We wish to acknowledge the excellent services of shipmaster Captain Terry Hansen and his capable crew.

Much help and stimulation was obtained through discussions with Dr. Theodore Enns, Dr. Andrew A. Benson, Dr. George G. Laties, and Dr. Anders Kylin. We wish to acknowledge also the fine cooperation received from the Secretaria de Marina and the Direccion General de Pesca e Industrias Conexas, who graciously permitted us to work in Mexican Waters, and from Dr. Richard Croker of the American Embassy in Mexico City.

* Contribution from the Scripps Institution of Oceanography, University of California (San Diego).

† John B. Pierce Foundation, New Haven, Connecticut.

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*ISOLATION OF THE GROWING POINT IN THE BACTERIAL CHROMOSOME**

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Communicated by Max Delbrück, May 25, 1964

Current ideas and evidence concerning the replication of the bacterial chromosome can be summarized as follows: (1) The chromosome consists of one piece of *double-stranded* DNA[1] (mol wt 2×10^9) which may exist as a closed circle.[1, 2] (2) It replicates *sequentially* from a starting point[3, 4] by forming a single growing point at a fork which moves along the structure.[1, 5] (3) Protein and/or RNA synthesis is required to initiate replication of the chromosome but the cycle can then be completed under conditions of protein synthesis inhibition.[6–8] (4) Replication is *semiconservative* and involves the separation of parental DNA strands "near" the growing point as complementary daughter strands are formed.[9–12]

In the autoradiographic analysis by Cairns,[1] the entire replicating chromosome was observed, but the linear grain density in the photographic emulsion (approximately one exposed grain per micron) limited the resolution of the growing point region to that of a 2×10^6 molecular weight segment of DNA. Preparatory to molecular characterization of the growing point region we have developed a procedure for the isolation of chromosome fragments containing this region, and we have defined some of the necessary conditions for the observation of these partially replicated DNA units.

Materials and Methods.—The thymine-requiring *E. coli* strain TAU-bar[13] was cultured aerobically in a glucose-salts synthetic medium at 37°C with required supplements as previously described.[6, 13] Exponential growth (mean generation time, 40 min) was maintained by periodic dilution into prewarmed medium to keep the cell concentration between 5×10^7 and 2×10^8 cells/ml.

The bacterial DNA was uniformly prelabeled with tritium by growth for 8–12 generations in medium containing H^3-methyl-thymine (New England Nuclear, Boston) at 0.85 μg/ml and a specific activity of 380 mc/mM.

Media changes were accomplished by the rapid filtration technique previously described.[6]

Density labeling involved the substitution of 5-bromouracil (5 BU) (2 μg/ml) in the growth medium in place of thymine. At 37°C the DNA synthesis *rate* in the presence of 5 BU was about half that for cultures growing with thymine.

P^{32}-pulse labeling of DNA involved the growth of 100-ml cultures in the minimal medium with the phosphate concentration reduced to 10^{-4} *M* and the subsequent addition of 6 mc P^{32}-orthophosphate (Radiochemical Centre, Amersham, England).

Preparation of cell lysates: Isotope incorporation and growth were stopped abruptly by the dilution of a 100-ml culture with an equal volume of ice-cold buffer (containing 0.1 *M* NaCl, 0.01 *M* EDTA, 0.01 *M* Tris (Sigma-121), and 0.01 *M* KCN at pH 8) abbreviated NET-CN. The cells were harvested by collection on a 9-cm diameter membrane filter (Schleicher & Schuell, Grade A coarse)[6] and rinsed with 100 ml cold NET-CN before resuspension in a 4-ml volume of NET-CN containing 1.5 *M* sucrose. The concentrated cell suspension was placed in dialysis tubing, and 0.1 ml of a 4 μg/ml solution of freshly prepared egg white lysozyme (Worthington, 2× recrystallized) was added. A 30-min incubation at 37°C with the dialysis tube suspended in NET-CN plus 1.5 *M* sucrose resulted in protoplasting of the cells. Attempts to use the Duponol procedure of Cairns[1] were unsuccessful for the subsequent isolation of newly replicated material in a CsCl density gradient. Up to this point in the procedure no appreciable shearing of the DNA should have occurred because the chromosomes were still contained within cell membranes.

