Multiple Periodicities in the Circadian System of Unicellular Algae

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Three periodicities in the circadian range are observed when measuring circadian parameters of unicellular organisms in long running experiments (more than 15 days). This is demonstrated for different organisms (*Chlamydomonas, Euglena, Chlorella*) and different parameters (autokinesis, extracellular pH, CO_2- and O_2-partial pressure). Having excluded analytical and experimental artefacts (*i.e.* filter leakage and subpopulation effects), the multiple periodicities have to be interpreted in a physiological model. The three periodicities always exhibit two common features: The locations of the side peaks are symmetrical to the middle peak and their energy contribution is always the same. We therefore favour the model of multiplicative coupling between the circadian oscillator and a low frequency oscillator modulating the amplitude of the circadian rhythm. Since the low frequency rhythm is not correlated to any exogenously running periodicity of the experimental surroundings, it is considered as generated by an endogenous oscillator. This shows the existence of different biological long time oscillators in one single cell and contradicts the so-called master-clock hypothesis stating that one cell has only one clock related oscillator.

1. Introduction

Many physiological processes show circadian rhythms, that is a daily periodic behaviour persisting in absence of synchronizing exogenous signals. The exact periodlength is considered to be a genetically fixed attribute of the organism looked at. Although the periodlength may vary in a range from 20 to 30 h for different organisms, the frequencies of different processes in *one* organism turned out to be the same. Based on these observations, a concept of a "master-clock" was developped [1] defining a central pacemaker. Due to this hypothesis, the different physiological processes are considered as "hands of the clock".

Studying the mechanism of circadian rhythms within the last decade, it became clear that this master clock hypothesis cannot hold for all organisms. In multicellular organisms, in animals [2] as well as in plants [3], the structure of the circadian system is far more complicated: It seems to be controlled by several independent circadian oscillators that may have different frequencies. When excluding external Zeitgebers, different physiological processes may run apart with different periods [2]. The question is still unanswered whether the existence of different oscillators is restricted to multicellular systems. Nevertheless, looking for the mechanisms of circadian rhythms, unicellular organisms are often used as a minimal system excluding interactions of possible different basic oscillators.

In the present paper, we show that in the circadian organization of unicellular algae, a coupling of several low frequency oscillators exists: Three different circadian periods are present, all running simultaneously. For recording the circadian rhythms, we used the pH of extracellular medium of populations of *Chlamydomonas, Euglena,* and *Chlorella.* In the case of *Chlamydomonas,* it has already be shown that this parameter exhibits a circadian rhythm [4]. Since the extracellular pH can reflect different physiological processes, *e.g.* photosynthesis, respiration, and cell membrane bound ion pumps [5], we confirm the existence of multiple periodicities in the CO_2-turnover and the autokinesis of *Chlamydomonas* too. A master-clock hypothesis therefore seems doubtful in the case of unicellular organisms.

2. Material and Methods

2.1. Organisms and culture techniques

We used the unicellular algae *Chlamydomonas reinhardii*, strain 11-32/89, *Chlorella fusca,* strain

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Table I. Multiple periodicities in the circadian range. The number of significant peaks in the periodogram analysis from filtered times series are given (f.i. +−+ means: middle peak below significance, side peaks are significant), and the middle peak of the periodogram. The modulation period was calculated from the two side peaks of the periodogram and the deviation of the calculated carrier periodicity of the model from that of the analysis is given, in percent. (Note that in the circadian range from 20 to 30 h 0.4 h < 2% < 0.6 h).

	Experiment	Number of significant peaks on the 95% threshold	Period length of the middle peak (h), and percent deviation of the calculated carrier period from the predicted one (≙ period length of middle peak)		Calculated modulation period (h)
A	pH measurements in a culture of *Chlamydomonas* (except 18, 19)				
1	pH	3	27.75	−0.583	750
2	pH	3	24	−0.208	575
3	pH	3	26	−1.269	368
4	pH	3	26.5	+0.377	355.8
5	pH	3	26.25	+0.152	339.2
6	pH	2 (+−+)	25.75	−0.583	252.4
7	pH	3	27	−0.148	216
8	pH	3	25.9	±0	212
9	pH	3	23.7	+0.04	200.1
10	pH	3	24.2	−1.57	192.2
11	pH	3	26.5	−2.08	169
12	pH	2 (++−)	22.8	+0.395	164.5
13	pH	1 (−+−)	27.5	−0.218	152
14	pH	3	22	+1.32	149.4
15	pH	3	25.25	−0.436	148.7
16	pH	2 (−++)	25.25	+0.119	145.76
17	pH	2 (++−)	24.25	±0	143.8
18	pH *Chlorella*	3	25.25	−0.713	133
19	pH *Euglena*	3	23	−5.652	106
B	Autokinesis measurements in a culture of *Chlamydomonas*				
1	AK	2 (+−+)	(26.78) [+]		533
2	AK	3	24.6	+1.79	441.56
3	AK	3	24.8	+0.847	418.51
4	AK [a]	3	24.8	+0.363	319.85
5	AK [b]	3	25.6	+0.078	314.87
6	AK [a]	3	24.8	+0.161	310.5
7	AK	2 (++−)	24.75	+0.364	310.5
8	AK	3	24.75	−2.22	277.47
9	AK	2 (−++)	24.8	−2.419	268.42
10	AK [b]	3	24.9	−3.293	254.18
11	AK	3	25.8	−0.116	232
12	AK	2 (−++)	24.5	+0.367	233
13	AK	3	24.5	+1.837	229.09
14	AK	3	23.7	−4.64	112.8
C	Simultaneously measured parameters in a culture of *Chlamydomonas* *Closed system*				
1	pH	3	24	+2.417	304.3
2	CO_2	3	24	+1.042	535.7
3	O_2	1 (−−+)	25	−2	291

