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*CELL-FREE PROTEIN SYNTHESIS: THE NATURE OF THE ACTIVE COMPLEX**

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In a previous communication[1] we presented evidence indicating that the addition of RNA from turnip yellow mosaic virus (TYMV) to purified *E. coli* ribosomes results in the formation of a complex containing one 70s ribosome and one molecule of RNA. We shall call such complexes monosomes. Indirect evidence, consisting of a correlation between the number of monosomes formed as a function of the RNA-ribosome ratio, and the extent of amino acid incorporation measured in parallel experiments, suggested that monosomes were the active complexes for protein synthesis in the cell-free system. However, the *direct* experiments were restricted to the demonstration that before protein synthesis had occurred, viral RNA was to be found only in monosomes. In this paper it will be shown that *after* protein synthesis has occurred, the newly formed protein appears predominantly in monosomes, and *while* viral RNA-directed protein synthesis is taking place, the only *activity* for amino acid incorporation is found in monosomes.[2] On the other hand, poly U promotes the formation of polysomes;[3–5] these are compared with monosomes with respect to the rate and extent of amino acid incorporation.

Materials and Methods.—Ribosomes and supernatant were obtained by alumina grinding of freshly grown log phase *E. coli* B, as described previously,[6] except that the buffer used to prepare the crude extract was changed to 0.014 *M* Mg^{++}, 0.06 *M* KCl, 0.01 *M* Tris pH 7.6, 0.006 *M* mercaptoethanol.[7] The crude extract was centrifuged for 20 min at 16,000 rpm, and the supernatant recentrifuged for 30 min at 16,000 rpm. To deplete endogenous messenger RNA, the supernatant thus obtained was supplemented with ATP, GTP, PEP, PK, and cold amino acids, and incubated for 80 min at 36°C.[8] After incubation the ribosomes were purified by several cycles of centrifugation at 38,000 rpm. The supernatant from the first of these ribosome centrifugations was concentrated by dialysis against polyethylene glycol, and then dialyzed against several changes of 0.01 *M* Tris, 0.006 *M* mercaptoethanol, 0.01 *M* Mg^{++}, 0.02 *M* KCl. P^{32}-labeled TYMV-RNA was obtained as described previously.[1] The poly U sample was part of a gift from L. Heppel to E. P. Geiduschek; it had $S_{20} = 5.3$ in 0.1 *M* NaCl. TMV-RNA was prepared by phenol extraction[9] of TMV (strain U1) purified by differential centrifugation in the presence of 0.01 *M* EDTA, pH 8.[10]

The complete system for amino acid incorporation contained, in 0.5 ml, 25 μmoles Tris pH 7.5, 25 μmoles KCl, 5 μmoles Mg^{++}, 5 μmoles mercaptoethanol, 2 μmoles PEP, 0.02 mg PK, 1 μmole ATP, 0.2 μmoles GTP, 0.25 μmoles of each amino acid except the label, ribosomes, and RNA as

indicated, and a saturating amount of supernatant, determined in advance for each preparation of supernatant. Reaction mixtures were worked up as before,[1] except that the final TCA insoluble pellets were taken up in dilute NH_4OH, transferred to ringed planchets, dried, and counted in a Nuclear-Chicago thin end-window counter. Labeled amino acids were obtained from New England Nuclear Co.; the C^{14}-proline and the C^{14}-phenylalanine had specific activities of 205 and 369 mC/mmole, respectively. Sucrose gradients were formed and analyzed as before.[1] Unless otherwise indicated, the gradients contained 0.05 *M* Tris, 0.05 *M* KCl, and 0.01 *M* Mg^{++}. We have had considerable trouble with mold and ribonuclease in sucrose; freshly dissolved, autoclaved, or even dialyzed sucrose solutions have at times been found to inhibit amino acid incorporation markedly. Occasionally, when incorporation activity was being assayed following sucrose gradient fractionation, considerable activity was found in the last fraction (corresponding to the meniscus). These fractions contained essentially no ribosomes; the activity was probably due to mold collected at the meniscus, and such experiments were discarded.

