Causal Relationships among Metabolic Circadian Rhythms in *Lemna*

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Ca$^{2+}$-Calmodulin, Circadian Rhythms, Glyceraldehyde 3-Phosphate Dehydrogenases (EC 1.2.1.12 & 13), NAD Kinase, NADP$^+$

Biochemical aspects of circadian rhythms were studied using a long-day duckweed, *Lemna gibba* G3 cultured in short day condition (9 h light at 3800 lux followed by 15 h darkness), which was transferred in continuous light (LL) at the end (LL 0) of the last night period. With such a system I have previously reported a rhythm of affinity for NAD$^+$ of cytoplasmic NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (Cyt-NAD-GPD) 180° out of phase with that of affinity for NADP$^+$ of chloroplastic NADP-dependent GPD (Chl-NADP-GPD) and that NADP$^+$ could increase in vitro the affinity for NADP$^+$ of Chl-NADP-GPD. I report here that NADP$^+$ can decrease in vitro the affinity for NAD$^+$ of Cyt-NAD-GPD as well, and furthermore, that the in vivo level of NADP$^+$ oscillates in phase with the rhythm of the affinity for NADP$^+$ of Chl-NADP-GPD.

Moreover, I found the existence of mirror-image circadian rhythms, of comparable amplitudes, of in vivo levels of NAD$^+$ + NADH (total NAD) (with peaks, as the ones of Cyt-NAD-GPD, at LL 0 and 24) and of NADP$^+$ + NADPH (total NADP) (with peaks, as the ones of Chl-NADP-GPD, at LL 12 and 36). Consequently, a circadian rhythm in the rate of net in vivo production of total NADP (or NAD) might be expected 90° in advance of that in the level of total NADP (or NAD). Indeed, I found oscillations in the activities of NAD kinase and of NADP phosphatase with peaks occurring, respectively, at LL 6 and at LL 18. Moreover, in vitro treatments with EGTA (a Ca$^{2+}$-chelator), chlorpromazine and W7 (both inhibitors of calmodulin) were able to both inhibit NAD kinase from its highest level of activity to its minimal one and activate NADP phosphatase from its lowest level of activity to its maximal one. I conclude, therefore, that the in vivo level of Ca$^{2+}$-calmodulin could oscillate in phase with the rhythm of NAD kinase activity and induce the mirror-image circadian rhythms of activities of NAD kinase and of NADP phosphatase.

I propose that the control sequence among the several circadian rhythms I studied could start with changes in Ca$^{2+}$-calmodulin, then proceed through oscillations in NAD kinase and NADP phosphatase activities, leading to changes in NAD$^+$, NADP$^+$, and NADPH levels, which would themselves induce the Chl-NADP-GPD and Cyt-NAD-GPD rhythms.

Introduction

One of the most important problems in studies on biological oscillations has been to determine the molecular mechanism of circadian clocks. An approach could be to demonstrate the existence of a chain of circadian rhythms, in which the earlier steps regulating the later ones might constitute a self-regulatory oscillating loop, that is, an integral part of the circadian clock. According to this view point, I have been studying the mechanism of the mutually inverse daily changes in activity of Chl-NADP-GPD and Cyt-NAD-GPD in the long-day duckweed, *Lemna gibba* G3 [1—4].

When the preliminary short-day cycles are followed by continuous light, the first peak of the Chl-NADP-GPD activity of *L. gibba* G3 occurs at LL 12, at which time the Cyt-NAD-GPD activity comes to its first minimum [1]. The mirror-image changes in these GPD activities have been ascribed to changes in the $K_m$ values, $V_{max}$ being unaltered [2].

Chl-NADP-GPD of *L. gibba* G3 [3] exists as two interconvertible oligomers, Chl-NADP-GPD I and II: Chl-NADP-GPD I (an allosteric enzyme of

Abbreviations: Chl-NADP-GPD, NADP-dependent glyceraldehyde 3-phosphate dehydrogenase exclusively localized in the chloroplast (EC 1.2.1.13), which always has NADP-GPD activity; Cyt-NAD-GPD, NAD-dependent GPD exclusively localized in the cytoplasm (EC 1.2.1.12); EGTA, ethyleneglycol-bis-(aminoethyl ether)-N,N$'$-tetraacetic acid; LL x, x hour after the onset of continuous light which comes at the end of the preliminary short day cycles; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide-HCl.

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520 K daltons, with a low affinity for NADP+) can dissociate into Chl-NADP-GPD II (a Michaelis enzyme of 170 K daltons, with a high affinity for NADP+) and “binding factor”. This dissociation occurs during the subjective day (LL 0 to LL 12) and increases the Chl-NADP-GPD affinity for NADP+; conversely, Chl-NADP-GPD II associates with the “binding factor” to restore Chl-NADP-GPD I during the subjective night (LL 12 to LL 24) and decreases the Chl-NADP-GPD affinity for NADP+ [3]. I have proposed, moreover, that ATP, dithiothreitol-like substance, NADP+, and NADPH were possible in vivo activators of Chl-NADP-GPD because we were able to show in vitro that these compounds could induce the dissociation of Chl-NADP-GPD I. Conversely, NAD+ could be a possible in vivo inhibitor of Chl-NADP-GPD, because it was able to induce, in vitro, the association of the two oligomers [3].

