we do not know whether the new peptides are strictly storage forms or may be the form of the secreted hormone. In this regard, it is important to note that a current radioimmunoassay technique (*16*) is unable to distinguish between synthetic arginine vasopressin and either of these compounds (*17*).

We were surprised to find two different molecules in the bovine neurohypophysis that may serve a similar function. Whether these are allotypic variants between members of the species or isotypes present in all individuals can best be answered by isolating the products from single rather than pooled pituitaries. If these two molecules are isotypes, a more interesting question remains. Are the two peptides a result of biosynthetic heterogeneity or a consequence of different anatomical sites of synthesis within the hypothalamus?

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3. On the evening before each study 0.1 mg of fludrocortisone was administered to the animal to minimize variability in endogenous mineralocorticoids and food, but not water, was withdrawn. On the morning of the study, 0.1 mg of fludrocortisone was given by mouth and the animals were anesthetized with pentobarbital. Test materials were dissolved in 5 ml of normal saline and administered over a 10- to 20-second period into the abdominal aorta above the renal arteries via an indwelling femoral-artery catheter. Urine collections (10 minutes in duration) were obtained by an indwelling bladder catheter and were terminated with distilled water and air rinses. Venous blood was obtained from the femoral vein for creatinine and electrolyte determinations. Blood pressure was monitored via the abdominal aorta catheter. The increment in sodium excretion produced by a test injection was expressed in microequivalents and used to calculate units of natriuretic activity. To calculate the incremental change in sodium excretion, we defined the baseline sodium excretion for each injection as the mean of the sodium excretion observed in the collection period immediately prior to the injection of test material and the sodium excretion during the last period obtained following the injection, when the effect of the injection had dissipated. In general, at least three 10-minute periods were obtained with test materials that produced no alteration in urinary flow rate and only slight changes in sodium excretion. When urinary flow increased in response to test material, we waited until the urine flow had returned to the preinjection rate or had stabilized at a new rate. Insofar as possible we attempted to use minimal doses of test materials throughout these experiments so that the effects of a given injection would be dissipated within six collection periods. Using this protocol, we observed a coefficient of variation in the range of 40 percent for a single injection in different animals.
4. Acetic acid extracts of bovine pituitary glands were fractionated on P-2 Biogel polyacrylamide beads equilibrated with 0.1*M* acetic acid. Portions of the column eluate were pooled, lyophilized, and dissolved in normal saline to test for natriuretic activity. Significant natriuresis occurred in fractions that emerged after the salt peak. This response with the pituitary extract was at least an order of magnitude greater than the response to similar fractions prepared from acetic acid extracts of cerebral cortex, kidney, liver, lung, and skeletal muscle. Subsequently, we observed that the posterior lobe of the pituitary, which accounts for only 20 percent of the weight of the whole gland, contains approximately 90 percent of the natriuretic activity [H. J. Gitelman and W. B. Blythe, *Clin. Res.* **20**, 594 (Abstr.) (1972).
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Circadian Rhythms in *Neurospora crassa*: Oligomycin-Resistant Mutations Affect Periodicity

Abstract. *Nuclear mutations conferring resistance to oligomycin, a mitochondrial inhibitor, shorten the period of the circadian conidiation rhythm of* Neurospora crassa *from the normal 21.5 hours to 18 to 19 hours and slow the linear growth rate by 30 percent. These* oli^r *mutations map very close to* frq, *a locus at which mutations affecting periodicity have been previously obtained. The possibilities are discussed that mitochondria are involved in circadian rhythm generation and that certain period-length mutations affect mitochondrial functions.*

The band (*bd*) strain of *Neurospora crassa* exhibits a spore-forming rhythm when grown on solid media in the dark (*1*). This conidiation rhythm is deemed circadian by three criteria: (i) it has a free-running period length close to 24 hours, (ii) the phase of the rhythm is sensitive to exposures to light, and (iii) the period length is relatively insensitive to growth temperature (*2*). In order to further the understanding of the biochemical basis of circadian timekeeping in *Neurospora*, the effects on circadian rhythmicity of a variety of auxotrophic and morphological mutations were previously investigated (*3*).

