A MODEL FOR RHYTHMIC AND TEMPERATURE-INDEPENDENT GROWTH IN 'CLOCK' MUTANTS OF NEUROSPORA

by

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(with 6 Figs.)

Introduction

One characteristic of circadian rhythmicities is a steadystate period which is almost constant over a range of temperatures. Such freedom from control by temperature is of great importance where regulation of physiological and biochemical processes is time-dependent (Sweeny & Hastings, 1960). However, aspects of living organisms other than rhythms may be temperature-compensated (Pittendrigh, 1960) although only a few have been shown to be so (cf. Burckhardt, 1959).

The isolation of a strain of *Neurospora crassa* exhibiting a growth rhythm in the form of mycelial bands produced approximately once a day (Sussman *et al.*, 1964) provided an organism in which the dependence of the rhythm upon temperature could readily be studied. Previously, a rhythm in the production of conidia in the 'patch' mutant of *Neurospora* had been shown to have a circadian periodicity relatively independent of temperature between 24 and 31° C (Pittendrigh *et al.*, 1959). The Q_{10} for the period of the rhythm in the formation of conidiophores of different height and density in *Monilia fructicola* is almost 1.0 in the range 17—26° C (Jerebzoff, 1961). Other fungal rhythms, however, are more influenced by temperature including sporulation in *Pilobolus* [$Q_{10} = 1.30$ (Schmidle, 1951) or 1.5 (Uebelmesser, 1954)] and *Alternaria tenuis* [$Q_{10} = 0.14$ (Jerebzoff, 1961)] and growth of *Ascobolus immersus* [$Q_{10} = 0.5$ (Chevaugeon, 1959a)]. The evidence of imprecision in the temperature regulation of fungal rhythms is accompanied by data which suggest that the period is markedly altered on certain media (Chevaugeon, 1959b; Jerebzoff, 1961).

The investigations described herein were carried out with two standard ('wild') strains and several morphological mutants in order to determine the effect of temperature on the growth rate and, more specifically, to study the banding and period of the 'clock' mutants in relation to temperature and growth rate.

Strains

A description of the strains used is provided below (the roman numeral following most of these descriptions designates the strain's linkage group):

4-121A(T^S), 4-137a(T^L): standard ('wild') strains of *Neurospora crassa*. Standard strains grow rapidly (approximately 8 cm a day for these strains on complete medium at 25° C), produce abundant aerial and subsurface growth and usually some conidia.

CL11A: 'clock'. a periodic colonial strain described by SUSSMAN *et al.* (1964). The growth bands or 'cycles' are approximately 1.0 cm in length at 25° C on complete medium. (V)

CL12a: 'modified clock', a double mutant which has, in addition to the clock allele, another mutant allele called 'mad'. 'Mad' is responsible for the shorter band length of CL12a which is approximately 0.5 cm at 25° C on complete medium. (V)

W85a: 'mad', a non-colonial (at temperatures above 15° C) morphological mutant which has the clock modifier-1 allele. (V)

E11200: 'osmotic', a strain which is inhibited by high osmotic pressure. (II)

P564: 'carpet', a colonial mutant which produces no aerial growth. (II)

Y8743: 'peach', a non-colonial strain which produces quantities of peach coloured conidia. (II)

L: 'fluffy', a non-colonial strain in which aerial growth is sparse. (II)

70007: 'colonial-4', a colonial which produces aerial sprays of hyphae, usually with much conidiation. (IV)

P628: 'fluffyoid'. a non-colonial strain which is aconidial. (IV)

R2371: 'shallow', a colonial which grows on the surface of the medium and is very aerobic. (V)

B132: 'spray', a semi-colonial strain which produces 'sprays' of aerial hyphae. (V)

B106: 'skin', a colonial which does not produce aerial growth or conidia. (VII)

These descriptions, strain designations and linkage data are taken from the summary of the first 'Neurospora Information Conference' (Publication 950, National Academy of Sciences, National Research Council, Washington, D. C., 1962).

Conditions of growth and inoculation

All cultures, including those used as stocks, were grown on 'complete' medium (RYAN, 1950). Blocks of mycelium at least 2

mm square were used as inoculum. Stocks were grown at 25° C and stored at 4° C. The temperature was maintained within ± 0.5° and high relative humidity was maintained by placing containers of water in the incubator.

