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Entrainment in solution of an oscillating NADH oxidase activity from the bovine milk fat globule membrane with a temperature-compensated period length suggestive of an ultradian time-keeping (clock) function

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Abstract

Entrainment in solution of an oscillating activity with a temperature compensated period of 24 min is described for a NADH oxidase (NOX) activity of the bovine milk fat globule membrane, a derivative of the mammary epithelial cell plasma membrane. The period of 24 min remained unchanged at 17°C, 27°C and 37°C whereas the amplitude approximately doubled with each 10°C rise in temperature ($Q_{10} \cong 2$). The periodicity was observed with both intact milk fat globule membranes and with detergent-solubilized membranes, demonstrating that the oscillations did not require an association with membranes. The periodicity was not the result of instrument variation or of chemical interactions among reactants in solution. Preparations with different periodicities entrained (autosynchronized) when mixed. Upon mixing, the preparations exhibited two oscillatory patterns but eventually a single pattern representing the mean of the farthest separated maxima of the two preparations analyzed separately emerged. The cell surface NOX protein is the first reported example of an entrainable biochemical entity with a temperature-compensated periodicity potentially capable of functioning as an ultradian or circadian clock driver.

Keywords: Ultradian rhythm; Temperature compensation; Circadian; Entrainment; Milk fat globule membrane; Bovine

1. Introduction

Our laboratory first described a growth factor- and hormone-responsive oxidation of NADH associated with plant [1] and animal [2] plasma membranes. In cancer, the hormone- and growth factor-responsiveness was lost and the activity became constitutively activated [3,4]. This constitutively activated oxidation of NADH was drug responsive and inhibited by both the antitumor sulfonylurea *N*-(4-methylphenyl)-*N*′-(4-chlorophenyl)sulfonylurea (LY181984) [5] or the antitumor vanilloid capsaicin (8-methyl-*N*-vanillyl-6-noneamide) [4,6]. With the plant plasma membrane, at least, the electron acceptor was shown to be either protein disulfides or molecular oxygen [7].

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The cancer form [8] and hormone stimulated forms [9] of the plasma membrane-associated NADH oxidase (NOX) also were inhibited specifically by thiol reagents and some function other than oxidation of NADH was sought. One such function, constitutively-activated in cancer and also inhibited by capsaicin and sulfonylureas [10], was that of protein disulfide–thiol interchange.

An unusual property of both the oxidation of NADH and of the protein disulfide thiol interchange activity catalyzed by the NOX protein in plants was that both activities alternate and oscillate with a period length of 24 min [11]. Oscillatory activity of the NOX protein has been documented for plants [11–13], and CHO [14] and HeLa cells [15,16] in culture. With both plants [11–13] and cultured cells [14–16] synchronous oscillations were observed with whole cells or tissues in addition to plasma membranes and the purified and even the bacterially expressed form of the cloned HeLa NOX protein (accession no. AF207881 [11,17]). The period length was temperature compensated (period length independent of temperature) [11–16] one of the hallmarks of biological timekeeping [18,19].

Because the oscillations are synchronous not only with isolated plasma membranes and the purified or recombinant protein but in whole plants and intact mammalian cells as well, opportunities for entrainment of the NOX period were sought. In plants, light entrainment is the major contributor to synchronous NOX oscillations [13]. However, the question remains as to how entrainment is achieved and maintained with isolated membranes or purified preparations of the protein.

In this report, the oxidation of externally supplied NADH was used as a convenient measure of the activity of the NOX protein and as a means to monitor the periodic modulation of the catalytic activity. The milk fat globule membrane of the bovine was used as an example of a non-transformed plasma membrane source derived from the apical surface of the lactating mammary epithelium [20]. The oscillations exhibited a ca. 24 min periodicity where the period was temperature compensated. Also, when mixed, the activities of two preparations from different animals and collection times and dates, where maxima and minima were displaced from each other, entrained to achieve a common period. Entrainment represents a second characteristic of biological time keeping which, along with a temperature-compensated period length, is reported here for the first time for a defined biochemical activity.

2. Materials and methods

2.1. Isolation of milk fat globule membranes

Milk fat globules were recovered by centrifugal flotation from fresh cow's (Holstein) milk at $200\times g$ for 15 min. Fat globules were washed twice to remove milk serum proteins. For each wash, fat globules were suspended in 2 volumes (based on original milk volume) of 0.9% NaC1 at 37°C with centrifugal flotation as above. Fat globules were suspended in 5 mM Tris–HCl (pH 7.4), 0.45% NaC1 at 8°C and the membranes were released by churning in a Waring blender. The churned mixture was melted at 40°C to release entrapped membranes, and fat globule membranes were collected from the aqueous phase by centrifugation at $120\,000\times g$ for 60 min at 2°C. Preparations were stored at −70°C prior to use in experiments.

