The Circadian Rhythm in Photosynthesis in *Acetabularia* in the Presence of Actinomycin D, Puromycin, and Chloramphenicol

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ABSTRACT Anucleate *Acetabularia crenulata* shows a circadian rhythm in photosynthesis. In this study, an oxygen electrode was employed to measure this photosynthetic rhythm in the presence and absence of the inhibitors, actinomycin D, chloramphenicol, and puromycin. High concentrations of the inhibitors were used: actinomycin D, 20–40 μg ml^{-1}; puromycin, 30 and 100 μg ml^{-1}; and chloramphenicol, 250 μg ml^{-1}. The effectiveness of these inhibitors on protein synthesis was also measured under the same conditions used for the determination of rhythmicity. In spite of large effects of all three inhibitors on the incorporation of ^{14}C leucine, no effect on the period or the phase of the photosynthetic rhythm was observed. The higher concentration of puromycin and chloramphenicol produced toxic effects which were expressed as a reduction in the amount of photosynthesis, but rhythmicity was still apparent. After 3 or 4 days' exposure to actinomycin, *Acetabularia* became resistant to its effect. Recovery was also observed in the ability to incorporate leucine. The implications of these results for theories of the basic oscillator responsible for circadian rhythmicity are discussed.

INTRODUCTION

In both plant and animal cells, regular fluctuations in the rate of one, or even of several physiological processes may occur, although the environmental conditions are not changing. If such patterns repeat at intervals of about 24 hr in constant light and temperature, these fluctuations are called circadian rhythms. At the present time it is not known what controls these oscillations, but it is quite unlikely that they are governed by any environmental cyclic cues arising from the rotation of the earth, since all such cues would of necessity show exactly a 24 hr period, while the period of many circadian rhythms can be appreciably different from 24 hr. Furthermore it is not uncommon for the period to change by some small though discernible amount when the temperature is changed. Therefore it seems likely that the

mechanism which determines rhythmicity is endogenous rather than exogenous. However, in spite of extensive effort, this mechanism has not been identified. Many inhibitors which are specific for certain metabolic pathways do not appear to affect rhythmicity. The processes by which we measure the rhythm may often be partially inhibited without any change in the period or phase of the rhythm itself. This lack of feedback from the processes which show rhythmicity to the underlying rhythmic mechanism is quite characteristic of many circadian rhythms. In *Gonyaulax* for example, rhythmicity is expressed in luminescence, photosynthesis, and cell division, and all these processes show identical behavior of period and phase, as if they were all cued from a single basic oscillator. However, specific inhibitors of photosynthesis and cell division have no effect on either period or phase of the rhythm in luminescence. With most inhibitors, fluctuations can still be seen as long as any measurable metabolism remains. One exception to this generalization comes from the experiments of Karakashian and Hastings (12, 13). They found that actinomycin D inhibited the rhythm in luminescent glow in *Gonyaulax* but did not entirely suppress the glow. From this evidence they suggested that DNA-dependent RNA synthesis may be involved in the mechanism underlying this rhythmicity. Whether the site of the effect was within the primary oscillator or in the coupling sequence which must intervene between the oscillator and the actual production of the glow was not determined. Rhythmicity in photosynthesis in *Gonyaulax* was not inhibited by the same concentration of actinomycin D nor in fact by a concentration ten times higher, and the rhythms in glow and photosynthesis were neither one inhibited by chloramphenicol. Thus the meaning of the effect of actinomycin on the glow rhythm was difficult to interpret.

The nucleus is certainly not required for the continuation of rhythmicity in *Acetabularia*, since Sweeney and Haxo (21) showed that removing the nucleus in this giant cell, the cytoplasm of which is so remarkably persistent, had not the slightest effect on the rhythm in photosynthesis which this alga shows very clearly (18, 19, 21). Of course, DNA-dependent RNA synthesis was not eliminated from consideration by these experiments since the chloroplasts of *Acetabularia* have been shown to contain DNA (10), and this DNA has been shown to function in the synthesis of RNA and protein (16). It is probable that mitochondria also contain functional DNA and RNA.