Table I (continued)

	Experiment	Number of significant peaks on the 95% threshold	Period length of the middle peak (h), and percent deviation of the calculated carrier period from the predicted one (≙ period length of middle peak)		Calculated modulation period (h)
	Open aerated system				
4	pH	3	24	+1	237.6
5	O_2	3	24	−1.25	169.1
6	CO_2	1 (−+−)	24	+0.333	292
7	pH − DCMU	2 (+−+)	(27.12)[+]		229.4
8	O_2 − DCMU	1 (−+−)	27.1	−0.074	193.1
9	CO_2 − DCMU	2 (+−+)	(27.12)[+]		229.4
10	pH − DCMU	2 (+−+)	27.1	−0.111	257.78
11	O_2 + DCMU	2 (−++)	27.25	±0.0	146.13
12	CO_2 + DCMU	2 (+−+)	(27.15)[+]		206.89

[+] The middle period was calculated from the side ones, periodogram analysis did not exhibit a middle peak, even after removal of side peaks.

Remarks for special experiments:

A) pH-measurements
17) Source culture for cloning. 1, 3, 15) are the results from this cloning; 5) gasing without CO_2; 8) changed L:D = 30:60 min; 6) reduced buffer capacity of the medium (20 µm: 10 µm = K_2HPO_4: KH_2PO_4); 4) DCCD was added abd 13) DCMU was added.

B) Autokinesis measurements
[a] 4, 6) are measurements with exactly the same conditions; [b] 5, 10) dto.

C) 1, 2, 3) and 4, 5, 6) are simultaneously measured; 7, 8, 9) and 10, 11, 12) is the measurement of pH and O_2 in the medium and CO_2 in the gas efflux of a culture before (−) and after (+) inhibition of photosynthesis by DCMU.

211-8b, and *Euglena gracilis*, strain 1224/5-9, all from the Algensammlung Göttingen, FRG. *Chlamydomonas* [6] and *Euglena* [7] are common objects for studying circadian rhythms. For both organisms different circadian processes are known [6, 7]. Endogenous rhythmic reproduction was described for *Chlorella* [8].

Cultures were grown in mineral medium; for *Chlamydomonas* and *Chlorella* the medium of Hartmann [9] was used (0.8 g/l KNO_3, 0.15 g/l $Ca(NO_3) \times 4H_2O$, 0.15 g/l $MgSO_4 \times 7H_2O$, 0.05 g/l KH_2PO_4, plus trace elements), and for *Euglena* the following medium (1.5 g/l NH_4NO_3, 0.1 g/l $MgSO_4 \times 7H_2O$, 0.1 g/l KCl, 0.5 g/l KH_2PO_4, 0.04 g/l $CaSO_4 \times 2H_2O$, plus trace elements, Fe-EDTA-Komplex, and vitamine B mixture (0.2 ml/l "Iloban MERCK")) [10].

The cultures were grown in 1.5 l bottles in temperature controlled waterbaths (25 °C) in constant light of ca. 2000 lux (Osram Fluora) and were aerated without enrichment of CO_2. At a cell number of approximately 5×10^6 for *Chlamydomonas* and *Chlorella*, and 1×10^6 for *Euglena*, cultures were transferred to the tests for circadian rhythmicity. In the case of pH measurements with *Euglena* and in some cases with *Chlamydomonas* (s. Table I) the medium was changed under axenic conditions for a 30 µM phosphate buffer (20 µm: 10 µm = K_2HPO_4: KH_2PO_4).