2.2. NADH oxidase activity

The assay for the plasma membrane NADH oxidase was in 50 mM Tris–Mes buffer (pH 7.0), 150 μM NADH and 1 mM potassium cyanide, the latter to inhibit any mitochondrial NADH oxidases contaminating the plasma membranes. The assay was started by the addition of 0.1 mg of plasma membrane protein (50 μl). The reaction was monitored by the decrease in the absorbance at 340 nm using paired Hitachi U3210 spectrophotometers or a SLM DW-2000 spectrophotometer with stirring and at 37°C. The change of absorbance was recorded as a function of time by a chart recorder. ΔA_{340} is given as the absolute value of the decrease in absorbance whereas increases in absorbance (due to small changes in light scattering (turbidity) at minima are represented as negative values. The specific activity of the plasma membrane was calculated using a millimolar absorption coefficient at 340 nm of 6.21 cm^{-1} [21].

Proteins were determined by the bicinchoninic acid

(BCA)/copper assay [22] using bovine serum albumin as standard.

2.3. Statistical analyses

Decomposition fits [23,24] were used to validate each of the oscillatory patterns. The procedure for decomposition analysis of an N dimensional cyclical time series with S being the number of pattern repeats or 'seasons' required seven steps [25].

In step 1, the moving averages of the raw data were computed by averaging S data points at a time. In step 2, the centered moving averages were used as an approximation of both trend and cycle components and were subtracted from the original data to obtain an approximation of the combined seasonal and irregular components. In step 3, the irregular components were removed by averaging the results of step 2 over each pattern repeat or season. As a result, S seasonal averages were obtained, one for each pattern repeat. In step 4, the seasonal estimates above were normalized and for step 5, the appropriate seasonal components were subtracted to deseasonalize the raw data. Step 6 was to estimate the trend component which was normally constant with the data reported here. This was done by linear regression of the deseasonalized data against a constant and a time trend. Step 7, the final step, was to reintegrate the trend, cycle, and seasonal component estimates to get a forecast or computed average of the original data. The final forecasts were obtained by adding the fitted values obtained in step 6 to the normalized seasonal components obtained from step 4 and provide a statistical analysis of the averaged pattern repeat for each data unit.

The actual time series analysis carried out used MINITAB For Windows Release 12.23. Data were entered into the field. Decomposition analysis was initiated by first selecting 'stat' tool followed by selecting 'decomposition' under the 'time series' options. Appropriate data columns were entered as variables for the analysis and the season length was determined by Fourier analysis (Sigma Plot). The model type was additive and the model component setting was selected to be seasonal only [24].

MAPE (mean average percentage error, a measure of the periodic oscillation), MAD (mean average deviation, a measure of the absolute average deviations from the fitted values) and MSD (mean standard deviation, the measure of standard deviation from the fitted values) were derived for each of the figures where changes in rates of NADH oxidation with time were reported.

Arrows were aligned with the maxima in each 24-min pattern repeat as determined from the decomposition fits. With mixtures to two different preparations prior to entrainment, maxima of both preparations as determined from each preparation separately were marked by arrows.

3. Results

The bovine milk fat globule membrane was characterized by an NADH oxidase activity of specific activity comparable to that of plasma membranes from other mammalian tissues and cell lines (Fig. 1). NADH oxidation was estimated from the decrease in A_{340}. Rates were monitored continuously over 1 min at intervals of 1.5 min. In Fig. 1 the cumulative decrease in A_{340} summed over 1 min at

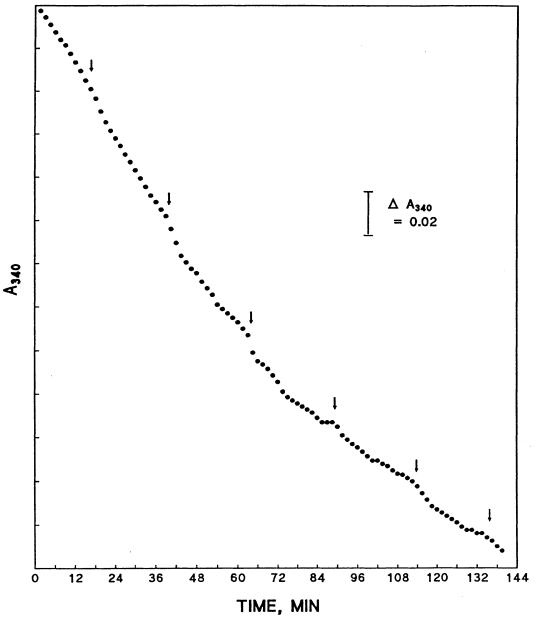

Fig. 1. Time course of NADH oxidation by bovine milk fat globule membranes (a derivative of the plasma membrane of the secreting mammary epithelia). Decrease in absorbance was measured at 340 nm over 1 min at 1.5-min intervals for 144 min as a measure of NADH oxidation. Transitions between minima and maxima in rates of NADH oxidation as indicated by the arrows occurred every 24 min.

