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*REPLICATION AND INHERITANCE OF MITOCHONDRIAL DNA**

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Several lines of evidence suggest that mitochondria of *Neurospora crassa* possess some degree of intracellular autonomy of growth, metabolism, and heredity. For example, mitochondrial density shifts[1, 2] and the labeling patterns and content of mitochondrial phospholipid[3] in logarithmically growing mycelium are in good accord with the view that mitochondria increase by growth and division. Furthermore, DNA of high molecular weight and of characteristic buoyant density, and an enzymatic activity resembling DNA-dependent RNA polymerase are associated with these[4] and other mitochondria.[5–8] These organelles therefore contain molecules capable of embodying genetic determinants in addition to the enzyme which catalyzes the presumed first step in gene expression. Finally, the mode of hereditary transmission of some changes in mitochondrial function has been shown to differ from that of the usual, presumably nuclear, genes.[9, 10]

The mere presence of DNA in mitochondria, although suggestive, does not prove that mitochondria possess a genetic apparatus or that mitochondrial DNA performs a genetic function. In this paper we present evidence concerning the physical conservation of mitochondrial DNA during replication in the course of vegetative growth and during sexual reproduction. The results add strong support for the postulate that this DNA functions genetically in determining some aspects of the phenotype of mitochondria, and demonstrate the maternal inheritance of mitochondrial DNA.

Materials and Methods.—The following strains of *Neurospora* were used: (*a*) for experiments performed with N^{15}: EM 5256 (*N. crassa*, wild type); (*b*) for interspecific crosses: 1090 (*N. sitophila;* thiamine$^-$) *poky*, F^+, choline$^-$ (*N. crassa;* derived from a cross 3627-3 × 34486) 6-486-2. (*N. crassa* strain obtained from Dr. Adrian Srb. This culture resulted from backcrosses for many generations of an *N. crassa* strain with *N. sitophila.* In these crosses *N. sitophila* always served as the paternal parent. Accordingly, 6-486-2 can be considered to possess a *sitophila* nucleus in *crassa* cytoplasm.) $N^{15}H_4$ $N^{15}O_3$ and $N^{15}H_4Cl$ (96–97.3%) were purchased from Isomet Corp., Palisades Park, New Jersey.

The details of methods for obtaining conidia, exponential culture of mycelia, isolation of mitochondria, preparation and characterization of mitochondrial and nuclear DNA's and for examining mitochondrial cytochrome content spectrophotometrically have been described previously.[3, 4, 11]

To study the replication of mitochondrial DNA using N^{15}, experiments were performed under conditions originally reported by Meselson and Stahl[12] in their studies of bacterial DNA. For these experiments conidia were obtained in the usual way except that $N^{15}H_4$ $N^{15}O_3$ (2 mg/ml) was the sole nitrogen source (satisfactory conidiation did not occur when $N^{15}H_4Cl$ replaced the $N^{15}H_4$-$N^{15}O_3$). Such N^{15} conidia were harvested, separated from mycelium, and inoculated into growth flasks under standard conditions, the medium having been modified so that $N^{15}H_4Cl$ (0.4 gm/liter) was the only source of nitrogen. The growth of cultures under these conditions was indistinguishable from usual growth under N^{14} conditions. DNA was isolated from mitochondrial and nuclear fractions as described previously[4] (in some cases the final postmitochondrial supernate was used as a source of nuclear DNA) at 10.5, 12, and 14 hr growth. Due to the large number of antecedent replication cycles, it was expected and found that the buoyant densities of DNA's extracted from the cultures at the three time points were identical.

