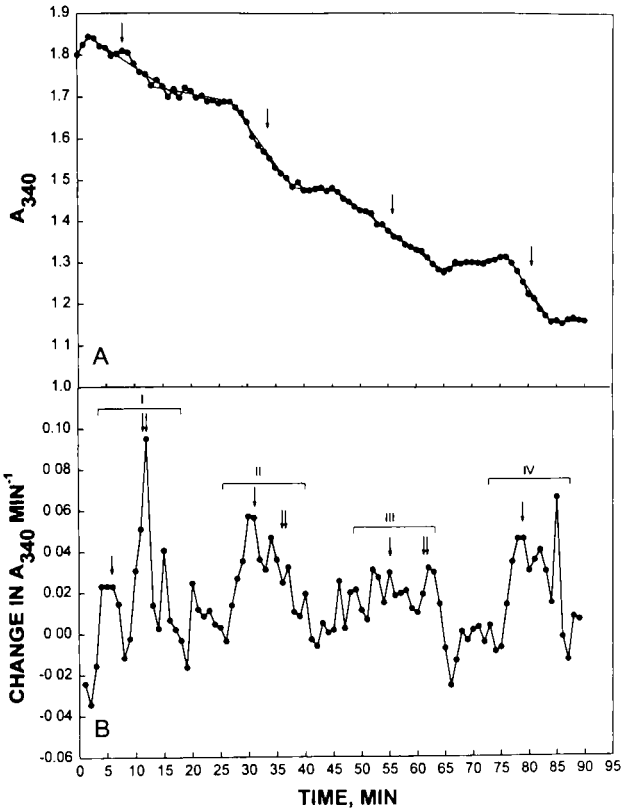

FIG. 2. NADH oxidation by living cells of *Tetrahymena pyriformis* (~1,100 cells/ml) in continuous light with maxima indicated at 9, 33, 57, and 81 min (arrows) for an average period length of 24 min. (**A**) Decrease in A_{340} averaged from two parallel determinations and phased to pronounced minima with midpoints at 18, 42, and 66 min. (**B**) Analysis of data of **A** by numerical averaging $[-(A_{t+1} - A_{t[t\ ]-1})]$ to obtain an absolute rate of change in A_{340} per min. Time segments corresponding to the maxima indicated by single arrows of **A** are indicated by the brackets centered at 9, 33, 57, and 81 min for an average period of 24 min. Within the bracketed regions, subperiods corresponding to intervals of 24 min (single arrows) or 26 min (double arrows) were identified by Fourier (Fig. 3) and by time series (Fig. 4) analyses.

added to start the reaction. A millimolar extinction coefficient of 6.22 was used to determine NADH disappearance.

Cell counts

Cell counts were made directly using a cytometer.

Protein determinations

Proteins were determined by the BCA assay (Smith *et al.*, 1985) using bovine serum albumin (BSA) as standard.

Statistical analyses

Line slopes were determined by linear regression using an Excel program. Statistical comparisons of starting and ending segments were by Student's two tailed *t*-test. Greater than 10 consecutive (usually greater than 15) measurements where the first and last points were not significantly different ($p < 0.01$) were classified as a minimum (no or insignificant average downward rate of NADH oxidation). Greater than 10 consecutive measurements where the first and last points were significantly different ($p < 0.01$) and where a consistent average downward trend (significant rate of NADH oxidation) was exhibited were classified as maxima. Arrows denote the numerically determined midpoints of the downward slopes. Numerical averaging according to the formula $[-(A_{t+1}\text{-}A_{t-1})/2]$ was used to obtain the rate of change in A_{340} per min.

RESULTS

The periodic pattern of oscillations of the cell-surface NADH oxidase activity of T. pyriformis

Cell-surface NADH oxidation by *Tetrahymena*, measured as the decrease in absorbance at 340 nm with organisms grown in darkness and transferred to incandescent light (Fig. 1) or grown in continuous light (Fig. 2), showed al-

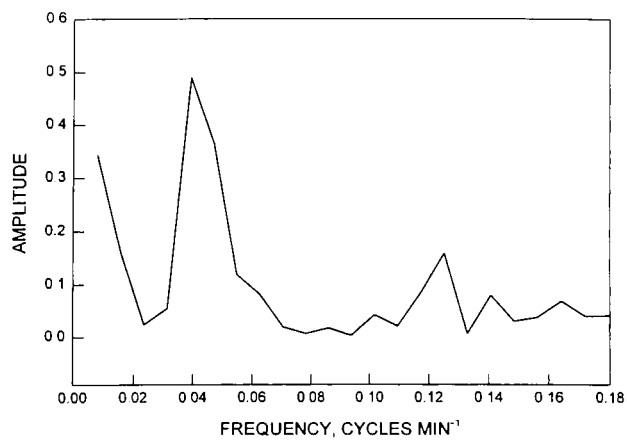

FIG. 3. Fourier transforms of the data of Fig. 2. The maximum amplitude coincides with a frequency of 0.036 cycles min^{-1} or a period length of 26 min. The shoulder at a frequency of 0.046 cycles min^{-1} and corresponds to a period >22 min and <26 min.