1.5-min intervals for 144 min was determined as a measure of the oxidation of NADH. The activity was seen to fluctuate with a period of 24 min. The oxidation of NADH, as determined from the decrease in absorbance at 340 nm, exhibited discernible periodic departures from linearity as the rate slowed or accelerated at regularly spaced intervals (Fig. 1).

When represented as rate of NADH oxidation, the dominant period of ca. 24 min was seen clearly with both the native milk fat globule membranes (not illustrated) and in preparations that had been stored for several months at −70°C (Fig. 2).

The rate of oxidation of NADH for Fig. 2A ≅ 0.25+0.1 sin(15.2πt)/180+200π/180) where t is time in minutes and the value of 0.25 is the mean rate of decrease in A_{340}. The sine function described by the equation (dashed sine function of Fig. 3A)

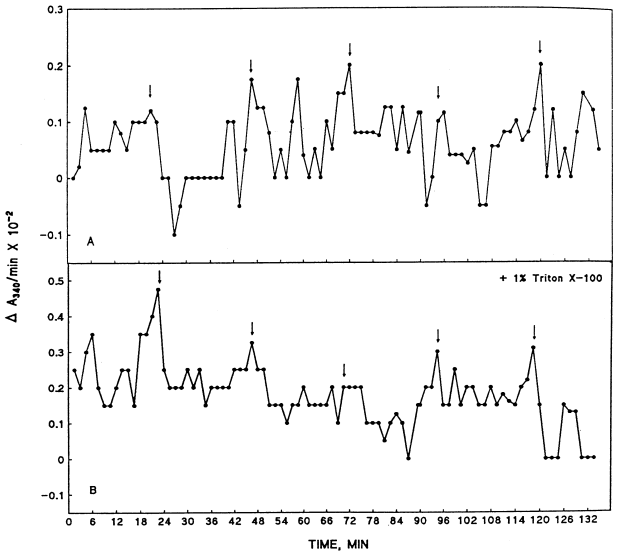

Fig. 2. Periodicity of NADH oxidase activity of bovine milk fat globule membranes as a function of time over 132 min at 37°C. (A) Intact milk fat globule membranes with maxima in rate of NADH oxidation at ca. 24-min intervals beginning at 13 min (maxima at 13, 37, 61, 85 and 109 min) (single arrows). (B) The milk fat globule membranes were dissolved in 1% Triton X-100 to solubilize the proteins. The activities of the two preparations were measured simultaneously using two side-by-side spectrophotometers to show that the positions of the maxima were unchanged by detergent treatment. Period length was unchanged at 24 min (maxima at 23, 47, 71, 95 and 119 min) (single arrows). A change in A_{340} of 0.1 = 0.16 nmol of NADH oxidized. The protein amount was 0.5 mg per reaction to give an average specific activity of 1.3 nmol/min per mg protein which approximates the specific activity of the NADH oxidase of plasma membranes of rat liver and other mammalian tissues.

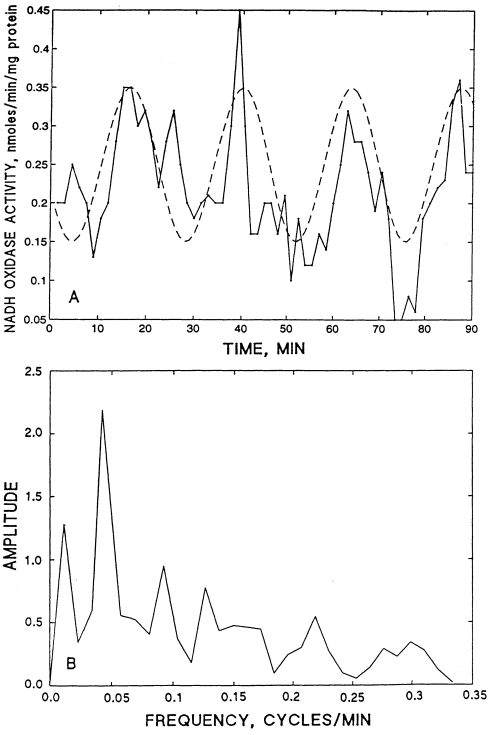

Fig. 3. Sine function and Fourier analysis of data of Fig. 6A. (A) A sine function was fitted to the data (dotted curve) according to the equation $t = 0.25+0.1$ sin(16πt/180+160π/180) where t = time in min and the value of 0.25 is the mean rate of decrease in A_{340}. (B) The maximum amplitude for Fourier analysis coincided with a frequency of 0.042 cycles/min and corresponded to a period length of 23.8 min.

recapitulated the period length of 24 min but only poorly approximated the experimentally determined values for individual experiments due to the complexity of the pattern of oscillations. When values were averaged from several experiments, the approximation to a sine function was somewhat better (Fig. 4, see also Fig. 6 of [12] as another example).

The corresponding Fourier transform of the oscillations of Fig. 3A shown in Fig. 3B further verified that the oscillations were periodic. The determined frequency was 0.042 cycles/min or a period length of 23.8 min.