The experiments reported here were designed to examine the effect of inhibitors of RNA and protein synthesis on the rhythm of photosynthesis in enucleated *Acetabularia*, in order to determine whether or not steps in this process in the cytoplasm could be implicated in the causation of rhythmicity.

MATERIALS AND METHODS

Acetabularia crenulata used in this study was collected either from the Florida Keys or from Jamaica, W. I. Plants with caps that had not yet formed reproductive cysts

were selected and were enucleated immediately by cutting off the basal portion. This procedure was adopted since plants showed a strong tendency to undergo nuclear division and form cysts within the cap on being brought into the laboratory, and, of course, such plants cannot be enucleated. Epiphytes were removed under the dissecting microscope. The plants, with caps about 1 cm in diameter and stems 1.5 cm long, were maintained in covered dishes containing autoclaved sea water in an artificial light-dark cycle (12 hr light at 250 ft-c and 12 hr darkness) at a temperature of 16–17°C. Plants were not used for experiments until at least 2 wk after enucleation and any with cysts at this time were discarded. In later experiments, plants grown from cysts in the laboratory were used. These plants were not contaminated with other algae, but were not bacteria-free.

The oxygen evolved in photosynthesis was measured by the method of Schweiger, Wallraff, and Schweiger (18), using either a Jarrell-Ash or a Beckman oxygen electrode. Four or eight *Acetabularia* cells were enclosed in a glass-stoppered tube to the base of which a stopcock and capillary had been attached. Sterile sea water, equilibrated for 15 min with 5% oxygen in nitrogen, was added and the tube was stoppered tightly. The plants were then exposed to light from a cool white fluorescent source (500 ft-c for 1 hr at 17°C). At the end of the light exposure, the oxygen in the tube was measured by introducing the water into a small closed chamber with a tiny magnetic stirrer and the electrode. The volume of this chamber was 3.5 ml and that of the water sample was 12 ml so that it was possible to rinse the electrode chamber thoroughly between measurements. This whole apparatus was immersed in a water bath at 16°C. Two oxygen measurements were made from each tube and two to three replicate tubes for each treatment were used. Three tubes without plant material but filled with sea water at the same time and measured at the same time as tubes containing plants served as blanks. The difference between the oxygen content of the tubes with *Acetabularia* and these blanks was used as the measure of the rate of photosynthesis. Measurements were usually made at 1100–1200 and at 2300–2400 each day during the course of an experiment. The method measures net photosynthesis. However, no correction was made for respiration since preliminary experiments had shown oxygen uptake in darkness to be small relative to photosynthesis and constant with time, not rhythmic.

That the measurements did not themselves affect rhythmicity was determined in experiments in continuous light when a different group of plants was used for each oxygen determination (Fig. 4).

When inhibitors were used, they were usually first added at the beginning of a dark period. This procedure was adopted to minimize inactivation by light in the case of actinomycin D. The oxygen measurement during the following night was omitted. Inhibitors were never present during the hour long measurement of photosynthesis. Thus colored inhibitors such as actinomycin D did not interfere with light absorption for photosynthesis. Fresh inhibitor solution was added at the end of each period of oxygen measurement.

Between measurements of photosynthesis, the plants, still in the stoppered tubes but immersed in fresh sea water or in the inhibitor solution in sea water, were returned to the maintenance conditions, either a light-dark cycle or continuous light (50 ft-c) at 16–17°C.

The incorporation of L-leucine uniformly labeled with ^{14}C into protein was measured by an adaptation of the method of Mans and Novelli (14). Young enucleated plants, with a total length of about 300 mm per aliquot, were pretreated with inhibitors for various times, either in alternating light and darkness, or in continuous light of 45 ft-c intensity at 16°C. They were then exposed to radioactive leucine, 0.5 mc in 10 ml sea water containing 5×10^{-4} M penicillin G, with or without inhibitors for 5 hr at 28°C in light (800 ft-c intensity). Plants were rinsed three times with sea water and homogenized in sea water. The volume of each homogenate was made up to 1 ml, and 0.1 or 0.2 ml aliquots were pipetted to discs of No. 5 Whatman paper impaled on pins. After drying briefly in air, the discs were placed in 10% cold TCA containing 0.1 M nonradioactive leucine and allowed to stand overnight at 4°C. The filters were then treated with 5% TCA at 90°C for 30 min to remove nucleic acids and subsequently dried in ethanol and ether. The radioactivity was counted in an Ansitron liquid scintillation spectrometer in 10 ml Bray's solution.