We now report that, upon crossing oligomycin-resistance mutations (*oli*^{r}) into *bd*, the normal circadian period of 21.5 hours for *Neurospora* is shortened to 18 to 19 hours. This result is of interest for two reasons. The first is that these *oli*^{r} mutations map very close to another set of circadian period mutants: those at the *frq* (frequency) locus (*4*). The second is that oligomycin resistance appears to be due to changes in the primary structure of a particular mitochondrial protein. Sebald has reported that similar *oli*^{r} mutations lead to changes in the primary structure of a small polypeptide component, subunit 9, of the F_0 membrane portion of the mitochondrial adenosine triphosphate (ATP) synthetase (*5*).

The *bd csp-1* strain of *N. crassa* was used for all genetic crosses. The *bd* mutation allowed the clear expression of a conidiation pattern in closed petri dishes (*1*). The *csp-1* mutation (conidial separation) allowed easy handling of the dishes without self-reinoculation (*6*). The period length was determined as reported (*7*). All petri dish cultures were grown at 22°C on Vogel's minimal medium with 0.5 percent maltose as the carbon source, and inositol at 0.05 mg/ml for those strains carrying the *inl* marker. Crosses were performed with SC media (*8*).

Four oligomycin-resistant strains, *oli*^{r} (16-1), *oli*^{r} (16-3), *oli*^{r} (16-14), and *oli*^{r} (16-16) and one revertant to oligomycin sensitivity, *oli*^{s} (16-16R45), were donated by Edwards (*9*). It is not known whether these strains represent different alleles of the *oli*^{r} locus or are independent isolates of the same mutation. These *oli*^{r} mutations were isolated in a background containing the Has, Azs, and *inl* mutations. The Has and Azs mutations block alternate respiratory pathways in *N. crassa* (*10*). When the *oli*^{r} strains were crossed to *bd csp-1*, all four *oli*^{r} mutants gave the same result. The *bd csp-1 oli*^{r} progeny all showed a shortening of the circadian pe-

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riod from the normal value of 21.5 hours (Table 1) to 18.5 hours and a slowing of growth rate from the normal 1.5 mm/hour to 0.9 to 1.1 mm/hour (Table 1). The presence or absence of the Has, Azs, and *inl* mutations did not significantly alter the effect of *oli*^{r}. All crosses showed an approximate 1:1 ratio in the progeny of *oli*^{r} to *oli*^{s} (oligomycin-sensitive), with *oli*^{s} progeny having normal growth rates and period lengths (Table 1). In many crosses and backcrosses, including those shown in Table 1, the slower growth rate and shorter period length segregated 143 out of 143 times with *oli*^{r}. Therefore, these characteristics appear to be due to the *oli*^{r} mutations and not to some other marker or factor in the genetic background. In addition, revertants to oligomycin sensitivity simultaneously reverted to the normal growth rate and period length. A cross of the revertant, Azs Has *inl oli*^{s} (16-16R45) to *bd inl oli*^{r} (16-16) gave ten progeny that were *bd inl oli*^{s} and seven progeny that were *bd inl oli*^{r}. The *oli*^{s} progeny all had normal 22-hour periods and growth rates of 1.3 mm/hour, whereas the *oli*^{r} progeny had 19.4-hour periods and growth rates of 1.0 mm/hour (Table 1).

Oligomycin-resistant mutants of *N. crassa* have been isolated and mapped by W. Sebald *et al.* (*11*). Their genetic localization shows that they are very close to the *oli*^{r} mutations used in our experiments, which were isolated and mapped by Edwards (*12*). To prove that the Edwards mutants and Sebald mutants are in the same gene (*13*) would require seeking recombinants between the mutants or sequencing subunit 9 from the Edwards set of mutants. If they are indeed in the same gene, then, our finding that the *oli*^{r} mutations confer a 19-hour period length on *Neurospora* would be the first report linking an alteration in a specific protein to a change in circadian periodicity.