We have studied in this paper the effects of the several compounds on GPD activities and have shown that the NADP+ was the only one having opposite effects on Chl-NADP-GPD and Cyt-NAD-GPD. We also report that the level of NADP+ not only oscillates but that the phases of its rhythm could explain the rhythms of GPD activities. We show in addition the NADP+ rhythm, in turn could be a consequence of the rhythmic changes in the rate of net in vivo production of total NADP (NADP+ + NADPH), themselves depending upon the mirror-image rhythms of activities of NAD kinase and of NADP phosphatase. The reverse sensitivities of these latter enzymatic activities to different inhibitors of Ca2+-calmodulin finally suggests an important role of the levels of Ca2+-calmodulin.

A part of the results was briefly presented in [4].

**Materials and Methods**

**Plant material**

The duckweed, *Lemna gibba* G3, a long-day plant, was always aseptically cultured in 100 ml of M medium supplemented with 1% sucrose [5] under a short day condition (9 h light at 3800 lux from white fluorescent lamps followed by 15 h darkness) at 26°C. About 14 day-old cultures were inoculated onto the fresh medium, exposed to approximately seven cycles of the short day circadian rhythms followed by the experimental continuous light period.

**Purification of Chl-NADP-GPD and Cyt-NAD-GPD**

Duckweeds collected in the early day phase of the short-day regime were homogenized with 50 mM phosphate buffer (pH 6.6) containing 3 mM EDTA and polyclar AT (1/10 weight of the duckweeds). The homogenates were filtered through nylon cloth, and the filtrates were centrifuged at 100 K x g for 30 min. Solid ammonium sulfate was added up to 80% saturation to the resultant supernatants. The precipitates were collected by centrifugation at 10 K x g for 30 min, and suspended in 50 mM phosphate buffer (pH 6.6) containing 3 mM EDTA. The precipitates, which were rich in Chl-NADP-GDP, were suspended in 20 mM phosphate buffer (pH 6.6) containing 3 mM EDTA, and desalted by a Sephadex G-25 column with the same buffer. The supernatants, rich in Cyt-NAD-GPD, were similarly desalted. These two desalted eluates were separately applied to a DEAE-cellulose column equilibrated with the same buffer as used in desalting. All the Cyt-NAD-GPD free from NADP-GPD activity was eluted with 50 mM phosphate buffer, and subsequently all the Chl-NADP-GPD free from Cyt-NAD-GPD with 200 mM phosphate buffer [1]. In order to obtain a single peak of Chl-NADP-GPD I [3], the combined Chl-NADP-GPD fractions adjusted to a final concentration of NAD+ equal to 0.2 mM was loaded on a Sephacryl S-300 column equilibrated with 50 mM phosphate buffer (pH 6.6) containing 3 mM EDTA and 0.2 mM NAD+. The elution buffer was the same as the one used for the equilibration. Ammonium sulfate up to 60% saturation was added to the Chl-NADP-GPD I and the precipitate, collected by centrifugation, was stored at −80°C before use. When used, it was thawed and suspended in 50 mM phosphate buffer (pH 6.6) containing 3 mM EDTA. Cyt-NAD-GPD was eluted as a single peak at the 130 K to 150 K daltons zone, irrespective of the presence or absence of NADP+.
NAD\(^+\), or dithiothreitol in the purification processes.

**Assays of GPD activities**

After treatment with different compounds at various concentrations for 20 min at 0 °C, the GPD activity was determined as follows.

The catalytic reaction was initiated by adding 25 \(\mu\)l of effector-treated GPD to 975 \(\mu\)l of the reaction mixture which contained 50 \(\mu\)mol Tris-HCl (pH 8.5), 15 \(\mu\)mol sodium arsenate, 20 \(\mu\)mol sodium fluoride, 4 \(\mu\)mol L-cysteine, 0.1 \(\mu\)mol (unless otherwise stated) NAD\(^+\) (or NADP\(^+\)), and 0.5 \(\mu\)mol DL-glyceraldehyde 3-phosphate. The reaction velocity was read from the initial linear portion (1 min) of the kinetic of reduction of NADP\(^+\) to NADPH measured at \(A_{340}\). The concentrations of compounds (Fig. 1 to 3) during the 20 min-treatment are indicated in the abscissa: They are reduced, in the assay, by 40 times.

**Extraction and determination of pyridine nucleotides levels in the tissues**

The extraction method was slightly modified from the method of Yamamoto [6]. Plants (300 mg fresh weight) were macerated in a glass-homogenizer with 2.5 ml of either 0.1 \(\times\) HCl (for NAD\(^+\) and NADP\(^+\)) or 0.1 \(\times\) NaOH (for NADH and NADPH), supplemented with 100 mg polyclar AT, for 2 min at 90 °C, then immediately cooled at 0 °C in an ice-bucket. HCl and NaOH, each at 0.1 \(\times\), completely destroyed respectively the reduced and the oxidized pyridine nucleotides. The homogenate, combined with 2.5 ml of washings of the homogenizer, was centrifuged at 10 \(K \times g\) for 20 min. Two-ml aliquots of the supernatant were washed four times with 4.0 ml of ethylether to remove the effectors of enzymes used in determining the levels of pyridine nucleotides. The final washings were separately kept overnight at below 4 °C, then used for the determination of pyridine nucleotides. The recoveries were always above 90%.

The determination of pyridine nucleotides was slightly modified from the method of Muto and Miyachi [7]. The reaction mixture (1.0 ml) for the determination of NAD\(^+\) or NADH contained 0.24 mmol of Tris-HCl (pH 8.5), 28 \(\mu\)g of 2,6-dichlorophenolindophenol, 360 \(\mu\)g of phenazinemethosulfate, 2 \(\mu\)mol of glucose 6-phosphate, 0.1 ml of the tissue extract (containing less than 0.2 nmol of the nucleotides), and 12 units of glucose 6-phosphate dehydrogenase (EC 1.1.1.49). Each reaction was started by addition of the enzymes: the reaction velocity was read from the initial linear portion of the kinetic of reduction of 2,6-dichlorophenolindophenol measured at \(A_{600}\).