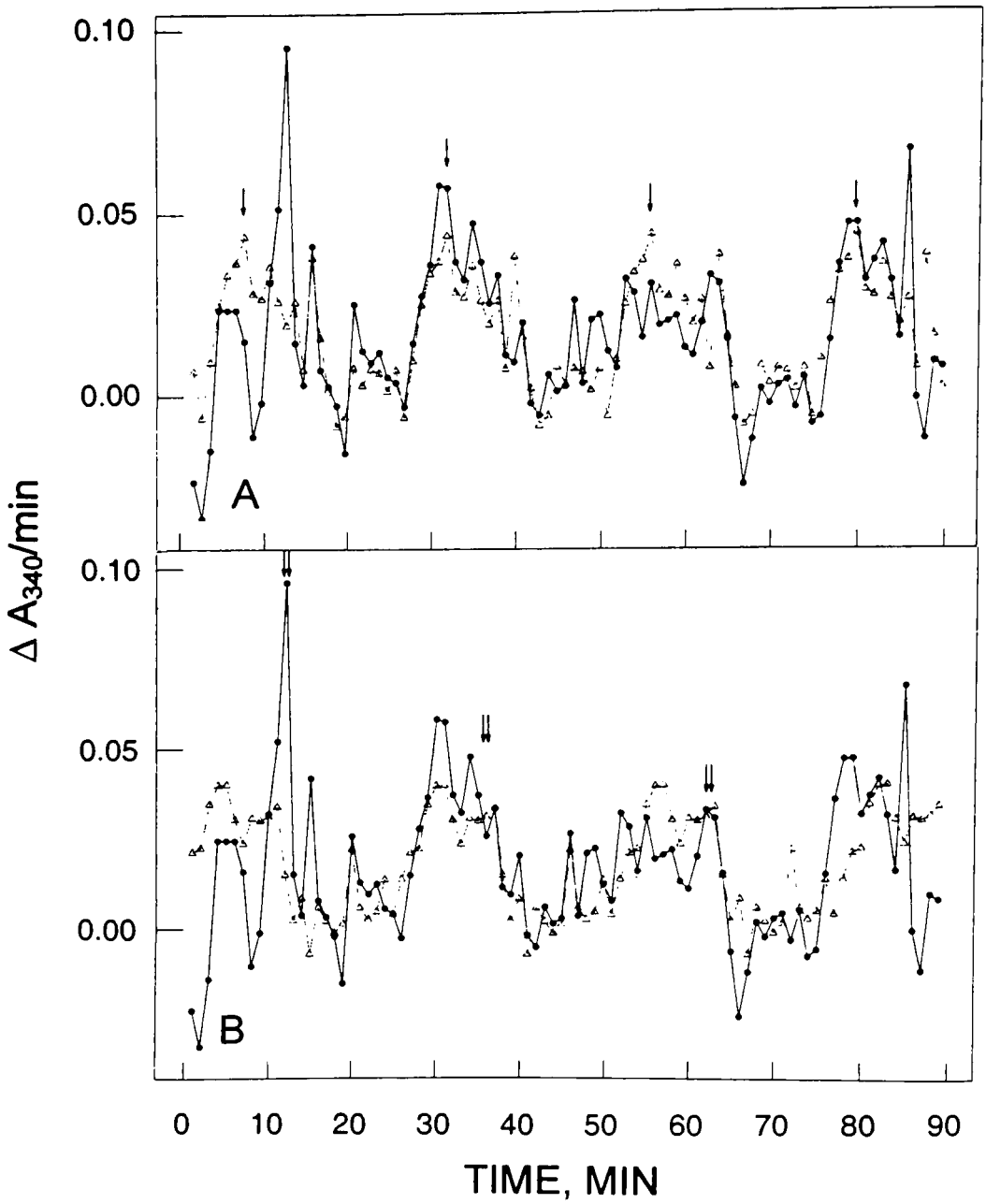

FIG. 4. Time series analysis of the data of Fig. 2. Decomposition fits (△, dotted lines) compared to the original data (▲, solid lines) reveal best fit patterns of the complex oscillatory behavior. (**A**) Decompression fit using a period length of 24 min align with the period maxima marked by single arrows of Fig. 2, indicating the subperiod of 24 min in length. (**B**) Decomposition fit using a period length of 26 min align with the maxima marked by double arrows of Fig. 2, indicating the subperiod 26 min in length.

