

Host Cell Participation in Small Virus Replication

I. Replication of M-13 in a Strain of *Escherichia coli* with a Temperature-sensitive Lesion in Deoxyribonucleic Acid Synthesis

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Received for publication 17 July 1968

The replication of M-13 in a strain of *Escherichia coli* with a thermosensitive lesion in deoxyribonucleic acid synthesis was studied. M-13 failed to replicate at the restrictive temperature, even when the parental replicative form was allowed to form at the permissive temperature. When cells which were actively producing phage at the permissive temperature were shifted to the restrictive temperature, phage production continued. The incorporation of radioactive label into phage particles at 42 C indicated that continued single-strand synthesis was unaffected by the lesion in the host cell.

The genome of the small deoxyribonucleic acid (DNA) bacteriophages, such as ϕ X174, S-13, and M-13, contains about 5,000 to 5,500 nucleotides (21, 25) and probably codes for only a few proteins. So far, six genes have been identified in M-13 (18), six in ϕ X (23), and seven in S-13 (25). This is probably close to the maximum number of genes which is possible (25). With such limited genetic information, these phages probably rely not only on the host for a supply of small molecular weight precursors, but also on the host cell enzymatic machinery for DNA replication. Since the formation of the first replicative forms (RF) of ϕ X174 and S-13 occur in the presence of 100 μ g of chloramphenicol per ml (15, 24), it can be inferred that this step is dependent upon host cell enzymes. Furthermore, it was recently shown *in vitro* that DNA polymerase, in conjunction with a polynucleotide-joining enzyme, can produce a fully covalent duplex circle from the single-stranded circular DNA of ϕ X174 (9, 17). Additional evidence for the participation of host enzymes has been presented by Denhardt et al. (6) who isolated a bacterial mutant (called *rep*⁻) which allows the formation of the first RF but not its subsequent replication. Phages M-13 and ϕ 1 also fail to replicate in this strain.

We began studying the replication of M-13 in *Escherichia coli* strains with a temperature-sensitive lesion in DNA synthesis (*ts* DNA) in an

attempt to clearly define the host-controlled steps in the viral life cycle and hopefully to identify the lesion in the host cells.

MATERIALS AND METHODS

Phage and bacteria. Coliphage M-13 was supplied by D. Pratt, University of Wisconsin, Madison. The preparation of high-titer stocks of purified phage has been described previously (4).

E. coli S-26 was the host normally used for plating M-13. This strain, which was originally described by Garen (8), was supplied by R. L. Sinsheimer, California Institute of Technology, Pasadena.

The strains of *E. coli* used in the experiments to be described were kindly supplied by F. Bonhoeffer, Max-Planck Institut für Virusforschung, Tübingen, Germany. Strain 1 is HfrH, *thy*⁻, *Str*^s; strain 5, a strain 1 derivative, is HfrH, *pro*⁻, *thy*⁻, *str*^r, *ts* DNA. In strain 5, DNA synthesis is normal at 35 C but ceases almost immediately upon shifting to 42 C (2). According to Bonhoeffer (2), DNA polymerase activity, protein and ribonucleic acid synthesis, and the levels of the trinucleotide phosphate pools are normal at the restrictive temperature, and the ultraviolet repair system is intact. Recently, Gellert et al. (Abst. Cold Spring Harbor Symp. Quant. Biol. XXXVIII) reported that this strain has DNA ligase activity at 42 C.

Media. Cells were grown in the defined medium of Maaløe and Hanawalt (16) supplemented with thymine, proline, leucine (40 μ g of each per ml), glucose (0.2%), and Casamino Acids (0.1%). This medium will be referred to as HM medium. Top and bottom agar and KC broth were described by Dowell and Sinsheimer (7), and phage dilution buffer was described by Brown and Dowell (4).

Chemicals. Radioactive methyl-³H-thymine (16.7

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c/m mole) was purchased from New England Nuclear Corp., Boston, Mass. Solid CsCl (optical grade) was obtained from the Harshaw Chemical Co., Cleveland, Ohio. Chloramphenicol was a gift from Parke, Davis & Co., Detroit, Mich.

Controls on bacterial strains. Under certain circumstances, cells of strain 5 can support growth of M-13 at the restrictive temperature; therefore, all cultures were checked for absence of phage prior to infection. In addition, all cultures of strain 5 were examined at the end of each experiment to ensure that they were still unable to grow at 42 C, i.e., they had not reverted.