**Preparation of extracts for the measurement of NAD kinase activity**

Duckweeds were homogenized with 50 \(\mu\)M Tris-HCl (pH 7.5) containing 0.25 \(\mu\)M sucrose and polyclar AT (1/10 weight of the duckweeds). They were then filtered through nylon cloth, and the filtrates were centrifuged at 100 \(K \times g\) for 30 min at 4 °C. An aliquot of the resultant supernatants (designated as the crude extract) was desalted through a Sephadex G-25 column using 50 \(\mu\)M Tris-HCl (pH 7.5) in the equilibration and elution. The eluates corresponding to the void volume were used as the desalted extract. Another aliquot of the crude extract was treated by solid ammonium sulfate up to 60% saturation, then centrifuged at 25 \(K \times g\) for 15 min at 4 °C. The pellet of the precipitates was suspended in 50 \(\mu\)M Tris-HCl (pH 7.5) saturated with ammonium sulfate, and then centrifuged as above. This washing with saturated ammonium sulfate solution was repeated 3 times and the final precipitate could be stored at \(-80\) °C for several weeks. An AS extract designates a clear suspension in 50 \(\mu\)M Tris-HCl (pH 7.5) of the precipitates, and a PS extract a suspension in 50 \(\mu\)M Tris-HCl (pH 7.5) of the precipitates obtained by centrifugation at 9 \(K \times g\) for 5 min of the crude extract mixed with the equal volume of 0.1% protamine sulfate in 50 \(\mu\)M Tris-HCl (pH 7.5).

**Assay of NAD kinase activity**

The reaction mixture (1 ml) contained 50 \(\mu\)mol of Tris-HCl (pH 7.5), 6 \(\mu\)mol of nicotinamide, 1 \(\mu\)mol of NAD\(^+\), 7 \(\mu\)mol of MgCl\(_2\), 2 \(\mu\)mol of ATP, and 50
to 300 μg protein of either desalted, AS, or PS extracts, unless otherwise stated. The reaction was started by adding the extract, continued for 60 min at 30 °C, and terminated by heating for 2 min at 90 °C.

Determination of the levels of NADP+

The reaction mixture (1.0 ml) for the determination of NADP+ produced by the NAD kinase reactions contained 0.24 mmol of Tris-HCl (pH 7.9), 28 μg of 2,6-dichlorophenolindophenol, 360 μg of phenazinemethosulfate, 2 μmol of glucose 6-phosphate, 12 units of glucose 6-phosphate dehydrogenase (G6PD), 0.2 ml of the test solution, and 0.2 ml of distilled water or pure NADP+. The reaction was started by adding glucose 6-phosphate dehydrogenase after the G6PD-less mixture was kept at 35 °C for 20 min. The reaction velocity was read from the initial linear portion of kinetic of reduction of 2,6-dichlorophenolindophenol. Three different mixtures of the test solution, pure NADP+, and water had to be tested in order to determine the amount of NADP+ present in the test solution: (a) 0.2 ml (0.2 nmol) of pure NADP+ and 0.2 ml of distilled water, (b) 0.2 ml (0.2 nmol) pure NADP+ and 0.2 ml of the test solution and (c) 0.2 ml of the test solution and 0.2 ml of distilled water. The amount of NADP+ in the test solution was calculated using the corrected absorbancy change: ΔA/I = (ΔAa − ΔAb).

Preparation of extracts for the measurement of NADP phosphatase activity

The crude and AS extracts were the same as those prepared for the measurements of NAD kinase activity.

Assays of phosphatases

In this paper, NADP phosphatase, β-glycerophosphate phosphatase, and p-nitrophenylphosphate phosphatase respectively indicate the phosphatase activity determined in the presence of either NADP(H) or β-glycerophosphate, and p-nitrophenylphosphate as substrates.

The reaction mixture (0.5 ml) contained 25 μmol of Tris-acetate (pH 5.5), and 0.25 μmol of either NADP(H), β-glycerophosphate, or p-nitrophenylphosphate, and the enzyme, unless otherwise stated. The reaction was started by adding the enzyme preparation, continued for 30 min at 30 °C, and terminated by adding 1.0 ml of 15% TCA. The liberated orthophosphate (Pi) was determined by the method of Chen et al. [8].

Biochemicals

NAD+, NADH, NADP+, NADPH, glucose 6-phosphate, and G6PD were purchased from Oriental Yeast Co., Osaka; DL-glyceraldehyde 3-phosphate and ATP from Boehringer, Mannheim; p-nitrophenyl phosphate and β-glycerophosphate from Merck, Darmstadt; chlorpromazine from Sigma, MO; calmodulin from Wako Pharmaceutical Co., Tokyo; and W 7 from Rikaken Co., Nagoya.

Results

Effect of NADH on Chl-NADP-GPD

The Chl-NADP-GPD I (520 K dalton aggregate of Chl-NADP-GPD having the lower affinity for NADP+) fraction from Sephacryl S-300 column was treated with various concentrations of NADH. NADH (4 mM) reduced the Chl-NADP-GPD I activity to 75% of the untreated Chl-NADP-GPD I (Fig. 1). In order to obtain Chl-NADP-GPD II (170 K daltons protomer of Chl-NADP-GPD having the higher affinity for NADP+) and the “binding factor”, the Chl-NADP-GPD I was treated for 20 min with 0.5 mM NADP+ in a test tube. The Chl-NADP-GPD II was then treated with various concentrations of NADH. In spite of the presence of “binding factor” which is required for the association of Chl-NADP-GPD II [3], NADH had little effect on Chl-NADP-GPD II (Fig. 1, upper curve).