Measurement of period and rate of linear extension

The mycelial front serves as a reliable marker of linear extension in most strains of *Neurospora* when grown in growth tubes (RYAN, 1950). However, the slow rate of extension and the irregular mycelial front characteristic of the 'clock' mutants at some temperatures requires more precise measurement. A technique, based on a method for measuring the area under a curve, has been evolved.
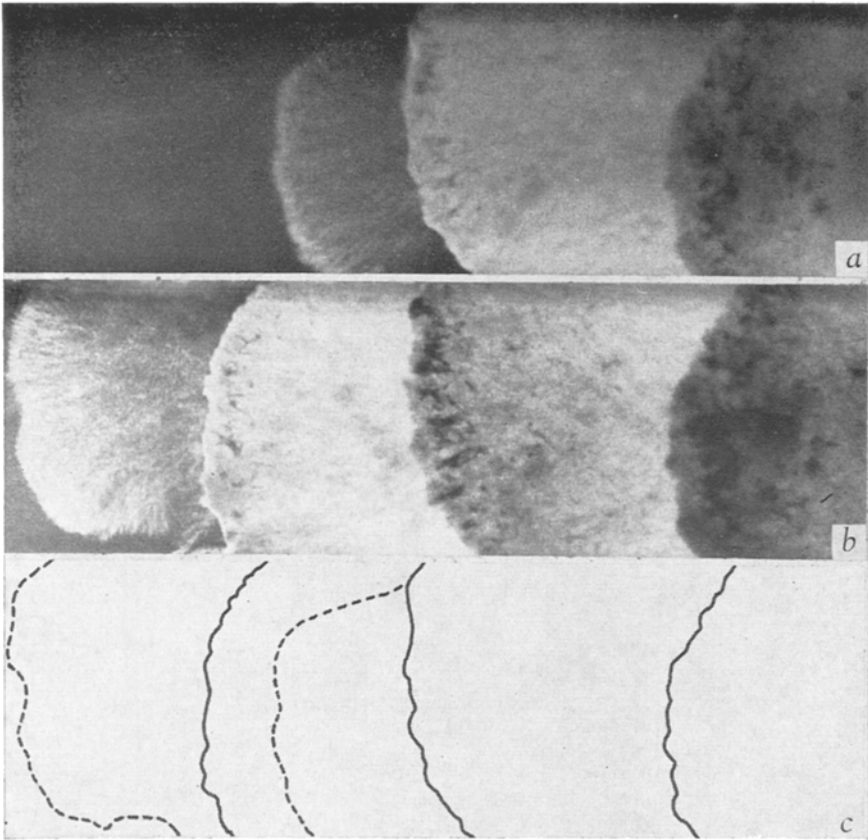

Fig. 1. Technique of recording the cycle and growing fronts of clock mutants. a. Strain CL11A of *N. crassa* on complete medium in a growth tube incubated at 25° C. b. The same tube 24 hours later. c. Tracings of the growing fronts (dotted lines) and cycle fronts (solid lines) made from a and b. Approx. 2,5×.

The organism is photographed at 24-hour intervals as it progresses down the growth tube. Then the negatives are projected onto tracing vellum (175H, Frederick Post Co.) at a magnification of 3 diameters and the mycelial front is recorded as in Fig. 1. The individual sectors representing the 24-hour increments of growth are cut out with scissors or a sharp blade and are folded and weighed. Areas are calculated by reference to average weights of pieces of vellum of known area and linear extension is calculated by dividing the area by the width of the colony. Band length may be determined by measuring the area between two band fronts rather than between two growing fronts.

Several sources of error should be noted. The paper must be handled with forceps or clean rubber gloves and placed only on clean surfaces. The paper should be covered when it is not being used. If a pencil is used as a marker it should have at least the hardness of a #3 lead and the marks must be light. The fine hyphae which appear at the initial stage of the band are difficult to photograph but darkfield lighting gives good results.

TABLE I

Effect of temperatures between 20 and 30° C on various parameters of growth of 'clock', and some non-colonial strains of N. crassa

Strain	Temperature			Q_{10} between		
	20° C	25° C	30° C	20—25	25—30	20—30
	Period (hours)					
CL11A	31.3	28.2	27.7	—	—	—
CL12a	45.3	26.9	22.2	—	—	—
	Frequency (cycles per 24 hours)					
CL11A	0.77	0.85	0.86	1.23	1.02	1.12]
CL12a	0.53	0.89	1.08	2.83	1.48	2.04
	Cycle length (cm)					
CL11A	1.36	1.05	0.66	—	—	—
CL12a	0.57	0.45	0.31	—	—	—
	Rate of linear extension (cm/24 hours)					
CL11A	1.05	0.89	0.57	0.7	0.4	0.6
CL12a	0.30	0.40	0.34	1.7	0.7	1.1
mW85a	1.5	3.8	5.1	6.4	1.4	3.4
T^L	5.6	8.1	10.6	2.2	1.7	1.9
T^s	6.1	8.3	10.3	1.9	1.5	1.7

Method of sampling:
Clocks — 5 increments for each of 3 tubes.
Wilds — 2 increments for each of 3 tubes (usually more depending on the temperature of incubation).