2.2. Test for rhythmicity and sampling of data

For all experiments the free running conditions consisted of L-D-changes of 1:1 h at a constant temperature of 20 °C (except: s. Table I, L:D = 30:60 min). Test conditions were started after 5 to 10 LD-cycles of 12:12 in 20 °C at the end of a 12 h light time.

pH and CO_2 measurements

Experiments were performed using 6 or 12 l fermenters (Biostat, Braun Melsungen, FRG) with 4.5 or 8 l medium. The usual condition was an open system, *i.e.* cultures were stirred and aerated, temperature controlled and illuminated (ca. 5000 lux at the surface of the vessel by Osram Fluora). The pH of the medium was continuously measured (pH electrode: Ingold 465, 180 mm; FRG; pH meter: Knick No. 645) with a precision of ca. $\pm 2 \times 10^{-3}$ pH units.

In one case, Fig. 5, the experimental condition was a closed system, *i.e.* without netto material (gas) exchange with the surroundings. The only energy support was light during the LD-cycles. In addition to pH, the CO_2 of the gasphase was measured by means of an infrared gasanalyzer (Binos, Leybold-Hereäus, FRG), and the O_2-content of the medium polarographically by a Clark-type electrode (Ingold, FRG).

The fermenter and the gasanalyzer were connected by glass and viton-B-tubes, material impermeable to CO_2 and O_2. The metabolism of green cells depends on the light conditions. Providing light intensities are sufficiently high, net photosynthesis will occur during light times, resulting in a photosynthetic CO_2-fixation during light and a respiratory CO_2-evolution in the dark. In our experiments it was possible to adjust the light intensity in the way that the average daily CO_2-fixation and consumption were balanced to maintain a steady state level of CO_2 in the closed system.

The pH of the medium is coupled with the CO_2 content *via* the CO_2/carbonate buffer system, causing an increase of pH with decreasing pCO_2 and vice versa. That this strict chemical coupling is not the reason for the actual circadian rhythm of extracellular pH however, is shown in section 3.2. All parameters were continuously measured and recorded by an analogue device. The analogous records were converted to digital data by digitizing values at the beginning and at the end of the light regimes. In the digital records of pH and CO_2 the circadian signal is superimposed by two hour cycles due to the applied light-dark regime (*cf.* Fig. 1 A).

Autokinesis

A detailed description of measuring autokinesis (AK) was previously given [10]. The principal is to measure the optical density (O.D.) of cell suspensions at a fixed horizon in 140 ml cuvettes, mounted in temperature controlled water baths. An increase in the optical density at the test horizon indicates an increase of motility [11].

Data were automatically and digitally recorded every two hours at the end of the dark time of the LD-1:1 h changes. In contrast to the pH/CO_2 measurements it was possible to run parallel measurements for autokinesis with exactly the same experimental and environmental conditions.

2.3. Time series analysis

All calculations were made on an IBM 370/168 at the RHRZ of the University of Bonn, FRG. Times series were analyzed using the interactive computer system TIMESDIA [4], that combines methods for digital filtering, for calculation of frequency structures, and for estimation of periodlengths and the stationary of phase and amplitude of periodic signals.

The analysis of time series by TIMESDIA has been described previously [12], but for better understanding, an example is given by the analysis of a pH time series, demonstrating the methods used in this paper (Fig. 1). First step is the estimation of the spectrum of the time series, *i.e.* an amplitude *versus* frequency representation of the time series. This allows testing whether there is a signal in the circadian range. Possible trends (=low frequency signals) and high frequency components, as induced by the LD-cycles or experimental noise, are removed by using digital filter techniques (Fig. 1 B, C). Estimation of the circadian periodicity is performed by high resolution periodogram analysis; the significance of all periods within the intervall looked at, is tested by comparison of the energy of each frequency to the total energy. Significance for the period estimation is based on a 95% threshold. The example shows one peak with a maximum at 25.1 h, indicating that there is *one* significant circadian periodicity occurring in the observed biological parameter (Fig. 1 D). But period estimation is only valid if a periodicity is stationary. Stationarity of a signal is confirmed or rejected *via* complex demodulation [13], giving an estimation of the amplitude and phase of the tested frequency against time (Fig. 1 D).