ternations of periods of active decline and periods of little or no decline at intervals of approximately 24 min. The light intensity was about 3 μmol of photons $m^{-2}cm^{-1}$. With NADH added directly to *Tetrahymena* cultures, rates were maintained in spectrophotometer cuvettes for up to 90 min. Blanks containing only the medium and no *Tetrahymena* (solid triangles) or autoclaved preparations (open circles) also showed a decrease in A_{340} upon addition of NADH, but at a much slower rate than with *Tetrahymena* present and with no obvious evidence of a rhythmic pattern (Fig. 1). The average period length determined in the light

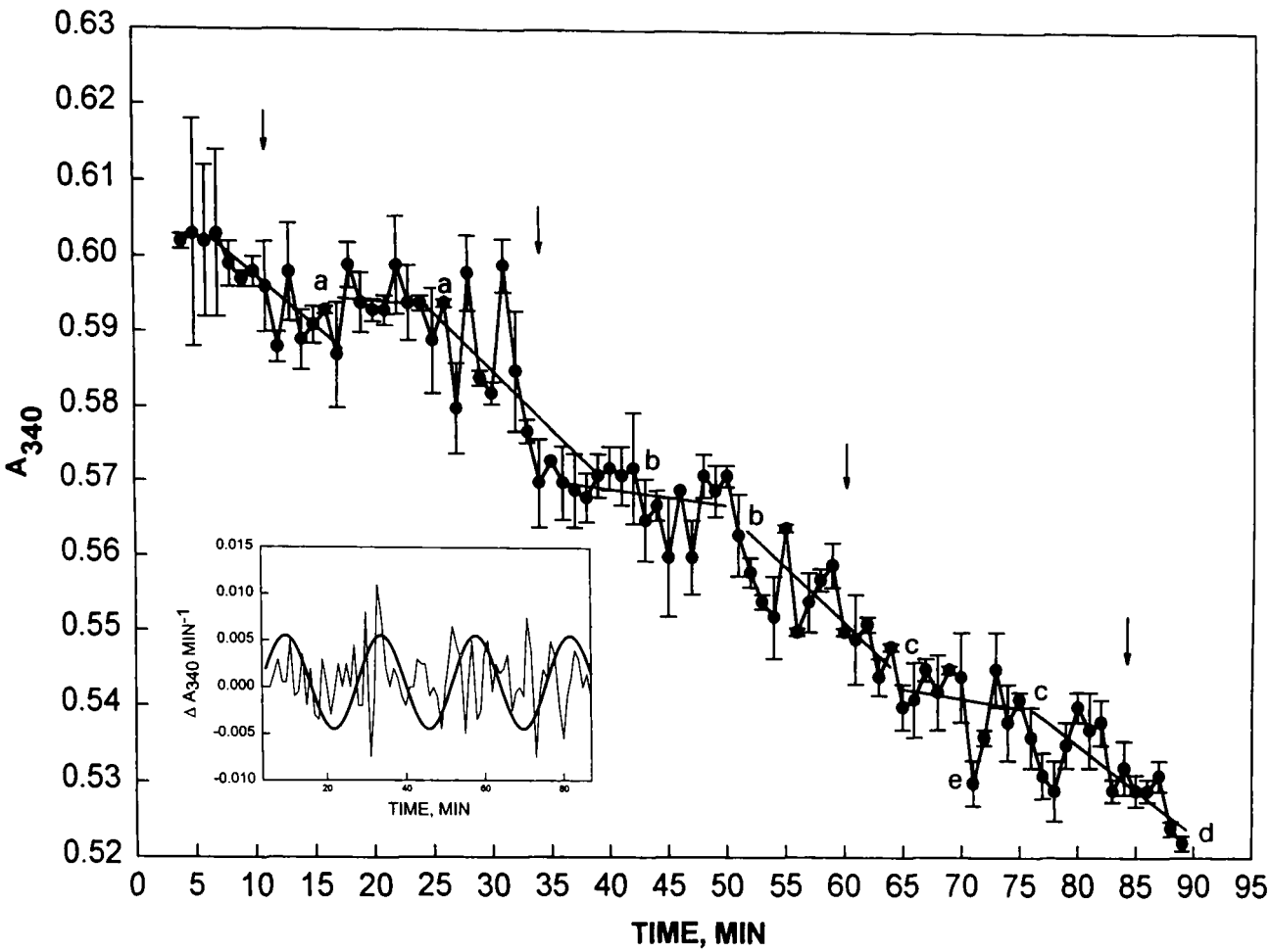

FIG. 5. NADH oxidation by living cells of *Tetrahymena pyriformis* (~500 cells/ml) in darkness following a 2-min exposure to incandescent light (3 μmol m^{-2} sec^{-1}) at $t = 0$. Average of three parallel determinations ± standard deviations. Values not followed by the same letter are significantly different ($p < 0.01$). Intervals of rapid NADH oxidation are indicated by the single arrows at 34, 58, and 82 min after the light interruption or an average period of 23.6 ± 0.6 min. The inset gives the rate changes by numerical averaging. A sine function indicating maxima and minima was fitted to the data. The apparently anomalous rate change at about 70 min ("e") was statistically significant ($p < 0.01$).

