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Studies on the Replication of DNA by *Escherichia coli* Polymerase

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The model of DNA proposed by Watson and Crick (19) accounts for the principal chemical and physical properties of DNA and also suggests how cellular replication of DNA might take place. That is, if the two chains of the double helix separate into two chains and a new one is formed complementary to each of them, the result will be two pairs of chains, each pair identical to the original parent duplex.

The density gradient technique developed by Meselson, Stahl, and Vinograd (14) made it possible to analyze the distribution of atoms of the labeled parental DNA among the progeny of dividing *Escherichia coli* and thus test the Watson and Crick proposal. In such an experiment, Meselson and Stahl (13) found that a DNA molecule consisted of two subunits. Following duplication, each daughter molecule received one subunit. The subunits were conserved through many duplications. Current studies by Shooter and Baldwin (16) strengthen the belief that these subunits are in fact single-stranded DNA chains.

Another approach to the problem of how DNA is replicated is through attempts to analyze and reconstruct the process in a cell-free system. Our earliest studies (11) showed that in extracts of *E. coli* there is an enzyme (DNA polymerase) which, in the presence of DNA, catalyzes the polymerization of deoxynucleotidyl groups. With a partially purified enzyme preparation it became clear that the exact replica-

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tion of DNA depends on the complementary base pairing of the deoxynucleotidyl substrate residues with those in the DNA template (9). What has not been understood and will be considered in this paper concerns the mechanism by which replication is initiated and the fate of the DNA template during the course of replication.

Further Purification of DNA Polymerase and Recognition of a New Component in the Preparation

Modifications of the earlier procedure and chromatography on a phosphocellulose column yielded a fraction which showed only one

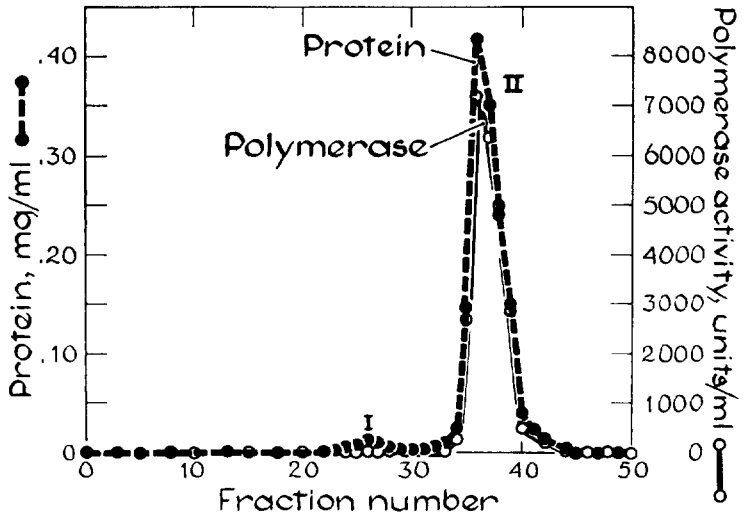

FIG. 1. Separation by hydroxylapatite chromatography of *E. coli* DNA polymerase, peak II, from another component, peak I.

band on starch gel electrophoresis and a unimodal symmetrical peak on sedimentation in the ultracentrifuge (Robert L. Baldwin, personal communication). Preliminary calculations give an estimate of 100,000 as the molecular weight of DNA polymerase.

When the phosphocellulose fraction was chromatographed on hydroxylapatite, 90% of the protein was eluted in a sharp peak containing 90% of the added polymerase activity (with dAT as primer) and having a constant specific activity throughout the peak (labeled II in Fig. 1). When calf thymus DNA was used as primer for assays, instead of dAT polymer, there was only a 20% recovery of polymerase activity. This activity could be fully restored by adding to the assay mixture very small amounts of protein from a barely detectable component (labeled

I in Fig. 1) which preceded the main peak. This new component also increased the reaction rate of the hydroxylapatite fraction by 2- to 10-fold when samples of *Bacillus subtilis*, *E. coli*, or T2 phage DNA were used as primer.

We considered, for reasons to be presented, that the most probable explanation for the stimulatory effect of component I was the exposure of 3′-hydroxyl groups as sites for the initiation of replication. This might be accomplished either by diester bond cleavage of intact DNA chains or by removal of any phosphate groups blocking terminal 3′-hydroxyls, if in fact such monoesterified groups were present.