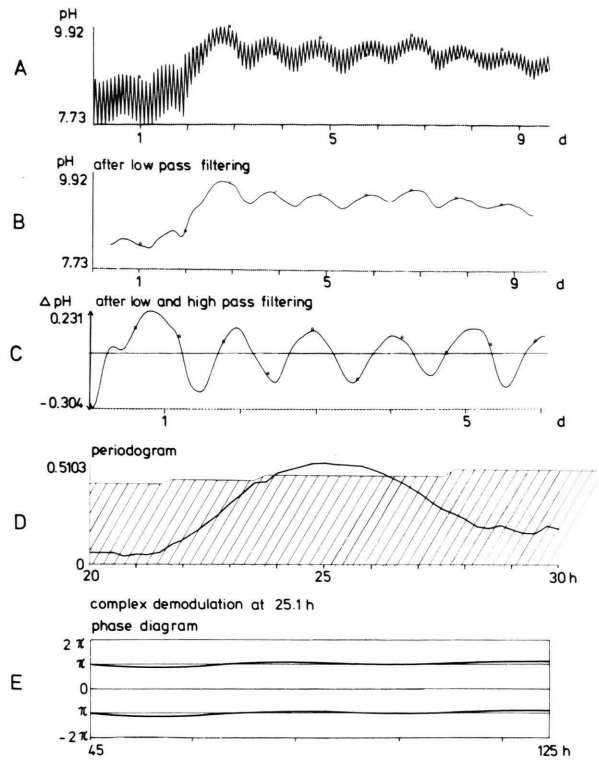

Fig. 1. Extracellular pH in a population of *Chlamydomonas reinhardii* under free running conditions of L:D = 1:1 h and 20 °C. A) In the observed time series, the circadian signal is superimposed by two hour cycles due to the LD-changes causing the pH to increase during light and to fall during dark times. B) pH after removal of the two hour cycles by low pass filtering. C) After additional removal of the trend by high pass filtering, the circadian signal is revealed. D) The periodogram of the filtered time series gives the location of the circadian periodicity; the estimated period length is 25.1 h. The shaded area of the periodogram gives the 95% F-test threshold for each frequency in the circadian range from 20 to 30 h. E) The stationarity of the time series is shown by the phase diagram of the complex demodulation at 25.1 h (pass period 22.5, stop period 15 h): Phase is statistically constant.

3. Results

3.1. pH-rhythm

Weakly buffered suspensions of photosynthesizing cells show light dependent pH-changes (increasing pH on illumination, pH drop in the dark). In *Chlamydomonas*, *Euglena*, and *Chlorella* this can be observed for short time (1:1 h) and long time (12:12 h) LD-changes (Fig. 1). In L:D = 12:12 h a daily pH-rhythm with maximum values at the end of the light time and minimal values at the end of dark is measured. When releasing cultures of *Chlamydomonas* from synchronizing L:D = 12:12 h cycles to free running conditions (L:D = 1:1 h) a phase shift of the daily component is indicated by maxima of pH located not further in the former light regime, but at the phase of the former dark regime [5]. Under L:D = 1:1 h *Chlamydomonas* shows free running circadian oscillation with an amplitude ranging between approximately 0.05–0.5 pH units depending on the mean pH-level. In a set of short time experiments (ca. 10 days) only one mean period is present ($\tau = 25.5 \pm 1.4$ h). That period is not altered by different constant temperatures (20, 25 °C) and the light regime (LD = 1:1 h, 30:60 min).

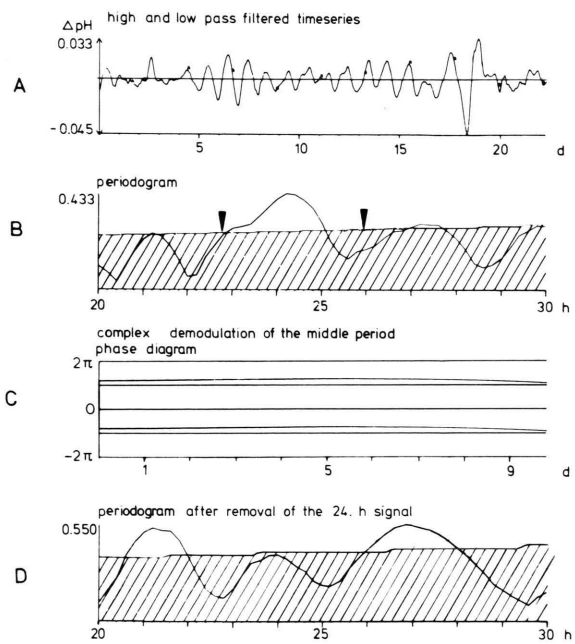

Fig. 2. Long time record of pH in a population of *Chlamydomonas* after filtering. A) A beat like varying amplitude, due to the three periods in the circadian range, can be recognized. B) The periodogram gives the location of the three peaks. The arrows indicate the location of the side lobe frequencies due to filter error. C) Complex demodulation at 24 h (pass periods 23.3, 24.74 and stop periods 21.9 and 26.55 h) reveals the stability of the major middle peak. (Though the side peaks at 21.1 and 27 h have only low energy, they are also stable in time, not shown here.) D) Periodogram of the long time record after removal of the major middle peak signal by bandpass filtering proves the existence of the side peaks. Shaded area of the periodogram gives the 95% F-test threshold for each frequency in the circadian range from 20 to 30 h.