In successive dialysis steps the sucrose concentration was reduced from 1.5 *M* to 0.75 *M* to 0, resulting in gradual lysis of the protoplasts. The dialysis tube was opened and the lysate was gently poured directly onto solid cesium chloride (Harshaw, optical grade) in a weighed beaker so that the density could be adjusted to 1.72 gm/cc without sampling. The pH of the cesium chloride solution was adjusted to 10.5 by the addition of 0.5 ml 0.5 *M* K_2HPO_4, pH 10.5, a step which greatly improved the yield of growing point regions, presumably by aiding in the high salt deproteinization of these regions. Use of alkaline CsCl was suggested to us by the paper of Vinograd *et al.*[14] The pH 10.5 is below that which would be expected to partially denature 5 BU hybrid DNA in CsCl.[12]

Density gradient equilibrium sedimentation: Four milliliters of the alkaline CsCl solution was slowly poured into a cellulose tube, layered with mineral oil, and subjected to 48 hr at 37,000 rpm and 20°C in the SW39 rotor of a Spinco Model L ultracentrifuge. After deceleration, drops were collected through a pinhole punched in the tube bottom, and one or two drop fractions were diluted with 0.5 ml 0.01 *M* NaCl, 0.001 *M* EDTA, 0.001 *M* Tris pH 8. For counting, 0.1-ml aliquots were added to 0.2 ml 1.0 *M* KOH and allowed to stand at room temperature overnight to hydrolyze RNA. Salmon-sperm DNA (10 μg) was added to each sample as carrier before the addition of 5 ml ice-cold 5% trichloroacetic acid. Samples were then collected by suction filtration on 23-mm diameter Millipore filters (Type HA, 0.45 μ pore size) and rinsed twice with 5 ml distilled water. After drying under a heat lamp the filters were placed in glass counting vials containing 5 ml toluene, 18 mg PPO, and 0.45 mg dimethyl-POPOP. The Packard TriCarb scintillation spectrometer was adjusted for two channel simultaneous assay of tritium and P^{32}. Overlap corrections were made by reference to freshly prepared standards.

Design of the experiments: In the random fragmentation of the bacterial chromosome during isolation, only one (if any!) of the isolated fragments would be expected to contain the fork, as illustrated in Figure 1*A*. When bacteria are transferred to a medium containing a density label (e.g., 5 BU) and the DNA is then extracted at an arbitrary time later, two classes of chromosomal fragment would be expected to have densities intermediate between normal (thymine-containing) and hybrid (5 BU replacement of thymine sites in one strand).[15] As shown in Figure 1*B*, these are the fragments containing *transition points* (i.e., the position of the growing point at the instant of transfer to 5 BU growth medium), and the *replication point* as caught at the instant growth was stopped. Fragments containing the transition points have previously been demonstrated.[5, 15] As far as the isolation of DNA is concerned, the transition point material should be sufficiently distant from the growing point region to behave as the bulk of the chromosomal fragments.[5] To distinguish between transition point regions and the growing point regions it is necessary to

demonstrate that the latter, but not the former, become hybrid within one generation period. Figures 1*C* and *D* illustrate a pulse and chase experiment to accomplish this.

Results.—Figure 2 shows the result of a P^{32} pulse and chase experiment to demonstrate the transfer of intermediate density material to the hybrid region. The bacterial culture was transferred to 5 BU medium 30 min before the addition of P^{32}. A rebanding of the intermediate density region from the chase (Fig. 3) shows that the P^{32} activity has been removed from that region but that some of the tritium activity remains, as expected for the fragments containing transition points. In Figure 4*A*, the rebanded intermediate density region from the pulse exhibits both P^{32} and H^3 activity in the intermediate region, but when an equal aliquot is subjected to further fragmentation by sonication (Fig. 4*B*), both P^{32} and H^3 activity are resolved in broader bands at the hybrid and normal densities. Note that no P^{32} appears in the normal band.

To improve the yield of DNA in a subsequent experiment, a portion of the cell lysate was treated with the enzymes lipase, trypsin, and chymotrypsin before the addition of alkaline CsCl. Equal portions of the lysate with and without such treatment are compared in Figure 5. Note that the enzyme treatment doubles