($n = 60$) was 23.6 ± 0.9 min. The maximum rate of absorbance was determined to be proportional to numbers of *Tetrahymena* cells over the range of 470 to 1,800 cells/ml (not shown).

Two experiments on successive days with *Tetrahymena* grown in continuous light were phased by alignment of pronounced minima at 18, 42, and 66 min and averaged. A background absorbance of 0.006 per min was subtracted. Periods of active NADH oxidation were observed as indicated at the arrows at 9, 33, 57, and 81 min, corresponding to an average period length of about 24 min (Fig. 2A). When analyzed by numerical averaging, broad maxima were seen as indicated by the bracketed regions (I–IV) centered at about 9, 33, 57, and 81 min with an average period of about 24 min. However, within these broad maxima, two subperiods, one of 24 min (single arrow) and another of 26 min (double arrows) were identified by Fourier (Fig. 3) and decomposition (Fig. 4) analyses.

To determine the duration of the period in darkness, the periodicity maxima in real time were determined first in continuous light and then in darkness without returning the organisms to light. In darkness, the average period was ~26 min (25.7 ± 0.2 min) whereas in the light the period was ~24 min.

The response of the NOX period to light in

TABLE 1. LIGHT SYNCHRONIZATION OF THE OSCILLATORY CELL-SURFACE NADH OXIDASE OF *TETRAHYMENA*

Time in minutes to midpoints of maxima following a 2-min exposure to light (n = 4)			
First maximum	*Second maximum*	*Third maximum*	*Fourth maximum*
11.6 ± 1.1[a]	36.3 ± 0.6	58.6 ± 1.5	82.6 ± 1.0

[a]The increased rate of NADH oxidation at 11.6 min was not significant.

Tetrahymena was investigated by first determining the positions in real time of the periodic maxima in darkness. Then some of the cells were exposed to 2 min of incandescent light (3 μmol photons m^{-2} sec^{-1}), and the positions in real time of the maxima were redetermined. This basic experiment was then carried out simultaneously in triplicate in three different cuvettes using the Beckman DU 640 spectrophotometer with measurements at 1-min intervals (Fig. 5). The absorbances were normalized to the same starting absorbance and averaged. Linear regression analyses showed statistically significant maxima at 34, 58, and 82 min (intervals of 23–24 min) after the beginning of the light exposure (Fig. 5). There may have been a maximum at 11 min as predicted from work with plants, but the change in rate at 11 min was insignificant. Similar findings were shown by the repetitions summarized in Table 1. The plateaus reached at 20, 44, and 68 min were highly significant as were the intervening downward slopes at ~36, 59, and 83 min. The maximum indicated for 11.6 min was not. The new period evident from 36 min after light exposure onward was set independently of the existing period as determined for the control in darkness (Table 2).

The periodic pattern of oscillations of the cell-surface NADH oxidase of human buffy coats

As a mammalian cell source for comparison with results from *Tetrahymena*, we used buffy coats prepared from human plasma. Buffy coats are a mixture of white cells and platelets that collect on top of the red cell fraction following centrifugation of whole blood.

The human buffy coat fraction oxidized NADH as indicated from the decrease in A_{340} with time (Fig. 6A). Measurements were collected at 10-sec intervals and each consecutive

TABLE 2. RESPONSE OF NADH OXIDATION PERIOD LENGTH TO LIGHT FOLLOWING 2 MIN OF IRRADIANCE WITH 3 μMOL $M^{-2}SEC^{-1}$ INCANDESCENT LIGHT

Material	*Experiment*	*Time in a period when irradiance was given (min)*	*Time to first maximum (min)**	*Elapsed time in light between start of irradiance and start of measurement (min)*	*Total elapsed time from end of light to first maximum (min)*
Tetrahymena	I	+2 to +4	39	2	37
	II	+17 to +19	40	2	38
	III	+0 to +2	38	2	36
	IV	+1 to +5	39	2	37
	Mean				37 ± 1
Buffy coats	I	+4 to +6	34	2	32
	II	+8 to +10	40	2	38
	III	+19 to +21	37	2	35
	IV	+23 to +25	37	2	35
	V	+22 to +24	40	2	38
	Mean				36 ± 2.5

*From beginning of light.