It was surprising to find that the *oli*^{r} mutants map so close to a second set of period-length mutants, those at the *frq* locus. The *oli*^{r} locus maps on linkage group VII of *N. crassa*, 8.5 map units from the *met-7* (*11*) and 11.5 map units from the *arg-10* markers (*12*). This places the *oli*^{r} locus very close to the *frq* locus, which we have found (*14*) is between *met-7* and *arg-10* and previously was reported to be 9 map units from *met-7* (*15*). The *frq* mutants have altered period lengths, some having short periods (*frq-1*, 16 hours) and others long (*frq-3*, 24 hours). The juxtaposition of these two classes of periodicity mutations, *oli*^{r} and *frq*, leads to speculation about the function of the *frq* locus. One possibility is that the *oli*^{r} mutations and the *frq* mutations are in the same gene. In this regard, our studies so far indicate that strains bearing the *frq-1*, *-2*, *-3* mutations are not as resistant to high concentrations (> 1 μg/ml) of oligomycin as are the *oli*^{r} strains (*16*). These mutants at the *frq* locus were not expected to be grossly resistant to high levels of oligomycin since they were selected on the basis of altered period lengths and normal growth rates on acetate-Casamino acid medium (*4*). On this medium, the highly resistant *oli*^{r} strains grow at approximately one-half the rate of *oli*^{s} strains (*16*), and thus might not have been selected as *frq* mutants in the initial screening. A second possibility is that the *frq* and *oli*^{r} mutations lie in nearby genes that code for separate interacting subunits of the ATP synthetase. In *Saccharomyces cerevisiae*, there are two pairs of linked mitochondrial loci in which *oli*^{r} mutations have been obtained (*17*). One locus, *oli 1*, codes for subunit 9 of the F_0 portion of the ATP synthetase (*11*), while *oli 3*, which is linked to *oli 1*, has been proposed to code for subunit 6 of the same hydrophobic complex (*18*). A third possibility is that the *frq* and oli^r mutations affect unrelated functions, although they map close to one another, and both lead to similar phenotypic effects.

In studies on oligomycin sensitivity, we have observed that strains carrying a different mutation, *cel*$^-$, are about threefold more sensitive to oligomycin inhibition than *cel*$^+$ strains (*16*). The *cel*$^-$ strains are severely deficient in fatty acid synthetase (*19*) and show a partial nutritional requirement for fatty acids. The introduction of the *cel*$^-$ mutation into the *bd csp-1* strain makes the period of this strain sensitive to exogenous unsaturated fatty acids (*7*); for example, supplementation with linoleic acid leads to 40-hour periods. It has been reported that the fatty acid synthetase from the *cel*$^-$ strain has only a small fraction of the normal level of 4-phosphopantetheine bound to the acyl carrier protein subunit (*19*). The prosthetic group, 4-phosphopantetheine, has also been reported to be bound to subunit 6 of the F_0 portion of

Table 1. Period length and growth rate of control parent strains and progeny from crosses with *oli*^{r} strains. Symbols: +, wild-type allele; −, mutant allele; r, oligomycin-resistant allele; s, oligomycin-sensitive allele; and (16-*x*), oligomycin resistance isolation number.

Genotype						Isolates (No.)	Periods (No.)	Period length (hours)	Standard error	Growth rate (mm/hour)
bd	*csp-1*	*oli*	*inl*	Azs	Has					
Control parent strains: bd, bd csp-1, bd inl										
−	+	s	+	+	+	2	54	21.7	0.2	1.4
−	−	s	+	+	+	2	68	21.5	0.2	1.5
−	+	s	−	+	+	2	68	22.3	0.2	1.3
Oligomycin-resistant progeny of crosses: bd csp-1 × oli^r inl *Azs Has*										
−	−	r (16-1)	+	+	+	6	124	18.5	0.2	0.9
−	−	r (16-3)	+	+	+	7	104	18.5	0.2	1.0
−	−	r (16-14)	+	+	+	3	61	18.5	0.3	0.9
−	−	r (16-16)	+	+	+	3	44	19.1	0.3	1.1
−	−	r (16-16)	+	−	+	6	127	18.4	0.2	1.0
−	−	r (16-16)	−	−	+	2	43	19.0	0.3	0.9
−	−	r (16-16)	−	+	−	1	16	18.6	0.6	0.9
−	−	r (16-16)	−	−	−	6	134	18.3	0.1	0.8
−	+	r (16-16)	−	+	+	7	112	20.1	0.2	0.9
Oligomycin-sensitive progeny of cross: bd csp-1 × oli^r *(16-16)* inl *Azs Has*										
−	−	s	+	+	+	2	57	21.7	0.2	1.5
−	−	s	+	+	−	2	54	22.1	0.2	1.5
Progeny of oli^s revertant backcross: bd inl oli^r *(16-16)* × bd inl oli^s *(16-16R45)*										
−	+	s (16-16R45)	−			10	129	22.2	0.2	1.3
−	+	r (16-16)	−			7	138	19.4	0.1	1.0