Results.—At first glance, it might appear that the most direct way to identify complexes carrying out protein synthesis is to locate complexes containing newly synthesized protein. An experiment illustrating this approach is shown in Figure 1. Here the complete system was incubated for 10 min at 36°C, chilled, and examined on a sucrose gradient. Nascent protein, identified by H^3-leucine, is found predominantly in the region of 80–100s, precisely where P^{32}-labeled TYMV-RNA was found *prior* to amino acid incorporation.[1] This correspondence suggests that these complexes, previously shown to contain one 70s ribosome and one molecule of RNA, are the site of protein synthesis.

However, such experiments leave open the possibility that a larger aggregate of RNA and ribosomes actually incorporates amino acids, the aggregate being broken down before examination on the gradient. This possibility was explored by incorporating cold amino acids in the complete system for five min, fractionating the resulting mixture on a sucrose gradient, and then assaying each fraction for its

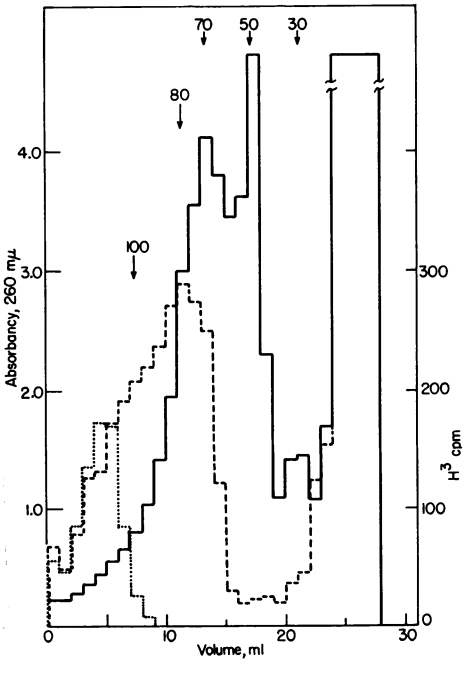

FIG. 1.—Sucrose gradient analysis of nascent protein. A complete reaction mixture containing TYMV-RNA and H^3-leucine was incubated at 36°C for 10 min, then chilled and examined on a 5–20% linear sucrose gradient. This run was for 200 min at 5°C, in the SW25 Spinco rotor. The dashed line represents TCA-precipitable H^3-leucine; the solid line absorbancy at 260 mμ. The dotted line near the bottom of the gradient represents P^{32} in whole TYMV (S_{20} = 116) added to the gradient as a sedimentation coefficient marker. The 10-min incubation results in about 60% of the incorporation obtained in 45-min incubation; in both cases approximately 70% of the TCA-precipitable radioactivity is ribosome-bound in 0.01 *M* Mg^{++}. In repeats of this experiment the only variations noticed are occasional valleys between peaks of nascent protein at 80 and 105s, and a smaller peak of protein just ahead of 50s. No significant amount of nascent protein has ever been observed in the pellet.

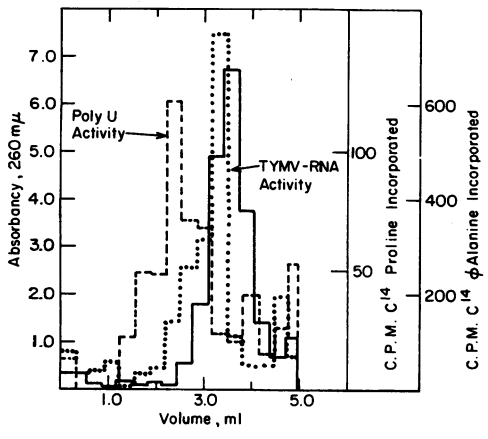

FIG. 2.—Activity gradients with TYMV-RNA and poly U. A complete reaction mixture containing 200 μg TYMV-RNA and 2 mg ribosomes in 0.4 ml was incubated at 36° for 5 min, then chilled in ice. 0.2 ml of this mixture was run on a 5–20% sucrose gradient in the SW39 rotor, 35,000 rpm, at 9°C, for 40 min. To each fraction of 0.3 ml *E. coli* supernatant, cofactors, and C^{14}-proline were added, and subsequent incubation at 36°C was for 30 min. A parallel experiment was run using in the initial reaction mixture 80 μg poly U and 2 mg ribosomes, and the subsequent incorporation utilized C^{14}-phenylalanine. The solid line represents absorbancy, the dotted line proline incorporation, and the dashed line phenylalanine incorporation. Recovery was 40% in the RNA experiment, and 80% in the poly U experiment, relative to equivalent aliquots not subjected to the fractionation procedure.