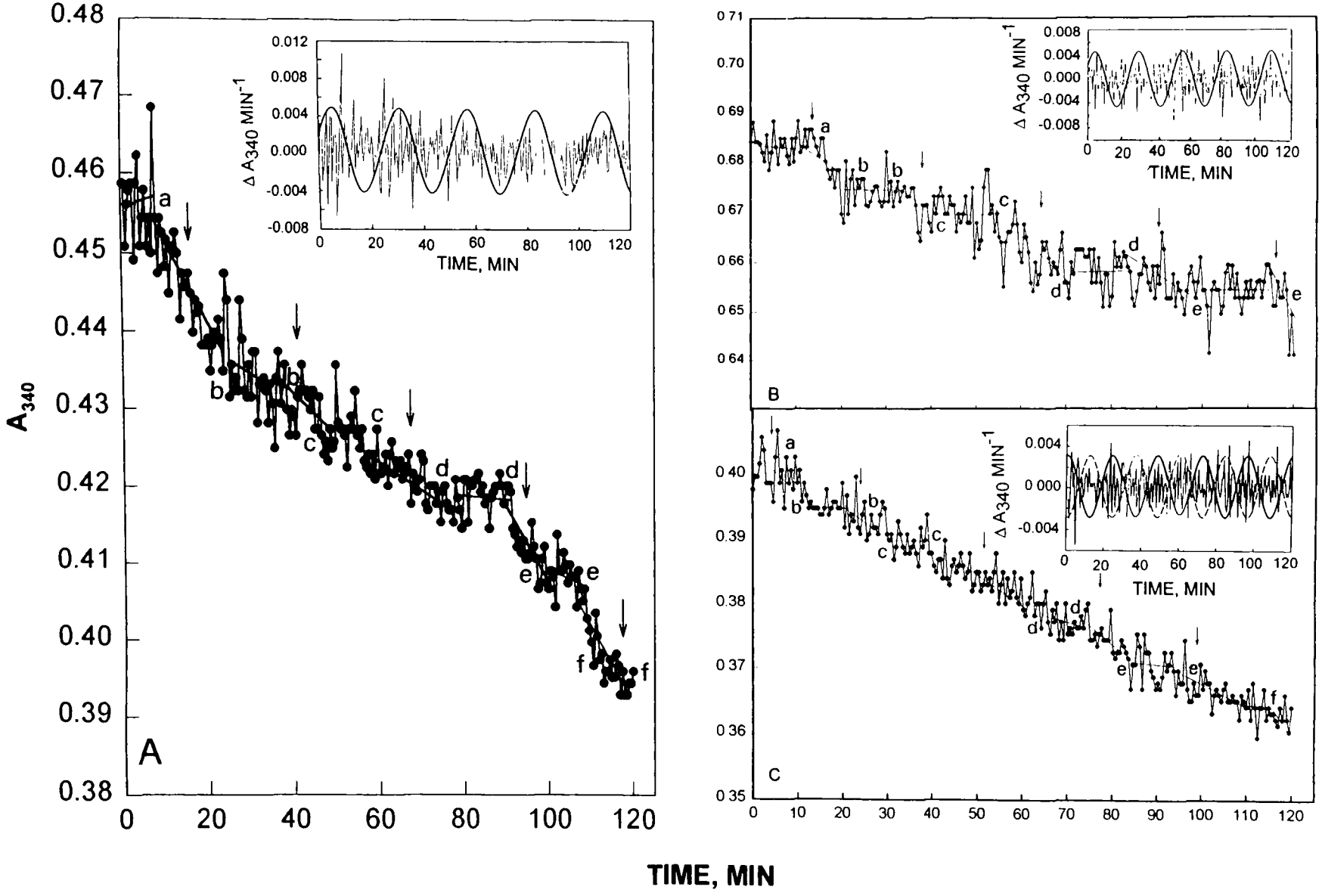

FIG. 6. NADH oxidation by ~10^8 human buffy coat cells. (**A**) In darkness, maxima in rates of NADH oxidation were observed at 15, 41, 67, 93, and 119 min, yielding a regular periodicity of 26 min. (**B**) As in **A** except assayed in the presence of 1 m*M* KCN with maxima in rates of NADH oxidation at 12, 38, 64, 90, and 116 min to generate an average 26-min period. (**C**) Following a 2-min light exposure at $t = -9$ min, a 24-min period with maxima at 28 (37 min after light), 52, 76, and 100 min is indicated (single arrows). Values not followed by the same letter are significantly different ($p < 0.01$). Insets provide rate changes by numerical averaging. The representations were fitted with a sine function as in Fig. 5. In **C**, a component of the original dark period remains (dotted sine wave). The two periodic oscillations were approximately 12 min out of phase as indicated by minima at points of sine wave convergence.