Effectors of Cyt-NAD-GPD

Cyt-NAD-GPD was incubated with ATP, dithiothreitol, NAD+, NADH, NADP+, or NADPH at concentrations ranging from 0 to 5 mM for 20 min at 0 °C; the catalytic reaction was then started by adding 25 μl of the effector-treated GPD to 975 μl of reaction mixture (Fig. 2). When NADH, NADPH, NADP+, and ATP were inhibitory on Cyt-NAD-GPD, NAD+ and dithiothreitol were ineffective. In a second set of assays, the inhibitory effects of NADH, NADPH, NADP+ and ATP were examined in the presence of various concentrations...
of NAD\(^+\) in the enzyme assays. The kinetic parameters (see the insert Fig. 3) were first determined by the method of direct linear plot by Eisenthal and Cornish-Bowden (cf. 9), then used to draw the Lineweaver-Burk plots (Fig. 3). In addition, the kinetic parameters characterizing Cyt-NAD-GPD at LL \(0\) (trough value) and LL \(12\) (peak value) were calculated by applying this method to results previously published [2]: \(K_m\) at LL \(0\) was \(47 \pm 3\) \(\mu M\); \(K_m\) at LL \(12\) was \(76 \pm 6\) \(\mu M\); \(V_{max}\) at LL \(0\) was \(0.68 \pm 0.01\) \(\mu mol/min/mg\) protein; \(V_{max}\) at LL \(12\) was \(0.70 \pm 0.03\) \(\mu mol/min/mg\) protein.

ATP, NADH, and NADP\(^+\) inhibited Cyt-NAD-GPD by decreasing its affinity for NAD\(^+\) without changing \(V_{max}\) (Fig. 3). The rise in concentration of exogenous ATP from 0 to 5 \(mM\) was not enough to explain the observed rhythmic change in \(K_m\) of Cyt-NAD-GPD from 47 \(\mu M\) at LL \(0\) to 76 \(\mu M\) at LL \(12\) (Fig. 3). Calculation (not shown) using data of both Figs. 2 and 3 showed that an addition of either approximately 0.25 \(mM\) NADH or approximately 0.75 \(mM\) NADP\(^+\) was sufficient to elevate \(K_m\) from the control level (49 \(\mu M\), not very different from the

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**Fig. 1.** Effects of NADH on Chl-NADP-GPD I and II. Chl-NADP-GPD I (•) was separated from Chl-NADP-GPD II using gel chromatography with a Sephacryl S-300 column, while Chl-NADP-GPD II (○) was obtained in a test tube by treating Chl-NADP-GPD I with 1.0 \(mM\) NADP\(^+\) sufficient to convert all of the Chl-NADP-GPD I to Chl-NADP-GPD II and “binding factor” (3). The treatment of Chl-NADP-GPD II and “binding factor” with NADH was then conducted in the presence of 0.5 \(mM\) NADP\(^+\). See Materials and methods for other detail. The mean of relative standard errors was 8.4%.

**Fig. 2.** Effects of ATP, dithiothreitol, NAD(H), and NADP(H) on Cyt-NAD-GPD. See Materials and methods for experimental procedures. The mean of relative standard errors was 3.1%.

**Fig. 3.** Kinetical studies of the effects of ATP, NADH, NADP\(^+\), and NADPH on Cyt-NAD-GPD. See Materials and methods and the text for experimental procedures.
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Fig. 4. Temporal changes in contents of pyridine nucleotides in continuous light. The standard errors for NAD$^+$ (●), NADH (○), NADP$^+$ (▲), and NADPH (▽) were indicated as the vertical bars attached to each points: the error bars lower than 0.2 were not drawn and those for both NADP$^+$ and NADPH were drawn as the half bars. Each points represented the averages of at least three independent extracts. The time of occurrence of the peaks of the rhythms of Cyt-NAD-GPD (+) and Chl-NADP-GPD (▲) activities were cited from [1] for the comparison.

<table>
<thead>
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<th>CONTENTS (nano moles per g fr wt.)</th>
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<tbody>
<tr>
<td>20</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>0</td>
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47 μM at LL 0) to the level 76 μM actually attained at LL 12.

Circadian rhythms in pyridine nucleotides contents in the tissues

Both NADP$^+$ and NADPH displayed circadian rhythms in phase and of comparable amplitudes (Fig. 4): peaks occurred at LL 12 and 36 and troughs at LL 0 and 24. In contrast, the level of NAD$^+$ oscillated with an amplitude twice as great and 180° out-of-phase with the levels of NADP$^+$ and NADPH, whereas NADH very slightly increased without displaying any rhythmicity. Consequently, the level of NADP$^+$ + NADPH (total NADP) oscillated 180° out-of-phase with the level of NAD$^+$ + NADH (total NAD), with both rhythms displaying comparable amplitudes. Thus, the total NAD$^+$ + total NADP level monotonically increased during the 40 h light timespan.

The curves of Fig. 4 also indicate the existence of a rhythm in the rate of net in vivo production of total NADP having a peak about LL 6 (12 pmol/min/g fresh weight) at the time the slope of total NADP variation was positive and maximal, and a trough at about LL 18 (−9 pmol/min/g fresh weight) when the slope was negative and minimal. The rates was equal to zero at LL 0, 12, 24, and 36. Likewise, the curves of level of NAD$^+$ and NADH indicate a circadian rhythm of the rate of net in vivo production of total NADP 180° out-of-phase with the one of net in vivo production of total NADP and presenting a peak at about LL 18 (10 pmol/min/g fresh weight), a trough at about LL 6 (−7 pmol/min/g fresh weight), and rates equal zero at LL 0, 12, 24, and 36. Since about 3 mg protein of the crude or desalted extracts were obtained from one gram fresh weight of *L. gibba* G3, the peak and the trough of the circadian rhythm of rate of net in vivo production of total NADP approximately corresponded 4 and −3 pmol/min/mg protein of the crude or desalted extract.