The notation of one mean period becomes doubtfull, however, when considering long time experiments (more than 20 days): The single period with the broad spectral peak (Fig. 1 D) can be resolved into different components. In these long running experiments the pH time series show beat like variation of the amplitude. The periodograms exhibit three periodicities within the circadian range. In most cases one middle peak with highest energy and two minor side peaks are observed (Fig. 2). Side peaks can be generated by filter errors [14], but we tested, that in all cases the period lengths of the side peaks in the time series differ from those periods predicted from filter errors (Fig. 2, arrows).

Hence the side peaks in the periodograms are not artefacts of digital filtering and have to be considered in a physiological interpretation.

The three different peaks may be due to instabilities of one circadian period (oscillatory free-run [15]). This possibility was excluded by applying complex demodulation to all three periods, showing the constant phase of the circadian signals. Furthermore, after removal of one period, the other ones were left unchanged (Fig. 2 C, D; Fig. 5).

As a second possibility, the three periodicities could be due to different subpopulations of clock mutants of *Chlamydomonas*, since mutants of the circadian period of *Chlamydomonas* may easily be selected [16]. Therefore a clone was isolated from a culture showing multiple periodicities and tested in long time pH measurements (Fig. 3).

Again three periodicities appeared, and the middle one ($\tau = 25.67 \pm 0.4$ h; $n = 3$) was not different from the average period of the original clone source (25.5 ± 1.4 h). Thus the hypothesis of three different subpopulations with different circadian periodlengths can be rejected.

The phenomenon of multiple circadian periods is not restricted to *Chlamydomonas*. The pH rhythm in populations of *Euglena* and *Chlorella*, as well, exhibits three peaks in the circadian range (Table I, Fig. 6).

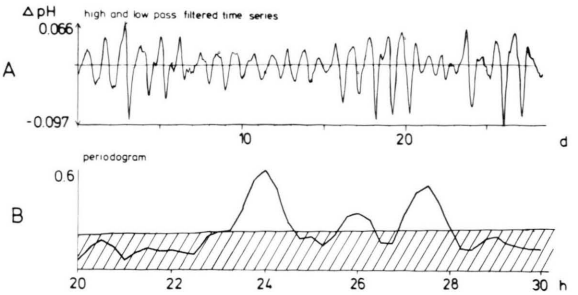

Fig. 3. Long time record of pH in a population of an isolated clone of *Chlamydomonas* after filtering. The clone was isolated from a culture that showed three periodicities. A) Time history plot of the filtered time series; a beat like varying amplitude can be recognized. It is due to three periods in the circadian range. B) The periodogram gives the location of the three peaks at 24.26 and 27.6 h. (The shaded area gives the 95% F-test threshold for each frequency in the total range from 20 to 30 h.) This example shows that the middle period may have lower energy than the side peaks.

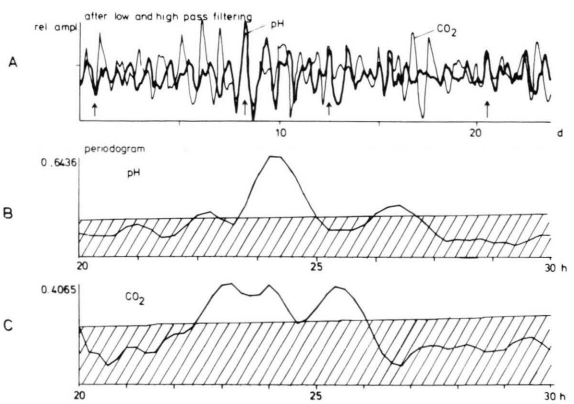

Fig. 4. Low and high pass filtered time series of pH (thick line) and CO_2 (thin line) in a population of *Chlamydomonas*, measured in a closed system. A) Time history plot: Arrows indicate parallel running of pH and CO_2, as the extreme deviation of the expected inverse behaviour. B) The periodogram of pH exhibits three periodicities at 22.75, 24, and 26.75 h. C) The periodogram of CO_2 exhibits peaks at 23.2, 24, and 25.4 h. Thus, concerning the multiplicative coupling model, a modulation periodicity of 300 h for the pH and 540 h for CO_2 are to be considered. (Shaded areas of the periodograms give the 95% F-test threshold for each frequency in the range from 20 to 30 h.)