Phage assay procedure. *E. coli* S-26 was grown with aeration to 3×10^8 /ml at 37 C in KC broth. A 0.4-ml amount of this culture was added to the appropriate phage dilution in 2 ml of melted top agar at 46 C, the mixture was poured over plates of bottom agar as described by Adams (1). The plates were incubated overnight at 37 C.

Trichloroacetic acid precipitation of labeled nucleic acids. Samples (0.1 ml) were added to tubes containing 0.1 ml of denatured herring sperm DNA (800 μ g/ml) and 1 ml of ice-cold 10% trichloroacetic acid. The samples were allowed to precipitate in the cold for 20 min and were then filtered through 0.45- μ m Millipore filters (Millipore Corp., Bedford, Mass.) which had been soaked overnight in 5% trichloroacetic acid. The filters were then washed with 20 ml of cold 5% trichloroacetic acid and placed in scintillation vials; 10 ml of Bray's solution (3) was added.

To prevent nonspecific adsorption of labeled thymine, the 10% and 5% trichloroacetic acid was supplemented with 50 μ g of unlabeled thymine per ml.

Determination of the number of infected cells. Infected cells, washed three times with ice-cold dilution buffer to remove extracellular phage, were resuspended in ice-cold dilution buffer. Samples (0.1 ml) were added to tubes containing 0.1 ml of lysozyme (2 mg/ml) and 0.8 ml of 0.033 M tris (hydroxymethyl)amino-methane (Tris), pH 8.1. After 10 min, samples were frozen (in a dry-ice acetone bath) and thawed (at 42 C) rapidly three times to break open the cells. Freezing-thawing does not affect the viability of unadsorbed phage (19) and, since Brown and Dowell (5) showed that there is no build up of intracellular phage in M-13 infected cells, the difference in the number of infective centers before and after treatment represents the number of infected cells.

Isolation of intracellular DNA. Intracellular DNA was extracted by the method of Brown and Dowell (4).

Sucrose density gradient centrifugation. Samples of 3 H-labeled DNA were layered onto a 5 to 20% linear sucrose gradient containing 0.0187 M sodium chloride and 0.00187 M sodium citrate; 14 C-labeled ϕ X174 DNA was added as a single-strand marker. The gradients were spun for 5.5 hr at 4 C and 37,000 rev/min in a Spinco SW-39 rotor. Fractions were collected directly into scintillation vials; 10 ml of Bray's solution was added.

CsCl equilibrium density gradient centrifugation of labeled phage. Solid CsCl was added (0.46 g/g) to phage-containing samples to give a mean density of 1.30 g/ml. The density was checked by weighing a known volume (500 μ liters) of each sample. Samples

prepared in this way were centrifuged for 24 hr at 4 C and 37,000 rev/min in a Spinco SW-39 rotor. Five-drop fractions were collected, alternate fractions being added directly to scintillation vials; 10 ml of Bray's solution was added. The remaining fractions were collected in glass vials (450 by 125 mm) containing 0.5 ml of dilution buffer and were assayed for phage infectivity.

Scintillation counting. All radioactive samples were counted for at least 10 min in a Packard Tri-Carb liquid scintillation counter. When necessary, corrections were made for quenching.

RESULTS

Infection with M-13 at the restrictive and permissive temperatures. To determine whether the *ts* DNA lesion in strain 5 prevented replication of M-13, cells which had been grown at 35 C were infected with M-13 at both the restrictive and permissive temperatures. From the results shown in Fig. 1, it is clear that there was no increase in infective centers in strain 5 at 42 C over a period of 2 hr, whereas there was a 500-fold increase with strain 1. Both strains showed a similar increase at 35 C. These results suggest that the lesion which prevents host-cell DNA synthesis at 42 C also