RESULTS

The effects of temperatures between 15-30° C

The rate of linear extension and, for the 'clock' mutants, band-length and period, were studied between 15 and 30° C. The results for the 'clock', 'mad' and standard strains are summarized in Figs. 2 and 4 and Table I.

The rate of linear extension of the standard strains, T^S and T^L, is temperature-dependent over the interval 15—30° C with Q_{10}'s that are close to two. The rate increases over the entire interval but maximum values are attained well above 30° C.
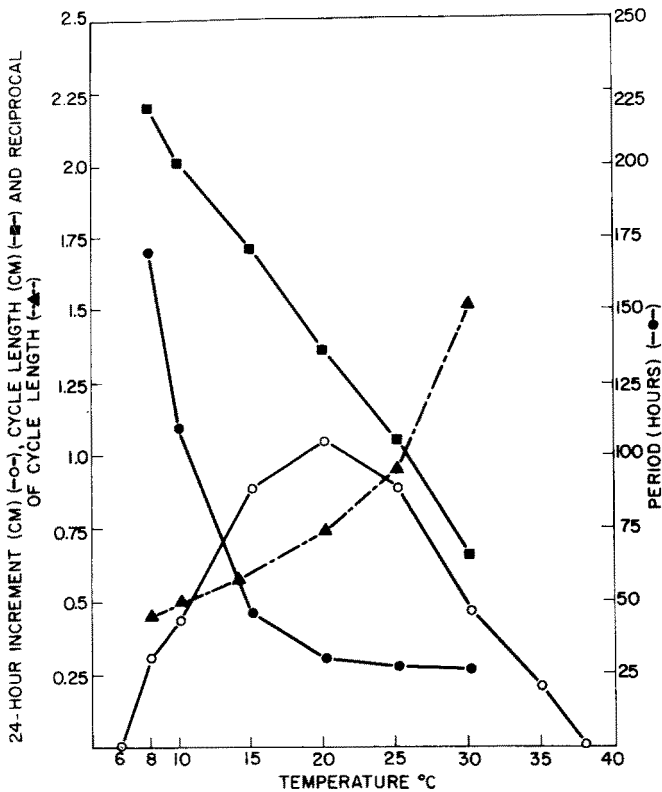

Fig. 2. Effect of temperature upon growth and cycling in strain CL11A of *N. crassa*.

'Clock' strains, like all colonial mutants, grow much more slowly than the standard strains. The growth rates of several colonial mutants are listed in Table II and their rates are comparable to those of CL11A in the range 10—20° C. CL11A differs in achieving its maximum rate at 20° C, well below the optimum temperature for most strains. The Q_{10}'s in the interval 20—30° C are less than one.

TABLE II

Effect of temperature on growth of various strains of N. crassa
(All measurements in cm)