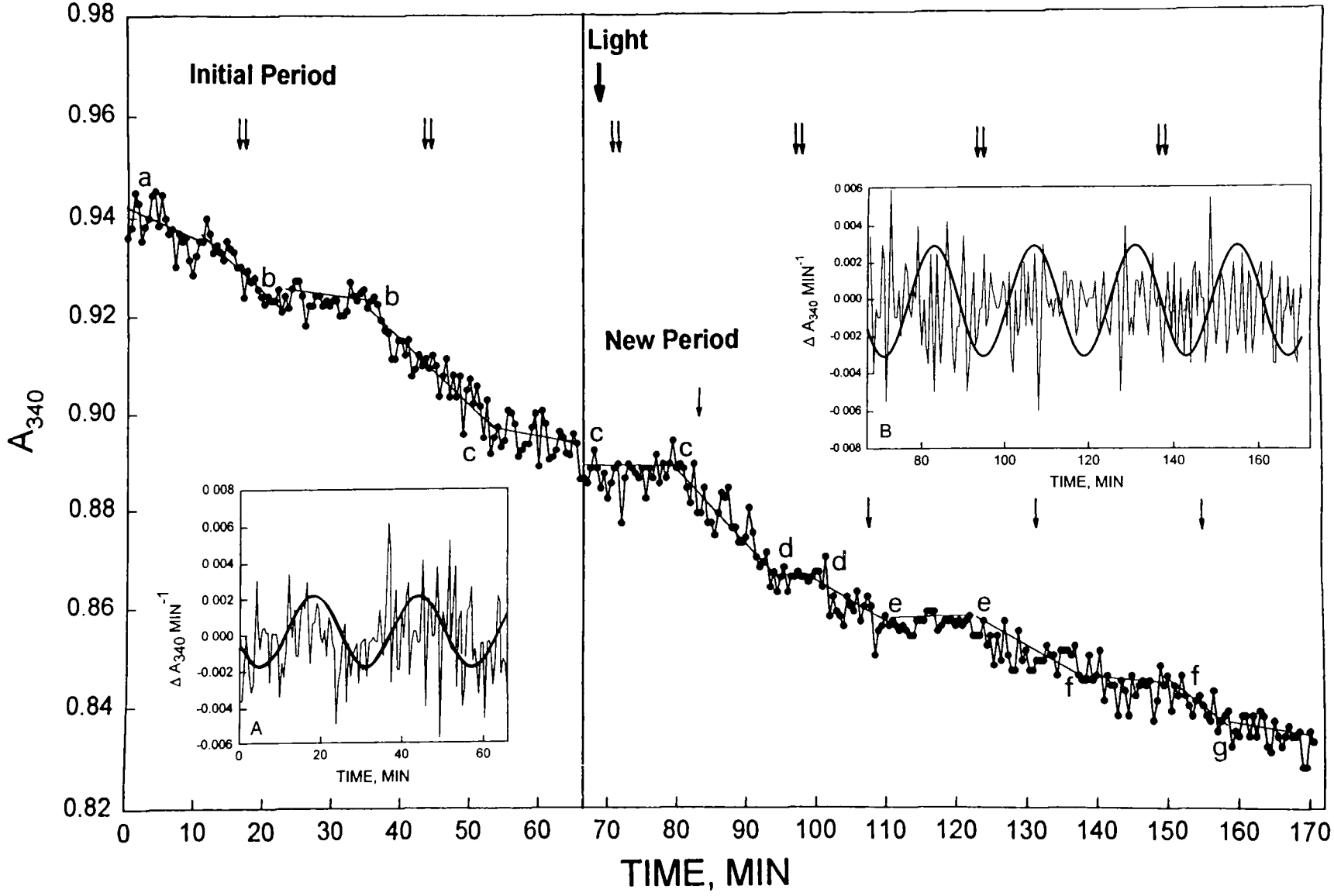

FIG. 7. NADH oxidation by 7.5×10^7 human buffy coat cells to show light entrainment. The initial period was determined in darkness. Midpoints of maximum rates of NADH oxidation are indicated at the double arrows at 18 and 44 min. At 70 min, a portion of the cells were irradiated for 2 min with white light and a new period with maxima indicated by single arrows at 106, 130, and 154 min for an average new period of 24 min beginning 36 to 38 min after exposure to light. The beginning of the 2-min light exposure is indicated by the large arrow. Following light exposure, the position of predicted maxima for the 26-min original dark period are indicated by the double arrows. Values not followed by the same letter are significantly different ($p < 0.01$). The insets show rate changes by numerical averaging. The representations were fitted with a sine function as in Fig. 5. In **A**, the maxima of the original period are represented. In **B**, the first two maxima of the new, light induced period are demarcated, but there is some indication, after 120 min, of a return to the original dark period as discussed in the text.