ability to incorporate additional amino acids when supplemented with supernatant and cofactors. An experiment of this type was first performed by Gilbert,[3] who found the *activity* for polyphenylalanine synthesis in the region of 150–200s when poly U was used to direct amino acid incorporation. The result of one of our experiments is shown in Figure 2. With TYMV-RNA as messenger, the activity for incorporating C^{14}-proline is seen to occupy the region of 80–90s. On the other hand, a parallel experiment run under identical conditions with poly U as messenger confirms Gilbert's result: the activity for C^{14}-phenylalanine incorporation is found in the region around 150s. The result with TYMV-RNA supports our previous assertion that the active complex formed with viral RNA *in vitro* is a monosome. Since the degree of polymerization of the viral RNA is at least ten times greater than that of the poly U used (S_{20} = 5.3), clearly the size of the active complex in the cell-free system is not simply proportional to the degree of polymerization of the messenger.

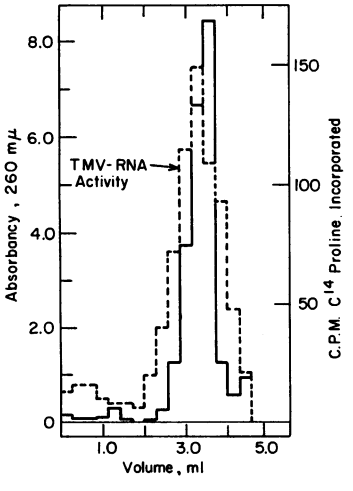

FIG. 3.—Activity gradient with TMV-RNA. Conditions identical to those for Fig. 2, except that the initial reaction mixture contained 165 μg TMV-RNA. Preincubation was for 5 min; recovery of activity was 63%.

TYMV-RNA is not unique in forming monosomes. A similar activity gradient using TMV-RNA as messenger is shown in Figure 3. Again the activity is found in the region around 80–90s. TMV-RNA can, however, be distinguished from TYMV-RNA. In agreement with Barondes and Nirenberg,[4] we find that TMV-RNA does not attach to *E. coli* ribosomes until the complete system is assembled, while TYMV-RNA requires only Mg^{++} for attachment.[11]

Having established that TYMV-RNA differs from poly U with respect to the size of the ribosomal aggregate that incorporates amino acids, it became of interest to compare the kinetics of amino acid incorporation in the two systems. For this purpose, the dependence of the rate of incorporation upon ribosome concentration was inves-

tigated. The data for the poly U-dependent incorporation of phenylalanine are shown in Figure 4. The concentration of poly U is in each case the same, being chosen to saturate the standard incorporation system (1 mg ribosomes, 30′ incorporation). Thus, at lower ribosome concentration poly U is in excess; at higher ribosome concentration poly U is limiting. At the two lowest ribosome concentrations, incorporation is linear for ten min; at the higher concentrations, since the deviation from linearity is not great at ten min, we have assumed the incorporation is linear for the first five min. Using these slopes to define the initial rate of amino acid incorporation, the rate is plotted as a function of ribosome concentration in Figure 5. The shape of the curve suggests that ribosomes participate in the rate-limiting step of amino acid incorporation as individuals, and this is seen to be so in the reciprocal plot in Figure 6.

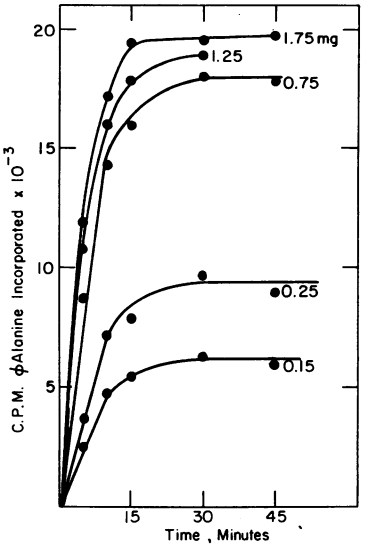

FIG. 4.—Kinetics of phenylalanine incorporation. Complete reaction mixtures were assembled in an ice bath. Each was distributed among five tubes, and then transferred to a 36°C water bath. At the indicated times, tubes were withdrawn and proteins precipitated with 3 ml of cold 5% TCA. The numbers refer to the concentration of ribosomes in mg per 0.5 ml, the standard reaction volume.

