

Effects of Chloramphenicol on the Circadian Rhythm of *Neurospora crassa*¹

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JOHN G. FRELINGER² HARVEY MOTULSKY³ AND DOW O. WOODWARD
Department of Biological Sciences, Stanford University, Stanford, California 94305

ABSTRACT

Chloramphenicol, an inhibitor of mitochondrial protein synthesis, shortened the period length of the circadian rhythm in the *Timex* strain of *Neurospora crassa* by 2 hours. Both the L(+) threo and D(-) threo optical isomers had the same effect on the period of the rhythm, whereas only the D(-) threo isomer significantly inhibited mitochondrial protein synthesis. Tetracycline, another inhibitor of mitochondrial protein synthesis, did not change the period of the circadian rhythm. The effect of chloramphenicol on the circadian rhythm is, therefore, presumably not directly related to inhibition of mitochondrial protein synthesis, suggesting that chloramphenicol has other effects.

MATERIALS AND METHODS

Growth Conditions. For these experiments, we used the *Timex* strain of *Neurospora crassa* which was isolated and characterized by Sargent *et al.* (15, 16). Glass tubes 60 cm long and 13 mm in diameter were half filled with 18 ml of medium containing Vogel's solution (20), 0.5% sucrose, 0.5% casamino acids, and 1.5% bacto agar. A chloramphenicol isomer (max. 2 mg/ml) or tetracycline (max. 0.75 mg/ml) was added to the medium after autoclaving. The chloramphenicol isomers were added directly to the medium. Tetracycline was dissolved in sterile H₂O which was then added to an equal volume of double strength medium at 50 C. The tubes were inoculated at one end with conidia from a 5-day-old culture. They were then placed in a constant temperature incubator inside a darkroom and were observed thereafter only with red light. The mycelia grew down the length of the tube at a rate of about 6 cm/day, periodically producing conidial bands. The conidial bands were produced approximately every 22 hr and this rhythmic expression is circadian as defined by Sussman *et al.* (17).

Calculation of Period. After inoculation, 2 days were allowed for germination and for the first conidial band to be formed. Every day thereafter the position of the growing front was marked on the tube. At the conclusion of the experiment, the position of the center of each conidial band relative to these "marks" was used to calculate when that band was produced. The times calculated were plotted against the band number. The slope of this linear plot, as determined by linear regression, gave the period of the rhythm for the five replicate tubes.

Mitochondrial Protein Synthesis Inhibition. We used a method similar to Lansman's (10) in which the incorporation of labeled leucine into mitochondrial protein was measured after treatment with cycloheximide. Flasks containing 500 ml of sterile medium (Vogel's minimal medium, 0.5% sucrose, 0.5% glucose) were inoculated with 10⁶ conidia/ml and were shaken at 200 rpm at 30 C for about 20 hr. The cells were separated from the liquid medium by filtering through Miracloth (a wide mesh filter) and were then resuspended in 500 ml of fresh medium containing the antibiotic. These solutions had been prepared 4 days earlier to control for any possible decomposition of the antibiotics during the circadian rhythm experiments. After waiting 10 min, cycloheximide was added to a final concentration of 25 μg/ml to inhibit cytoplasmic protein synthesis. The cultures were then again shaken at 200 rpm at 30 C. Two min later, 100 μCi of ³H-leucine were added and the cultures were left on the shaker. After 18 min, the mycelia were filtered onto Miracloth and protein synthesis was halted by the addition of ice water. The mycelial mats were homogenized with 0.5-mm glass beads in STE buffer in a Braunmill cell homogenizer. The homogenate was centrifuged twice at 2000g for 10 min to remove cell debris. The 2000g supernatant was centrifuged at 20,000g for 30 min to isolate the mitochondria. The mitochondrial pellets were suspended in STE buffer and centrifuged for 20 min at 30,000g.