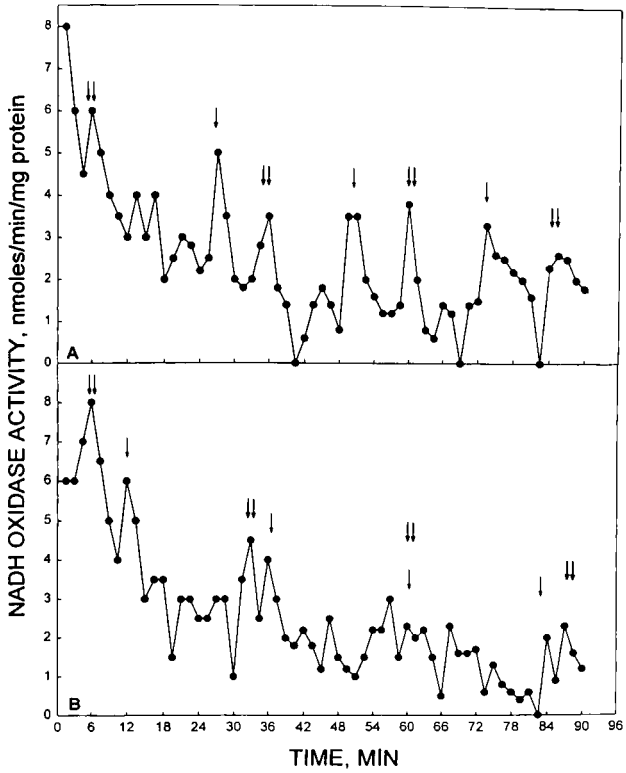

FIG. 8. Rate of NADH oxidation by solubilized and partially purified cell-surface NADH oxidase preparations released from human buffy coats by treatment for 1 hr with 0.1 *M* sodium acetate, pH 5, as described (Del Castillo-Olivares *et al.*, 1998). (**A**) Periodicity in the absence of retinol. (**B**) Periodicity in the presence of 1 μM retinol. The periods were determined from rates measured over 1 min at intervals of 1.5 min. Two periodicities were observed, one with maxima at intervals of 24 min (single arrows) and one with maxima at intervals of 26 min (double arrows). The rates of NADH oxidation were reduced 30–40% by retinol addition for both activities after the first full 24- or 26-min period (Fig. 5B).

six 10-sec readings were averaged for every 1-min interval. Linear regression analyses of line segments show periods of strong NADH oxidation activity at 15, 41, 67, 93, and 119 min, with an average period of about 26 min. This activity was resistant to potassium cyanide, as is characteristic of NOX activities (Fig. 6B). The buffy coat activity also appeared to exhibit at least a partial response to light (Fig. 6C; see also Fig. 7 and Table 2 for comparisons with dark controls). With 2 min of light given at between $t = -7$ and $t = -9$ min, new maxima were observed at 28, 52, 76, and 100 min. The first significant maximum was at ~36 (12 + 24) min after light exposure. The expected maximum at 2–4 min (11 and 13 min after the beginning and the end of the light exposure, respectively) was insignificant (Fig. 6C). In this experiment, a background of activity corresponding to the phase of the dark rhythm was retained, as revealed from rate analyses by numerical averaging (Fig. 6C, inset).

The shift in response of the cell-surface NADH oxidase of human buffy coat preparations to light is documented more clearly by data of Fig. 7. Here the positions of maxima of the initial periodic pattern in darkness were determined over 68 min. A 2-min light interruption was given (large arrow) and the measurements were continued in darkness for an additional 100 min. Regression lines identify segments exhibiting alternating fast and slow rates of NADH oxidation, as confirmed by rate analyses using numerical averaging. The maxima corresponding to the original period are the rapid rates of NADH oxidation centered at 18, 44, 70, 96, 122, and 148 min as indicated by double arrows. Following the light interruption, a new period was established at approximately 104 min (68 + 12 + 24 min) after the light interruption and continued at intervals of about 24 min thereafter, as indicated by the single arrows of Fig. 7. Again the expected initial light-induced maximum 12 min after the light interruption was not clearly demarcated, possibly due to overlap with the position of the original period. Also with time in darkness, the light-induced phase appeared to begin to dampen after two full cycles, possibly suggesting a return to the original or to a new dark period length. As with *Tetrahymena*, a response to light was independent of the phase of the rhythm at the time of the light impulse (Table 2).