For the transfer experiments described in this paper, N^{15} conidia were grown in N^{15} medium for 10.5 hr, at which point mycelia were filtered and shifted to a dilution medium, modified to contain $N^{14}H_4Cl$ (2.7 gm/liter), and N^{14} adenine, cytosine, uracil, and guanine (30 mg/liter of each compound). The addition of the purines and pyrimidines to the medium did not alter the growth pattern or mass-doubling time, nor did they significantly influence the pattern of DNA replication. Under these growth conditions the dry mass-doubling time of the culture was 2.5 hr. In the case of cultures allowed to grow for 3 τ in the dilution medium, the mycelia were filtered from 50 ml of N^{15} medium and transferred to 150 ml of N^{14} medium. Control experiments, in which mycelial DNA content was determined colorimetrically, revealed that the DNA-doubling and the mass-doubling times were identical during the three doubling cycles under observation.

Crosses of different *Neurospora* stocks were performed using synthetic crossing medium[13] supplemented where necessary with appropriate growth factors. Efforts were made to control the polarity of the cross through the use of selective biochemical supplements, and by careful observation during the period after conidia were applied to the protoperithecia. For most crosses, protoperithecia were allowed to form for 5 days, and ascospores were isolated 10–12 days after application of conidia.

Results.—Replication of mitochondrial DNA: In studying the replication of *E. coli* DNA following transfer of bacteria from N^{15} to N^{14} medium, Meselson and Stahl[12] observed the following sequence of changes in buoyant density of native DNA: (*a*) At the time of transfer the density of native DNA corresponded to that of a fully N^{15}-containing polynucleotide ("heavy"). (*b*) Coincident with growth in N^{14} medium a DNA species of "hybrid" density (intermediate between

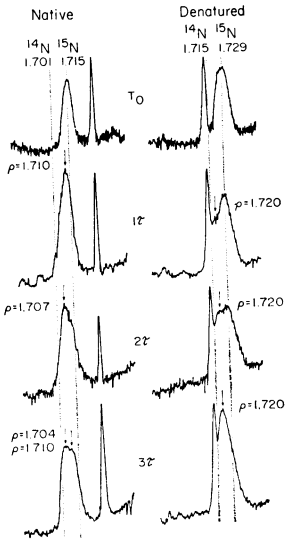

FIG. 1.—Equilibrium density gradient centrifugation in CsCl of *N. crassa* mitochondrial DNA isolated following transfer of mycelium from N^{15} to N^{14} medium. The marker for centrifugation of native DNA was native bacteriophage SP-8 DNA; the marker for denatured DNA was native *E. coli* DNA. Denaturation was accomplished either by incubating a solution (0.15 *N* NaCl) containing DNA in a boiling water bath for 10 min, followed by cooling in ice; or by exposure to 0.04 *N* NaOH for 30 min, followed by reneutralization.

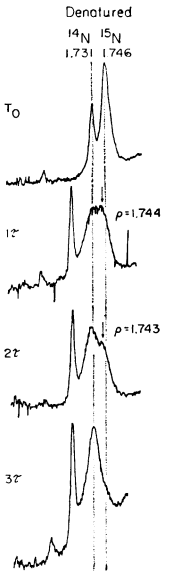

FIG. 2.—Equilibrium density gradient centrifugation in CsCl *N. crassa* nuclear DNA isolated after transfer of mycelium from N^{15} to N^{14} medium. The marker at T_0 was native *Micrococcus lysodeikticus* DNA, that at the other time points was native *E. coli* DNA.

"heavy" and "light") appeared. During the first replication cycle in N^{14} medium the DNA of "hybrid" density progressively replaced the "heavy" DNA. (*c*) After the completion of the first replication cycle a third DNA species ("light") appeared; its density corresponded to that of N^{14}-DNA. (*d*) No DNA species of "intermediate" density (i.e., density falling between "heavy" and "hybrid," or between "hybrid" and "light") were observed.

The changes in buoyant density of *N. crassa* mitochondrial (Fig. 1) and nuclear (Fig. 2) DNA which occur when exponentially growing mycelia are shifted from N^{15} to N^{14} medium differ somewhat from those observed for bacterial DNA under similar conditions, as shown by the following results:

(1) Most of the mitochondrial DNA synthesized during the first mass-doubling cycle consists of strands containing wholly or largely undiluted N^{15}; this is shown by the density distribution of the native DNA (modal density higher than expected "hybrid" density, $\rho = 1.708$) which on denaturation is found to consist mainly (80%) of pure N^{15} strands ($\rho = 1.729$). Even the lighter components contain an appreciable fraction of N^{15}, but the exact proportion cannot be calculated accurately from the available data.