Actinomycin D was the gift of Merck, Sharp and Dohme Research Laboratory, chloramphenicol was obtained through the courtesy of Parke, Davis and Company, and puromycin was purchased from Sigma Chemical Company. L-Leucine uniformly labeled with ^{14}C, specific activity 94.7 mc/mM, was obtained from Calbiochem.

RESULTS

When grown in a light-dark cycle, *Acetabularia* showed a marked rhythm in photosynthesis as previously described (21). Light-dark cycles are not necessary for the manifestation of this rhythm (18, 21), but they serve to entrain the rhythm so that the phase can be predicted. Previous experiments have shown that on a light-dark cycle (12 hr light; 12 hr darkness), the maximum photosynthesis occurs in the middle of the light period. The first experiments on the effect of inhibitors on the photosynthesis of *Acetabularia* were thus carried out using plants on this light-dark cycle (Fig. 1). The rate of photosynthesis was reduced by the time the first measurement was taken after the addition of actinomycin D (20 μg ml^{-1}), an indication that the inhibitor was able to penetrate into the chloroplast. The amount of inhibition appeared to be greater during the day and this effect was progressive, so that little rhythm was observed on the 3rd day after addition of the inhibitor. On the 4th day, however, although fresh actinomycin D was added after each measurement as usual, the effect of the inhibitor began to diminish, and by the 7th day an appreciable rhythm was restored. This recovery could not be attributed to destruction of actinomycin in the sea water solution since freshly prepared solutions were no more inhibitory than those stored at 4°C in the dark before use. Doubling the actinomycin D concentration produced neither greater nor more persistent inhibition. Lower concentrations of actinomycin D (0.1, 0.5, and 1.0 μg ml^{-1}) inhibited neither photosynthesis nor rhythmicity.

Puromycin was used as an inhibitor in one experiment in which the plants were kept on light-dark cycles. At the concentration used (30 μg ml^{-1}), this inhibitor showed no effect on either photosynthesis or rhythmicity. Chlor-

amphenicol (250 μg ml^{-1}) did not affect rhythmicity in *Acetabularia* kept on a light-dark schedule, but like actinomycin, brought about a reduction in photosynthesis.

Parallel experiments in which the incorporation of labeled leucine into protein was measured after 3 or 7 days' exposure to actinomycin D (20 μg ml^{-1}), puromycin (30 μg ml^{-1}), and chloramphenicol (250 μg ml^{-1}) in alter-