The ratio of NADPH to NADP$^+$ shown in Fig. 5 was deduced from the results shown in Fig. 4. The curve connecting mean values of the ratios shown in the figure showed the circadian rhythm in the ratio 90° out-of-phase with the rhythms of NADP$^+$ and NADPH levels (Fig. 4). Both the curve connecting the upper limits of the ratios except for the ratio at LL 36 and that connecting the lower limits (curves not drawn) also showed the same rhythm. It is suggested that the rate of net in vivo reduction of NADP$^+$ might display a circadian rhythm advanced by 90° with the rhythm of the NADPH/NADP$^+$ ratio. The ratio of NADH to NAD$^+$ also showed a circadian rhythm 180° out-of-phase with the NAD$^+$ level rhythm (Fig. 4). Inasmuch as the NADH level displayed no rhythmical change (Fig. 4), the NADH/NAD$^+$ rhythm was induced only by the rhythm in NAD$^+$ level.

Circadian rhythm of NADP phosphatase activity

The temporal change in activity of NADP phosphatase in continuous light was then examined using both the crude extracts and the desalted extracts prepared at the different times. The NADP
phosphatase activity in the crude extracts changed with a circadian periodicity displaying a peak at LL 18 and troughs at LL 6 and 30 (Fig. 6). The enzyme activity in the crude extract, prepared at any given time of the continuous light period, was consistently increased by desalting: the ratio of maximum (at LL 18) to minimum (at LL 6) activity was reduced from 4.1 (= 18.5/4.5) to 1.7 (= 32.0/18.6) when the amplitude (the peak minus the trough level) was unchanged at about 14 nmol/min/mg protein. The fact that the rhythmicity occurred even in the desalted extract indicated that none of the factors which could be dissociated by the desalting procedure used induced the rhythmicity.

The rhythm was also obvious in the AS extract, although the ratio of maximum to minimum activity and the amplitude were reduced respectively, to 1.15 and 4 nmol/min/mg protein, indicating that the factor inducing the rhythmicity was not completely dissociated from NADP phosphatase by the repeated treatment with ammonium sulfate. The AS extract was treated with 0.6% protamine sulfate, and then centrifuged. The resultant supernatant was able to decompose NADPH as well as NADP: The affinity of NADP phosphatase to NADP+ ($K_m = 336 \pm 12 \mu M$) was approximately twice lower than its affinity to NADPH (163 $\pm$ 17 $\mu M$), whereas $V_{max}$ for NADP+ and for NADPH were almost the same (respectively 55.9 $\pm$ 1.1 and 51.9 $\pm$ 2.2 nmol/min/mg protein present in this preparation).

Similar circadian rhythms were found for both $\beta$-glycerophosphate phosphatase (Fig. 6) and $p$-nitrophenylphosphate phosphatase activities (Table I). Both $\beta$-glycerophosphate and $p$-nitrophenylphosphate are known substrates for the non-specific

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**Fig. 5.** Temporal changes in the ratios of NADH to NAD+, NADPH to NADP+, and total NADP to total NAD. The ratios of NADH to NAD+ (○), NADPH to NADP+ (□), and total NADP to total NAD (△) were calculated on the data shown in Fig. 4. The upper limits and the lower ones of vertical bars were calculated by the formula, respectively [the upper limits of NADPH (Fig. 4)]/[the lower limits of NADPH (Fig. 4)] and [the lower limits of NADP (Fig. 4)]/[the upper limits of NADP (Fig. 4)].

**Fig. 6.** Temporal changes in activities of NADP phosphatase and $\beta$-glycerophosphate phosphatase in continuous light. Crude extracts (47.8 $\pm$ 0.8 mg protein per 0.5 ml of reaction mixture) (○), desalted extracts (●), and AS extracts (□) were used for NADP phosphatase activity measurement. The same amount of crude extracts (△) and desalted extracts (▲) were used for the determination of activity of $\beta$-glycerophosphate phosphatase. The means of relative standard errors were 14.6% for ○, 5.4% for ●, 2.5% for □, 23.9% for △, and 7.9% for ▲.
Table I. Temporal change in the activity of p-nitrophenyl-phosphate phosphatase in duckweeds under continuous light at 26 °C.

<table>
<thead>
<tr>
<th>Enzyme preparations</th>
<th>nmol Pi liberated/min/mg protein ± S.E.</th>
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<tbody>
<tr>
<td></td>
<td>LL 6</td>
</tr>
<tr>
<td>Crude extracts</td>
<td>23.7 ± 3.5</td>
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<tr>
<td>Desalted extracts</td>
<td>33.5 ± 1.8</td>
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acid and alkaline phosphatases. The type of phosphatase that we measured has to be characterized, since no phosphatase able to attack NADP(H) alone has so far been identified.

Circadian rhythm of NAD kinase activity

The temporal changes in the NAD kinase activity in desalted extracts of L. gibba G3 under continuous light are shown in Fig. 7. The activity changed with a circadian rhythmicity eliciting a peak at LL 6 and a trough at LL 18. The NAD kinase activities in the AS extracts prepared at both LL 6 and 18 were respectively 104 ± 4 and 62 ± 6 pmol/min/mg protein of the AS extract. Thus, as it was the case for the factor inducing the rhythmicity in the NADP phosphatase activity, the factor inducing the rhythmicity in the NAD kinase activity cannot be a factor dissociable either by the desalting through Sephadex G-25 column or by the repeated ammonium sulfate treatment.