| Designation	Incubation temperature							Q_{10}				
	10° C	15° C	20° C	25° C	30° C	35° C	40° C	10–15	15–20	20–25	25–30	30–35
Ts	1.8	3.3	6.1	8.3	10.3	11.8	11.0	3.4	3.4	2.2	1.7	1.3
TL	1.8	3.3	5.6	8.1	10.6	11.8	9.7	3.4	2.9	1.9	1.5	1.2
fluffy (45)		3.8	5.5	7.5	10.3	10.6			2.1	1.9	1.9	1.1
fluffyoid (554)		3.4	5.4	7.2	9.6	10.6			2.5	1.7	1.7	1.2
peach (37)		2.7	4.3	5.8	9.2	8.9			2.5	1.8	2.5	0.9
osmotic (34)		1.1	3.5	5.6	6.7	7.2			10.1	2.6	1.4	1.1
modifier–1 (W85a)			1.5	3.8	5.1	4.5	1.1			6.4	1.4	0.8
spray (70)		0.5	1.0	3.5	4.4	7.9			4.0	12.3	1.5	3.2
shallow (13)		0.7	1.4	2.0	4.0	4.9			4.0	2.0	4.0	1.5
colonial–4 (67)		0.4	0.5	0.9	2.1	3.4			1.6	3.2	5.4	2.6
carpet (104)		0.3	0.6	0.9	1.4	2.1			4.0	2.3	2.4	2.3
skin (276)		0.8	1.3	1.3	1.4	1.4			2.6	1.0	1.2	1.0
clock (CL11A)	0.4	0.9	1.1	0.9	0.6			5.1	1.5	0.7	0.4	
mod-clock (CL12a)			0.3	0.4	0.3					1.7	0.7	
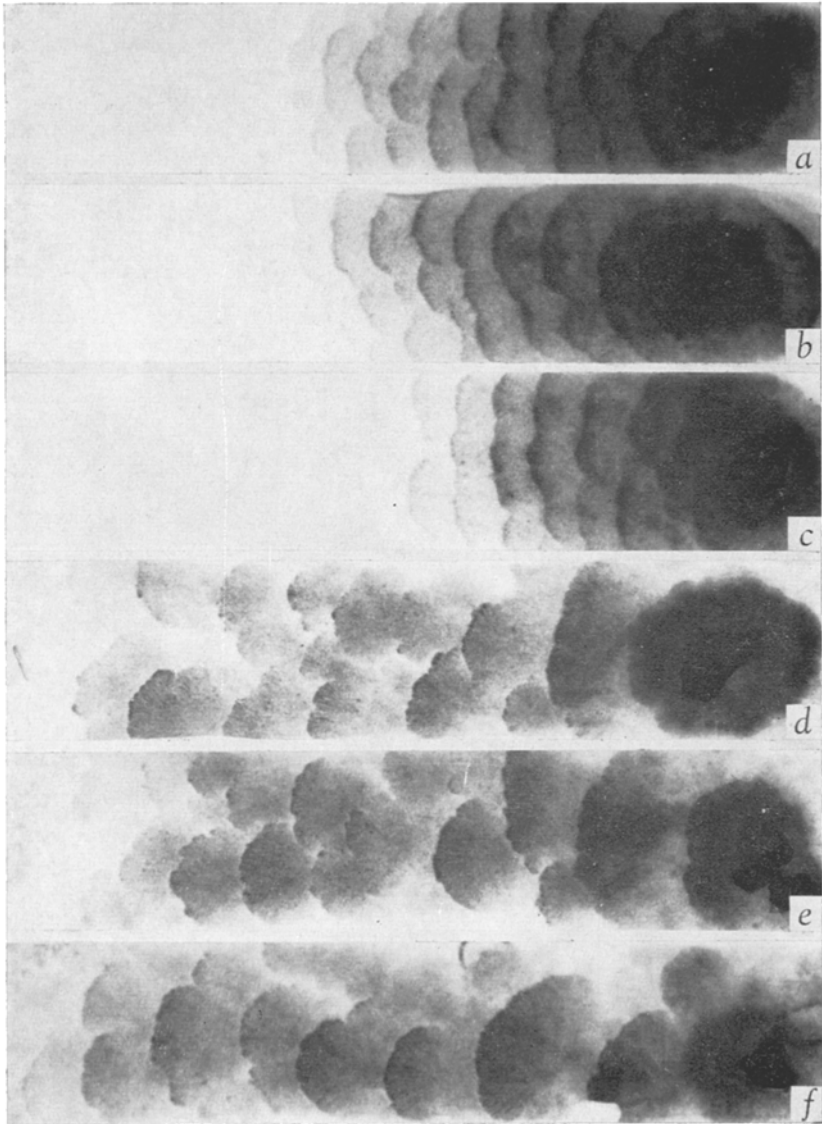
Fig. 3. Replicate cultures of strains CL12a (a, b, c) and CL11A (d, e, f) on complete medium at 30° C. Approx. 1.75×.

In the same range the period and the frequency of CL11A vary little. The period begins to increase below 20° C and the Q_{10} for the interval 15—20° C is 2.2. The length of the bands, however, decreases almost linearly over the entire range from 15 to 30° C.

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Aerial growth, which is found in the terminal region of the band at
15° C, is not produced at 30° C and the front of the bands becomes
much less uniform (Figs. 1 and 3) at the latter temperature.

