STNV RNA by pancreatic ribonuclease contains a pyrophosphate at the 5′ hydroxyl end. Thus, the presence of seven negative charges on the original terminal oligonucleotide and the resultant two negative charges on the product of dephosphorylation can only be explained on the basis of a trinucleotide-5′ pyrophosphate (ppNpNpNp) in peak −7 of Fig. 1.

This interpretation is further supported by the radioactive counts. Assuming 1200 nucleotides in STNV RNA (*13*) the number of counts recovered in the minor peak of Fig. 3 is 73 percent of the number of counts theoretically expected for a trinucleotide (*14*). This high recovery and the absence of any measurable minor peaks of radioactivity in fractions −6 and −8 precludes the possibility of the 5′ pyrophosphate having originated from a random degradation of a 5′ triphosphate, or from a 5′ triphosphate that chromatogramed in an intermediate position between fractions −7 and −8.

It should be noted that a specific enzyme in *E. coli,* capable of removing the γ phosphate of an ATP terminus of a synthetic RNA, has been purified and investigated (*15*). In addition to this enzyme the presence of other enzymes which hydrolyze γ phosphate nonspecifically with respect to the nature of the base and which yield pyrophosphate-terminated RNA's has also been reported (*15*). Any of these enzymes, if present also in tobacco plants, could have generated a 5′ terminal pyrophosphate in STNV RNA.

Finally, subsequent chemical studies of the 5′ terminal oligonucleotide in this laboratory established its base sequence as AGU and further verified its trinucleotide composition (*16*).

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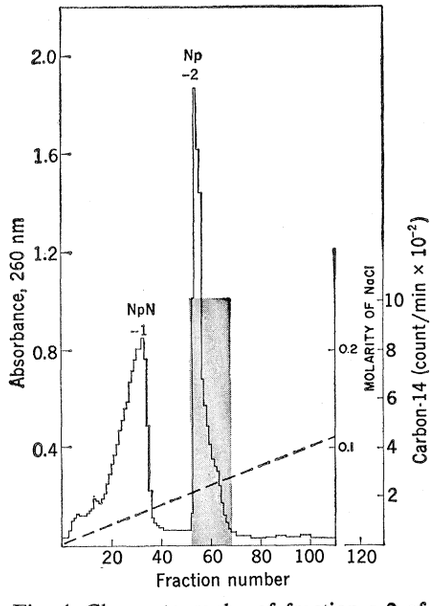

Fig. 4. Chromatography of fraction −2 of Fig. 3. Test tubes 20 to 23 of Fig. 3 were pooled, diluted with 15 ml 7*M* urea and, after the addition of 14 O.D. units of ApA (NpN) and 13 O.D. units of Cp (Np), applied to a DEAE-cellulose column (chloride form, 0.5 × 40 cm) equilibrated with 7*M* urea and 5 m*M* tris HCl, *p*H 7.5. The elution was carried out with a gradient (broken line) of NaCl (0 to 0.2*M*, total volume 300 ml), in 7*M* urea containing 5 m*M* tris HCl, *p*H 7.5. Fractions of 1.5 ml were collected and measured for absorbance at 260 nm (solid line). The eluents from tubes 10 to 38 and 52 to 68 were combined and desalted as described in the text. Aliquots of fraction −1 and −2 were counted for radioactivity (shaded area). After desalting the total counts in peak −2 were 1060 count/min (73 percent of peak −2 of Fig. 3).

References and Notes

1. The 5′ terminus is that end of a nucleic acid the terminal nucleoside of which is linked by its 3′ hydroxy group to the nucleotide chain.
2. Abbreviations used: RNA, ribonucleic acid; pp, pyrophosphate; p, phosphate; N, nucleoside; A, adenosine; G, guanosine; U, uridine; C, cytidine; p on the left of N indicates a 5′ phosphate; p on the right, a 3′ phosphate; STNV, Satellite Tobacco Necrosis Virus; O. D. unit, that amount of RNA per milliliter solution which produces an absorbance of 1 in a 1-cm light path cell at 260 nm; DEAE, diethylaminoethyl; TAC, triethylammonium carbonate; tris, tris (hydroxymethyl) aminomethane.
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Circadian Rhythmicity in Amino Acid Incorporation in Euglena gracilis

Abstract. *A daily rhythm of amino acid incorporation exists in nondividing cultures of* Euglena *kept in a light-dark cycle. This rhythmicity persists for the two cycles measured in cultures transferred to constant conditions and is therefore probably circadian in nature.*

Circadian rhythms are a manifestation of an endogenous timing mechanism, a biological clock, which allows an organism to restrict a given activity to a particular part of the solar day. These clocks exist in nearly all eucaryotic organisms including unicellulars. In *Euglena gracilis,* the circadian clock controls phototactic response (*1*), motility in the dark (*2*), and, under certain conditions, cell division (*3*). In other organisms many biochemical rhythms have been found. Of particular interest are the reports of a circadian oscillation in RNA metabolism (*4*). Although such fluctuations do not in themselves indicate whether RNA metabolism is part of the clock or simply another parameter controlled by the clock, they may eventually acquire added significance in light of studies with actinomycin D which suggest that RNA synthesis is required for the normal operation of the clock (*5*).