The activity of NOX protein still oscillated with bovine milk fat globule membranes solubilized by treatment with 1% Triton X-100 to disrupt the integrity of the membrane and to release the protein (Fig. 2B). The oscillations retained the 24 min period length after Triton X-100 solubilization.

With the reagents alone (no enzyme source) or the

enzyme plus all reactants but lacking NADH, no periodic fluctuations were observed. Several different spectrophotometers were compared with similar results.

The periodicity and period length of about 24 min were highly reproducible (Fig. 4). In three experiments carried out in parallel on different days beginning the measurements each day at the same clock time (i.e., 09:30 h), maxima and minima occurred at regularly spaced intervals of 24 min and the differences between maxima and minima were highly significant (Fig. 4). With the averaged data, the fitted sine function (dashed) more closely approximated the experimentally determined values than for Fig. 3.

The period was unchanged with temperature, being the same at 17°C (Fig. 5A), 27°C (Fig. 5B) or 37°C (Fig. 2A and Table 1). Fourier analysis, applied to these data provided period lengths of 23.8, 23.8 and 23.5 min at 17°C, 27°C and 37°C, respectively with an average period length of 23.7 ± 0.17 min (Table 1). At all three temperatures,

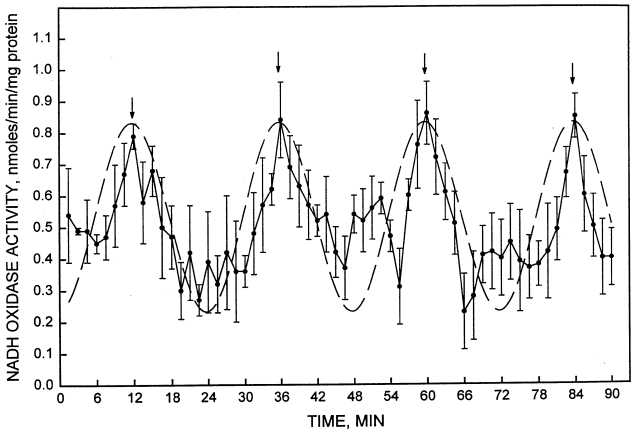

Fig. 4. Three parallel determinations of the rate of NADH oxidation by a single preparation of milk fat globule membranes with time over 90 min. The determinations were carried out on different days but begun at the same time each day to be in phase. There appears to be no daily entrainment with the isolated membranes and no relationship of period to time of freezing or time of thawing. The activity oscillated with a 24-min period even when the preparations were frozen. Rates of NADH oxidation were measured over 1 min at intervals of 1.5 min. Results are given as means ± standard deviations among the three determinations to show the reproducibility of the pattern of the periodicity. The amplitudes at cycle maxima coincided with a period length ca. 24 min and were significantly different ($P < 0.001$) from the amplitudes at cycle minima. A sine function was fitted to the data as described for Fig. 3.

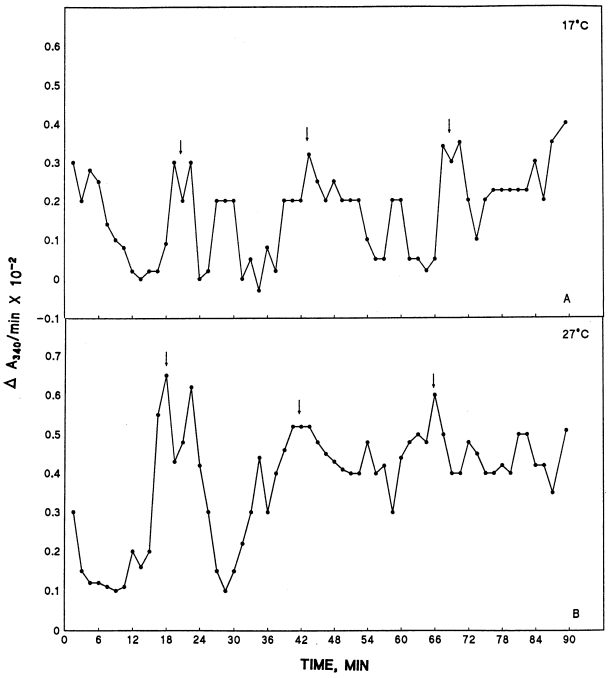

Fig. 5. The periodicity of the bovine milk fat globule membrane was temperature compensated (independent of temperature). The activities were determined as in Fig. 2 but not simultaneously since each was at a different temperature. (A) Temperature 17°C with maxima spaced 24 min apart at 21, 45, and 69 min (single arrows). (B) Temperature 27°C with maxima spaced 24 min apart at 18, 42, and 66 min (single arrows). Other conditions were as in Fig. 2.

a ca. 24-min period was maintained despite the fact that the reaction rate approximately doubled with each 10°C rise in temperature (Table 1). A fully temperature-compensated periodicity (period length independent of temperature over a wide range of temperature) has been a hallmark of circadian rhythms and the most crucial identifier of a potential driver of either ultradian or circadian rhythms. The NOX protein is the first biochemical entity to be described that exhibits this important characteristic.