Strain CL12a, a 'modified clock', attains its maximum rate of
linear extension at 25° C, 5° above the optimum for CL11A. How-
ever, CL12a grows more slowly than the unmodified strain,
usually at less than half its rate. The appearance of the colony
changes considerably at 15° C (Fig. 5) and bands are not produced.
The aerial hyphae form a compact mat at this temperature and,
as with CL11A, the quantity of aerial growth decreases as the
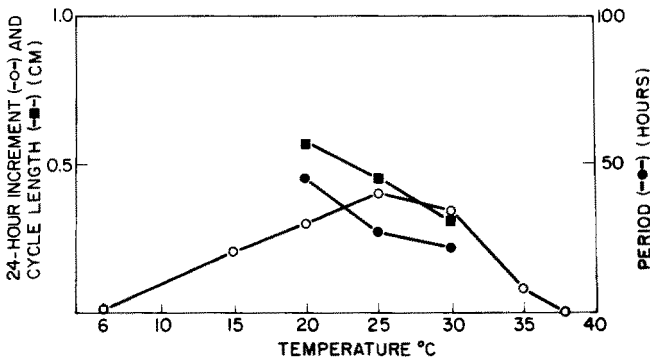temperature is increased until at 30° it disappears.

Fig. 4. Effect of temperature upon growth and cycling of strain CL12a of *N. crassa*.

The period of CL12a is reduced by about half when the temper-
ature is increased from 20 to 30° C (Table I and Fig. 4). The Q_{10}
of 2.0 for frequency over this range reflects the decrease which
is, however, not linear. An upward deflection is apparent in the
curve below 25° C so that most of the decrease in period occurs
between 20 and 25° C. A similar deflection in the curve for the period
of CL11A is seen below 20° C. The Q_{10}'s for frequency of the two
strains are similar in the interval where the marked increase in
period begins.

Strain W85a, which contains the modifier of 'clock' band size,
is a non-colonial (above 15° C) morphological mutant. W85a grows
more slowly than the standard strains (Table I)and forms a
depauperate mycelium (Fig. 5) which is readily distinguishable
from that of standard strains. The growth rate of W85a is compar-
able to that of 'spray' and it displays a similar sensitivity to low
temperatures. At 15° C the growth rate of W85a is greatly reduced
and a restricted compact mycelium replaces the loose spreading
growth characteristic at higher temperatures (Fig. 5). The high
Q_{10} over the interval 15—25° C reflects the transition from a
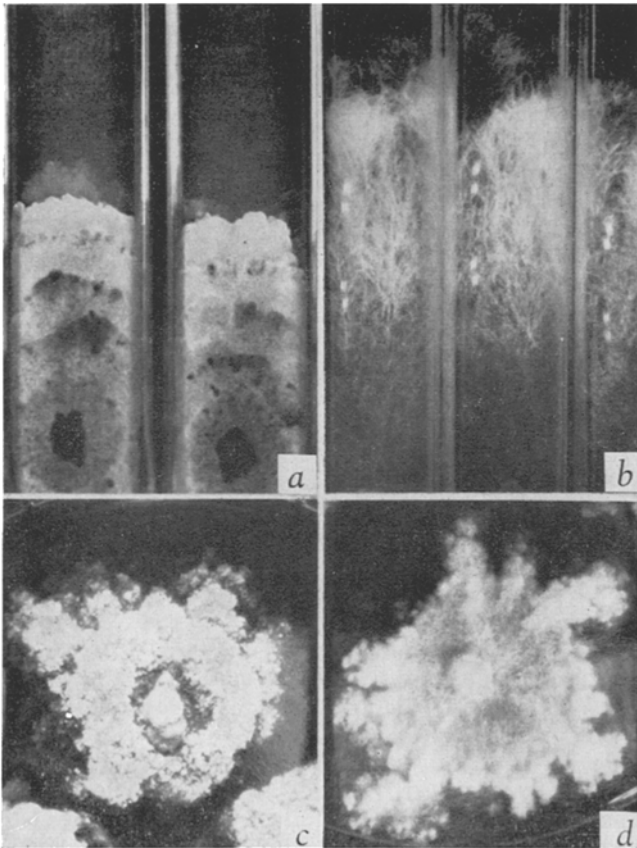colonial to a semi-wild type of mycelium.

Fig. 5. Gross morphology of strains CL12a (a, c) and W85a (b, d) grown on complete medium at 25° C (a, b) and at 15° C (c, d). Approx. 1.1×.