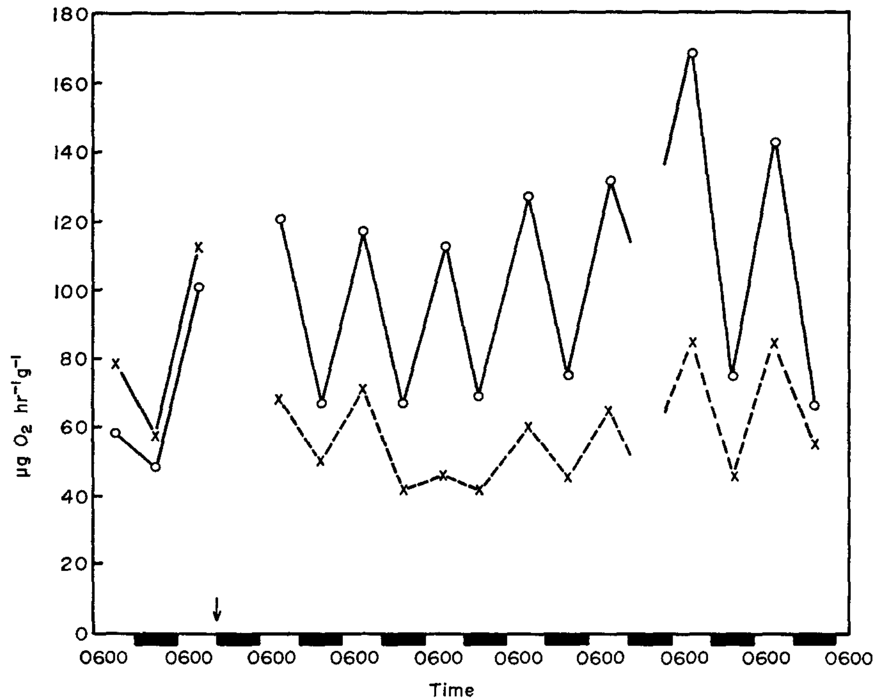

FIGURE 1. The effect of actinomycin D on the rhythm in photosynthesis in *Acetabularia crenulata* in alternating light and darkness. Plants with actinomycin D, 20 μg ml^{-1} (×), control plants without actinomycin D (open circles). The actinomycin was first added at the arrow and again after every subsequent oxygen determination. The black bars on the abscissa each represent a 12 hr dark period ending at 0600 eastern standard time. Ordinate is net oxygen evolution at 500 ft-c and 17°C. The points represent averages of three groups of four plants each. All plants had caps and were anucleate.

nating light and darkness (Table I, Experiment I), showed that protein synthesis had been inhibited by these substances 46, 37, and 44% respectively. The smaller effect of puromycin is consistent with the lack of inhibition of photosynthesis by this inhibitor at this concentration. After about 7 days' treatment in actinomycin, no inhibition of incorporation was found, in keeping with the recovery also observed in photosynthesis.

That the inhibitors at the concentrations employed did not prevent the light-dark cycle from entraining the rhythm in photosynthesis was shown in experiments in which plants received one double light period in the presence

and absence of inhibitors. This procedure brings about an inversion of the light-dark cycles. Also none of the inhibitors prevented the rhythm from becoming reversed. Thus in experiments in which the plants were exposed to a light-dark cycle, even in the presence of inhibitors the phase will be held fixed by this cycle. Complete inhibition of rhythmicity would be observable. In order to be able to observe changes in period or phase, however, an experimental regime without entraining signals is necessary. Hence, experiments were carried out in which *Acetabularia* was maintained in continuous light and this light was of relatively low intensity (45–50 ft-c) to prevent the dis-

TABLE I

THE INCORPORATION OF L-LEUCINE $u^{14}C$ INTO PROTEIN BY ENUCLEATED *Acetabularia crenulata* IN THE PRESENCE OF ACTINOMYCIN D, PUROMYCIN, AND CHLORAMPHENICOL

The figures are for the incorporation by 300 mm of fragments at 28°C. in light (800 ft-c) for 5 hr in the presence of 5×10^{-4} M penicillin G.

Experiment:	IA-LD		IB-LD		II-LL	
Pretreatment duration, *hr*	63		159		87.5	
Inhibitor	*cmp*	−%	*cmp*	−%	*cmp*	−%
None	39,685		55,460		35,800	
Actinomycin D						
20 μg ml^{-1}	21,655	46	66,925	0	16,900	53
40 μg ml^{-1}	—		—		20,300	44
Chloramphenicol						
250 μg ml^{-1}	22,140	44	—		19,200	46
Puromycin						
30 μg ml^{-1}	25,155	37	—		—	
100 μg ml^{-1}			—		6,750	81

appearance of rhythmicity (Figs. 2 and 3). Actinomycin D (20 μg per ml), puromycin (100 μg/ml), or chloramphenicol (250 μg per ml) was introduced and replaced as in the experiments described above. In the presence of actinomycin D, photosynthesis was at first inhibited (Fig. 2), more during the day phase than during the night phase, so that rhythmicity was almost lost during the second cycle. Recovery from the effect of actinomycin D also again occurred. Chloramphenicol inhibited photosynthesis but rhythmicity continued in the presence of this inhibitor.

In the course of the experiment shown in Fig. 2, measurements were made every 12 hr. Since the same plants were used repeatedly it was possible that exposure to the bright light might alter the subsequent course of the rhythm in photosynthesis. To test this possibility, an experiment was carried out in which new plants were used for the determination of each point and the resulting curve was compared with one obtained by repeated measurement of the same plants (Fig. 4). No major phase discrepancy was observed. In both this

experiment and that of Fig. 2 the period of the rhythm in photosynthesis was unusually long, almost 40 hr. Perhaps this is because the temperature is abnormally low for such a tropical species.