A second characteristic of ultradian or circadian phenomena has been entrainment. For the NOX protein molecules within or among different cells or plasma membrane preparation to remain in synchrony, some mechanism might be anticipated to synchronize individual NOX protein molecules within a population or to respond to environmental signals.

Fig. 6 shows preparations of milk fat globule membranes from two different sources with maxima

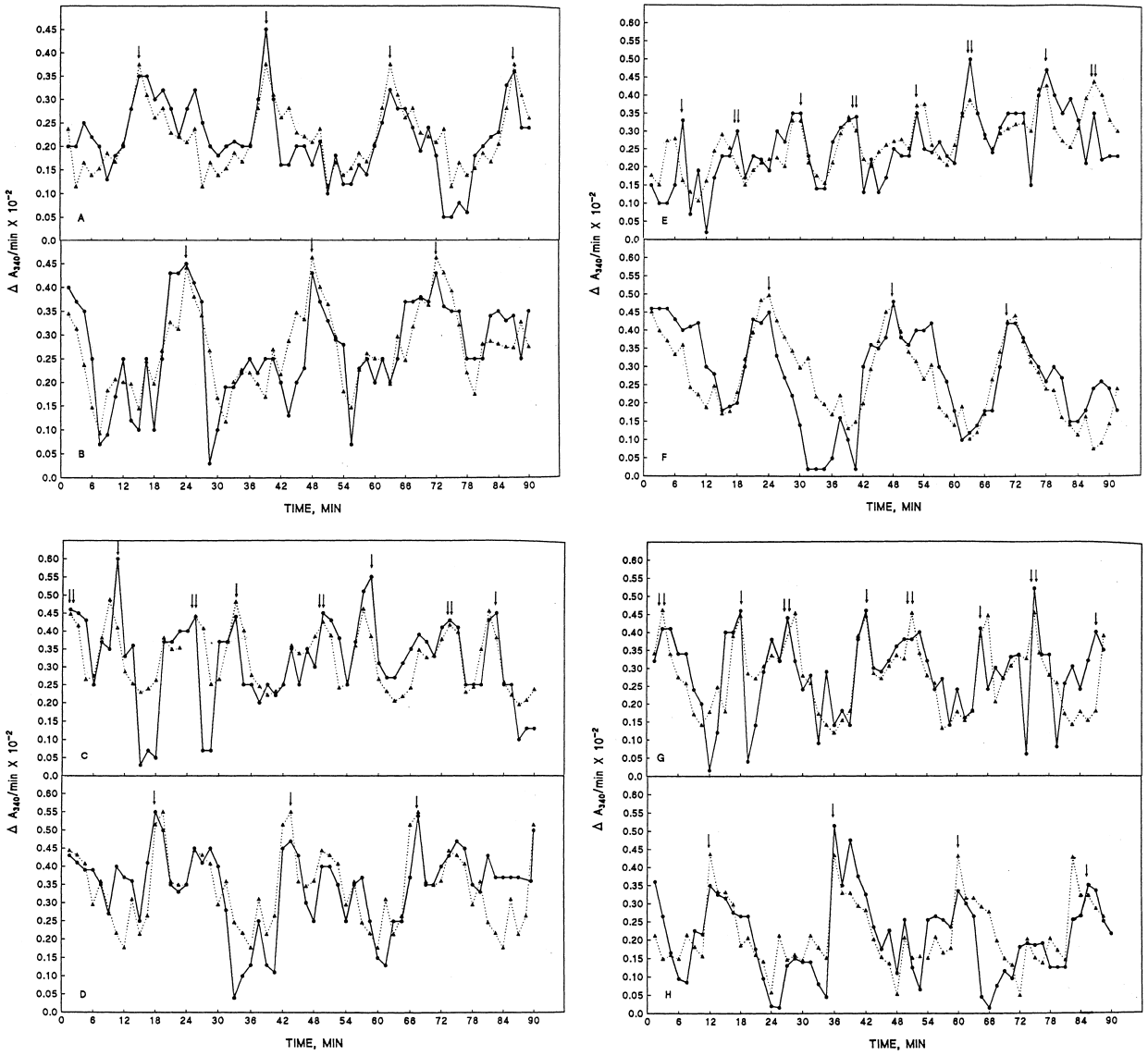

Fig. 6. Entrainment of NOX periodicity using two different preparations of milk fat globule membranes. Experimental values are given by the solid circles and lines. Decomposition fits according to the additive model (closed triangles, dotted lines) show the reproducibility of the pattern of periodicity inherent in the experimental values. The amplitudes at cycle maxima were significantly different ($P < 0.001$) from the amplitudes at cycle minima and the periodicity patterns were highly autocorrelated (Table 2). (A) Milk fat globule membrane preparation with maxima at 15, 39, 63, and 87 min (single arrows). (B) A second milk fat globule preparation with maxima displaced by 9 min at 24, 48, and 72 min (single arrows). Activities in A and B were determined simultaneously using two spectrophotometers in parallel. (C) Equal amounts of A and B mixed together just prior to assay. Two distinct 24-min periods (single and double arrows) were observed. (D) A and B mixed together for 24 h at −20°C prior to assay to permit entrainment. Maxima at 19.5, 43.5, and 67.5 min (single arrows) represent the means of the furthest separated maxima of the two preparations analyzed separately (C). The activities in C and D were determined simultaneously using two spectrophotometers in parallel. The only differences were that the preparations of C were stored separately and combined at the time of assay while those of D were combined and stored for 24 h at −20°C prior to assay. When combined at the time of assay, two separate oscillations each with 24-min periods were observed (C). When incubated together for 24 h or longer prior to assay only a single entrained oscillation with a 24-min period was observed (D). (E–H) This experiment was repeated two additional times with identical results. (E) As in C except combined and assayed in parallel with F. (F) As in D except combined at −20°C for 48 h prior to assay. (G) As in C except combined and assayed in parallel with H. (H) As in D except combined at −20°C for 144 h prior to assay.

Table 1
Fourier analysis of NADH oxidase periodicities comparing 17°C, 27°C and 37°C

Temperature (°C)	Amplitude (nmol/min/mg protein)	Frequency (cycles/min)	Period (min)
17	0.3	0.042	23.8
27	0.6	0.042	23.8
37	1.0	0.0425	23.5
Mean		0.042 ± 0.0003	23.7 ± 0.17

The period was determined from the frequencies observed at maximum amplitude.

(and minima) displaced by about 9 min (Fig. 6A,B). Both preparations had been stored frozen. The preparation in A exhibited activity maxima at 15, 39, 63 and 87 min. Activity maxima of preparation B were displaced by 9 min at 24, 48 and 72 min. These preparations, when mixed together and assayed immediately, exhibited a complex pattern that incorporated the combined periodicities of the two individual preparations (Fig. 6C). This was an important control in that it demonstrated that the periodicity was inherent in the membrane preparations themselves and was not due to instrument variation or substrate or chemical cycling in the reaction mixture.