Inhibitory Effect of Terminal 3′-Phosphoryl Groups on DNA Replication

The decrease of polymerase activity measured with native DNA as primer had also been encountered earlier in the purification procedure. This difficulty was attributed to the removal of *E. coli* endonuclease (12), which liberated terminal 3′-hydroxyl or 5′-phosphoryl groups for the initiation of replication.[2] This decrease of polymerase activity could be circumvented by pre-treating the DNA with purified *E. coli* endonuclease or with crystalline pancreatic DNase or by substituting dAT polymer as primer.

A demonstration of the capacity of purified *E. coli* endonuclease to increase the priming activity[3] of *B. subtilis* DNA is shown in Fig. 2. Within certain limits, the increased priming activity is proportional to the extent of hydrolysis of phosphodiester bonds as judged by the decrease in viscosity of the DNA. With prolonged exposure to the endonuclease (for several hours) or to larger amounts of this enzyme, the priming activity may increase up to 30-fold and then decrease progressively until it is destroyed completely.

The hydrolysis of DNA by *E. coli* endonuclease and pancreatic DNase produces 3′-hydroxyl groups and 5′-phosphoryl groups. What would the effect on priming activity be of an endonuclease which splits DNA to produce 3′-phosphoryl and 5′-hydroxyl groups? As seen in Fig.

[2] The studies of Adler *et al.* demonstrated the addition of deoxynucleoside triphosphate monomers to the 3′-hydroxyl ends of primer DNA chains. This result, as mentioned below, may be due to the repair of one strand of a frayed double helix. However, the synthesis of new chains not covalently linked to the primer may depend on end groups which are either 3′-hydroxyl or 5′-phosphoryl. At present, we cannot conclude which of the two end groups is important for the initiation of replication.

[3] Relative capacity of a given amount of DNA to serve as primer for a given enzyme preparation.

2, the spleen endonuclease described by Koerner and Sinsheimer (8), which has this action, concomitantly reduces the priming activity and viscosity; more extensive enzyme action eliminates both properties altogether. Another endonuclease, from micrococcus (2), which splits diester bonds in the same way as the splenic enzyme, has identical effects in destroying priming activity.

The spleen or micrococcal endonucleases actually reduce priming activity, whereas the original concentration of 3′-hydroxyl and 5′-phos-

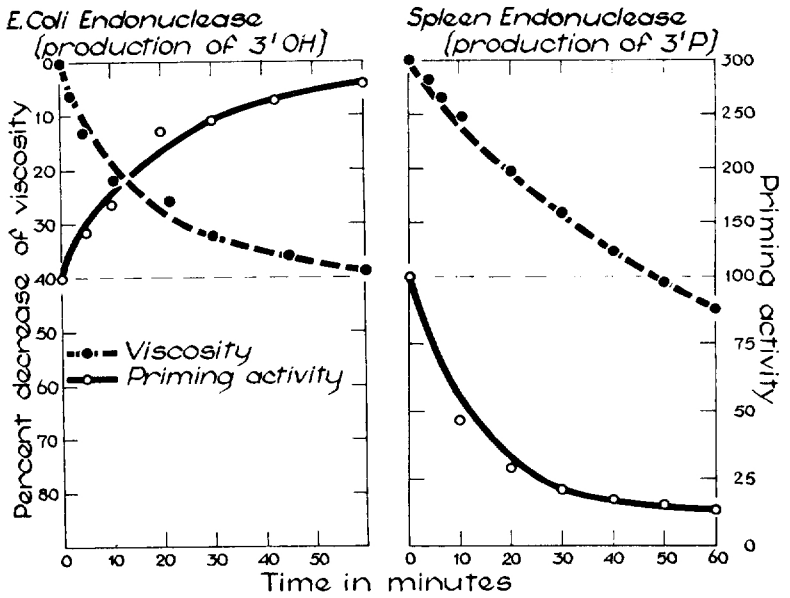

FIG. 2. The effect of endonuclease action on priming activity. *Bacillus subtilis* DNA was incubated with either *E. coli* endonuclease, producing 3′-hydroxyl ends in the DNA chain or with spleen endonuclease, producing 3′-phosphoryl ends. The endonuclease action was followed in each case by the percentage decrease in the relative viscosity. The priming activity of aliquots of the endonuclease-treated DNA was determined under routine assay conditions for DNA polymerase and the priming activity expressed relative to native *B. subtilis* DNA given a value of 100%.

phoryl groups in the DNA chain remains unchanged. This reduction in priming activity might, therefore, be attributed to inhibition by 3′-phosphoryl end groups. This suggestion has also been made to explain similar findings in DNA synthesis with mammalian extracts (6, 7). In support of this is the finding that a nuclease-treated DNA containing many such 3′-phosphoryl groups will actually decrease the priming activity of a native DNA and that this apparent inhibition can then be destroyed by the enzymatic removal of the 3′-phosphoryl groups.