3.2. *Comparison of pH and CO_2 rhythm*

Photosynthesis and respiration contribute to the extracellular pH *via* CO_2-exchange. One might expect that in equilibrium with CO_2 the pH-series indicate the time course of balance between photosynthesis and respiration. The best way to prove correlated rhythms of photosynthesis and respiration is to measure the time course of CO_2, O_2 and pH in a closed culture system as shown in Fig. 4. According to the gas equilibrium hypothesis one should expect a strict 180° phase coupling of the CO_2 and pH recordings. From the filtered time series (Fig. 4) it becomes obvious that a 180° phase angle is not confirmed, obviously the phases do not

lock at all. This has to be predicted assuming both parameters show multiple periodicities with different side frequencies even if the middle period is the same. Indeed, these multiple periodicities are observed in all experiments, in the closed as well as in the open aerated system. Multiple periodicities were also detected in the O_2-records and the locations of the side peaks differed from those of the pH and CO_2 [17]. From this picture, it appears almost impossible to evaluate the exact phase relation-ship of the circadian (middle) component of pH and CO_2. On the other hand, a strict 180° phase coupling between pH and CO_2 should always hold, if the pH is completely determined by the CO_2 partial pressure. (It was proved that, when sampling values with $\Delta t = 3600$ s, a phase difference due to diffusion can be neglected.) Thus one conclusion gets clear: The pH is not only caused by CO_2.

3.3. *Autokinesis rhythm*

The complex interrelation of several metabolic activities in the pH, CO_2, and O_2-signals rised the question whether multiple periodicities are also present, if measuring an activity parameter of the cell. Such a parameter is autokinesis as indicated by the sedimentation behaviour of stationary populations (*cf.* sect. 2.2). In preceeding experiments, we tested that the shape and volume [18], as well as the pigment content of *Chlamydomonas* do not vary in free running circadian oscillations. Thus we consider the rhythm of optical density in the test horizon of a population as entirely indicating autonomous variations of motility, *i.e.* autokinesis. In time records not longer than about 10 days the circadian period (25.55 ± 0.77 h, $n = 10$) did not differ from that of the pH-rhythm (25.5 h).

In long time experiments (up to 45 days) triple periodicities were again observed (Fig. 5). All periodicities are stable in time and the middle peak of these records reveals the same period (25.1 ± 0.48 h, $n = 11$) as the broad mean peak of the short records. The locations of the side peaks differ, they even differ in simultaneously running experiments. This excludes the involvement of external synchronizing factors.

3.4. *Multiple periodicities*

The existence of stable mutiple periodicities is confirmed by different organisms and different

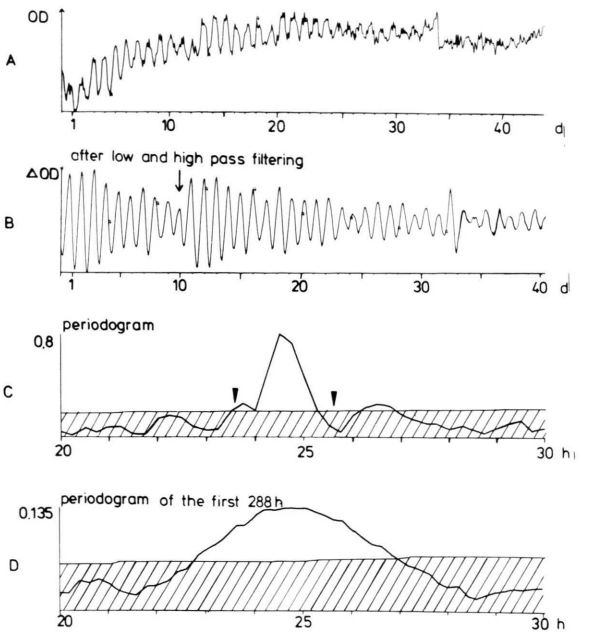

Fig. 5. Autokinesis of *Chlamydomonas*, L : D = 1 : 1 h, 20 °C. A) Time history plot (original data). B) Time series after filtering (high and low pass). C) Periodogram of the total record, exhibiting three peaks at 23.7, 24.6, and 26.5 h. The arrows indicate the location of side peaks due to filter errors. D) Periodogram of the first 288 h of the time series (indicated by the arrow in B) shows a broad significant peak, covering the range in which all peaks of the total time series are located. (The shaded areas of the periodogram give the 95% F-test threshold for each frequency of the range from 20 to 30 h.)

observed parameters. But the location of the middle shows a high variation, and especially the side peaks vary from experiment to experiment. Even within the same experiment, different observed parameters (pH/CO_2 and autokinesis for instance), have different locations of the side peaks. Surveying some 40 time series exhibiting multiple periodicities (Table I), common features become obvious: The distance of both side peaks to the middle peak is the same measured in the frequency domaine and the energy of both side peaks is statistically identical (± 10%). But the energy relation of the middle peak to the side peaks is varying. The energy of the middle peak may be lower than that of the side peaks (s. Fig. 3) and may even be below significance.