Entrainment of the combined preparations, however, occurred with time after mixing as shown in Fig. 6C–H. If the combined preparations were stored at −20°C for 24 h and then assayed, a single major period representing the mean of the furthest separated maxima of the two preparations analyzed separately began to emerge (Fig. 6D). The single major period became even more evident after 48 h of incubation (Fig. 6F) whereas the two preparations when mixed together just prior to assay still exhibited a complex pattern of periodicity representative of the starting preparations (Fig. 6E). The periodicity of the individual preparations stored separately and then combined just prior to assay was unchanged by storage for 144 h (Fig. 6G) whereas the two preparations stored combined for 144 h continued to show a single dominant period (Fig. 6H).

All of the periodicity data were significantly autocorrelated (Table 2 and Fig. 7). The fitted values by the Holt–Winters seasonal exponential method using an additive model, agreed closely with the experimental data (cf. Fig. 6). Amplitudes at cycle maxima were significantly different ($P < 0.001$) from the amplitudes at cycle minima and the periodic patterns of rate of NADH oxidation were highly autocorrelated (Table 2 and Fig. 7). Rate troughs also generally were complementary. This is shown by the correspondence of decomposition fits and experimental data of Fig. 6, for example.

The timing of the oscillations of the milk fat globule membranes and of the milk fat globule membrane preparations continued from one day to the next at an average period of about 24 min. Preparations stored frozen continue to keep time without interruption with an average period of 23.7 min even at temperatures as low as −70°C. For example, for a preparation with a measured maximum rate of NADH oxidation at 16:30 h that was frozen starting at 16:45 h, thawed the next morning starting at 08:30 h and then placed in the spectrophotometer at 09:00 h, the next maximum was recorded at 09:06 h, approximately 42×23.7 min later. If freezing (c.a. 17:00 h) stopped the oscillations and thawing (ca. 08:35 h) restarted the oscillations, the oscillations would have been 11 min out of phase with the observed maximum of the following day. Clearly the oscillations do not cease nor does period length change markedly as temperatures are lowered below

Table 2
Measures of the accuracy of the statistically fitted values

Data set	Decomposition fit			Holt–Winters additive model		
	MAPE	MAD	MSD	MAPE	MAD	MSD
Fig. 2A	53.1	0.041	0.003	62.8	0.047	0.003
Fig. 2B	24.0	0.055	0.006	26.3	0.048	0.004
Figs. 3 and 6A	28.9	0.043	0.003	29.6	0.047	0.003
Fig. 5A	81.9	0.071	0.009	83.9	0.068	0.006
Fig. 5B	42.7	0.096	0.014	33.9	0.089	0.011
Fig. 6B	37.1	0.056	0.005	45.8	0.066	0.007
Fig. 6C	49.9	0.067	0.009	43.8	0.079	0.009
Fig. 6D	32.0	0.067	0.008	30.7	0.063	0.007
Fig. 6E	38.5	0.061	0.006	36.0	0.059	0.006
Fig. 6F	92.0	0.073	0.009	57.9	0.072	0.009
Fig. 6G	56.9	0.062	0.008	54.0	0.078	0.009
Fig. 6H	94.6	0.068	0.008	89.1	0.077	0.009