Quite different results were obtained with TYMV-RNA. Figure 7 shows that approximately three min at 36°C are required before the maximal rate of amino acid incorporation is attained. This lag is reproducibly observed with TYMV-RNA as messenger, and is not observed with poly U. The same lag is observed with proline, alanine, lysine, arginine, or an algal hydrolysate. To examine this question more carefully, the following experiment was performed: reaction mixtures, complete except for amino acids, were assembled in the cold and then divided in half. One half received amino acids and was incubated at 36°C; the other half was incubated for three min at 36°C and then received amino acids. The results of this experiment are shown in Figure 8. First we note that the lag is dependent upon the concentration of ribosomes. Secondly, preincubation eliminates most, but not all, of the lag. We interpret these results to mean that the lag is comprised of two steps: one is the association of RNA and ribosomes, which proceeds in the absence of protein synthesis; the second requires amino acids, and could be peptide chain initiation.

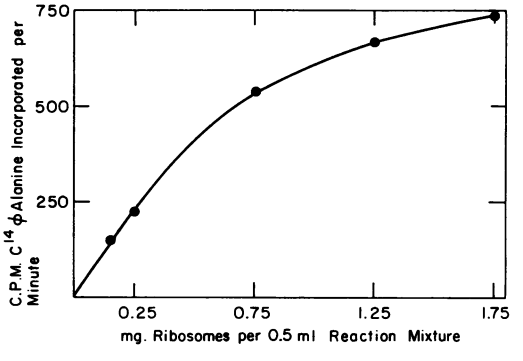

FIG. 5.—Dependence of the rate of phenylalanine incorporation upon ribosome concentration. Data from Fig. 4; see text for details.

Another kind of comparison between monosomes and polysomes may be made by examining the extent of amino acid incorporation per ribosome as a function of

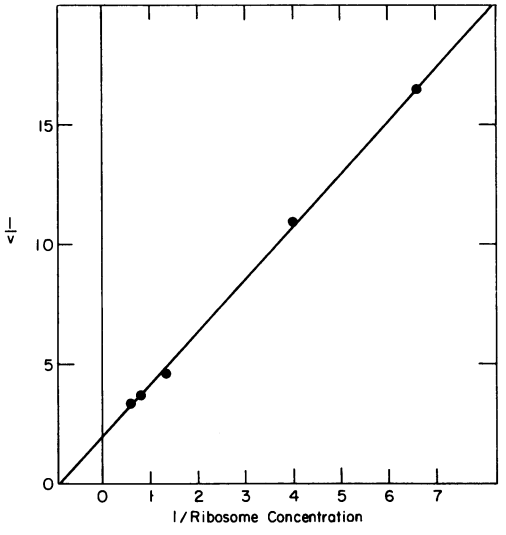

FIG. 6.—Reciprocal plot of data in Fig. 5.

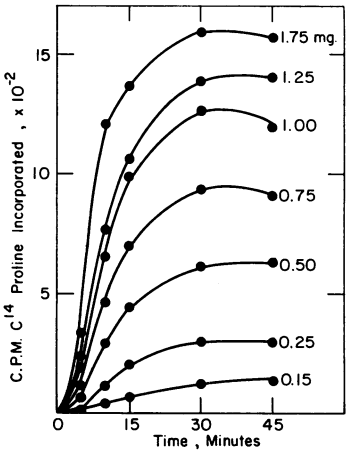

FIG. 7.—Kinetics of proline incorporation. Procedure the same as indicated in Fig. 4.

ribosome concentration, using a single messenger. If the ribosomes are functioning independently, as the kinetic data suggest, then the extent of incorporation per ribosome should be a constant as long as messenger is in excess. This is seen to be essentially so in the monosome case; the data for the TYMV-RNA-directed reaction are shown in Figure 9*A*. On the other hand, the higher the concentration of ribosomes in the poly U-directed reaction, the fewer amino acids each ribosome incorporates on the average (see Fig. 9*B*). Such "inhibition" could be due to a limitation on the number of poly U sites available for initiation of polypeptide chains, or a limitation on the average excursion of poly U across active ribosomes. In either event, at higher ribosome concentrations, poly U sequences are being read less efficiently than they would be if fewer ribosomes were present.