(2) During the second doubling cycle following transfer to N^{14} medium, an increasing proportion of N^{14} is incorporated into mitochondrial DNA. The modal density of native DNA (1.707) is close to that of N^{15}–N^{14} hybrids, and the distribution is skewed toward densities corresponding to high N^{15} content. On denaturation slightly over half of the DNA is found to consist of strands containing undiluted N^{15}. Therefore, even after one complete doubling cycle, a small amount of relatively undiluted N^{15} continues to be incorporated into DNA. The density of the less dense denatured strands formed during this period (1.720) indicates that they also contain some N^{15} (approximately 30%).

(3) By the end of the next doubling cycle, two distinct and almost equal peaks are visible in native mitochondrial DNA. The density of both is slightly greater than

"light" and "hybrid," respectively, suggesting that, even after three doubling cycles, N^{15} is still being incorporated into DNA, although at high dilution. This is confirmed by the density profile following denaturation, which reveals a main peak (1.720) corresponding to a content of approximately 30 per cent N^{15}, and a shoulder corresponding to the pure N^{15} strands.

(4) By extrapolating the densitometric profiles of Figure 1 on magnified tracings, we have made a rough estimate of the proportion of N^{15} strands in DNA at 2 τ and 3 τ; these are equal, respectively, to $\sim$55 per cent and $\sim$22 per cent. The change in ratio N^{15}/N^{14} between 2 τ and 3 τ suggests that mitochondrial DNA has actually undergone approximately one doubling in this interval. Therefore, mitochondrial DNA appears to double at a rate similar to that of mycelial dry mass, total cell DNA, mitochondrial number, and phospholipid.[1, 3]

(5) Although the N^{15} content of "heavy" strands is not diluted in the course of replication, the fraction of "heavy" strands in the total population of mitochondrial DNA molecules decreases during growth in N^{14} medium. However, N^{15} continues to be incorporated into mitochondrial DNA throughout the period under observation, and the amount of N^{15} in mitochondrial DNA increases substantially between T_0 and 3 τ. If undiluted N^{14} flowed into mitochondrial DNA starting at T_0, the expected N^{15} content at 3 τ would be equal to 12.5 per cent of the total; the observed N^{15} content is about 40 per cent; thus the amount of N^{15} in mitochondrial DNA has increased threefold during growth in N^{14}.

The behavior of nuclear DNA under these conditions differs in some ways from that of mitochondrial DNA in the same cell. From the data in Figure 2, it can be seen (1) that the buoyant density of denatured nuclear DNA at T_0 corresponds to that of a fully N^{15}-containing polynucleotide (native N^{14}-*N. crassa* nuclear DNA ρ = 1.713, native N^{15} nuclear DNA ρ = 1.729).

(2) During the first doubling cycle in N^{14} medium, the great majority of newly formed strands are N^{14}, and by the end of this period pure N^{14} and pure N^{15} strands each comprise approximately one half the total amount of DNA. This is in contrast to mitochondrial DNA where pure N^{14} strands were not seen even after three doubling cycles. The pattern at 1 τ suggests the presence of a small fraction of mixed N^{14}–N^{15} strands in nuclear DNA, but the proportion of such strands is too small to alter significantly the density of native nuclear DNA. At 1 τ the modal density observed was 1.721 corresponding to the expected density of the true hybrid (ρ = 1.721).

(3) During two further replication cycles in N^{14}, pure "light" strands continue to accumulate and a corresponding decrease in the proportion of "heavy" strands results. However, N^{15} strands are present both at 2 τ and 3 τ; in the former case the expected fraction appears as the smaller of two peaks in denatured DNA, whereas at 3 τ the N^{15}-strands continue to band at the same "heavy" density and appear as a skewed distribution pattern in the densitometric profile.