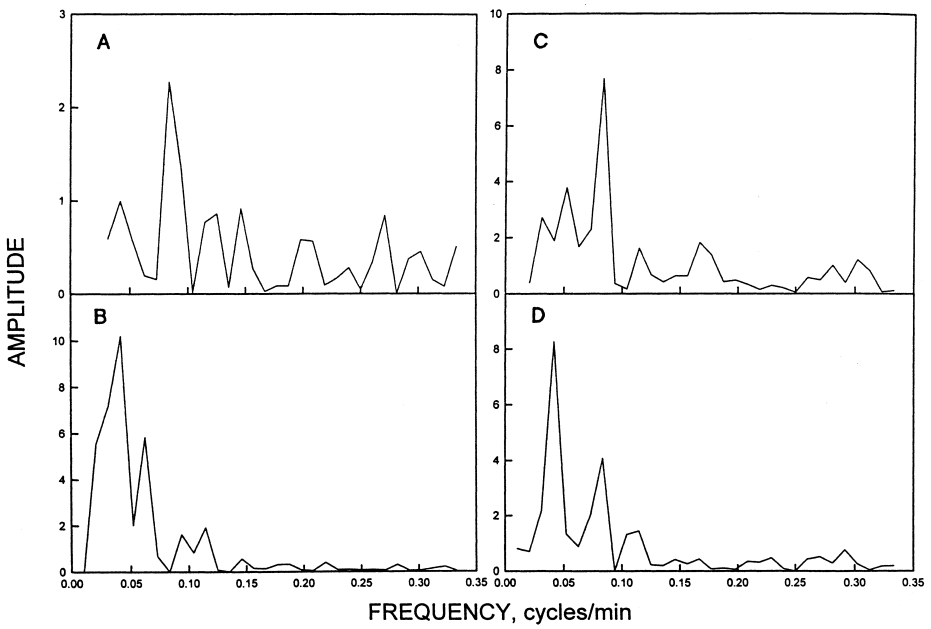

Fig. 7. Fourier analyses of data of Fig. 6E–H demonstrating entrainment of NOX activity using two different preparations of milk fat globule membranes. (A) Data of Fig. 6E of two preparations freshly mixed yielded a frequency of 0.084 cycles/min or an average period length of ca. 12 (11.9) min. (B) Fourier analysis of the same two preparations after having been combined at −20°C for 48 h prior to assay after which the two preparations were assayed in parallel. The result of the two preparations being combined for 48 h at −20°C was a single period with a frequency of 0.042 cycles/min corresponding to a period length of 23.8 min. (C) As in A except data for Fig. 6G. Again, the two preparations when freshly mixed show the result of two different sets of oscillations with an average period length of about 12 min corresponding to a frequency of 0.082 cycles min by Fourier analysis. (D) As in B for data of Fig. 6G where the preparations of Fig. 6H were combined for 48 h at −20°C and then assayed in parallel with the same two preparation freshly mixed (Fig. 6G). After 48 h at −20°C, the data were represented by oscillations with a single frequency from Fourier analysis of 0.042 cycles/min corresponding to a period length of 23.8 min.

room temperature nor do the oscillations stop with freezing. Additionally, time of freezing, length of freezing or time stored frozen were without effect.

4. Discussion

Despite knowledge of a biological clock for many years, there have been no candidate molecules for biochemical oscillators capable of functioning as ultradian timekeepers [18]. A critical feature of the timekeeping function is that of temperature compensation of the period length. The period length of the circadian biological clock is largely unaffected by changes in temperature (temperature compensated) [19].

The oxidation of NADH by plasma membranes including the fat globule membranes of bovine milk reported here is periodic and oscillates with a period of about 24 min, i.e., the activity cycles 60 times per 24-h day. The actual circadian period may vary such that precise 24-h cycles require entrainment. A free running day of 23.7 h has been reported for mice [26]. The intrinsic period of the human circadian pacemaker was determined to be 24.18 h [27] but also may vary up to ±0.8 h or more from 24 h [28].

For the bovine milk fat globule membranes reported here, the length of the period was unaffected by temperature even though amplitude increased by a factor of about two for each 10°C rise in temperature between 17°C and 37°C. This extraordinary degree of temperature compensation provides the major impetus for suggesting that the NADH oxidase protein may represent an ultradian oscillator possibly functioning as a driver responsible even for the ca. 24-h biological clock [11–16].