Since NADP phosphatase reaction requires NADP+ while NAD kinase requires ATP, and since NADP+ is a product of NAD kinase reaction while ATP is not a product of NADP phosphatase reaction, the NADP phosphatase reaction can proceed as NAD kinase produces NADP+ in our routine assay of NAD kinase activity, while the NAD kinase reaction cannot proceed in the assay of NADP phosphatase activity. It was possible, therefore, that the changes in the NAD kinase activity observed shown in Fig. 7 merely corresponded to the mirror-image of the previously described change in the NADP phosphatase (Fig. 6). In other words, it might be that NAD kinase activity was really unchanged but the higher activity of NADP phosphatase might yield the lower amount of NADP+ in the assay of NAD kinase activity and then produce an apparent lower activity of NAD kinase. The data presented in Table II, however, confirmed that there was a real circadian rhythm in NAD kinase activity. When 0.15 mg protein of crude extracts was added to 0.5 ml of the assay mixture for NADP phosphatase activity, no NADP phosphatase activities were detected, indicating that the crude extracts contained the inhibitors of

Table II. NAD kinase and NADP phosphatase activities in extracts from duckweeds at LL 6 and 18. Both enzymes were assayed at pH 8.0 (Tris-HCl) at 30 °C. NAD kinase was determined in the presence of 1 mM NAD+, 2 mM ATP, and 7 mM MgCl2, and NADP phosphatase in the presence of 1 mM NADP+. Specific activities (pmol/min/mg protein) are shown. Figures in brackets show the specific activity on the basis of the initial protein content of the tissue extracts before the protamine sulfate-treatment. Figures in parentheses show mg protein per ml reaction mixture.

<table>
<thead>
<tr>
<th>Enzyme preparations</th>
<th>NAD kinase</th>
<th>NADP phosphatase</th>
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<tr>
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<td>LL 6</td>
<td>LL 18</td>
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<tr>
<td>Crude extract (0.31)</td>
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<td>PS extract (0.44)</td>
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Fig. 7. Temporal changes in activity of NAD kinase in continuous light. The desalted extracts were used. The mean of relative standard errors was 4.9%.
NADP phosphatase. Even in the condition where all NADP phosphatase activities were suppressed by the inhibitors in the crude extracts, the NAD kinase at LL 6 showed more than twice the activity detected at LL 18, both activities being slightly higher than the ones measured using desalted extracts (Fig. 7). Furthermore, similar results were obtained when using the PS extract as enzyme preparations, which contained much less NADP phosphatase than the desalted extracts but all the NAD kinase normally present in the desalted extracts (Table II).

**Effects of calmodulin and its inhibitors on NAD kinase and NADP phosphatase activities**

Calmodulin, a Ca\(^{2+}\) binding protein of relatively low molecular weight (for example, 17 k daltons for bovine brain calmodulin), regulates many types of cell functions in almost all eukaryotes: it is active only when bound with Ca\(^{2+}\) [10].

The effects of EGTA, a Ca\(^{2+}\)-chelator, on NAD kinase in the AS extracts prepared at LL 6 and LL 18 are shown in Table III. The NAD kinase reaction was run in the presence of 6 mM Mg\(^{2+}\), 5 mM ATP, 1 mM NAD\(^{+}\), and with or without EGTA. A saturating dose of EGTA (10 \(\mu\)M) reduced the activity of NAD kinase at LL 6 [LL 6 corresponds to a peak point of the NAD kinase rhythm (see Fig. 7)] to the same level as that of NAD kinase at LL 18 corresponding to a trough value (Table III). This inhibition was partially removed by the addition of 100 \(\mu\)M Ca\(^{2+}\) (Table III). Conversely, EGTA at 10 \(\mu\)M (or even at higher concentration up to 500 \(\mu\)M, data not shown) had little effect on NAD kinase activity at LL 18 (Table III).

![Table III. Effects of calmodulin, EGTA, and W7 on NAD kinase.](image)

**Table III. Effects of calmodulin, EGTA, and W7 on NAD kinase.**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Activity [pmol/min/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LL 6</td>
</tr>
<tr>
<td>None</td>
<td>436 ± 9</td>
</tr>
<tr>
<td>10 (\mu)M EGTA</td>
<td>282 ± 28</td>
</tr>
<tr>
<td>10 (\mu)M EGTA and 100 (\mu)M CaCl(_2)</td>
<td>379 ± 36</td>
</tr>
<tr>
<td>200 (\mu)M W7</td>
<td>353 ± 25</td>
</tr>
<tr>
<td>200 (\mu)M W7 and 20 U calmodulin</td>
<td>451 ± 38</td>
</tr>
</tbody>
</table>

NAD kinase reactions were run in the presence of 5 mM ATP, 6 mM MgCl\(_2\), and 1 mM NAD\(^{+}\) at 30 °C for 60 min.

These results suggest that the AS extract from the duckweeds at LL 6 still contained Ca\(^{2+}\) in spite of the repeated desalting treatment by ammonium sulfate and that the higher amount of Ca\(^{2+}\) in the AS extract was responsible for the higher activity of NAD kinase. In addition, Table III shows that W7, a calmodulin inhibitor [11], reduced the activity of NAD kinase at LL 6 to almost the same level as that of NAD kinase at LL 18, and that this inhibition could be completely removed by 20 units of calmodulin (from bovine brain).