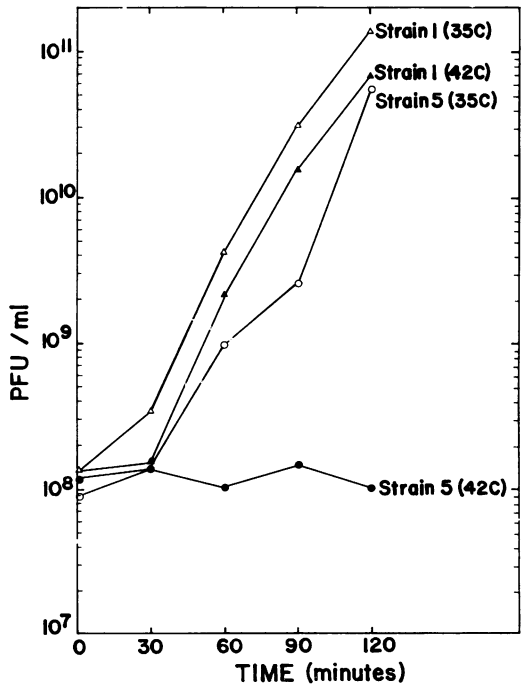


FIG. 1. Cells of both strains were grown in HM medium to 3×10^8 /ml, centrifuged, and resuspended in HM medium at 35 and 42 C; M-13 was added at a multiplicity of infection of 1. Samples were removed at regular intervals for assay of infective centers.

affects the synthesis of viral progeny. However, a similar result would be obtained if the phage failed to adsorb to strain 5 at 42 C. To examine this possibility, the number of infected cells of strain 5 after incubation for 2 hr at 42 C was determined. The number of infective centers before freeze-thawing was 7.50×10^6 ; after freeze-thawing, the number of infective centers was 8×10^4 ; therefore, the number of infected cells was 7.42×10^6 . This represents 13.9% of the viable cells, thus confirming that strain 5 is capable of being infected at the restrictive temperature but is unable to produce phage.

Phage multiplication after temperature shifts. The infective cycle of M-13 is distinguished by the fact that progeny phage are released through the wall of the infected cell without lysis (4, 10, 11, 19). It was thus of interest to see whether cells of strain 5 which were actively producing phage at 35 C could continue to do so after being shifted to 42 C. Figure 2 clearly demonstrates that production of phage by strain 5 was not affected by the shift. However, continued synthesis of phage after the shift could result either from

depletion of a pool of viral single strands synthesized prior to the shift or from de novo synthesis of single strands. By shifting cells into a labeled medium at 42 C, it is possible to distinguish between these alternatives. De novo synthesis of single strands demands that label be incorporated into intracellular phage components and into progeny phage particles at 42 C, whereas no label incorporation would be observed if an intracellular pool of single strands was being depleted. Whereas uninfected cells incorporate only a small amount of label at 42 C, as would be expected for a *ts* DNA strain, infected cells show a marked incorporation of label which parallels phage production (Fig. 3); almost all of this label is in phage-specific components (Fig. 4). Under the ionic conditions used in this experiment, the faster-sedimenting component is RF I and the slower-sedimenting component is RF II (5), whereas host DNA sediments to the bottom of the tube (L. R. Brown, unpublished data). The absence of a peak of radioactivity at the bottom of the gradient shows that host DNA was not labeled, indicating that phage infection does not restore the capacity of the cells to synthesize DNA at 42 C. In the CsCl gradient shown in Fig. 5, the peak of radioactivity coincides with the peak of phage infectivity, demonstrating that labeled phage were produced and, therefore, that de novo

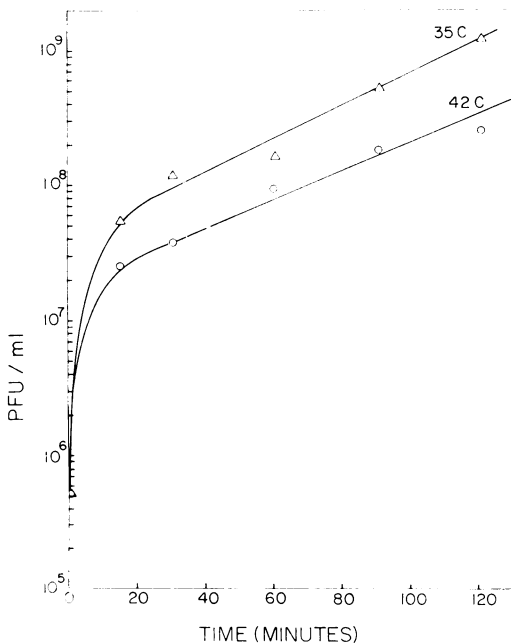


FIG. 2. Cells were grown at 35 C in HM medium to 10^8 /ml, infected with M-13 at a multiplicity of infection of 10, and incubated for 2 hr at 35 C. The cells were washed thoroughly with cold HM medium and resuspended in HM medium at 35 and 42 C. Samples were removed at regular intervals for assay of infective centers.