The other morphological mutants which were used as a basis for comparison can be divided into several groups based on their morphology and their response to temperature. (1) 'Fluffy', 'fluffyoid' and 'peach' are similar to standard strains in appearance but grow more slowly. The Q_{10}'s for these mutant strains in the interval 15—30° C are between 1.7 and 2.5. These Q_{10}'s, which are similar to those of the 'wild' strains, are characteristic of most biological processes. (2) 'Shallow', 'osmotic' and 'spray' show a growth habit that is intermediate between that of 'wild' and colonial strains and their rates of linear extension are also intermediate. 'Osmotic' and 'spray' show marked sensitivity to low temperatures as is reflected in the Q_{10}'s of 10.1 ('osmotic' over the interval 15—20° C) and 12.3 ('spray' over the interval 20—25° C). (3) 'Colonial-4', 'carpet' and 'skin' are truly colonial in morphology

as is reflected in their slow growth rates. 'Clock' and 'modified clock' also belong to this group although their maxima are at lower temperatures. 'Skin' is of particular interest because the rate of extension is almost completely independent of temperature over the interval 20—35° C.

Cardinal temperatures for growth and rhythmicity

Strains CL11A and CL12a have unusually low maxima as compared with the other strains listed in Table II. Both cease to grow near 38° C which is slightly above the optimum for linear extension of 'wild' strains. At the opposite end of the temperature scale, CL11A and CL12a stop growing at 6° C although growth is still perceptible in the 'wild' strains (Fig. 6).

Strain CL11A reaches its maximum rate of extension at 20° C but thereafter the growth curve declines. Bands are formed over the entire range from 6 to 38° C but above 30° they become increasingly irregular and asynchronous. The period which is about 7 days at 8° decreases as the temperature rises until at 30° it is little more than a day in duration. Above 30° C the asynchronous nature of the bands prevents the accurate determination of the period.

Strain CL12a is sensitive to low as well as high temperatures and it ceases to be rhythmic below 15° C. It also differs from CL11A in attaining its maximum rate of extension at 25° C and in forming well defined bands above 30° C. Synchronous banding continues until growth stops near 38° C.

Strain W85a grows optimally at 33° C but below 15° C it is colonial. The rate of growth of 'mad' colonies at lower temperatures is, like that of 'spray' at higher temperatures, not consistent and may vary as much as several millimeters a day. The rate of extension of W85a decreases rapidly above 33° C and the maximum temperature for growth is slightly above 40° C.

Effects of temperatures upon variability

It was noted above that the regularity of the banding of strain CL11A as well as its linear extension were adversely affected by temperatures above 25° C. Therefore, experiments were carried out wherein cultures were grown at 20, 25 and 30°C and the standard deviation and covariance of band length and growth rate were determined. As can be seen in Table III, the size of the bands and the rate of linear extension of replicate cultures of strain CL11A grown at 30° C were much more variable than were those for cultures grown at lower temperatures. On the other hand, the opposite effect was obtained with strain CL12a where the growth rate was much more variable at 20° C although there was not much difference in the variability of band length at the temperatures used.

TABLE III

*Variability of "clock" strains of Neurospora crassa grown at different temperatures.
Values represent the average of 15 replicates. All cultures were inoculated from cultures
maintained at 25° C on "complete" medium.*

Strain	Temperature of incubation (°C)	Cycle length (cm)	St. dev [a]	Covar [b]	Growth cm/day (cm)	St. Dev [a]	Covar [b]
CL11A							
	30	0.66	0.20	30.0	0.57	0.24	42.8
	25	1.03	0.17	16.0	0.92	0.17	18.6
	25	1.05	0.16	15.4	0.89	0.20	22.4
	20	1.36	0.24	17.4	1.05	0.21	20.4
CL12a							
	30	0.31	0.07	22.5	0.34	0.06	17.7
	25	0.45	0.09	20.4	0.40	0.10	23.9
	25	0.45	0.08	17.8	0.40	0.08	20.0
	20	0.57	0.12	20.7	0.30	0.14	46.2

[a] Standard deviation.
[b] Covariance.

DISCUSSION

The rate of linear extension of the 'clock' mutant of *Neurospora crassa*, which differs from standard strains by a single gene (SUSS-MAN *et al.*, 1964), has a very low Q_{10} in the range 20—30° C (Table I). Although the rates of various chemical and biological processes increase by 2—3 fold when the temperature is raised 10° (viz., the Q_{10}'s for linear extension of the standard strains and most morphological mutants listed in Table II over the interval 20—30° C), the rate of extension of strain CL11A *decreases* by almost one-half over such a range ($Q_{10} = 0.55$). A similar decrease would be expected in the period of band formation but a decrease of only 12% occurs over the same 10° interval, suggesting a relative lack of temperature-dependence. These data underscore the fact that while period changes little over this range, the rate of growth is much reduced. Inasmuch as the rhythmic form which characterizes the clock mutant is a function of the organism's linear extension, it follows that the effect of temperature upon the period of this strain is related to the mechanism which determines the low Q_{10} for extension. Therefore a mechanism to explain the latter will be proposed and the relative lack of temperature-dependence will then be discussed within the framework of this model.