Whereas 2 min of exposure to white light initiated a new set of oscillations out of phase with those of the dark period prior to light exposure, the period length of the buffy coat cells varied as was observed with *Tetrahymena*. The dark period length appeared to be about 26 min, but after light exposure both a 26-min (Fig. 6C) and a 24-min (Fig. 7) period length were observed. More correctly, it may be that after the light exposure, the new period length was set at 24

min, but after several cycles, the 26-min dark period length was restored (Fig. 6C), as also indicated for data of Fig. 7.

Thus, either the NOX proteins of the buffy coat fraction appear to exist in at least two populations or the period length of a portion of the NOX proteins is light sensitive. These possibilities are indicated from data of Fig. 8 where the cell-surface NADH oxidases were released from the buffy coat cells by treatment with 0.1 *M* sodium acetate, pH 5, according to the protocol of del Castillo-Olivares *et al.* (1998). Two experiments are shown, one in the absence of retinol (Fig. 8A) and one in the presence of 1 μM retinol (Fig. 8B). Retinol at the 1 μM concentration inhibited NADH oxidation by the external plasma membrane NADH oxidase (Dai et al., 1997). The inhibition occurred after a lag of one full cycle (Wang, 1997; Wang *et al.*, 1997). The NOX activity of the solubilized and partially purified NOX protein exhibited an oscillatory behavior most evident after the initial equilibration phase of about 18 min, but with a complex periodicity indicative of two periods of unequal length. One period length of 24 min was represented by maxima indicated by the single arrows and the second period length of 26 min was represented by maxima indicated by the double arrows. Retinol inhibited the activity by the third and fourth cycle but the inhibition was not complete (Fig. 8B). Components with activity maxima separated by both 24 min and by 26 min remained.

DISCUSSION

Our laboratory has described a cell-surface NADH oxidase related to the growth of cells (Morré, 1998) that oscillates with a regular period of about 24 min in both plants (Morré and Morré, 1998) and HeLa (human cervical carcinoma) cells (Wang *et al.*, 1997; Morré, 1998). The oscillations are highly synchronized and capable of autosynchrony (Wang, 1997; Morré, 1998). In plants growing in darkness or following exposure to far red illumination, the period is set by light with the first maximum following light exposure coming exactly 12 min (one-half period length) following the beginning of the exposure to white or red light (Morré *et al.*, 1999).

In these experiments, we have attempted to determine if the real-time positions of the periodic maxima of cell-surface NADH oxidation also can be determined by light in cells of animal origin. With *Tetrahymena*, the cells showed a considerable variation most likely due to random swimming behavior of the cells. This source of variability also was encountered previously with brine shrimp (Chalko *et al.*, 2000). Nevertheless, the rate of NADH oxidation was observed to exhibit regular oscillations with a period length of ~24 min.

If the cells were returned to darkness and if the period length was redetermined some hours later, the period length was always greater than 24 min. In three experiments, the average dark period length in darkness was determined to be 25.7 min.

When dark-grown *Tetrahymena* were exposed to incandescent light, a new maximum was consistently encountered between 36 and 38 min after the light exposure (average about 37 min). This is about 1 min longer than with plants and consistent with the new maximum appearing after an interval equivalent to one-half of the length of the dark period of about 26 min plus 24 min (13 + 24 min). Once the new maximum was established, the NOX protein continued to oscillate with a 24-min period in the light beginning from the new maximum.

The absence of a clear maximum at 12–14 min following the light exposure is problematic. With plants, the new maximum is observed clearly 12 min after exposure to white or red light (Morré *et al.*, 1999). The next maximum occurs at 36 min, 24 min later. *Tetrahymena* apparently senses the first half-period following light exposure without a readily perceptible change in the rate of NADH oxidation. In this respect, plants and *Tetrahymena* appear to differ.

A light response also was observed with buffy coat cells approximately 37 min after the beginning of the light exposure, but the subsequent oscillations were more variable and sometimes appeared to be spaced more than 24 min apart. This was subsequently traced to the buffy coat cells exhibiting at least two sets of

oscillations, one with a period length of about 24 min and one with a period length of about 26 min.

At present, we have not determined the molecular basis for the two periods of unequal length nor have we determined the spectral qualities of the light required to synchronize the oscillations. We do not know if the oscillating NADH oxidase activities at the surface of *Tetrahymena* are in any way related to the ultradian rhythms in respiratory activity and cell division seen in this organism (Kippert, 1996) nor do we know if the oscillating activities seen both in *Tetrahymena* and buffy coat cells are in anyway related to the classical light-regulated clock of animals in general (for example, Winfree, 1980).

ACKNOWLEDGMENT

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ABBREVIATIONS

BCA, bichinchoninic acid/copper; BSA, bovine serum albumin; GSH, reduced glutathione; MES, 2-(*N*-morpholino)ethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; NOX, cell-surface hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity.

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