Circadian rhythms provide one type of eucaryotic cell regulation and have been described in a variety of organisms. Despite much work, very little is known about the biochemical basis of the "biological clock." One experimental approach has been to use chemicals and drugs to alter the rhythm. Heavy water (D₂O) (2), ethanol (5), lithium (4), and cycloheximide (6) have all been reported to increase the period of the rhythm in at least one organism. Many biological processes are presumably affected by these chemicals, and it is, therefore, difficult to make inferences regarding the basic biological mechanism of the circadian clock from such studies. There have been no previous reports of chemicals decreasing the period of a circadian rhythm.

Circadian rhythms have not been clearly demonstrated in procaroytic organisms, although all studied eucaryotic organisms do exhibit a rhythm. Because only eucaryotes have mitochondria, there has been speculation that mitochondria have a role in circadian rhythms (1). We, therefore, studied the effect of an inhibitor of mitochondrial protein synthesis, chloramphenicol, on the period of the circadian rhythm in *Neurospora crassa*.

There are L(+) threo and D(-) threo⁴ optical isomers of chloramphenicol. D(-) Chloramphenicol inhibits mitochondrial protein synthesis (9, 13, 19), but L(+) chloramphenicol does not (7). In this paper, we report that both isomers of chloramphenicol decrease the period length of the circadian rhythm in *Neurospora crassa* as expressed by periodic conidia formation.

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Present address: Division of Biology, California Institute of Technology, Pasadena, California.

³ Present address: University of Washington School of Medicine, Seattle, Washington.

⁴ Abbreviations: D(-) chloramphenicol: D(-) threo chloramphenicol; L(+) chloramphenicol: L(+) threo chloramphenicol.

The acid-insoluble mitochondrial proteins were separated from free leucine by solubilization in 0.2 N NaOH and subsequent precipitation in a slight excess of trichloroacetic acid. This procedure was repeated four times. After the last precipitation, the proteins were dissolved in 0.2 N NaOH to a concentration of about 10 mg/ml and filtered through a Millipore filter to exclude any precipitated proteins. Radioactivity was determined with a scintillation counter using a mixture of 0.2 ml of the mitochondrial protein solution, 2 ml of NCS tissue solubilizer, and 10 ml of Omnifluor toluene. The protein concentration was determined by the Lowry method (12). The amount of radioactivity/mg of mitochondrial protein is a measure of mitochondrial protein synthesis during the 18-min labeling period.

Degree of Racemization. To test the possibility of racemization, the optical activity of chloramphenicol solutions was measured before and after autoclaving. The solutions were Vogel's minimal medium with a chloramphenicol concentration of 3.5 mg/ml. The measurement of optical activity was made in a polarimeter using the sodium D line. All solutions were equilibrated to 20 C inside the machine. Water lost through evaporation during autoclaving was replaced.

RESULTS

Alteration of the Period of the Circadian Rhythm. We measured the period of the circadian rhythm of *Neurospora* as