The dose responses of NAD kinase at LL 6 and LL 18 to chlorpromazine [12] and W7, both calmodulin inhibitors, are shown in Fig. 8. Both reduced the activity of LL 6 level of NAD kinase to the same level as that found at LL 18. The concentrations \(t_{50}\) of chlorpromazine and W7 responsible for the inhibition of half of the maximal calmodulin action were about 8 and 28 \(\mu\)M, respectively. In addition, 20 units of calmodulin, only in the presence of 100 \(\mu\)M Ca\(^{2+}\), activated LL 18-level of NAD kinase to almost the same as that
found at LL 6 (Fig. 8). On the contrary, the calmodulin inhibitors had almost no effect on the low activity of the LL 18-level of NAD kinase (Fig. 8).

The effects of EGTA, chlorpromazine, and W7 on NADP phosphatase activity in extracts prepared from the duckweeds at LL 6 (trough value) and LL 18 (peak value) are shown in Fig. 9. Similar to the results found for NAD kinase activity, each of these reagents either had little effect if assayed on a peak value extract (LL 18) or was able to activate a trough value of NADP phosphatase extract (LL 6) to almost the same level as the peak value found at LL 18. The $K_m$'s of chlorpromazine and W7 were respectively about 100 and 50 $\mu$M. It is interesting to notice that while the calmodulin inhibitors activated NADP phosphatase they inhibited NAD kinase. Therefore, Ca$^{2+}$-calmodulin might be not only an activator of NAD kinase but also an inhibitor of NADP phosphatase.

**Discussion**

Miyata and Yamamoto [13] were the first to show the mirror-image circadian rhythms in the activities of NAD-GPD and NADP-GPD in *L. gibba* G3. Later, Wagner's group reported similar rhythms of both GPDs in *Chenopodium rubrum* and suggested that they could be induced by circadian rhythms in the values of the ratios NADPH/NADP$^+$ [14, 15]. In order to determine what might induce these mirror-image circadian rhythms, it was important to know: (i) the molecular nature of the activity changes; (ii) if effectors exist which could induce the changes; (iii) if such effectors exist, do they change in the expected way.

**Opposite effects of NADP$^+$ on Chl-NADP-GPD and Cyt-NAD-GPD activities**

I have demonstrated previously in *Lemma* [2] that the affinity ($1/K_m$) for NADP$^+$ of Chl-NADP-GPD oscillates with a circadian periodicity eliciting a peak at LL 12 and a trough at LL 24 while the affinity of Cyt-NAD-GPD for NAD$^+$ displays a circadian rhythm 180° out-of-phase with the Chl-NADP-GPD affinity rhythm. Furthermore, I have shown [3] that ATP, dithiothreitol, NADP$^+$, and NADPH can mimic the *in vivo* activation of Chl-NADP-GPD, whereas NAD$^+$ can mimic its inhibition. In the present study, I have demonstrated that NADH plays no role in the *in vivo* regulation of Chl-NADP-GPD for the following reasons. The activation of Chl-NADP-GPD *in vivo* is accompanied by the conversion of the more inactive form of the enzyme (Chl-NADP-GPD I) into the more active form (Chl-NADP-GPD II), while the *in vivo* inhibition is associated with the conversion of Chl-NADP-GPD II to Chl-NADP-GPD I [3]. Because NADH was uneffective on Chl-NADP-GPD II and inhibitory on Chl-NADP-GPD I (Fig. 1), it could not be an *in vivo* activator of Chl-NADP-GPD, and because an *in vivo* inhibitor of Chl-NADP-GPD must attack on Chl-NADP-GPD II and then convert it to Chl-NADP-GPD I, NADH could not be an *in vivo* inhibitor of Chl-NADP-GPD. At the present, we do not have any idea of the *in vivo* role of the inhibitory effect of NADH on Chl-NADP-GPD I.

Furthermore, it was found (Figs. 2 and 3) that NADP$^+$ and NADH (contrary to ATP, dithiothreitol, NAD$^+$ and NADPH) mimicked the *in vivo* inhibition of Cyt-NAD-GPD. NADP$^+$ could then be not only an *in vivo* activator of Chl-NADP-GPD, but also an *in vivo* inhibitor of Cyt-NAD-GPD. The simplest explanation, therefore, for the mirror-
Causal Relationships among Metabolic Circadian Rhythms in *Lemna*

image circadian rhythms of affinities of Chl-NADP-GPD (for NADP⁺) and Cyt-NAD-GPD (for NAD⁺) would be to assume circadian rhythms of NADP⁺ level, both in the chloroplasts and in the cytoplasm, in phase with the rhythm of Chl-NADP-GPD affinity for NADP⁺, that is, with a peak at LL 12 and a trough at LL 24.

**Circadian rhythms in NADP⁺ level in vivo**

A circadian rhythm in NADP⁺ level was first shown in *Neurospora* [16]. Wagner and Frosch also reported a rhythm of NADP⁺ level in *Chenopodium* displaying a 15 h periodicity [15]. They found that 15 h rhythms of both NADP⁺ and NADPH levels could generate a circadian rhythm in the ratio of NADPH to NADP⁺ and suggested that this latter rhythm might induce the GPD rhythm. However, their suggestion is questionable because on the one hand the rhythm in the ratio NADPH/NADP⁺, in their material, ran out-of-phase with the GPD rhythms, and on the other hand, no evidence had ever been presented so far in any plant of a regulation of GPD’s by the value of the NADPH/NADP⁺ ratios.