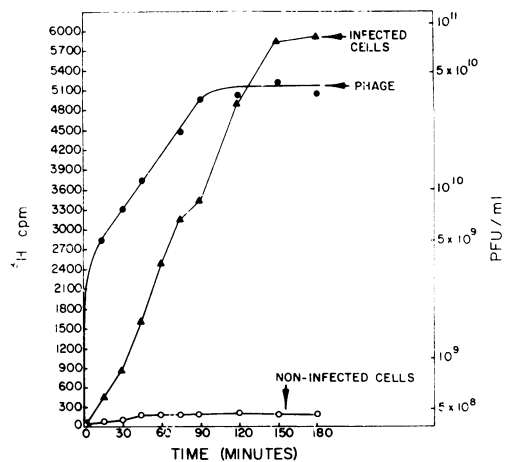


FIG. 3. Cells were grown at 35 C to a density of 10^8 cells/ml and infected with M-13 at a multiplicity of infection of 10. After a further 2-hr of incubation at 35 C, the cells were washed three times and resuspended in HM medium warmed to 42 C and containing $10 \mu\text{C}$ of ^3H -thymine/ml. Samples were removed at regular intervals and assayed for phage and trichloroacetic acid-precipitable counts. Samples (20 ml) were removed 30 min after the shift and were used for analysis of intracellular phage components (see Fig. 4).

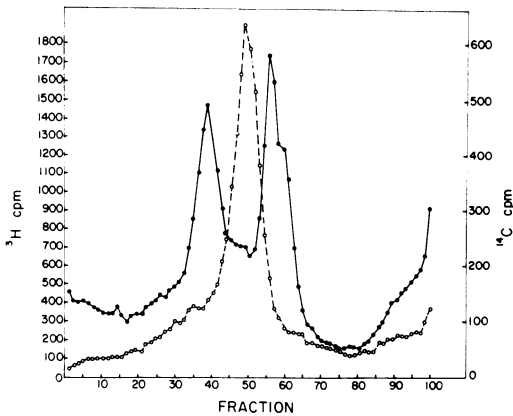


FIG. 4. The 20-ml samples described in the legend to Fig. 3 were centrifuged to pellet the cells, and both the cell and the supernatant fractions were retained. The cells were washed three times with cold HM medium, and the DNA was extracted and sedimented in a sucrose density gradient as described. Symbols: \circ , ^{14}C -labeled single-stranded DNA; \bullet , ^3H -labeled intracellular forms of M-13 DNA.

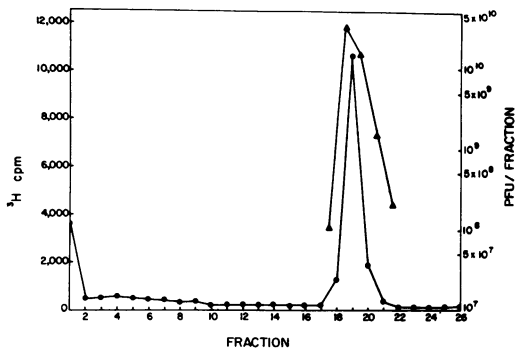


FIG. 5. The supernatant fraction described in the legend to Fig. 4 was spun at 4 C and 27,000 rev/min for 16 hr in a Spinco model L-2 fitted with the SW-30 rotor. The phage pellet was washed once with dilution buffer and banded in a CsCl density gradient. Fractions were collected and assayed for phage and radioactivity as described. Symbols: \blacktriangle , PFU; \bullet , ^3H cpm.

synthesis of viral single strands must have taken place.

Replication of M-13 after infection in the presence of chloramphenicol. When cells are infected with S-13 or ϕX174 in the presence of 100 μg of chloramphenicol per ml, the incoming viral DNA is converted to RF, but subsequent replication of this RF is blocked (15, 24). Pratt and Erdahl (*in press*) obtained similar results with M-13. By infecting cells at 35 C in the presence of chloramphenicol and observing whether phage is produced after the shift to 42 C, it is possible to determine whether formation of the first RF bypasses the block to phage production in the

host cell. The data presented in Fig. 6 indicates that the step blocked is subsequent to the formation of the first RF (Fig. 6) and confirms that the lesion in the host cell at 42 C prevents phage replication.