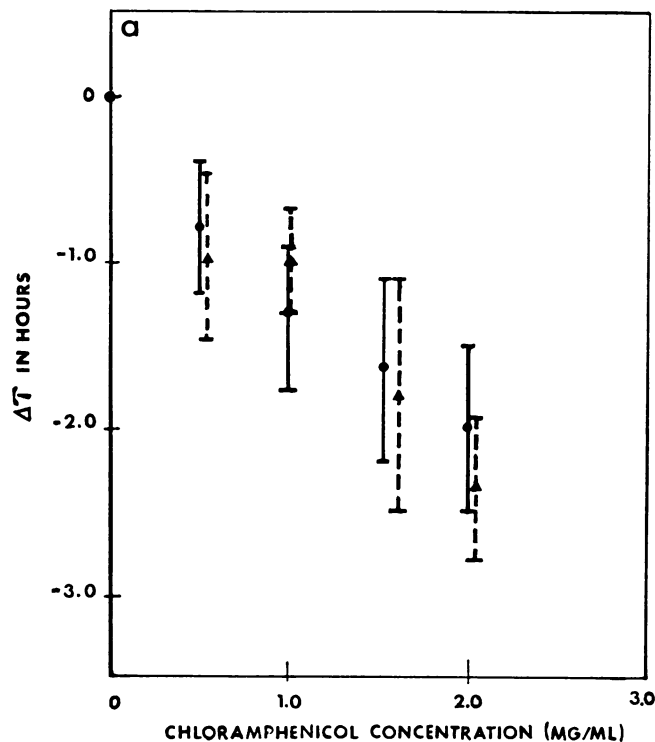


Fig. 1. a: Effects of L(+) and D(-) isomers of chloramphenicol on the period of the circadian rhythm in *Neurospora*. Experiments were run with five replicate tubes for 7 days. Approximately nine experiments were run at each concentration of the D(-) isomer and four experiments were performed at each concentration of the L(+) isomer. The results obtained at 28 and 32 C were not significantly different, and the results were pooled. ΔT is defined as the period of the circadian rhythm in the experimental tubes minus the period of the rhythm in paired controls. The average period change is indicated by (●) for the D(-) isomer and (▲) for the L(+) isomer. The bars represent the 95% confidence limits. b: Effects of L(+) and D(-) isomers of chloramphenicol on mitochondrial protein synthesis. Cells were grown for 20 hr and then transferred to 4-day-old media containing the chloramphenicol isomer. Cycloheximide was added to a final concentration of 25 $\mu\text{g/ml}$ to inhibit cytoplasmic protein synthesis, followed in 2 min by an 18-min pulse of 100 μCi of ^3H -leucine. The mitochondrial proteins were extracted and the amount of ^3H -leucine incorporated was determined. The amount of incorporation/mg of mitochondrial protein is a measure of how much protein synthesis occurred in the mitochondria. Each point represents the average of at least two experiments. The per cent inhibition is defined as follows:

$$\% \text{ inhibition} = 100 \times \left[1 - \frac{I(\text{experimental})}{I(\text{control})} \right]$$

I = the amount of ^3H -leucine incorporation/mg of mitochondrial protein.

expressed by periodic conidiation in the presence of various concentrations of chloramphenicol (Fig. 1a). In the presence of D(-) chloramphenicol, this period was decreased, suggesting that the biological clock was "running faster." The experiments were carried out at both 28 and 32 C with similar results. Experiments were not reported at other temperatures because at lower temperatures, growth was too drastically inhibited by the drug, and at higher temperatures, too few conidia were produced. The drug effect was dependent on concentration; the largest average effect occurred at a concentration of 2 mg/ml and changed the period by about 2 hr (10%). The largest change observed in a single experiment was 3.2 hr.

The effect of L(+) chloramphenicol on the circadian rhythm of *Neurospora* is also shown in Figure 1a. The D(-) and L(+) isomers had similar effects on the rhythm at all concentrations and at both temperatures. During the course of the experiment, there was no discernible change in the length of the altered period.

Inhibitory Effects of Chloramphenicol. The effects of chloramphenicol isomers on mitochondrial protein synthesis are shown in Figure 1b. The D(-) isomer inhibited mitochondrial protein synthesis almost completely at all concentrations used. The L(+) isomer, in the range of concentrations used to affect the circadian rhythm, decreased protein synthesis in mitochondria by less than 15%.