It should be noted that while denatured nuclear DNA at T_0 formed a band at a density ρ = 1.746, the density of "heavy" strands at the later time points was decreased to 1.743 and 1.744. This change, although minimal, exceeds that to be expected from random variations in method, and raises the possibility that a small amount of N^{14} was incorporated into pre-existing heavy strands by an undefined process during the first replication cycle following transfer to N^{14}-medium.

The following conclusions may be drawn:

(1) As in the case of other, previously studied systems,[12, 14, 15] the synthesis of mitochondrial and nuclear DNA in *N. crassa* is associated with conservation of intact pre-existing polynucleotide. No evidence has been obtained in these experiments suggesting the involvement of a cycle of depolymerization and resynthesis in DNA replication. The physical continuity of polynucleotide which is maintained throughout several replication cycles is a basic requirement for genetic activity. Both mitochondrial and nuclear DNA satisfy this requirement.

(2) The nitrogenous precursors for mitochondrial DNA synthesis are drawn from a pool which is effectively large in relation to the amount of mitochondrial DNA, turns over slowly relative to the rate of mitochondrial DNA synthesis, and resists dilution by exogenous nitrogen sources. The contrary is true for nuclear DNA. Therefore, the replication of the two DNA species is at least metabolically independent, and perhaps topographically isolated, and a precursor-product relationship between the two is excluded. Based on the flow of N^{14} into mitochondrial DNA during the period T_0 to 3 τ, and assuming normal turnover kinetics and constancy of pool size per unit mass, the effective size of the mitochondrial DNA precursor pool can be estimated to be manyfold greater than the amount of mitochondrial DNA present. Since the existence of such a large pool of soluble deoxynucleotides would be surprising, it may be that in mitochondria, as in some other systems, the turnover of RNA provides the immediate precursors for DNA synthesis.

(3) Since "hybrid" DNA molecules were not observed at the anticipated times, the present experiments do not establish whether the mechanism of replication of mitochondrial DNA is semiconservative. However, the data are entirely consistent with such a mechanism, and the appearance of a distinct band with near-"hybrid" density (ρ = 1.710; theoretical ρ = 1.708) in native mitochondrial DNA at 3 τ is strongly suggestive of it.

Inheritance of mitochondrial DNA: There are several examples in *Neurospora* of the transmission of mitochondrial phenotypes by a uniparental pattern of inheritance.[9, 16, 17] For the determinants inherited in this way, reproduction is essentially asexual and recombination is normally excluded. If mitochondrial DNA were also transmitted under conditions excluding recombination, then factors which promote changes in base ratio of DNA could lead to a situation in which closely related species possessed mitochondria whose DNA's differed in base composition. In such a favorable case, the inheritance pattern of mitochondrial DNA itself might be examined, if viable progeny could be obtained from a cross.

Two species of *Neurospora* provide the opportunity for such an experiment. As seen in Figure 3, the mitochondrial DNA of *N. crassa* contains two DNA populations with buoyant densities in CsCl corresponding to 1.698 and 1.702, respectively. The mitochondria of the closely related *N. sitophila* contain DNA's of these densities, but possess in addition a unique major component banding at ρ = 1.692.[18] We have studied the inheritance pattern of $DNA_{1.692}$ in crosses performed between selected strains of both species.