I now report a circadian oscillation in amino acid incorporation into that fraction of *Euglena gracilis* which can be precipitated with hot trichloroacetic acid (TCA). Although this result, as with the fluctuations in RNA metabolism, does not in itself imply that protein synthesis is part of the basic clock mechanism, it is significant since the "slowing down" of the *Euglena* clock by an inhibitor of protein synthesis indicates that in some way the clock requires continued protein synthesis (*6*).

Several experiments demonstrated a daily fluctuation in the rate of amino acid incorporation in autotrophic nondividing cultures of *Euglena gracilis* Z maintained at constant temperature (25°C) in a cycle of 12 hours light (cool white fluorescent, 3850 lumen/m²) and 12 hours dark (LD 12:12). The cultures had been grown axenically in an inorganic salt medium under the same conditions of light and temperature (*6, 7*). Samples were removed from these cultures 10 minutes before a light-dark or dark-light transition. Amino acid incorporation was then measured either in the light (cool white fluores-

cent, 3850 lumen/m^2) or in the dark after the addition of DL-phenylalanine-3-^{14}C (0.1 μc/ml, 4.5 μc/μmole, New England Nuclear Corporation) to the cultures. At the end of 1 hour, the samples were precipitated with an equal volume of 10 percent TCA, heated for 15 minutes at 90°C, and cooled; the precipitates were collected on Whatman glass fiber filters (type GF/A) and counted in a Packard Tri-Carb Scintillation Counter (Fig. 1). When the samples were kept in the dark during the 1 hour, cells taken at the end of the day phase (hour 12) had a much higher rate of incorporation than those taken at the end of the night phase (hour 0). On the other hand, when the samples were kept in the light during the 1 hour, there were no substantial differences—the rates at both times in the light were similar to those obtained in the dark with the cells taken at hour 12.

The fact that these fluctuations are under the control of an endogenous circadian clock rather than an immediate result of the imposed light-dark cycle was demonstrated by the persistence of rhythmicity in cultures maintained in continuous darkness (DD) for several cycles. Amino acid incorporation was measured under the conditions normally used for assaying the circadian rhythm of phototactic response. The apparatus and procedure for preparing the cells were the same as previously described (*6, 7*). All cultures exhibited the normal circadian rhythm of phototaxis throughout the experiments.

The protocol for a typical experiment was as follows: Cultures were transferred to the phototaxis recording apparatus and monitored for two cycles of LD 12:12. The following day was the beginning of DD; that is, the lights were not turned on thereafter. Four pairs of cultures were used; each pair was given ^{14}C-phenylalanine (additions to each pair were made at different times), and samples were taken 6 to 12 hours after addition of the isotope. Pair 1 was exposed to isotope from hour 0 to hour 12 on the 1st day of DD (day 1) (hour 0 is the time the lights would have come on if the previous LD cycle had been continued). Pair 2 was exposed from hours 12 to 24 of day 1, pair 3 from hours 0 to 12 of day 2, and pair 4 from hours 12 to 24 of day 2. Figure 2 (solid line) shows the rate of incorporation for each 6 hours of the 48-hour experiment. There is a clear rhythm in which the values for hours 6 to 12 on both days are highest and those for hours 18 to 24 are lowest. The values