The oscillations in activity are inherent in the cell surface NADH oxidase protein itself and are not dependent on a more complex environment [11]. NOX proteins released from the milk fat globule mem-

brane by detergent exhibited an oscillating NOX activity essentially unchanged from that of the isolated milk fat globule membranes. Machine variation and/or cycling of reagents [29] do not explain the NOX oscillations [12]. Blanks lacking NADH or blanks prepared without a source of NOX enzyme did not exhibit oscillating changes in absorbance. Additionally, when solubilized and partially purified preparations were mixed from different batches of milk fat globules exhibiting maxima 24 min apart but displaced in real time by several minutes between the two preparations, the mixtures displayed both sets of maxima (Figs. 6 and 7). This phenomenon would not be expected if the oscillations were the result of interactions among chemical reactants rather than inherent in the protein molecules themselves. Similarly, oscillations with period lengths of 24 min have been observed with solubilized and extensively purified preparations of the plasma membrane NADH oxidase from plant sources [12,13] and from CHO [14] or HeLa plasma membranes [15,16] as well as by a recombinant form of the protein expressed in bacteria [11,17]. In contrast, NADH cytochrome *c* reductase of pig liver microsomes assayed under conditions identical to those of the NADH oxidase (disappearance of NADH) showed no periodic oscillations in enzymatic activity.

The periodicity data for the milkfat globule membranes were significantly auto-correlated. In the regression analyses of the time series data, the deviation from the mean showed a recurrent pattern where values were positively correlated over time (autocorrelated or serially correlated). Decomposition fit analyses and Holt–Winters seasonal exponential smoothing using the additive model also were applied to validate both trend (normally constant, i.e., no slope) and the periodic pattern.

The fat globule membranes of bovine milk provide a convenient source of plasma membranes from a non-transformed source [30–33]. This feature of the experimental system presently cannot be duplicated even with the recombinant protein which is a tumor-associated NOX form.

Secretion of milk fat by mammary epithelium involves envelopment of newly synthesized cytoplasmic fat droplets by the apical plasma membrane during milk formation [20,30]. In apical cell regions lipid droplets interact with and are enveloped progressively by plasma membrane [31]. Eventually the plasma membrane pinches off behind the budding droplet, leaving the droplet surrounded by plasma membrane in the alveolar lumen [33]. This secretory mechanism was first described by Bargmann and Knoop [30]. Since then, a number of electron microscopic studies have provided evidence supporting this secretory mechanism [31,33]. The biochemical evidence obtained to date provides support for the interpretation that the milk fat globule membrane is derived from the apical plasma membrane [31–34].

In the secretion of the lipid droplet, the apical plasma membrane of the cell does not rupture but rather fuses under the protruding droplet so that the plasma membrane is pinched off below the droplet freeing the lipid into the gland lumen surrounded by plasma membrane derived from the mammary epithelium [20]. Thus, milk represents a physiological source of isolated plasma membrane [32]. One needs only to strip the membrane from the fat globules. Fat globules are separated from raw milk of individual animals by centrifugation and washed one or several times to remove milk serum [31,34]. The membranes are released by freezing and thawing, sonication, or churning using a blender [32,34]. After release, the membranes are isolated as a pellet by high-speed centrifugation. Further washing removes extraneous contaminants. Because of their ready availability and ease of manipulation, the membrane surrounding the milk fat globule of the bovine provides a convenient source of the cell surface NOX protein as well.

Transcriptional models have been presented to explain clock function [35,36]. The cell surface NADH oxidase does not depend on transcriptional control to account for its oscillatory behavior as the period length of 24 min is given by the solubilized and detergent-solubilized cell surface NOX protein in the absence of protein synthesis.

How a ca. 24-min ultradian oscillation could regulate a 24-h periodicity would require that the ultradian oscillation function as the balance wheel in a mechanical watch, in conjunction with a mechanism to 'count' oscillations up to 60 to mark each 24-h day. The mechanism whereby the plasma membrane NOX protein maintains an intrinsic 24-min rhythm is, at the moment, speculative but may reside within the secondary or tertiary structure of the NOX pro-

tein. Both quinone reduction [37] and protein disulfide–thiol interchange [38] both catalyzed by NOX proteins, exhibit oscillations in activity with a period length of 24 min. One possibility is that NOX dimers undergo spontaneous and reversible random coil or α-helix to β-sheet transformations that repeat every 24 min. Upon reaching a certain conformation by 12 min, the process would then reverse and, after an additional 12 min, begin anew. The extent of conformational change could be temperature dependent but the duration of change would need to be temperature-independent in order to achieve temperature compensation of period length.

To explain synchronization or entrainment, synchronous NOX subunits might at some point in the cycle tend to dissociate more readily than asynchronous subunits. The dissociated subunits would then reassociate with other less synchronized subunits. As a result, a membrane population or solution of NOX complexes would become and remain synchronized. The NOX protein is membrane associated but not transmembrane or anchored [39]. As such, the NOX protein is free to associate and dissociate from the membrane and to enter the circulation. Exchange of NOX subunits between cells would then contribute to a synchronous NOX population not only in individual cells but also in tissues, organs and perhaps even in organisms.

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