To follow more closely the course of the rhythm in continuous light in the presence of inhibitors, plants were exposed to actinomycin D (20 μg ml^{-1}), puromycin (100 μg ml^{-1}), and chloramphenicol (250 μg ml^{-1}) for 85 hr at 45 ft-c light intensity before oxygen measurements were made. Frequent

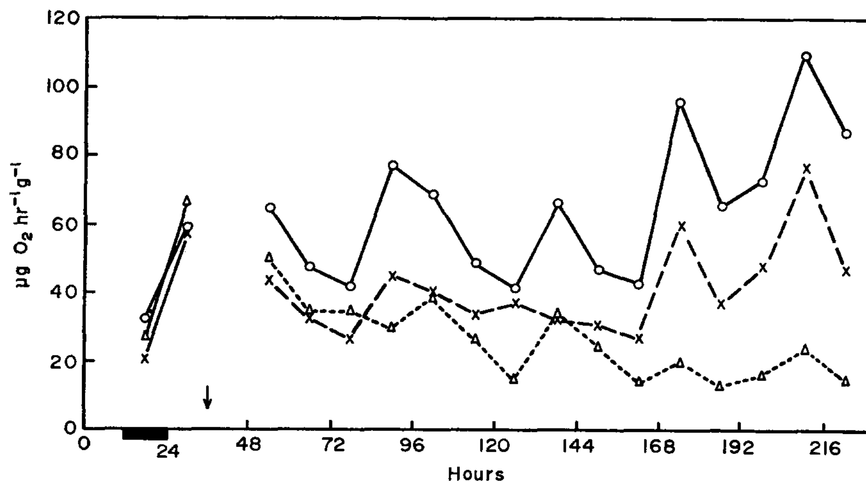

FIGURE 2. The effect of actinomycin and chloramphenicol on the rhythm in photosynthesis in anucleate *Acetabularia crenulata* in continuous light, 50 ft-c. Actinomycin D, 20 μg ml^{-1} (x), chloramphenicol, 250 μg ml^{-1} (open triangles), control (open circles). Inhibitors were first added at arrow and again after every subsequent oxygen determination. The arrow also represents the beginning of the exposure to continuous light of 50 ft-c intensity, ordinate is net oxygen evolution at 17°C and 500 ft-c. The points represent averages of three groups of four plants each.

determinations were then made over the course of several days with special attention to determining the phase in each case. The results are shown in Fig. 3. It is clear that the phase of plants pretreated with actinomycin D and chloramphenicol is not different from that of untreated plants. In an experiment carried out in conjunction with that of Fig. 3, the incorporation of leucine after 87½ hr exposure to the same inhibitors was appreciably inhibited (Table I, Experiment II). Inhibition was in no case complete, however. Doubling the concentration of actinomycin D did not increase the inhibition, as was to be expected from the observation reported above that the inhibition of photosynthesis was no greater at 40 than at 20 μg ml^{-1} actinomycin.

To summarize, then, protein synthesis in enucleated *Acetabularia crenulata*,

as in other kinds of cells which have been investigated, appears to be inhibited by actinomycin D, chloramphenicol, and puromycin. The effect of actinomycin is assumed to be indirect via an inhibition of the synthesis of mRNA.