the yeast mitochondrial ATP synthetase complex (*20*). It is not yet known whether, like yeast, *Neurospora* also has 4-phosphopantetheine bound to subunit 6 and whether the *cel*$^-$ strains are deficient in 4-phosphopantetheine bound to subunit 6. However, the sensitivity of the *cel*$^-$ strains to fatty acids and to oligomycin may be related to a common defect in the addition of 4-phosphopantetheine to two proteins: the acyl carrier protein and subunit 6. Therefore, the basis for the effects of *cel*$^-$ mutation on circadian rhythmicity may be a defect in the F_0 portion of the ATP synthetase complex, as is the case of the *oli*^{r} mutations.

It still remains to be elucidated how the *oli*^{r} mutations affect the mechanism of circadian timekeeping. One possibility is that the *oli*^{r} mutations in *Neurospora* may affect proton transport in the mitochondria, since subunit 9 purified from yeast *oli*^{r} mutants shows reduced and oligomycin-resistant proton transport (as compared to wild-type subunit 9) when purified and incorporated into liposomes (*21*). Subunit 9 from wild-type and *oli*^{r} strains of *Neurospora* has yet to be tested in such an isolated system. However, the *Neurospora* protein has 50 percent sequence homology with yeast subunit 9 (*5*) and, therefore, might be expected to act similarly in liposome preparations. The analogous subunits of both chloroplast and bacterial ATP synthetase have also been shown to translocate protons in liposome preparations (*22*). Another possible effect of *oli*^{r} mutations is that they may lead to secondary effects on mitochondrial membrane structure, as has been proposed for yeast *oli*^{r} mutations (*23*).

Our finding that a change in mitochondrial function can lead to a periodicity change suggests three possible approaches to the study of the biochemistry of rhythmicity. (i) Mitochondrial functions and sensitivity to inhibitors could be analyzed in the strains carrying period-altering mutations. (ii) Existing strains of *Neurospora* that bear mutations in the mitochondrial genome could be analyzed for their effects on circadian rhythms. This approach has already proved worthwhile in that the *poky* mutation, a maternally inherited mutation, when introduced into the *bd* strain, was 200-fold less sensitive than the normal *bd* strain to the inhibition of the conidiation rhythm caused by exposure to light (*24*). (iii) New mutants selected on the basis of defective mitochondrial function could be obtained and tested for their rhythmic properties. For example, a broad class of mitochondrial mutants could be isolated as nongrowers on glycerol (*25*), and mutants with defects in ATP synthetase could be selected on the basis of resistance to compounds that specifically inhibit this complex, such as aurovertin, rutamycin, and venturicidin (*26*). The three lines of investigation listed above, coupled with biochemical studies of the ATP synthetase complex in wild-type and mutant strains such as *oli*^{r}, *frq*, and *cel*, may lead to the understanding of the role of mitochondria in generating circadian periodicity.

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Feedback Control of Juvenile Hormone Synthesis in Cockroaches: Possible Role for Ecdysterone

Abstract. *Inactive female corpora allata implanted into adult males become active and continue to synthesize juvenile hormone at high rates. However, when an ovary is implanted together with the corpora allata, rates of juvenile hormone synthesis decline as the oocytes complete maturation. Injections of ecdysterone mimic the effect of an implanted ovary.*

Ovarian development in the viviparous cockroach *Diploptera punctata*, as is the case in many insects, is dependent on the synthesis and release of juvenile hormone (JH) by the corpora allata (CA) (*1*). In other cockroach species, JH stimulates the fat body to produce vitellogenins (yolk precursors) and also stimulates the oocytes to take up and store these as yolk (*2, 3*). The synthesis of JH correlates precisely with the cycle of egg maturation, as demonstrated by in vitro radiochemical assay of *D. punctata* CA (*4*). Also, in other species, ovaries need not be present for the synthesis of vitellogenin by the fat body (*2, 5*), and therefore it could be hypothesized that the ovaries do not influence the CA. However, we have found evidence that ovaries do influence the CA: after ovariectomy the cycle of JH synthesis is suppressed; and, when two pairs of CA of different ages are implanted into a host, both pairs simultaneously terminate their cycle of JH synthesis in close correlation with the maturation of oocytes (*6, 7*), suggesting that termination of the cycle is related to the presence of a mature ovary or a physiological event associated with the completion of vitellogenesis.

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