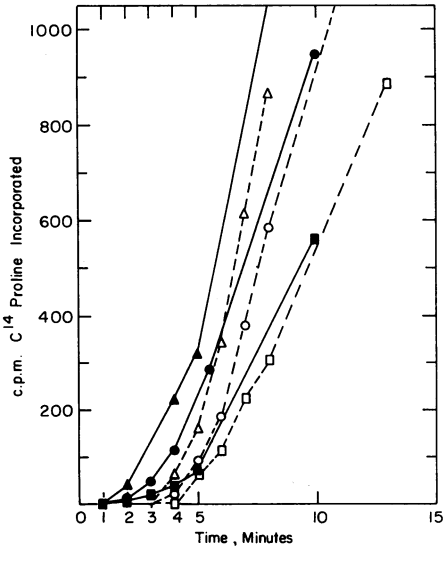

FIG. 8.—Kinetics of proline incorporation. This is an expanded view of the lag period in Fig. 7. See text for experimental procedure, which differs from that in Fig. 7. Solid lines indicate no preincubation; dashed lines preincubation for 3 min. Δ, ○, □ = 1.5, 1.0, 0.5 mg ribosomes per 0.5 ml, respectively.

Discussion.—It has been widely assumed that ribosomes support the growth of one polypeptide chain at a time. The strongest single fact favoring this view is Cannon, Krug, and Gilbert's finding that ribosomes contain a single site for binding sRNA.[12] Our observation that ribosomes, whether single or in clusters, enter the rate-limiting step in protein synthesis as individuals, is

consistent with this view. Our data are also compatible with a number of peptide chains per ribosome greater than one, provided the number of chains per ribosome is independent of the ribosome concentration and the poly U/ribosome ratio.

The over-all kinetics in the reactions directed by RNA and by poly U can be accounted for in the following scheme:

$$M + nR \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR_n \underset{k_4}{\overset{k_3}{\rightleftharpoons}} MR'_n \xrightarrow[aa]{k_5} \times$$

$$MR'_nP$$

in which M = messenger, R = ribosome, aa = amino acids and/or their activated intermediates, and P = protein. k_1 is the forward rate of association of messenger with ribosomes; for poly U k_1 is at least five times greater than for TYMV-RNA. In preliminary experiments it appears that the binding constant for poly U is also greater than for TYMV-RNA. The reaction MR $\rightleftharpoons$MR′ could be the reversible attachment of the first amino-acyl sRNA or the formation of the first peptide bond.

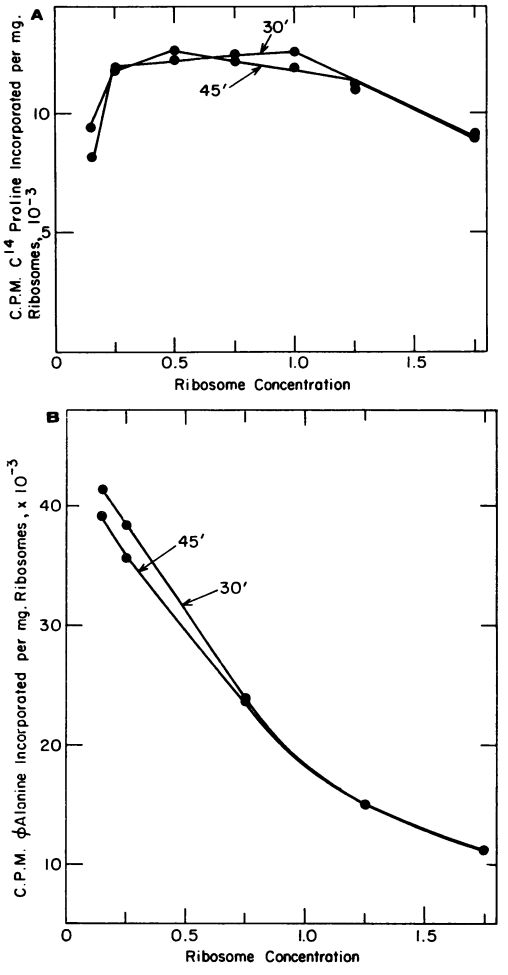

FIG. 9.—Extent of amino acid incorporation per ribosome as a function of ribosome concentration. (*A*) TYMV-RNA, data from plateaus in Fig. 7. (*B*) Poly U, data from Fig. 4.