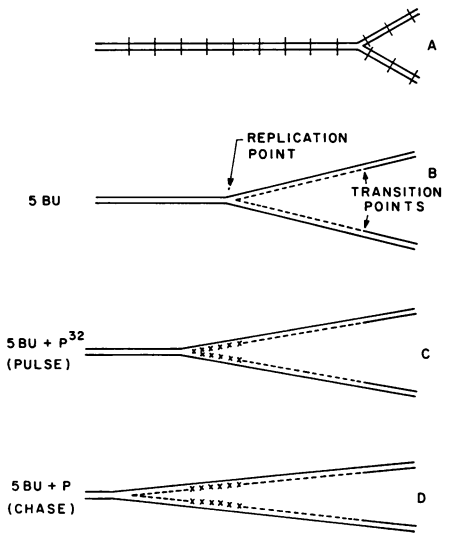

FIG. 1.—Schematic representation of a segment of the bacterial chromosome containing the growing point. (*A*) Fragmentation of the DNA during extraction. Single strands shown as solid lines. Short cross lines indicate lateral scissions and would be expected also for the three lower diagrams. (*B*) The same chromosomal segment after a period of density labeling with 5 BU. The dashed lines are 5 BU containing strands. (*C*) Same segment after short pulse incorporation of P^{32} to label growing point fragment during growth with 5 BU. P^{32} containing regions indicated by *x*'s. (*D*) Transfer of P^{32} containing regions into hybrid fragments during subsequent growth with 5 BU and unlabeled phosphate.

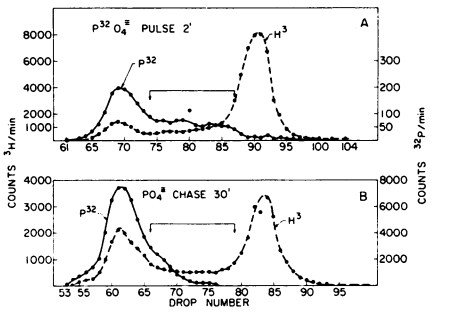

FIG. 2.—Density distributions of DNA fragments in alkaline CsCl following bacterial growth with 5 BU. A 30-min period of 5 BU incorporation preceded the addition of P^{32} so that the replication point but not the transition point would be labeled during the P^{32} pulse. (*A*) After 2′ pulse of P^{32} as indicated in Fig. 1*C*. (*B*) After 30-min chase with unlabeled phosphate as shown in Fig. 1*D*. ●----●, tritium activity, from uniform prelabeling of DNA (see *Methods*). Peak on the right is at the position of normal unreplicated DNA. ●——●, P^{32} activity. Peak on the left is at the position of hybrid, completely replicated DNA fragments. Intermediate density fractions for rebanding (Figs. 3 and 4) are indicated by bracketed arrows.

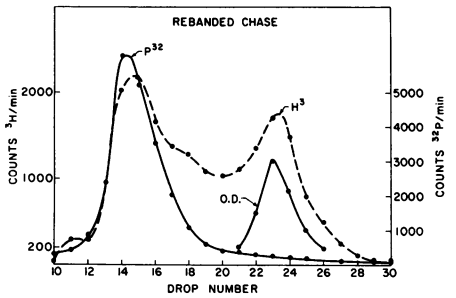

FIG. 3.—Rebanding of intermediate density fractions from Fig. 2*B*. Normal unlabeled TAU-bar DNA added as optical density (O.D.) marker. O.D. scale not shown.

the yield of normal and hybrid fragments but that it more than triples the yield of partially replicated material.

In a third preparation (not shown), the lysate was held at 60°C for 30 min before adding the alkaline CsCl. This treatment also doubled the yield but did not change the *relative* yield of intermediate density material.

It might be predicted that the fork in the replicating DNA would be particularly sensitive to shear and that this would explain, in part, why it has not previously been isolated in density gradients. An experiment to test this is illustrated in Figure 6. The relatively mild shearing in a vortex mixer removes essentially all of the P^{32} activity from the intermediate density region while leaving some H^3 activity, as expected if replication points are more sensitive to shear than transition points. To determine the size of the DNA fragment which is being observed in these experiments and the extent of fragmentation by the vortex mixer, the sucrose gradient procedure of Burgi and Hershey[16] was used as shown in Figure 7. The molecular weight distribution observed in Figure 7*A* represents a *lower* limit for this isolation procedure, since drop collecting from the preparative CsCl gradient probably shears the DNA also. Yet our rebanding experiments show that at least some fragments containing replication points survive the drop-collecting procedure.