HASTINGS & SWEENEY (1957) have pointed out that Q_{10} values below 1.0 for the period of rhythmic processes such as those found in the sporulation of *Oedogonium* (BÜHNEMANN, 1955) and luminescence in *Gonyaulax* (HASTING & SWEENEY, 1957) can be explained

by temperature compensation through two temperature-dependent reactions: A → B (1) and C → D (2). This model, with some modification, may be used to explain the effect of temperature upon strain CL11A. Assume that the rate of reaction (1) determines the rate of linear extension and that D is an inhibitor of this reaction. Extension should then be constant over a certain range if the two reactions have equivalent temperature coefficients. However, if the coefficient of reaction (2) is higher than that of reaction (1), an apparent Q_{10} of less than 1.0 for the rate of extension will result. Conversely, if the temperature coefficient of reaction (2) is lower than that of reaction (1) a Q_{10} greater than 1.0 would result. If this model holds for linear extension in CL11A, then the former situation would obtain below 20° and the latter above.

The model explains the relation of period to the rate of extension in CL11A over the range 20—30° C. Assume that a band forms only when a certain threshold concentration of D accumulates per unit of hypha or of protoplasm. Then slower extension at the higher temperature would not result in a longer period for the concentration of D would increase more rapidly than at 20°C. Conversely, faster growth at the lower temperature would not shorten the period for D would accumulate more slowly than at 30° C. These assumptions are reasonable in that the greater amount of inhibitor at the higher temperature is produced in a smaller area, thereby raising the effective concentration.

The temperature-compensation model leads to an explanation of the data obtained at temperatures above and below the 20—30° C range. It follows from these assumptions that the temperature coefficients for reaction (1) and (2) differ and that the optima for the two reactions may differ as well, except in the unlikely event that the coefficients for denaturation of enzymes limiting both reactions are such as to render the descending arms of both curves congruent at higher temperatures.

An inhibitor with the properties of D has not yet been isolated so that the kinetics of its formation have not been studied. However, a theoretical curve may be plotted using the data in Fig. 2. If the assumption is made that the concentration of inhibitor is inversely proportional to the cycle length at each temperature, then a curve is obtained which represents the formation of inhibitor (dotted line in Fig. 2). This curve rises markedly above 20° C at almost exactly the temperature where the rate of extension of strain CLIIA peaks. Below 20° C the rate of extension appears to be limited by temperature alone. That an inhibitor, or a restraint other than higher temperatures, limits the rate of growth of 'clock' is suggested by the fact that all of the other strains listed in Table II, including the colonial mutants, have much higher temperature optima and maxima.

The theoretical curve predicts that the production of D will be markedly reduced below 20° C, thereby extending the duration of

the period. The rates of extension of CLIIA are, in fact, equivalent at 15 and 25° C but the periods at these temperatures differ considerably. Although the reduced production of D at 15° C limits the rate of growth less than at 25° C, the lower temperature also slows reaction (1) proportionately. On the other hand, reaction (1) is speeded at 25° C but so is the production of D. The net result is that the rate of linear extension at 25° C is the same as at 15° C. However, the rates at which D accumulates differ and consequently the periods are unequal in duration. The lower rate of accumulation of D at 15° C results in a longer period (46 hours) while at 25° C the higher rate causes a shorter period (28 hours).

The tendency for the period to increase as temperature decreases is maintained until growth stops at 6° C. At higher temperatures the model predicts that growth gradually will be overcome by the increased production of the inhibitor D. In fact, the low maximum temperature of CLIIA (Fig. 2) as compared with most other strains (Fig. 6 and Table II) confirms this hypothesis.
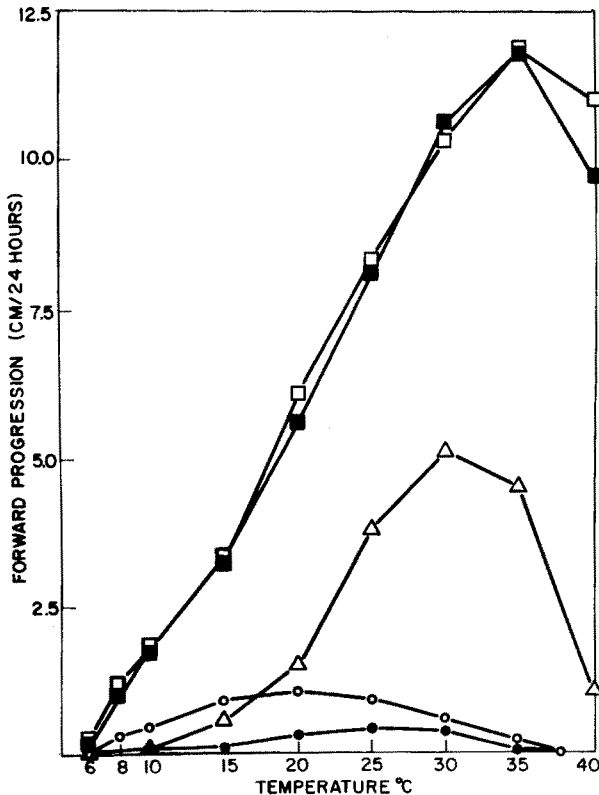