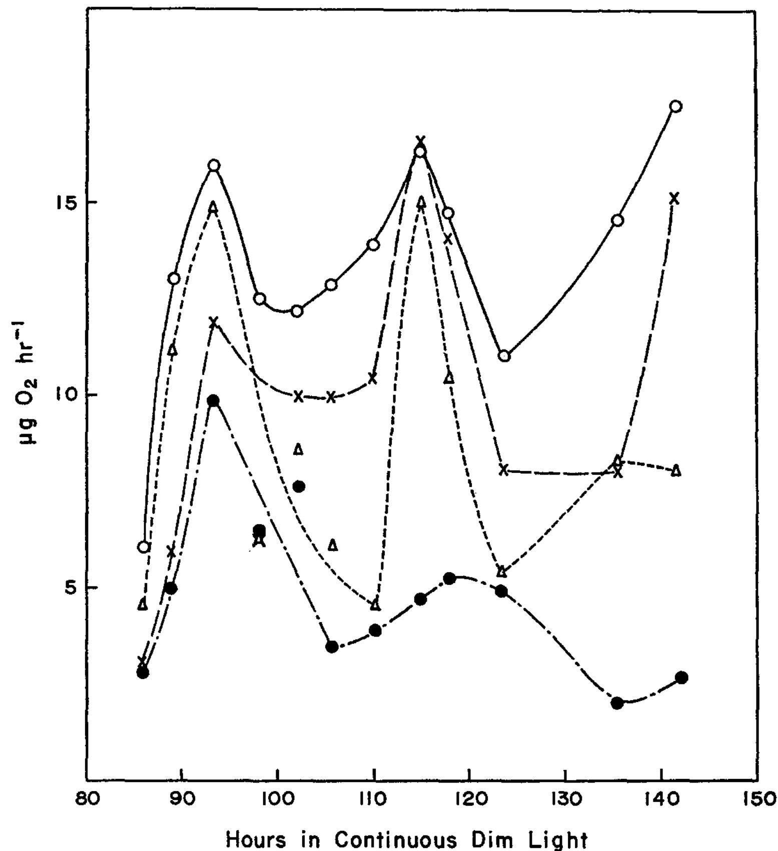

FIGURE 3. The rhythm in photosynthesis in anucleate *Acetabularia crenulata* in continuous light of 45 ft-c intensity during the 4th, 5th, and 6th days in the presence of actinomycin D, 20 μg ml^{-1}(×); puromycin, 100 μg ml^{-1} (filled circles); or chloramphenicol, 250 μg ml^{-1} (open triangles). Control without inhibitors (open circles). Abscissa, time in hours since the plants were placed in continuous light and the inhibitor added. Each point is the average oxygen evolution of two groups of young plants without caps. Each group originally contained a total of 300 mm of plants.

The presence of these inhibitors, however, neither brings about the loss of rhythmicity, nor is the period or the phase of the rhythm in photosynthesis appreciably altered.

DISCUSSION

The most interesting problem in the study of circadian rhythms at the present time is the identification of an oscillator at the molecular level which all evidence indicates must exist within the individual cell and by which the rhythms

which we observe in various physiological functions are controlled. There is almost no positive evidence concerning the nature of this oscillator, but it is insensitive to most specific inhibitors as well as to temperature level. However, the evidence of Karakashian and Hastings (12, 13) that actinomycin D inhibits rhythmicity of the luminescent glow in *Gonyaulax* without completely

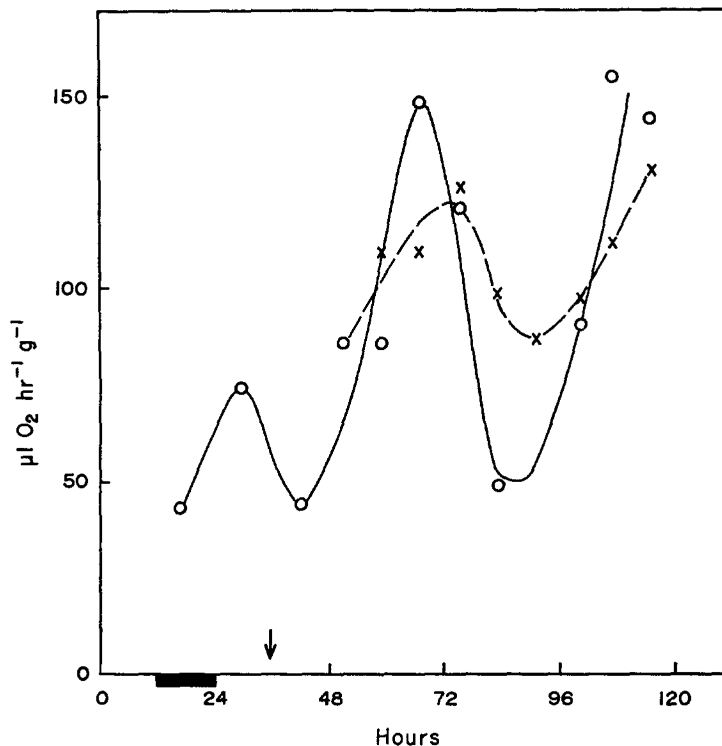

FIGURE 4. A comparison of the rhythm in photosynthesis in anucleate *Acetabularia crenulata* plants with caps measured repeatedly with one group of plants as in experiments shown in Figs. 1–3 (×); or using a different group of plants for each point (open circles). Plants were placed in continuous light, 50 ft-c, at the arrow on the abscissa. Each group contained eight plants with caps.