Gilbert has shown that the average chain length of polyphenylalanine increases only slightly between the fifth and twentieth minute of amino acid incorporation.[3] This means that most of the incorporation beyond the fifth minute represents the initiation of new peptide chains, and for a given sample of poly U the plateau level of incorporation is a measure of the number of chains initiated. The lower extent of incorporation per ribosome we observe at high ribosome concentration is then probably due to the blocking of poly U sites which would otherwise be available for chain initiation. It is not due to a more efficient use of the poly U at low ribosome concentration, since the level of poly U was chosen to be saturating at high ribosome concentration.

The TYMV-RNA which, as we have seen, forms only monosomes *in vitro* has a molecular weight of 2×10^6.[13] Poliovirus RNA, which has a similar molecular weight, forms 400s polysomes *in vivo*, containing at least 50 ribosomes.[14] Bacterial ribosomes are certainly capable of forming polysomes with messenger RNA *in*

vivo.[15-18] Failure to observe polysome formation with RNA *in vitro* could be due solely to the loss of some essential orienting surface, such as a membrane,[16] but we are strongly persuaded that our observations are due at least in part to the equilibrium conformation of RNA in solution. We can distinguish the following kinds of interaction between polyribonucleotides and ribosomes *in vitro:* poly U forms polysomes; TYMV-RNA forms monosomes with Mg^{++} the only additional requirement; TMV-RNA forms monosomes, requiring Mg^{++}, energy, and *E. coli* supernatant; *E. coli* ribosomal RNA does not associate with ribosomes under any conditions we have been able to devise, including partial reaction with formaldehyde, dialysis from low Mg^{++} into high Mg^{++}, and centrifugation of ribosomes onto a pad of ribosomal RNA. Recently, however, Okamoto and Takanami have used a more extensive reaction with formaldehyde to permit ribosomal RNA to bind to *E. coli* ribosomes.[19] Poly U is known to be devoid of secondary structure above room temperature,[20] while the RNA's are listed above in order of increasing secondary structure, as determined by the fraction of groups available for reaction with formaldehyde.[21, 22] Thus, the equilibrium conformation of messenger RNA in solution is considerably different from the extended one found in polysomes, and it is the former that governs the interaction with ribosomes *in vitro*. It appears to be impossible at present to reconstruct a polysome from purified RNA in its equilibrium conformation and single ribosomes. If this argument is correct, it follows that the cell contains specific structures capable of extending messenger RNA to facilitate polysome formation, and we are currently testing the hypothesis that such a structure might be DNA itself.

One less important corollary of the importance of RNA secondary structure in protein synthesis *in vitro* is the possibility that nonrepresentative nucleotide sequences may be found in the open regions that are predominantly "read" *in vitro*. This may well be the explanation for the inordinately high proportion of coat protein synthesis directed by f2 RNA,[23] and the low proportion of coat protein synthesis directed by TMV-RNA.[24]

In this connection, the specific effect of polynucleotide conformation on the coding properties of such polymers was pointed out some time ago by Singer *et al.*,[25] who found that a G-rich copolymer of U and G, which was highly ordered, exhibited sharply diminished priming activity for several amino acids compared to U-rich copolymers.

Summary.—TYMV-RNA and TMV-RNA associate with *E. coli* ribosomes *in vitro* to form complexes (monosomes) containing single ribosomes. In a complete reaction mixture, such monosomes contain all the activity for amino acid incorporation. Poly U associates with ribosomes to form polysomes, but the ribosomes in such polysomes participate in the rate-limiting step of polyphenylalanine synthesis as individuals. The efficiency with which ribosomes incorporate amino acids in poly U polysomes varies inversely with the number of ribosomes per unit volume of reaction mixture. The ability of polynucleotides to form monosomes or polysomes *in vitro* appears to be correlated with the secondary structure of the polynucleotide.

We should like to thank L. Johnson for technical assistance, and E. P. Geiduschek for many helpful discussions.

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*ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID, XVI. OLIGONUCLEOTIDES AS TEMPLATES AND THE MECHANISM OF THEIR REPLICATION**

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Communicated December 30, 1963

DNA[1] in the molecular weight range of a million or more serves as a primer for replication by DNA polymerase but the minimal size of a DNA molecule that will function has not been determined. Extensive digestion with pancreatic deoxyribonuclease destroys the priming capacity of a DNA preparation,[2] and the use of partial digests has failed to establish the range and relative effectiveness of molecules of intermediate size. With the availability of synthetic deoxyribopolynu-