Fig. 6. Effect of temperature on growth of 'clock' and some non-colonial strains of *Neurospora crassa*.

Other mechanisms to explain the lack of temperature-dependence of the period of biological clocks have been proposed, including rhythmic and uncontrolled environmental factors (BROWN, 1960) and diffusion-limited processes (EHRET & BARLOW, 1960). However, there are reasons for believing that an exogenous factor does not determine the rhythm of clock mutants of *Neurospora*, including the variability of the period at a single temperature and the deviation of the Q_{10} from one. Moreover, unpublished experiments in our laboratory reveal that cultures inoculated 4 to 12 hours out of phase maintain the phase-difference indefinitely. Finally, the period can be altered on certain media (DURKEE, unpublished; NEURATH & BERLINER, 1964). The diffusion-limited model cannot explain how a Q_{10} of less than 1.0 can be obtained so that it is not generally applicable.

Due to some confusion in the literature concerning the definition of "circadian" rhythms, we will define this term arbitrarily and use this as a framework for discussion. We consider the following properties to characterize circadian rhythms:
a. temperature-independence
b. persistence (free-running)
c. responsiveness of phase to light
d. consistency of period

Our work has revealed that the 'clock' mutants of *Neurospora* are free-running and temperature-independent but are unresponsive to phase-shifting. Also, the period can be changed markedly by several means including changes of medium, temperature and genotype. Therefore, we conclude that the 'clock' mutants display a non-circadian free-running endogenous rhythm, a conclusion reached by NEURATH & BERLINER (1964) as well.

The lack of response of 'skin' to temperatures between 20 and 35° C (Table III) underscores PITTENDRIGH's (1960) reminder that rhythms are not the only aspects of organisms that are temperature-compensated. Put another way, the ability to compensate for changes in temperature does not ensure rhythmicity. In *Neurospora*, several unlinked genes appear to determine temperature-independence, including 'clock', 'patch' (PITTENDRIGH *et al.*, 1959) and 'skin' (PERKINS, 1959), although only the first two are rhythmic.

The modifier in strain CL12a produces three major changes in the type of growth characteristic of strain CL11A including, 1) The rate of linear extension and band size are decreased by half or more at all temperatures. 2) The rate of linear extension begins to decline at a temperature 5° higher for CL12a than for CL11A. 3). The cyclic nature of CL12a is masked below 15° C, whereas above 30° C, at which temperature CL11A becomes asynchronous, uniform banding is maintained.

Further evidence that the mutation which converts CL11A into CL12a affects its response to temperature is provided by the data

for strain W85a. When the modifier is introduced into a standard strain, as in W85a, the Q_{10}'s for extension deviate considerably from those for strains T^S and T^L. The Q_{10} between 20 and 25° C is 6.4 for W85a as compared with 2.2 and 1.9 for T^S and T^L. Consequently either the temperature coefficient of reaction 2 is affected or a second reaction, which influences the production of B or D, is altered by the modifier.

Summary

The Q_{10} for the frequency (number of bands per 24 hours) of the 'clock' mutant (strain CL11A) of *Neurospora crassa* over the range 20—30° C is close to 1.0. By contrast, that for the double mutant, 'wrist watch' (strain CL12a), is closer to 2 over this temperature range. Strain CL12a differs from 'clock' in other ways as well, including 1) decreased rate of linear extension and band size, 2) greater sensitivity of growth rate to high temperatures and, 3) masking of rhythmic growth below 15° C. The response to temperature of several colonial mutants and standard ('wild-type') strains was studied and it is shown that some strains are temperature-independent yet arhythmic. A temperature-compensation model is presented to explain the response of 'clock' mutants to temperature and it is concluded that they demonstrate a non-circadian free-running endogenous rhythm.

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