These results explain why most purified enzyme preparations have barely detectable activity on native DNA which can be presumed to have only one or a very few 3′-hydroxyl and 5′-phosphoryl groups per strand. They also may account for the variable priming activity of different DNA preparations on the basis of the number of 3′-phosphoryl groups relative to 3′-hydroxyl or 5′-phosphoryl groups.

Component I is readily distinguished from *E. coli* endonuclease by physical separation of the two activities and by failure of RNA or endonuclease antiserum to inhibit component I while inhibiting the endonuclease completely. Furthermore, component I acts differently from endonucleases: the levels required to increase priming activity result in little or no decrease in viscosity, no release of acid-soluble fragments, and no detectable loss in *B. subtilis* DNA transforming activity. Even when used in a 100-fold excess over levels optimal for activating DNA as a primer, there is no decrease in priming activity of the DNA, comparatively little decrease in transforming activity, and less than 10% conversion of the DNA to acid-soluble fragments. These results encouraged us to consider the alternative possibility that component I exerted its activating effect by removing 3′-phosphate residues. In support of this was the observation that the priming activity of a DNA sample reduced to only 10% of its original value by splenic or micrococcal nuclease was raised on exposure to component I to a level of 300% or more of the original level.

A Phosphatase for 3′-Phosphate Esters Terminating a DNA Chain (Fig. 3)

In order to increase the sensitivity of detecting the action of the postulated phosphatase, a P^{32}-labeled DNA was first incubated with micrococcal nuclease to the point where about 30% of the radioactivity was rendered acid-soluble. After dialysis and upon exposure to component I, a fixed amount of acid-soluble radioactivity was released which was not adsorbable to Norite and which chromatographed on a Dowex 1 ion-exchange column identically with inorganic orthophosphate. *E. coli* alkaline phosphatase (4) released the same amount of radioactivity. Unlike the alkaline phosphatase, component I does not act on a simple phosphate ester such as 5′-dAMP, does not remove the 5′-phosphate groups produced by *E. coli* endonuclease cleavage of DNA, and does not remove the phosphate groups from the dialyzable oligonucleotides of a micrococcal nuclease digest. Current studies are directed toward the purification of component I, its characterization as a "DNA phosphatase" and the possible significance of this enzyme in the replication of DNA.

Fate of the DNA Template in the Course of Replication

B. subtilis DNA extensively labeled with N^{15}, H^2, and H^3 was used as primer; the deoxynucleoside triphosphate substrates contained P^{32} as a marker. The N^{15} and H^2 labels serve for buoyant density sep-

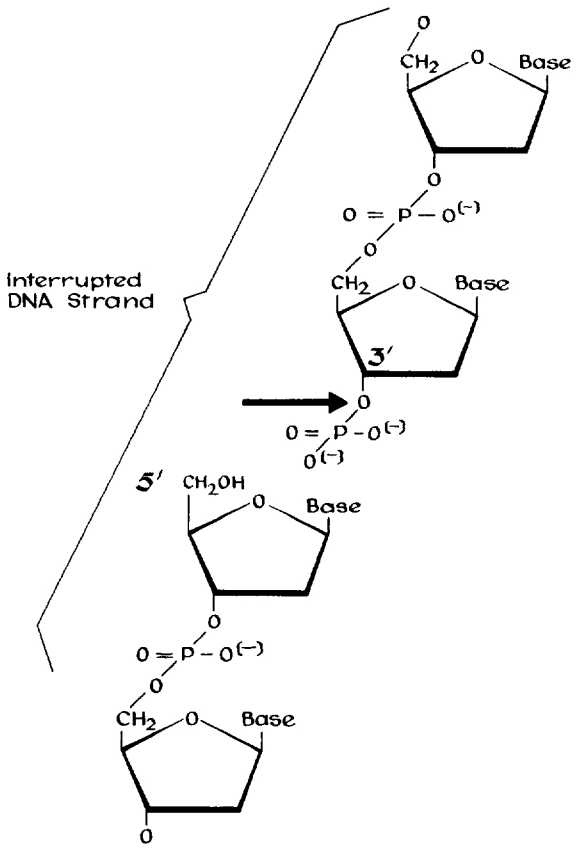

Fig. 3. The site of action of the DNA phosphatase as discussed in the text is at a 3′-phosphoryl-ended DNA chain resulting in the formation of a free 3′-hydroxyl group and the liberation of inorganic orthophosphate. The arrow indicates the site of action of the enzyme on a 3′-phosphoryl-ended polynucleotide occurring at a break in a DNA strand.