extinguishing this glow provided a suggestion that the synthesis of messenger RNA on a DNA template might have some connection with the postulated oscillator, although the lack of inhibition by chloramphenicol and other inhibitors of protein synthesis which act at sites removed from the synthesis of RNA seemed to rule out the intervention of protein synthesis as a whole.

Two other studies have yielded evidence of the involvement of an actinomycin-sensitive step in the primary oscillator. Strumwasser (20) found that an isolated neuron from the parietovisceral ganglion of the sea hare *Aplysia* in constant conditions produces a burst of spikes of intracellular electrical potential about the time that the light would have gone on in the light-dark

cycle to which the animal had been accustomed, and another smaller spike about 24 hr later. Hence this cell shows circadian behavior. When actinomycin is injected into the neuron before the first spike has occurred, this spike is induced at once, and the next appears 24 hr after the injection of actinomycin. Adding the inhibitor after the first spike has no effect. Strumwasser interprets his results to mean that actinomycin displaces prematurely from its DNA template an RNA which carries the information for the synthesis of a depolarizing substance. Since the rhythm in spike output started afresh after the actinomycin injection, the cyclic production of this mRNA could be the basic oscillator. This possibility has not yet been examined further, however.

One additional study concerns the effect of actinomycin on rhythmicity. This is an unpublished investigation of Vanden Driessche on the photosynthetic rhythm in *Acetabularia mediterranea*, which Brachet mentions very briefly (3, 4). He states that when rhythmicity is lost after long exposures to bright continuous light, it cannot be reinstated if actinomycin is present. Such a finding could mean only that new mRNA is necessary for rebuilding some component of the basic oscillator, but is not required for its day-to-day function. Incidentally, Vanden Driessche also reports that she is able to correlate changes in chloroplast shape with the photosynthetic rhythm in *Acetabularia mediterranea* (22). However, we have not been able to find rhythmic morphological changes in *Acetabularia crenulata* by light microscopy and photomicrography.

The results of the experiments reported here show that in *Acetabularia crenulata* without a nucleus, rhythmicity can continue essentially unchanged in the presence of high concentrations of the inhibitors, actinomycin D, puromycin, and chloramphenicol, concentrations which do affect protein synthesis in this alga. In the face of evidence to the contrary, is it justified to cite this as evidence that steps in the synthesis of mRNA and protein are hence not a part of the basic oscillator responsible for this rhythm?

The first problem concerns the type and degree of inhibition brought about by these inhibitors in this tissue. Eisenstadt and Brawerman (8) have shown that the incorporation of labeled amino acids into protein in isolated chloroplasts from *Euglena* is sensitive to puromycin, less so to actinomycin, and insensitive to chloramphenicol. In *Acetabularia*, actinomycin C and D (10–40 $\mu g\ ml^{-1}$) and puromycin (3–30 $\mu g\ ml^{-1}$) cause marked reduction in growth and cap formation (2, 5, 6, 17, 23). The morphology of the nucleus and chloroplasts appears to be modified by the presence of actinomycin D (10–20 $\mu g\ ml^{-1}$) and puromycin (10 $\mu g\ ml^{-1}$) (1, 3, 6). Therefore there seems to be no doubt that these inhibitors can penetrate into the cytoplasm of this alga. The chloroplasts of *Acetabularia* have been shown to contain DNA (10), and this DNA can function as an RNA template in isolated chloroplasts, since RNA can be synthesized in such chloroplasts (15). That this RNA synthesis is inhibited by actinomycin D is suggested by the inhibition of uracil incor-

poration in the presence of this inhibitor (16). Brachet and Goffeau (7, 11) have demonstrated that chloroplasts isolated from anucleate fragments of *Acetabularia mediterranea* do incorporate labeled amino acids, and that this incorporation is inhibited by actinomycin D, chloramphenicol, and puromycin. However, high concentrations were required and the inhibition was incomplete. Our experiments on the incorporation of label from leucine ^{14}C by intact anucleate cells also show that all these inhibitors at the concentrations employed cause marked although not complete inhibition.