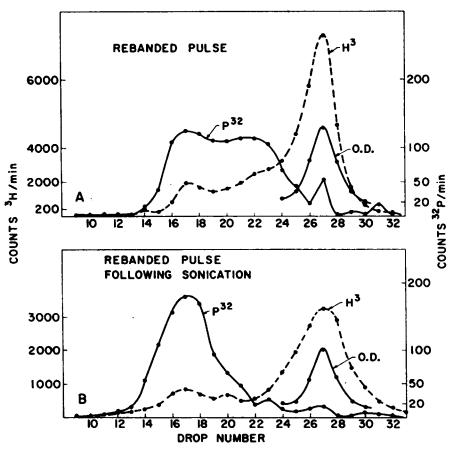

FIG. 4.—Rebanding of intermediate density fractions from Fig. 2*A*. Normal unlabeled TAU-bar DNA added as density marker as in Fig. 3. (*A*) No further treatment. (*B*) Following sonication for 30 sec under conditions previously described.[15]

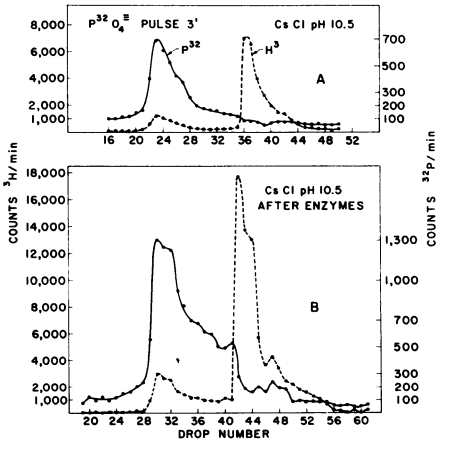

FIG. 5.—Effect of enzyme treatment on recovery of pulse-labeled DNA in the cesium chloride gradient. Culture treatment same as for Fig. 2*A*, but 3-min pulse of P^{32} was used. ●- - - -●, tritium activity; ●——●, P^{32} activity. (*A*) Same isolation procedure as for Figs. 2*A* and *B*. (*B*) Lysate subjected to 37°C incubation with lipase [(wheat germ) B grade, Calbiochem]. 50 γ/ml for 10 min, then trypsin 20 γ/ml and α-chymotrypsin 20 γ/ml for 15 min before adding to CsCl.

The DNA preparation as shown in Figure 7*A* is very heterogeneous (molecular weight range roughly 3×10^7 to 20×10^7) but the mean molecular weight is 10^8, considerably higher than that usually obtained from bacteria.[5, 18] Further fragmentation in the vortex mixer reduces this to a more homogeneous preparation in the molecular weight range of roughly 3×10^7 to 6×10^7, which is in the range generally obtained[5, 18] (Fig. 7*B*).

Resistance to isolation of newly replicated DNA: The importance of quantitating recovery of DNA in studies on its physical state *in vivo* has recently been emphasized by Cohen.[19] Experimental evidence that newly replicated DNA differs in state from the bulk of cellular DNA has come from the studies of Goldstein and Brown[20] in which pulse-labeled bacterial DNA was found to be selectively resistant to release from aggregates by sonication. Also, it has been demonstrated that newly synthesized DNA remains in the interface after a chloroform-octanol extraction which releases 90 per cent of cellular DNA from a rabbit kidney cell preparation.[21]

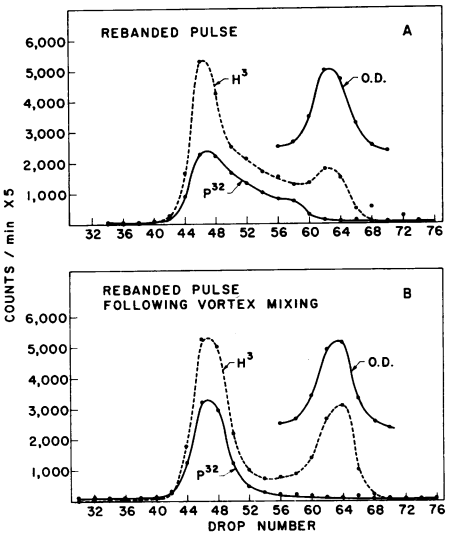

FIG. 6.—Rebanding of intermediate density fractions 36–40 inclusive from Fig. 5*B*. Unlabeled TAU-bar DNA added as density marker. (*A*) No further treatment. (*B*) Following 5 min in Cyclomixer (Clay-Adams, N. Y.)

We have observed a similar resistance to isolation of newly replicated DNA from bacterial preparations. In Figure 5, the lipase and proteolytic enzyme treatment increased the yield of DNA from 22 to 40 per cent while also increasing the *proportional* yield of pulse-labeled intermediate density DNA in the cesium chloride gradient. (The unrecovered DNA is found in the meniscus at the end of a density gradient sedimentation run, presumably because it is still bound to protein.)