aration of heavy (primer) from light (product); the H^3 and P^{32} allows a sensitive radiochemical assay of primer and product atoms. At several points during the course of replication, samples were analyzed for P^{32} incorporation into DNA (as a measure of the extent of synthesis) and for buoyant density distribution in a CsCl density gradient. The results

are shown in Fig. 4. The "heavy" primer, at the top of the figure, forms a band at a density of 1.755 gm.cm^{-3}; the position at which "light" *B. subtilis* DNA would be expected to band is given by the reference line at a density of 1.703 gm.cm^{-3}. It can be seen that as synthesis proceeds, the DNA present in the reaction mixture assumes a bimodal distribution. The primer peak decreases with increasing synthesis but a residual fraction persists even after 2 replications. The major band approaches but

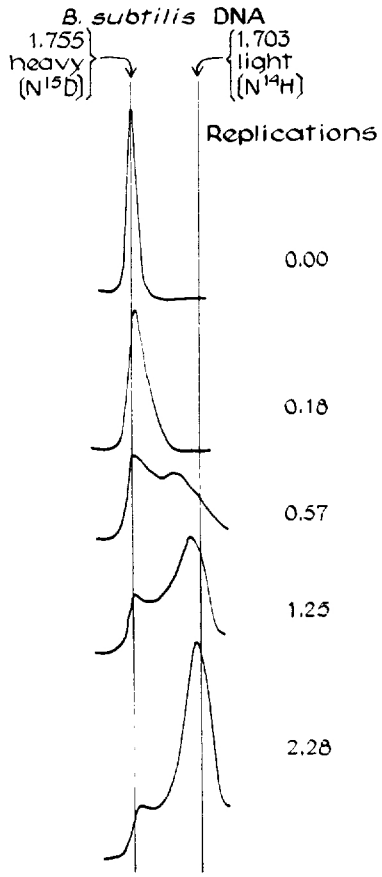

FIG. 4. Replication of *B. subtilis* DNA with *E. coli* polymerase: untreated primer. Aliquots were taken during the progress of an enzymatic synthesis and centrifuged in a CsCl density gradient formed at 44,770 rpm. Microdensitometer tracings of ultraviolet absorption photographs of synthesized DNA samples are shown above. The ordinate is proportional to the concentration of DNA at any point in the gradient; the abscissa represents the buoyant density. The number of replications is defined as the ratio of moles of P^{32} (synthesized DNA) to the number of moles of phosphorus in the primer DNA.

does not reach the density of light DNA, and we may presume that it contains some of the parental atoms among the daughter molecules, contrary to the simplest expectations of the semi-conservative replication scheme.

It may be recalled that the DNA phosphatase activates *B. subtilis* DNA as a primer and that this result was interpreted as due to an unmasking of 3′-hydroxyl groups to serve as sites for priming. A replication experiment in which the heavy *B. subtilis* DNA primer was first treated with the DNA phosphatase, but otherwise like that described in Fig. 4, was carried out. The reaction proceeded at 3 times the rate observed with untreated DNA. The patterns obtained are shown in Fig. 5. In this experiment the primer band disappears completely but the main band representing the new DNA is at every stage of the reaction more dense than in the preceding experiment (compare Figs. 4 and 5). This shows clearly that more of the heavy primer has been incorporated into it.

What is the nature of the association between primer and product that accounts for the buoyant density patterns in Figs. 4 and 5? Is there a covalent linkage between them? Earlier studies by Adler *et al.* (1) showed that in reactions limited by the presence of only a single deoxynucleoside triphosphate monomer, a covalent phosphodiester bond was formed between the monomer and the 3′-hydroxyl end of the primer chain. In subsequent experiments with dAT-primed reactions, Schachman *et al.* (15) showed that only dATP or dTTP and not dGTP or dCTP participated in such a limited reaction. One explanation for this result may be that such reactions represent the extension (or repair) of the shorter strand of a pair of DNA strands of unequal length and may be entirely distinct from reactions that initiate new chains and extensive synthesis.

In order to determine whether a covalent linkage connects the primer and product during extensive replication, we subjected a 3.0-fold replication sample (similar to the 2.0-fold replication sample shown in Fig. 5) to pH 12 in CsCl. At this pH, the guanine and thymine bases of native DNA are known to be titrated; the double helix collapses and shows a buoyant density increase over that of the native double helix (18). The mixture of primer and newly synthesized DNA at pH 12 yields two distinct bands, one at the density corresponding to single-stranded heavy primer and the other at the density expected for single-stranded light *B. subtilis* DNA. These results show that, whatever the linkage between primer and product, it was split by alkaline treatment. Another replication sample was heated at 100°C for 5 minutes, quickly cooled and then analyzed for buoyant densities. Once again two sharp

bands, widely separated, were obtained (Fig. 6). This ready separation of primer and product by heat or by alkaline treatment makes it more likely that the association of primer and product in the hybrid helix does not involve a stable covalent linkage between the new and old strands.