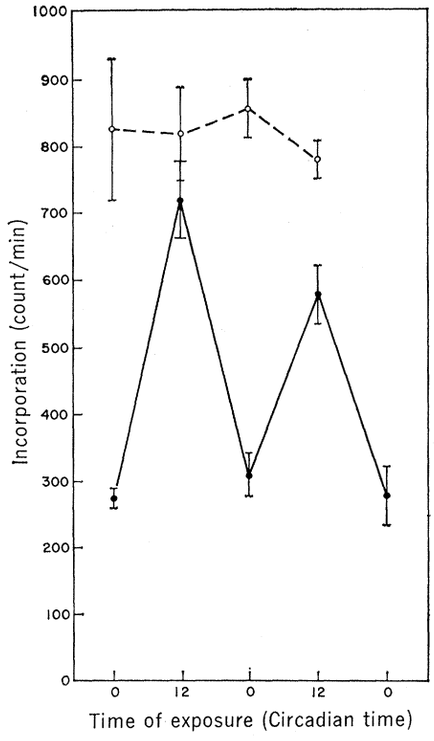

Fig. 1. Daily rhythm in amino acid incorporation in *Euglena gracilis*; cultures maintained in cycle of 12 hours light, 12 hours dark (lights on 0 to 12). (Solid line) Incorporation of ^{14}C-phenylalanine (1 hour exposure) in darkness. (Broken line) Same as solid line, except incorporation measured in the light. Bars above and below data points represent total range of two replicate values for each pulse. Kinetics of incorporation were linear for the 1 hour.

for the second 6 hours of each 12-hour exposure were obtained by subtracting the value for the first 6 hours from that of the total 12 hours.

A second type of experiment was carried out which involved shorter exposures to isotope; isotope was added to separate cultures (in duplicate) every 6 hours throughout the cycle (Fig. 2, broken line). Even though the exposures were shorter and the values for hours 6 to 12 and 18 to 24 were obtained directly (rather than by difference as in a 12-hour pulse), the results of the two sets of data agree. In addition to the data shown in the graph, in this same experiment samples were also taken from these cultures 2 and 4 hours after addition of the isotope. The 2-hour values also showed an oscillation with a maximum from hours 10 to 12 (380 count min^{-1} ml^{-1}) and a minimum from 16 to 18 (140 count min^{-1} ml^{-1}). All other points (except one) fell on a "smooth" curve.

These experiments demonstrate, therefore, a circadian oscillation in the rate of amino acid incorporation in *Euglena*. This rhythmicity could represent changes in the rate of protein synthesis, that is, in the rate of peptide bond formation. It could also result, however, from circadian oscillations in amino acid uptake or in the size of endogenous amino acid pools. Nevertheless, it is interesting that light can supply to

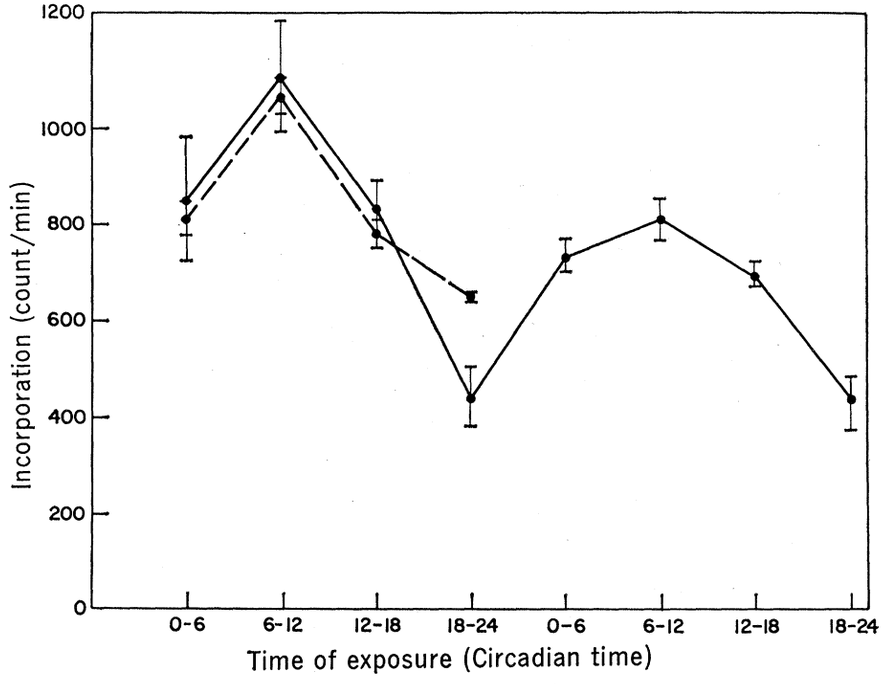

Fig. 2. Circadian rhythm in phenylalanine incorporation; cultures maintained in continuous darkness. (Solid line) Cultures exposed to isotope every 12 hours. (Broken line) Cultures exposed every 6 hours (see text). Bars above and below data points represent total range of four replicate values for each pulse.

the organism whatever limiting factor falls to a low level at hour 0 (see Fig. 1). Since the primary function of light for these autotrophic cells is to provide energy, this result may indicate a depletion of the energy pools of the cells during the night phase. Further experiments along these lines are necessary to distinguish these alternatives. Further experiments will also be necessary to determine what relationship, if any, this oscillation has to the retardation of the clock by cycloheximide and hence to the mechanism of the circadian clock.