Adsorption of M-13 to host cells is almost eliminated at cell concentrations below $10^8/\text{ml}$ (26). Therefore, it is unlikely that the increases in infective centers seen in the control cultures are due to reinfection of the cells after release from chloramphenicol inhibition, since the final concentrations used in this experiment were 2×10^5 to $3 \times 10^5/\text{ml}$. However, as an added control, the ability of M-13 to adsorb to *E. coli* at low cell and phage concentrations was examined. Even at phage concentrations of 10^6 particles per ml, which was the maximum reached in the experiment shown in Fig. 6, there was no detectable increase in phage titer over a period of 90 min, but rather a marked decrease (Fig. 7). Thus, it is unlikely that reinfection occurred after release from chloramphenicol inhibition.

DISCUSSION

From the data presented, it is clear that the lesion which prevents DNA synthesis in the host at 42 C also inhibits the replication of M-13. In agreement with this, Dowell and Dapper (*in*

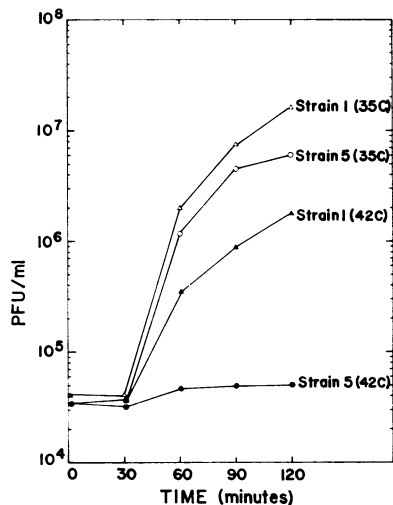


FIG. 6. Cells were grown at 35 C in 20 ml of HM medium to $3 \times 10^8/\text{ml}$, centrifuged, and resuspended in 20 ml of HM containing 150 μg of chloramphenicol per ml. After 5 min, M-13 was added at a multiplicity of infection of 1,000, and incubation continued at 35 C for 1 hr. The cells were washed three times with cold HM to free them of unadsorbed phage, resuspended in 20 ml of cold HM, and diluted 1000-fold into HM warmed to 35 and 42 C. Samples were removed at 30-min intervals and were assayed for infective centers.

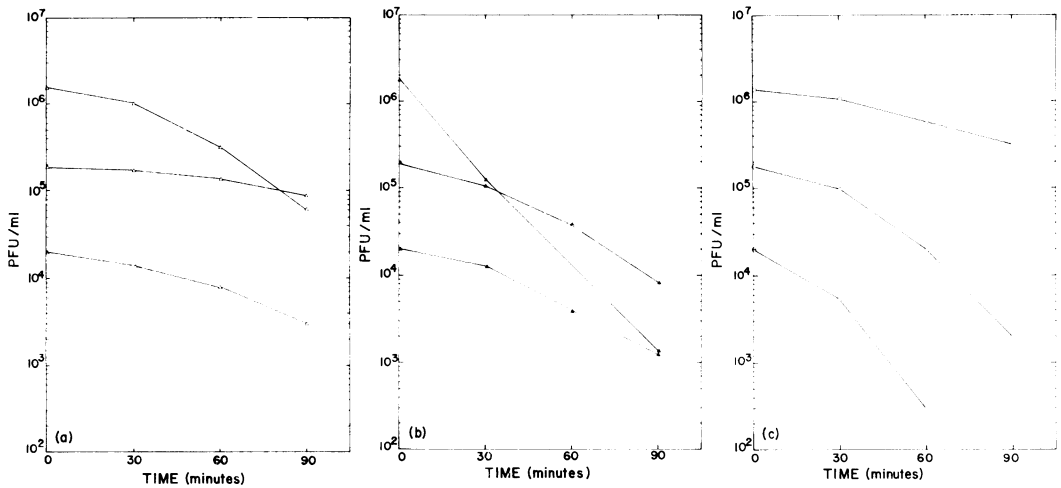


FIG. 7. Cells were grown and treated in the same manner as the experiment outlined in Fig. 6, except that phage was not added after resuspension in the medium containing chloramphenicol. After the 1,000-fold dilution, M-13 was added to give final concentrations of 10^3 , 10^5 , and 10^6 particles/ml. (a) Strain 1 at 35 C; (b) strain 1 at 42 C; (c) strain 5 at 35 C.