The deproteinization of a lysate (before addition to alkaline cesium chloride) by shaking 5 min on the vortex mixer with an equal volume of chloroform-octanol 9:1 (v/v)[22] was observed to lose P^{32} pulse-labeled (i.e., newly replicated) DNA from the aqueous phase selectively, even though over-all yields as high as 90 per cent of the total DNA could be obtained. The treatment of such a lysate with lipase, trypsin, and chymotrypsin (as for Fig. 5) before shaking with chloroform-octanol gave a 6 per cent increase in yield of uniformly labeled H^3-DNA in the aqueous phase, but a 38 per cent increase in recovery of P^{32} pulse-labeled DNA. In another experiment, the yield of alkaline resistant P^{32} activity in the aqueous phase was improved from 32 to 50% when the lysate was treated with the enzymes prior to chloroform-octanol extraction. Thus far, it has not been possible to obtain a quantitative recovery of newly replicated DNA. The detergent and phenol methods for deproteinization have also been found to select against newly replicated DNA.

Discussion.—We have shown that P^{32} pulse-labeled DNA during density labeling with 5BU appears in the intermediate density region between normal and hybrid

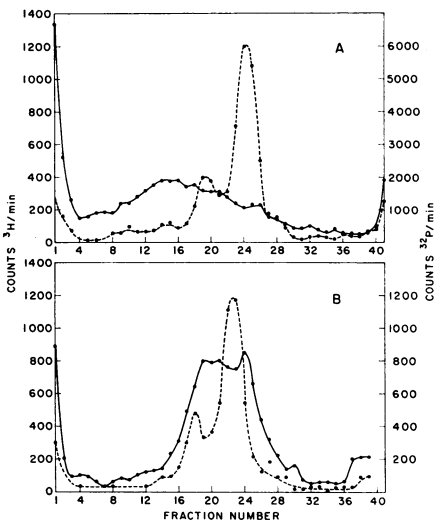

FIG. 7.—Molecular weight distribution of DNA isolated by our method and the effect of mild shearing on this distribution. P^{32}-labeled DNA was isolated from the hybrid band in a preparation such as shown in Fig. 5*B*. A 0.03-ml (less than 5 γ/ml DNA) volume was added with a 0.07-ml volume containing H^3-DNA (phage λ DNA at 50 γ/ml) to the top of a 5–20% linear sucrose gradient. Fractions were collected after 4-hr centrifugation at 28 K and assayed for H^3 and P^{32} activity. (See *Methods* and refs. 16 and 17.) ●----●, tritium activity, λ DNA marker (31 × 10^6 MW); ●——●, P^{32} activity. The small H^3 peak probably represents a dimer of λ DNA molecules, as calculated from its position relative to the main H^3 peak. Such dimers have been reported by Hershey *et al.*[17] and have been shown to occur at high DNA concentrations. The small peak was not observed in a sucrose gradient run in which the λ DNA concentration was reduced by a factor of two (Ray and Hanawalt, to be published). Velocity sedimentation studies on this particular λ DNA preparation by Dr. William Studier indicated that no fragments of the whole λ DNA were present. The high concentration of the λ DNA required a correction in the observed sedimentation rate. (*A*) Unsheared preparation. (*B*) Following 5 min on Cyclomixer (see text).

densities and that it leaves this region (presumably by becoming completely hybrid-upon chasing with unlabeled phosphate. It could be argued that the lighter) than-hybrid density is due to protein binding to newly replicated hybrid segments and that this protein is released as the growing point moves beyond these segments. We consider this explanation unlikely for a number of reasons. In P^{32} pulse-labeling experiments in which no density label was used (not reported in this paper), the newly synthesized DNA appeared in a symmetrical band at the same density as the bulk of the isolated DNA. In Figure 5, we show that the relative yield of intermediate density DNA is *increased* following proteolytic enzyme treatment of the cell lysate. A comparison of Figures 6*A* and *B* shows that the effect of mild shearing on the intermediate density material is to remove the P^{32} counts to the hybrid band but simultaneously to remove tritium activity (i.e., unreplicated fragments) to the normal band. Thus, the shearing involves a separation into normal and hybrid density fragments as expected if the intermediate density region contained the fork in partially replicated DNA units.

The isolation of partially replicated DNA units is just a first step in the physical characterization of the growing point region in the bacterial chromosome. However, in reporting the isolation of such fragments we can define two important problems which may explain why they have not previously been observed.