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Cigarettes: Chemical Effects of Sodium Nitrate Content

Abstract. *Although addition of sodium nitrate to cigarettes is reported to reduce the components and properties of cigarette smoke that are associated with tumorigenicity, we find that the additive nevertheless significantly increases the levels of certain vapor-phase constituents of smoke that are known to inhibit ciliary movement; and also it produces other effects of questionable value.*

Cigarettes with $NaNO_3$ added produce smoke that is less tumorigenic and toxic (in mice) and contains less particulate matter, benzo[*a*]pyrene, nicotine, and phenol than does smoke from standard cigarettes (*1*). These effects are attributed partly to the thermal decomposition of the nitrate into oxygen and nitrogen oxides, the former enhancing combustion of tobacco and the latter inhibiting free-radical reactions leading to formation of benzo[*a*]pyrene and other polynuclear aromatic hydrocarbons.

Table 1. Yields of the components of the vapor phase of smoke (fifth puff, 35 ml) from cigarettes without or with $NaNO_3$ added; averages of ten or more determinations. The method did not distinguish between NO and NO_2; the lower limit of detection of N_2O was 1.0 μg.

Component	Without $NaNO_3$	With $NaNO_3$
	Mole percentages	
H_2	1.38	1.64
O_2	13.46	12.46
CO	3.00	3.55
CO_2	7.43	7.86
	Micrograms	
$NO+NO_2$	22.9	48.7
N_2O	<1.0	11.0
HCN	23.0	23.3
H_2S	4.5	0.3
CH_4	107.5	90.0
C_2H_6	37.8	36.4
CH_3CHO	74.0	146.3
CH_3COCH_3	40.6	55.3
CH_3CN	14.3	29.3
$CH_2=CHCHO$	9.2	18.4
CH_2O	5.3	5.6

Compounds (*2*) other than those cited above, however, are believed to contribute to the reported biological activity (*3*) of cigarette smoke. Several of these, including ciliostatic agents (*2*) and irritants (*4*), are present in the vapor phase of cigarette smoke, and their concentrations may be affected adversely by the addition of $NaNO_3$ to cigarettes. Moreover, thermal decomposition of the additive may increase the concentrations of nitrogen oxides in smoke and lead to formation of other compounds (*5*) of questionable activity such as nitroalkanes.

We have studied the effect of $NaNO_3$ on the qualitative and quantitative composition of the vapor phase of tobacco smoke. Table 1 compares the concentrations of several vapor-phase constituents of smoke from cigarettes with and without addition of 8.3 percent $NaNO_3$. The data show that addition of $NaNO_3$ leads to significantly (at least twice) greater quantities of undesirable vapor-phase constituents such as nitrogen oxides, acetaldehyde, acrolein, and acetonitrile. Acetaldehyde and acrolein strongly inhibit ciliary movement (*2*). Certain nitrogen oxides (*6*) have been implicated in ciliostasis. From the nature of the additive one expects higher concentrations of nitrogen oxides; of them, however, N_2O apparently occurs only in the smoke of treated cigarettes (11.0 μg per 35-ml puff); if it is produced by normal cigarettes, it is below the level of detection (<1 μg per 35-ml puff). Our data also show that concentrations of CO, CO_2, HCN, and HCHO remain essentially unchanged, while that of H_2S is significantly reduced. Although not indicated in Table 1, levels of low-boiling olefinic hydrocarbons are increased in proportion to decrease in levels of paraffins.

Our findings lead one to conclude that, although $NaNO_3$ reduces properties of cigarette smoke that are reportedly associated with tumorigenicity and toxicity, it increases the concentrations of certain vapor-phase constituents that play important roles in inhibition of ciliary movement; it may also lead to formation of new compounds of questionable activity.

We used commercial cigarettes (without filters and 85 mm long) that were smoked by machine. Each puff passed through a Cambridge filter before being collected in a bulb for subsequent analyses. All analyses were conducted on fifth puffs (volume, 35 ml; duration, 2 seconds; interval between puffs, 60 seconds). Reported colorimetric methods (*7*) were used for determination of HCN, H_2S, HCHO, and nitrogen oxides. Analyses by gas chromatography, with appropriate molecular-sieve or polyaromatic-resin columns, were used to determine the other components. During the smoking, the temperatures of burning were monitored with a thermocouple. Cigarettes containing $NaNO_3$ burned at 788°C; normal cigarettes, at 850°C.

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