In the first cross (cross I, Table 1), *N. crassa poky* F^+ $chol^-$ $a(DNA_{1.698 + 1.702})$ served as the protoperithecial, and *N. sitophila* A thi^- ($DNA_{1.692}$) as the fertilizing parent. Ascospores were isolated in order from 19 asci, all of which yielded com-

plete tetrads. The nuclear segregation pattern was abnormal in that none of the progeny were thiamine-deficient;[19] however, the segregation of the genes determining choline requirement and mating type was normal. On the basis of spectrophotometrically detectable excess cytochrome *c* and deficient cytochromes *a* + *b*, all the progeny were *poky*, reflecting the phenotype of the protoperithecial parent. Mitochondrial DNA was isolated from cultures corresponding to each spore pair of two asci, two spore pairs of a third, and one spore pair from a fourth ascus. None of the progeny contained $DNA_{1.692}$ (Fig. 4 shows results for a single ascus). In this respect the maternal phenotype is reproduced and a perfect correlation between the inheritance of mitochondrial cytochrome pattern and that of mitochondrial DNA is observed.

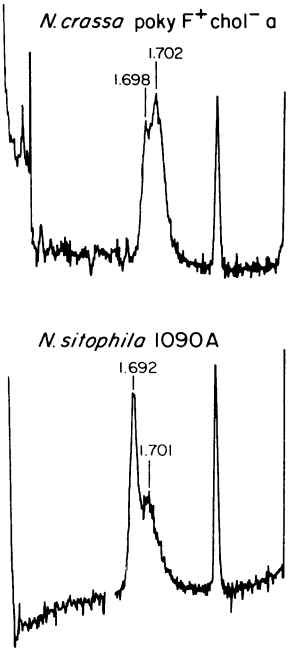

FIG. 3.—Equilibrium density gradient centrifugation in CsCl of mitochondrial DNA from *N. crassa* and *N. sitophila*.

Numerous attempts were made to obtain a reciprocal cross, but these all failed. The progeny of cross I were repeatedly crossed with *N. sitophila* parent; however, the strains isolated from each subsequent cross rather surprisingly showed decreasing ability to fertilize protoperithecia of *N. sitophila*. This approach was therefore discontinued.

In order to observe the heritability of $DNA_{1.692}$ when this component was present in the protoperithecial parent, compatible strains were isolated by the following route: conidia of *Neurospora* strain 6-486, containing $DNA_{1.698 + 1.702}$ (kindly provided by Dr. A. Srb) were used to fertilize protoperithecia of *N. sitophila* 1090. Of four tetrads obtained and studied from this cross (Table 1, cross II), one contained $DNA_{1.692}$ (the results from ascus 1 are shown in Fig. 5). Two strains selected from ascus 1 and 2, respectively, were subjected to reciprocal crosses. In the first of these (cross III, Table 1), strain 1090-I-(1-7) A thi$^-$ $DNA_{1.692}$ was the protoperithecial parent; $DNA_{1.692}$ was found in cultures derived from all spore pairs in two of five asci investigated. In the reciprocal cross, with strain 1090-I(2-3)a $DNA_{1.698 + 1.702}$ as the protoperithecial parent, none of four tetrads examined contained $DNA_{1.692}$. In all asci obtained from crosses II, III, and IV (Table 1), the thi$^-$ marker showed the usual nuclear segregation pattern.

The observations concerning the inheritance of mitochondrial DNA may be summarized as follows: (1) $DNA_{1.692}$, like some other mitochondrial properties, is inherited in a uniparental manner, exclusively through the maternal parent.

TABLE 1

CROSSES PERFORMED BETWEEN *Neurospora* STRAINS WITH DIFFERENT MITOCHONDRIAL DNA'S

Cross	Protoperithecial parent	Conidial parent
I	*N. crassa poky* F$^+$chol$^-$($DNA_{1.698 + 1.702}$)	*N. sitophila* 1090 A thi$^-$($DNA_{1.692}$)
II	*N. sitophila* 1090 A thi$^-$($DNA_{1.692}$)	*N. crassa* 6-486-2a($DNA_{1.698 + 1.702}$)
III	*N. sitophila* 1090-I-(1-7)A thi$^-$($DNA_{1.692}$)	*N. sitophila* 1090-I-(2-3)a($DNA_{1.698 + 1.702}$)
IV	*N. sitophila* 1090-I-(2-3)a($DNA_{1.698 + 1.702}$)	*N. sitophila* 1090-I-(1-7)A thi$^-$($DNA_{1.692}$)