The finding that actinomycin, puromycin, and chloramphenicol do not inhibit protein synthesis 100% is almost universal (11, 16). In most cases, the maximum inhibition is about 50%, and increasing the inhibitor concentration does not increase the inhibition. An exception is the inhibition of the incorporation of label from amino acids by very high concentrations of puromycin (167 $\mu g\ ml^{-1}$) which Goffeau and Brachet (11) report may reach 100% under special conditions. Several explanations for a maximum inhibition of 50% have been suggested. Schweiger and Berger (16) find that a part of the label from uracil is not rendered soluble by RNAase and hence may be in substances other than RNA. Long lived mRNA's have been postulated in *Acetabularia* to account for the long survival of this cell in the anucleate condition. Such mRNA's may be protected from attack in stable complexes.

The presence of bacteria in all the plant material except that of Gibor (9, 10) renders the interpretation more difficult, although infection was controlled by penicillin and the use of short exposure times. The fact that Gibor (9) obtained inhibitions of similar magnitude with actinomycin D in bacteria-free *Acetabularia* suggests that the intervention by bacteria is not quantitatively important.

The second question concerns what one would expect to observe were the basic oscillator inhibited by inhibitors of protein synthesis. Effects at the level of the oscillator must be distinguished from those on processes which are only controlled by this oscillator, photosynthesis in this case. Thus only changes in period and phase are usually considered significant in this respect. In certain cases, however, the disappearance of rhythmicity may be detected, provided the process by which rhythmicity is measured is not itself completely inhibited, as in the experiments of Karakashian and Hastings with actinomycin D (12, 13). Our experiments on plants in a light-dark cycle (Fig. 1) were designed with the hope of detecting a similar effect of actinomycin. However, in *Acetabularia* the complete inhibition of rhythmicity was not observed, and partial inhibition under these conditions cannot be considered of any significance for the basic oscillator so long as any rhythmicity can be detected. The effects indicate rather an inhibition of photosynthesis.

The rather unprecedented recovery from the effects of actinomycin shown by both photosynthesis and leucine incorporation warrants brief comment. Possibly an enzyme or microorganism capable of breaking down actinomycin

is induced after 4–5 days in the presence of this substance. As a result of this phenomenon, it is not possible to observe the inhibitory effects of actinomycin for an unlimited time.

The failure to observe 100% inhibition of rhythmicity is not surprising, in view of the observation that protein synthesis shows only partial inhibition. But what sort of effect might one expect to observe were the basic oscillator, like protein synthesis, 50% inhibited? Such an inhibition in a step basic to the oscillator might be expected to slow its operation so that its period would be lengthened. The experiments using plants kept in continuous light were designed to detect changes in period in the rhythm of photosynthesis. A period 50% longer than usual would certainly have been detected, but no such change was observed. If the basic oscillator is compensated with respect to chemically active substances, as it appears to be for temperature, then a much smaller increase or even a decrease in period might result from 50% inhibition. Small changes in period accumulate over the course of a number of cycles, however, and hence would be detected in an experiment running a relatively long time, like those reported here. Such changes were not found.

What then can be concluded from these experiments concerning the nature of the basic oscillator? Since the synthesis of protein is not completely inhibited by any of the inhibitors used, the participation of a special inhibitor-resistant path of protein synthesis in the clock cannot be eliminated. However, it seems unlikely that this resistant path could involve protected mRNA complexes, since if their activity is cyclic, they would be expected to become unprotected at some time in this cycle. Participation of an inhibitor-sensitive step in protein synthesis in rhythmicity should be manifest in changes in period, and none was observed. Therefore it seems likely that we should look elsewhere than in the steps leading to RNA and protein synthesis for the primary oscillator which determines the circadian rhythm in photosynthesis in *Acetabularia crenulata*, and perhaps other circadian rhythms as well.

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