The present study has shown that the in vivo levels of both NADP⁺ and NADPH oscillated with a circadian periodicity in phase with the rhythm of affinity for NADP⁺ of Chl-NADP-GPD and 180° out-of-phase with that of affinity for NAD⁺ of Cyt-NAD-GPD. These phase-relationships between the rhythms of NADP⁺ level and GPD affinities are quite in agreement with the hypothesis that NADP⁺ might induce the mirror-image rhythms of Chl-NADP-GPD and Cyt-NAD-GPD affinities. However, it must be noted that our results for in vivo NADP⁺ levels do not necessarily reflect in-phase rhythms in both chloroplast and cytoplasmic compartments.

**Circadian rhythm of rate of net in vivo production of total NADP**

The present study reports that the rate of net in vivo production of total NADP oscillated with a circadian periodicity 180° out-of-phase with the rate of net in vivo production of total NAD and that both rhythms presented comparable amplitudes (Fig. 4). Consequently, total pyridine nucleotides levels displayed no rhythmical change. If this is the case, the rhythmicities in total NADP and NAD might be ascribable to rhythms in activities of enzymes catalyzing the formation of NADP⁺ from NAD⁺ or the breakdown of NADP(H) to NAD(H), or both. We corroborated such an hypothesis by demonstrating that NAD kinase and NADP phosphatase activities displayed circadian rhythm with the expected phases (Figs. 6 and 7; Table II).

The fact that the in vitro activities of NADP phosphatase (Fig. 6) were higher, by a factor 2 to 3, than those of NAD kinase (Fig. 7) implies that the tissue must contain either much lower activities of NADP phosphatase or much higher ones of NAD kinase than those observed in vitro, in order to display the circadian rhythm of rate of net in vivo production of total NADP (Fig. 4). We have compared in this paper the activities measured using desalted extracts (Fig. 6) with those measured using crude extracts (Fig. 6; Table II). We can conclude that the tissue contained enough inhibitor(s) of NADP phosphatase, dissociable by a desalting procedure through a Sephadex G-25 column, to suppress almost all the NADP phosphatase activities. This point is still under investigation.

**The cause of circadian rhythms of NAD kinase and NADP phosphatase activities**

The next question was then what could induce the mirror-image circadian rhythms of activities of NAD kinase and NADP phosphatase. Since these rhythmicities could be observed both in desalted extracts and AS extracts (Figs. 6 and 7), it is suggested that either the inducing factors for the rhythms might remain bound to the enzymes after these treatments or that the rhythmicities might be due to those in the amounts of enzymes. Although the rhythmicities of other several enzyme activities have been attributed to rhythmic changes in their amounts such as alanine dehydrogenase of *Euglena* [17], phosphoenolpyruvate carboxylase of *Kalanchoe* [18], nitrate reductase of *Wolffia* [19], and luciferase of *Gonyaulax* [20], we propose, for the following reasons, that the activities of NAD kinase and NADP phosphatase in *L. gibba* are not due to the level of the enzymes. The activities of NAD kinase in sea urchin [21] and many plants [7, 22, 23] have been shown to be activated by Ca²⁺-calmodulin, and we have demonstrated here that the activity of NAD kinase of *L. gibba* G3 was also activated by Ca²⁺-calmodulin (Table III and Fig. 8). In addition, we have shown that the inhibitors of Ca²⁺-cal-
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modulin inhibited NAD kinase prepared in its maximal activity (at LL 6), while they were ineffective on the trough activity of NAD kinase at LL 18. The inhibitors of Ca\(^{2+}\)-calmodulin activated NADP phosphatase when it was prepared at the time of its trough activity (LL 6), but again were ineffective on the peak activity of NADP phosphatase at LL 18. These results suggest that the *in vivo* level of Ca\(^{2+}\)-calmodulin oscillates with a peak occurring at LL 6 and a trough at LL 18 inducing the mirror-image rhythms of NAD kinase activity (peak at LL 6) and of NADP phosphatase activity (peak at LL 18).

Our working hypothesis is that a circadian rhythm in the level of Ca\(^{2+}\)-calmodulin induces that in the rate of *net in vivo* production of total NADP by regulating the activities of NAD kinase and NADP phosphatase. The latter rhythm might generate the circadian rhythm in the *in vivo* level of NADP\(^+\)*, which in turn would induce the mirror-image circadian rhythms of activities of Chl-NADP-GPD and Cyt-NAD-GPD. Inasmuch as the chloroplast membranes are not permeable to NADP\(^+\)* [24], these events must occur both in the chloroplast and in the cytoplasm and must run in phase with each other. The nature of the induction of this presumed circadian rhythm of Ca\(^{2+}\)-calmodulin and the possibility that Ca\(^{2+}\)-calmodulin and NAD\(^+\)* may be part of the self-sustaining circadian oscillator are under investigation (cf. 4).

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Note added in proof:

Wagner's group [S. de Loop and E. Wagner, *Physiol. Plant.* 57, 238–242 (1983)] now seems to have abandoned their previous idea [15] of the potential role of the ratio of NADPH/NADP\(^+\) for generating the rhythms in GPD activities and, instead, speculates that the ratio of NADP(H)/NAD(H) may be responsible. These workers also appear to think, mistakenly, that I have proposed [3] that the circadian rhythm in the activity of Chl-NADP-GPD was due to that in the \(K_m\) of Chl-NADP-GPD I (or II, or both), which they consider to be impossible. I agree with this impossibility because my previous proposal, in fact, was that the rhythm in the activity of Chl-NADP-GPD was due to that in the \(K_m\) of Chl-NADP-GPD II, and that this rhythm in \(K_m\), in turn, was due to the rhythmic interconversion between Chl-NADP-GPD I and II, whose \(K_m\)'s were both unchanged during LL [3].