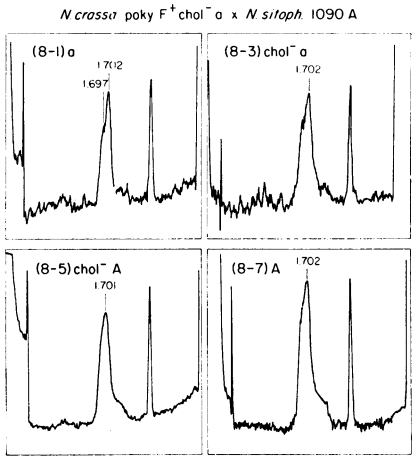

FIG. 4.—Equilibrium density gradient centrifugation in CsCl of mitochondrial DNA isolated from cultures derived from one member of each spore pair in ascus 8, cross I (Table 1). The reference marker is bacteriophage SP-8 DNA.

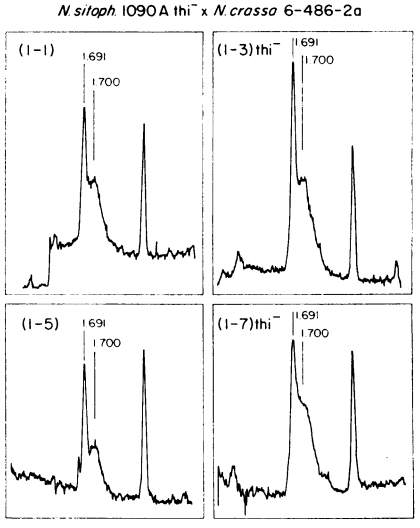

FIG. 5.—Equilibrium density gradient centrifugation in CsCl of mitochondrial DNA isolated from cultures derived from one member of each spore pair in ascus 1, cross II (Table 1). The reference marker is bacteriophage SP-8 DNA.

(2) When a cross permits the maternal transmission of $DNA_{1.692}$, the distribution of this component in the spores of any given ascus is all-or-none.

Although these findings are in accord with the cytoplasmic inheritance of other mitochondrial phenotypes, several reservations are apparent. The first of these concerns the fact that $DNA_{1.692}$, despite its maternal inheritance pattern, is found in fewer than half of the progeny asci, whereas cytoplasmic characters, such as *poky*[9] or SG,[20] are normally transmitted maternally at close to 100 per cent efficiency. This finding could be explained in one of several ways, but presently available data permit no choice between alternative possibilities. For example, two distinct mitochondrial populations, the smaller of which would possess $DNA_{1.698 + 1.702}$, may be present in strains with $DNA_{1.692}$, and these could be inherited independently. Another possibility is that the mechanism which normally assures uniparental inheritance may be operating imperfectly in these strains; such a mechanism could be based on some precise structural compatibility between the trichogyne and fertilizing conidia, and might not be fulfilled in the organs produced by offspring of interspecific crosses. In this case, paternal inheritance might occasionally be observed, provided a suitable mitochondrial marker could be followed. Both of these possibilities would require the additional hypothesis that during sexual cycles, a reduction in the number of mitochondrial genomes occurs, permitting the "selection" of a minority mitochondrial population. A second unavoidable weakness pertains to those crosses conducted with maternal parents containing $DNA_{1.692}$ in which neither parent strain possessed an easily identifiable mitochondrial physiological marker, such as *poky*. Unlike the situation in cross I, therefore, the inheritance of a given mitochondrial DNA species and an enzyme defect characteristic of the same parent could not be associated in these cases. Although

the correlation of the inheritance of mitochondrial enzyme patterns and mitochondrial DNA is at present incomplete, it is clear that the results, so far as they go, are in accord both with the maternal inheritance and genetic activity of mitochondrial DNA. Further work will be required to eliminate the presently remaining uncertainties.