A triple of periodicities, two side peaks with same energy to a middle one, could be the realization of an amplitude-modulated oscillator. Accord-

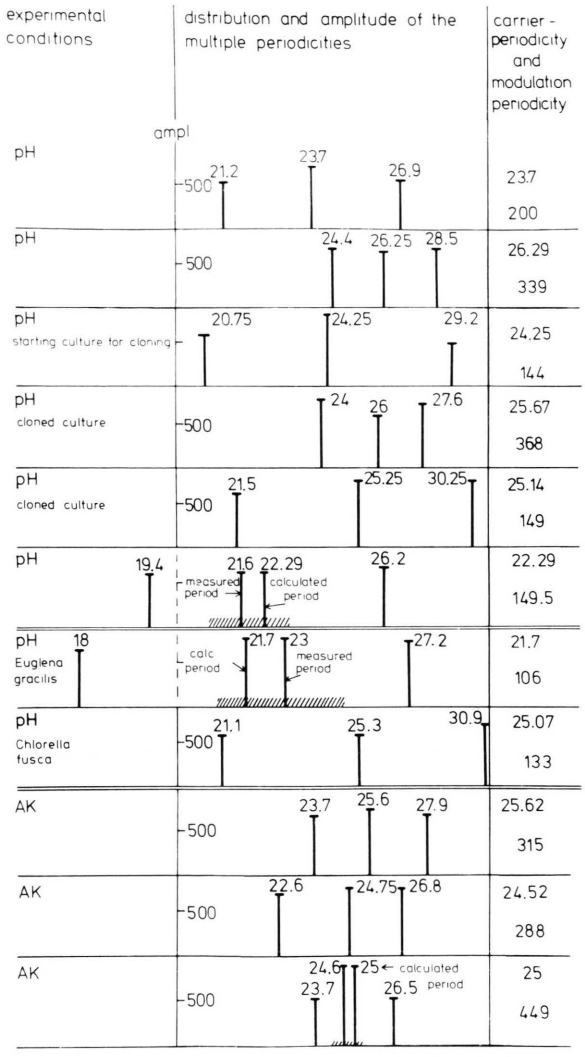

Fig. 6. Multiple periodicities and amplitude relation, as derived from the periodograms, in different experiments and for different organisms. Indicated period lengths (in h) are drawn on a frequency scale to show the symmetrical location. According to the multiplicative coupling model the carrier periodicity and the modulation periodicity are given.

ing to the relation

$$(b + a \sin \lambda t) \sin \omega t = b \sin \omega t - \frac{a}{2} (\cos (\omega - \lambda) t - \cos (\omega + \lambda) t) \quad (1)$$

we cannot distinguish between the two possibilities: The additive superimposition of three different circadian periodicities or the multiplicative coupling of the circadian period with an even longer periodicity, *i.e.* an amplitude modulation of the circadian period. It seems rather unlikely to meet three independent oscillators with varying locations of their actual frequencies from experiment to experiment, but always fitting just the scheme of an amplitude modulation. Therefore, we favour the multiplicative coupling model. Having located the triple peaks of a biological time series by periodogram analysis, the parameters of the multiplicative coupling model were calculated (*cf.* Table I, Fig. 6), giving estimates of the carrier frequency ω and the modulation frequency λ. It becomes obvious that the modulation period of the circadian oscillator ranges within 100 to 750 h, an impression of the wide spread variation of the side peaks gives Fig. 6.

Furthermore, it is seen from (1), that the energy of all peaks depends on a and b. Hence, if b becomes smaller the middle peak drops down (*cf.* Table I, Fig. 3) and may be even below significance in a corresponding periodogram analysis. Table I summarises those time series indicating an amplitude modulated circadian oscillation and gives the parameters for the amplitude modulation model as were derived from periodogram analysis: 28 time series showing clear three significant peaks; in 11 cases one or two of the side peaks and in six cases the observed middle peaks were below significance. But they became significant after removal of the middle peak or the side peaks, resp. Since all statistics for the single experiments have been done on the 95% threshold, one must take in account that in 2–3 cases of all experiments (5% out of 100%) the acception of multiple periodicities may not be valid. Probably due to differential experimental conditions the data set A (pH) shows a higher variability than set B (autokinesis). A comparison of the two sets suggests, however, that they do not significantly differ.

4. Discussion

Multiple periodicities were observed in the circadian range of 20 to 30 h under free running conditions. If the experiment is long lasting (more than 15 days), the phenomenon is found in the rhythm of extracellular pH of *Chlamydomonas*, *Euglena*, and *Chlorella*, and also in the autokinesis and long time recordings of CO_2 and O_2 turnover in cultures of *Chlamydomonas*. In most cases three periodicities are present; the two side peaks are symmetrical to the middle one and have identical

energy. In most cases, this energy is lower than the energy of the middle peak. Having excluded artefacts stemming from experimental methods or mathematical procedures (filter leakage in the analysis), we have to discuss the following possible explanations of the multiple periodicities:

A The existence of *one* oscillator, that may have time varying frequencies (oscillator freerun [15]).

B The existence of *multiple* oscillators with stable frequencies.