Racemization. The effects of the chloramphenicol isomer on

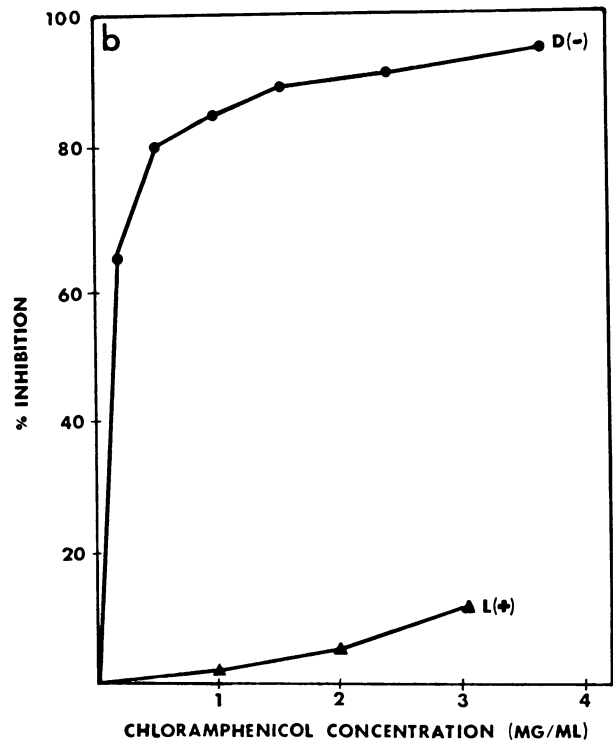


Table I. Growth Rate

	0 mg/ml	mg/ml Chloramphenicol			
		0.5 mg/ml	1.0 mg/ml	1.5 mg/ml	2.0 mg/ml
28 C					
L+	43.8(3.7) ¹	36.2(6.3)	28.9(5.5)	24.3(7.3)	19.9(8.2)
D-	43.8(3.7)	37.8(5.1)	30.4(4.9)	29.8(6.1)	22.3(5.9)
32 C					
L+	55.7(3.0)	54.0(5.2)	50.9(6.1)	32.6(7.4)	26.1(6.2)
D-	55.7(3.0)	56.7(3.8)	50.1(4.3)	45.6(4.4)	35.8(5.5)

¹ Numbers in () represent standard deviation.

the rate of growth in race tubes are shown in Table I. Since optical isomers were used, it was necessary to show that no racemization occurred. Autoclaving did not significantly change the optical activity of the D(-) or L(+) isomer of chloramphenicol (data not shown). In all other experiments, chloramphenicol was always added to the media after autoclaving, providing even less possibility for racemization.

These experiments would not detect any *in vivo* enzymic racemization. Racemization *in vivo* could significantly alter the conclusions of these experiments only if it occurred on the agar growth tubes during measurements of the circadian period and not in the liquid cultures during determination of mitochondrial protein synthesis inhibition.

Effects of Tetracycline. Tetracycline at a concentration of 0.75 mg/ml inhibited protein synthesis in mitochondria by 60% yet did not significantly change the period of the circadian rhythm (data not shown).

DISCUSSION

We have shown that both optical isomers of chloramphenicol decrease the period of the circadian rhythm in *Neurospora* equally as expressed by periodic conidiation. Since the effects of the two chloramphenicol isomers on mitochondrial protein synthesis are quite different, the simplest interpretation is that chloramphenicol does not exert its effect on the circadian rhythm by inhibiting mitochondrial protein synthesis. This explanation is supported by fact that tetracycline inhibits mitochondrial protein synthesis while leaving the period of the circadian rhythm unchanged.

Although we have shown that only the D(-) isomers of chloramphenicol significantly inhibits total mitochondrial protein synthesis, it is conceivable that both isomers inhibit the synthesis of a specific small class of mitochondrial proteins involved with the circadian clock. This hypothesis would be consistent with our results only if the synthesis of such a class of mitochondrial proteins were unaffected by tetracycline.

Since Sweeney (18) previously reported that chloramphenicol had no effect on the circadian rhythm of *Acetabularia*, chloramphenicol may not be altering a component common to all circadian mechanisms. Alternatively, the measurements of the circadian period in *Acetabularia* may not be sensitive enough to detect moderate changes.

In yeast, both isomers of chloramphenicol have been shown to interfere with electron transport (9), as well as possibly having more general membrane effects (8, 11) not related to inhibition of protein synthesis in mitochondria. Therefore, an alteration of the electron transport system or some membrane change may be the mechanism by which chloramphenicol alters the circadian rhythm. The destabilization of membranes possibly caused by chloramphenicol is consistent with both Bünning's (3) Njus' (14) models that membranes are involved in the circadian rhythm.

Preliminary experiments with valinomycin, an ionophore, indicate that it has a qualitative effect similar to chloramphenicol (unpublished results).

Since chloramphenicol apparently accelerates the biological clock, it may interfere with a rate-limiting modulator that normally slows down the clock.

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