Discussion and Summary.—The observations reported in this paper are consistent with and strongly support the idea that mitochondrial DNA of *N. crassa* performs genetic functions. The DNA component of unique buoyant density and high molecular weight, the RNA-polymerase activity associated with it, and the RNA-polymerase product all appear to be localized within the mitochondria since they are unaffected when the intact organelles are suspended in elevated concentrations of nucleases.[4, 6, 7] The present experiments demonstrate: (1) that the DNA of mitochondria possesses physical continuity throughout several cycles of replication in the course of vegetative growth; (2) that the replication of mitochondrial DNA and nuclear DNA are at least in some respects metabolically independent since the precursors for the synthesis of the two species are drawn from pools which differ in their rates of turnover and dilution by exogenous sources of nitrogen; and (3) that the pattern of inheritance of mitochondrial DNA species themselves is predominantly that usually associated with many abnormal mitochondrial phenotypes, namely, uniparental by way of the maternal parent.

Additional, but still indirect, evidence concerning the involvement of mitochondrial nucleic acids in mitochondrial heredity is found in the behavior of other ascomycetes (*saccharomyces*) which yield a high frequency of mutations affecting mitochondrial functions when exposed to acridines,[10, 21] UV light,[22] and 5-fluorouracil.[23] Moreover, the multiplication of mitochondria by growth and division ensures the continuity of a distinct mitochondrial compartment within the cytoplasm and thus provides a structural basis for the physical segregation and independent activity of mitochondrial DNA.

These characteristics of mitochondrial DNA are those expected of genetically active polynucleotides, but are not in themselves proof of such activity. The fortuitous association of these properties with an inert, nonfunctional species of DNA would be remarkable and unexpected; however, the B chromosomes of maize,[24] and the "crab dAT"[25, 26] may be precedents for such behavior, which should be kept in mind pending the identification of the precise functions and/or the gene product determined by mitochondrial DNA.

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[18] This component was found also in English *N. sitophila* (a wild strain isolated by Dr. A. Srb) and in *N. sitophila* 299, but not in North Africa (NSA), or in Nigeria (Nig 9-A-1) wild-type *N. sitophila*'s. Thin sections of mycelia from different strains of *N. sitophila* were examined in the electron microscope, and sections through mitochondrial pellets obtained from such cultures were similarly examined. No differences were observed in structure of mitochondria from strains which possessed $DNA_{1.692}$ and those which did not. The cytochrome pattern of mitochondrial fractions containing $DNA_{1.692}$ did not differ from those with $DNA_{1.698 + 1.702}$.
[19] Cross I was actually performed twice. Few fruiting bodies contained ripe spores, and numerous perithecia had to be opened for each ascus isolated. These experiences are in agreement with previous accounts [Fincham, J., *J. Genetics*, **50**, 221 (1950); see also ref. 20] which emphasized the difficulty of obtaining viable progeny from interspecific crosses and reported that normal Mendelian segregation was encountered only when the *crassa* parent had been back crossed with *sitophila* many times.
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*THE SYNTHESIS OF INFECTIOUS RNA WITH A REPLICASE PURIFIED ACCORDING TO ITS SIZE AND DENSITY**

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In agreement with the earlier study of the MS-2-RNA coliphage,[1] the RNA replicase isolated from *E. coli* infected with Watanabe's[2] Qβ phage has been demonstrated[3, 4] to require intact homologous RNA as a template. It was further shown that the RNA synthesized is physically,[5] chemically,[6] and biologically[7] indistinguishable from the strands found in mature particles of the Qβ virus.

The *in vitro* serial transfer experiments,[7] which established that the newly synthesized RNA was a self-propagating and biologically competent entity, required infectivity assays of the reaction mixtures. A technical complication was introduced by the presence of viable virus particles in the replicase preparation. Their chemical contribution to the RNA content was trivial compared to the amounts synthesized. However, because they have a far greater infective efficiency than free RNA, even moderate contamination with intact particles cannot be tolerated