B_1 *Additive* superposition of *three* different independent oscillators due to either subpopulations in the testcultures, with the possibility of clock mutants of the *Chlamydomonas* system, or three physiological processes with different frequencies running in single genetically identical *Chlamydomonas* cells.

B_2 *Multiplicative* coupling of the circadian frequency with a lower frequency due to either endogenous factors, or exogenous factors.

Period length of circadian rhythms need not be time independent, their lengths may vary. This so-called oscillatory freerun has been known for the activity of animals since 1967/68 [15, 19]. Oscillatory freerun is a non-stationary phenomenon and can be checked by an appropriate time series analysis [13]. Applying these methods, in all experiments, we could not reject the hypothesis of stationarity. Hence, there is no hint for an oscillatory freerun. The origin of the multiple periodicities must therefore be due to the existence of different oscillators with stable periods. An additive superposition or a multiplicative coupling are feasable.

If thinking of an additive superposition, we have to consider the existence of a class of subpopulations in our test cultures with different period lengths. At least for *Chlamydomonas*, this seems likely, since clock mutants are known, and mutation rates are easily enhanced [16]. But cloning *Chlamydomonas* test cultures did not support the idea that each periodicity is due to a subpopulation: A newly cloned strain as well exhibited triple periodicities with a middle period not different from that of the source cultures. It seems quite unlikely that immediately after cloning, mutants with the same properties as before will occur again. Furthermore, though the middle period is relatively fixed for each organism, the locations of the side peaks differ from experiment to experiment. Mutants of definite period lengths should not show this variation. Thus we have to accept that multiple oscillators caused the different frequencies in *one* organism, and, since we investigated unicellular algae, in a single cell.

For higher organisms it is known that physiological processes being clocked by a circadian system can differ in period length: Under free running conditions they may become desynchronized [2, 3]. A parameter, as pH or CO_2 can reflect different physiological processes, as photosynthesis or respiration. Providing each of these processes shows a circadian rhythm and their period lengths are different, this should lead to an additive superposition in the observed parameter. Considering the pH rhythm only, we note that the range of the locations of the side peaks is widespread, whereas the middle peak is relatively fixed. This argues already against the additive superposition hypothesis: One should expect a more fixed distribution of the different physiological processes contributing to the external pH or that all processes should vary in the same unpredictable manner.

Looking now at the circadian rhythms of pH, CO_2, and O_2 in one culture of *Chlamydomonas*. (*cf.* [17]), all differ in the occurrence and location of the side peak frequencies, while, usually, the middle period is identical. Thus, no correlation between different physiological processes and periods can be revealed.

The best way to explain these results seems to consider a multiplicative coupling model. According to equation (1) the existence of three periodicities can result either from the addition of *three* circadian oscillators or from an amplitude modulated oscillations, *i.e. two* oscillators are coupled multiplicatively. Regarding our experimental results the symmetric distance of the side peak frequencies from the mid-frequency, as well as the equal height of the side peak amplitudes fit the multiplicatively coupling model (Fig. 6). Since it is rather unlikely that in the case of the additive superposition all three oscillators always happen to fit just the conditions of an amplitude modulated oscillation, it seems reasonable to favour the multiplicatively coupling model. Here, we have to consider the interaction of a circadian oscillator with an unknown external or internal oscillator of longer period. As can be seen from Table I, its lengths may range between 100 and 750 h. This range, the

variability within and the wide scope of possible oscillators restrict the opportunity of investigating the unknown process.

Exogenous factors, spec. exogenous CO_2-fluctuations of the atmosphere might cause the low frequency amplitude modulation. Indeed, we have experimental hints that the daily pCO_2 shows a similar amplitude modulation. But we could not see any correlation with our experimental results: Different period lengths were found in parallel measurements of pH, CO_2 and O_2 and for autokinesis, whereas in these simultaneously running experiments the same external influence is present. And even in measurements without CO_2 or O_2-exchange with the surrounding (closed system, Fig. 5; *cf.* [17]), there are present the amplitude modulations of the circadian rhythm. Hence, we conclude that the multiple oscillations are produced endogenously.

An endogenous origin of the multiple periodicities, however, is still a matter of speculation. Besides the recently discussed circaseptian rhythm in *Acetabularia* [20], only circatidal and circadian processes are known for unicellular algae; but in the case of the multiplicatively coupling model we have to assume long time processes with different period lengths. It may be possible that the phenomenon of multiple periodicities is not produced by a periodic process at all, but by pseudoperiodic or chaotic signals, which would lead to the same biological realization [21].

The multiple periodicities of the described properties bring up a new problem when investigating circadian rhythms of unicellular organisms. As we have shown they are not restricted to one organism, but seem to be common in other cells as well. It seems reasonable that in many experiments multiple periodicities were not detected as jet, since in short time experiments they are masked and could be considered as a damping out of the circadian oscillation.

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