(1) The growing point is very sensitive to shear, evidently more so than the bulk of the DNA. Any isolation procedure which results in shearing to the 30 million molecular weight range will probably preclude the observation of partially replicated DNA fragments. Thus, shearing of the bacterial chromosome during isolation is not random with respect to the replication point.

(2) The growing point region is more resistant to isolation than the bulk of the DNA and will be selectively lost at the meniscus in cesium chloride density gradi-

ents. The use of alkaline cesium chloride and our pretreatment of lysates with lipase and proteolytic enzymes to disaggregate DNA from complexes has been shown to help in recovery of this material. (The specific and independent functions of the lipase and/or proteolytic enzymes in this disaggregation have not been ascertained.)

The nature of the two problems above poses a dilemma. If one uses a very gentle method for isolation and deproteinization of the DNA (e.g., the detergent method of Cairns[1]), then the newly replicated DNA will not be recovered in a cesium chloride gradient. On the other hand, vigorous shaking and extraction with chloroform-octanol may improve the yield of newly replicated material but will certainly shear all of it at the replication point.

We cannot estimate the size of the partially replicated fragments from the relative amount of P^{32} in hybrid and intermediate regions following a pulse of known duration, because of the difference in sensitivity to shear and resistance to isolation of these fragments. However, the mere existence of such fragments rules out that replication is an all-or-none event within defined regions along the chromosome and that breakage necessarily occurs at the ends of such regions. The resistance to isolation of partially replicated DNA regions might have been predicted since the polymerase (or polymerases) engaged there, or perhaps structural proteins involved in the "zipper," should require more stringent deproteinization procedures than for the bulk of the chromosomal DNA.

Our studies have little bearing on the reported observation of prereplicative states of DNA in bacteria by Rolfe,[23] Rosenberg and Cavalieri,[24] and Lark.[25] We have not obtained any detectable amounts of DNA at positions corresponding to denatured DNA components in P^{32} pulse-labeling experients in which no density label was used. It is possible that a denatured portion of the chromosome ahead of the growing point would have a chance to renature in the course of our lysate preparation, as it is perhaps equally possible that the DNA strands might continue to unwind without synthesis at the growing point in other isolation procedures. Continuing studies are designed to examine these possibilities.

Summary.—A thymine-requiring bacterium was transferred to a medium containing the thymine analogue, 5-bromouracil, as a density label for newly replicated DNA. Following a gentle lysis procedure, the density distribution of the DNA molecules was examined in cesium chloride density gradients. A small fraction of the DNA molecules were isolated at densities intermediate between normal (unreplicated) and hybrid (5-bromouracil in one strand) and these were characterized as containing transition points (i.e., the replication point at instant of transfer to 5-bromouracil medium) or replication points (as caught at the instant growth was stopped). The isolation of replication points was specifically shown by P^{32} pulse-labeling during 5-bromouracil incorporation and the demonstration that the intermediate density P^{32} activity could be "chased" into the hybrid density region by subsequent growth with unlabeled phosphate. The extracted DNA was shown to have a mean molecular weight of 10^8, and mild shear which reduced this to the 3 to 6×10^7 range sheared all replication point containing molecules into hybrid and normal density fragments. The resistance to isolation and the sensitivity to shear of partially replicated DNA molecules is discussed.

*This work was supported by a grant GM 09901 from the U.S. Public Health Service. We are indebted to Dr. V. Bode for supplying H^3-labeled λ phage DNA.

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*THE ENVIRONMENTAL CONTROL OF INSULAR VARIATION IN BIRD SPECIES ABUNDANCE**

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Communicated by Ernst Mayr, May 27, 1964

Theory.—It can be assumed that the number of species found breeding on a given island is a resultant of (*a*) the chances of reaching the island by dispersal, and (*b*) the chances, once there, of becoming reproductively established by the finding of vacant or available ecological niches or spheres.[1] Isolation in this instance may have two roles: either a direct one which influences the probability of dispersing individuals reaching islands, or an indirect one influencing the success of any particular colonization attempt by effects on the previous filtering of species of plants and animals upon which new colonizers may be dependent for ecological support.[2] Information on the relative importance of the two facets of the colonization problem (viz., isolation and ecologic diversity) may be gained by the quantifying and testing of the influence on species numbers of various factors of the insular environment by multiple regression and variance analysis. Thus it is possible to determine the ability of an environmental variable to predict species numbers in-