preparation), Sinsheimer (*personal communication*), and Denhardt [cited by Sinsheimer (22)] found that ϕ X174 fails to replicate in a Bonhoeffer strain 5 at 42 C. It is also apparent with M-13 that the lesion at 42 C can be bypassed by allowing infected cells to reach the phase of single-strand synthesis, and then shifting them to the restrictive temperature. However, with ϕ X174, phage production stops after the shift to 42 C (22).

The replication of an RF molecule probably requires the participation of a polymerase to make the two daughter strands and a ligase to join the 3' and 5' ends. Possibly a third enzyme could be required to open one of the strands to permit their separation during replication. Another protein could be involved, since ϕ X174 RF does not replicate unless it is attached to a 'site' on the host-cell membrane (12, 27). However, this 'site' could represent the cellular location of some of the enzymes referred to above (12, 27). Since the *E. coli* chromosome is also a double-stranded circle, similar enzymes, if not the same ones, are probably involved in its replication.

The replication of RF appears to require both phage- and host-specified functions. A 100- μ g amount of chloramphenicol per ml is known to prevent RF replication (15, 24). A similar effect was obtained by infecting cells with mutants in gene 2 of M-13 (Pratt and Erdahl, *in press*), gene IV of S-13 (24), and gene VI of ϕ X174 (23). The experiment reported here, which shows that the lesion in strain 5 at 42 C cannot be bypassed by infecting cells at 35 C in the presence of chlor-

amphenicol, indicates that a host-specified function is also required. Denhardt et al. (6) and Friedman and Tessman [cited in (20)] isolated mutants which do not allow RF replication, but these may differ from strain 5 since these mutations are not lethal for the host cell. At the present moment, it is not possible to correlate any of the known mutations in either the host or the phage with any of the necessary enzymatic steps.

With M-13 infection, no precursor relationship has yet been shown between any of the RF molecules and single strands but, by analogy with ϕ X (13, 14), it is reasonable to assume that such a relationship exists.

The data presented here show that, although single-strand synthesis can occur at 42 C (Fig. 5), M-13 is unable to replicate at this temperature even after RF formation at 35 C (Fig. 6). If M-13 replication is identical with ϕ X174, then this suggests that either RF replication or the initiation of single-strand synthesis is blocked in strain 5 at 42 C. The isolation of labeled RF I and RF II after a temperature shift (Fig. 4) demonstrates that synthesis of these components continues at 42 C. However, this does not necessarily imply that RF replication is unaffected by the lesion in the host cell, since it is possible that once RF synthesis is initiated at 35 C, it will continue after a shift to 42 C. Alternatively, labeled RF could be obtained after the shift, even in the absence of net RF synthesis, if one of the strands of the preexisting RF molecules is replaced by a displacement mode of replication in an analogous manner to ϕ X single-strand synthesis (13, 14).

This is currently being examined and should determine whether RF is indeed a precursor of viral single strands.

According to Brown and Dowell (5) and Pratt and Erdahl (*in press*), there is no large intracellular pool of single-strand DNA; the sedimentation profile shown in Fig. 4 is in agreement with their results.

An ancillary consequence of these experiments is the development of a technique for producing radioactively labeled phage with 10 times as many counts per minute per phage than has been described previously (4). If cells of strain 5 which are actively producing phage at 35 C are shifted to 42 C into medium containing 10 μ c of 3 H-thymine per ml as the sole thymine source, it is possible to obtain phage with an activity of 1 count per min per 10^5 phage particles.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI 07657 from the National Institute of Allergy and Infectious Diseases, and by National Science Foundation grant GB-7164. During the investigation, S. B. Primrose was in receipt of a University of California Regents Fellowship and a Fulbright Scholarship from the U.S.-U.K. Educational Commission. L. R. Brown was supported by Public Health Service Fellowship 5-F1-GM-30, 403-03.

We gratefully acknowledge the excellent technical assistance of Diane Dapper. We thank D. Pratt for communicating his results prior to publication.

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