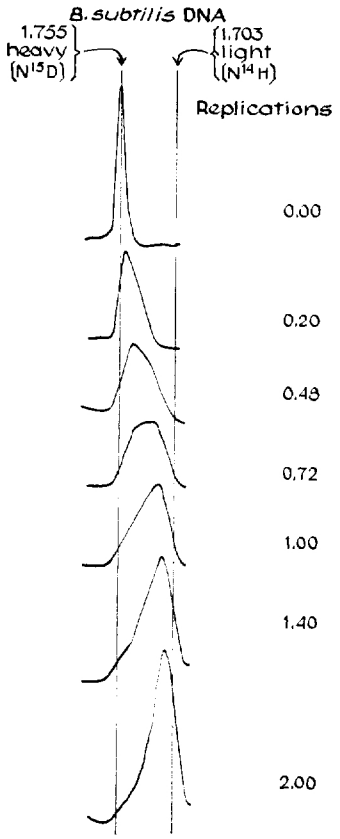

FIG. 5. Replication of *B. subtilis* DNA with *E. coli* polymerase: primer pretreated with DNA phosphatase. Microdensitometer tracings as described in Fig. 4. Duplicate aliquots, taken at 1.0 and 2.5 replications and shaken with an equal volume of water-saturated phenol, showed no significant change in band profile; there was a density increase of about 0.004 gm.cm^{-3}.

The buoyant density values recorded for the two bands of the heated replication sample deserve special notice. The denser band at 1.769 gm.cm^{-3} showed the increment of 0.015 gm.cm^{-3} characteristic for heat-denatured DNA (17). However, the lighter band was found at 1.703 gm.cm^{-3} corresponding exactly to that of native *B. subtilis* DNA!

(See Fig. 6). This lack of hysteresis in enzymatically synthesized DNA was previously noted by Zimmerman and Kornberg (20) in spectrophotometric studies of DNA melting as a function of heating and quick cooling; it had also been observed by buoyant density analyses in unpublished studies of Schildkraut, Marmur, Doty, Josse, and Kornberg (5). A third criterion, the susceptibility to Lehman's *E. coli* exonuclease (10) which acts only on denatured DNA, has further confirmed that heated

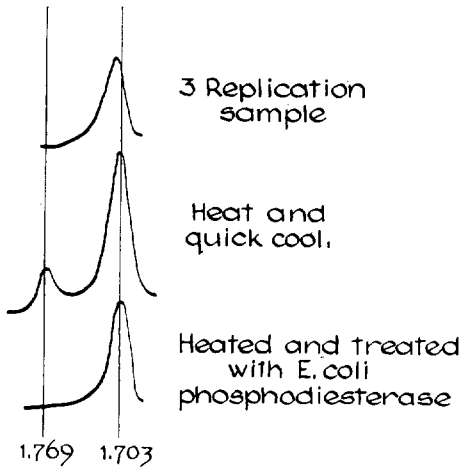

FIG. 6. Effect of heat on the banding pattern of a replication product. The samples were treated as described in the text and banded in CsCl. Microdensitometer tracings shown are as described in Fig. 4.

and quickly cooled synthetic DNA appears to be fully renatured. The structural basis for this significant distinction between synthesized DNA and samples isolated from nature deserves serious study.

ATTEMPTS TO ASSESS THE GENETIC ACTIVITY OF SYNTHESIZED DNA

Genetic specificity is an important aspect of the identity of DNA. The following experiments were designed to test the transfer of genetic information to newly synthesized molecules of DNA. As will be seen, they help to accent the possible pitfalls of study in this area.

The labeling procedures described above make it possible (a) to separate primer (H^2 and N^{15}) from product (H^1 and N^{14}) molecules by density gradient separation; and (b) to verify the extent of separation by sensitive radiochemical assay, H^3 versus P^{32} of each fraction. The genetic activity of each fraction could then be correlated with its composition in terms of primer and product atoms.

As described above, labeled DNA was isolated from a *B. subtilis* prototroph grown on an $N^{15}H^2$ medium with added H^3 thymidine. This DNA, pretreated with the DNA phosphatase, was used to prime a 5-fold synthesis product. This retained about 70% of the control genetic activ-

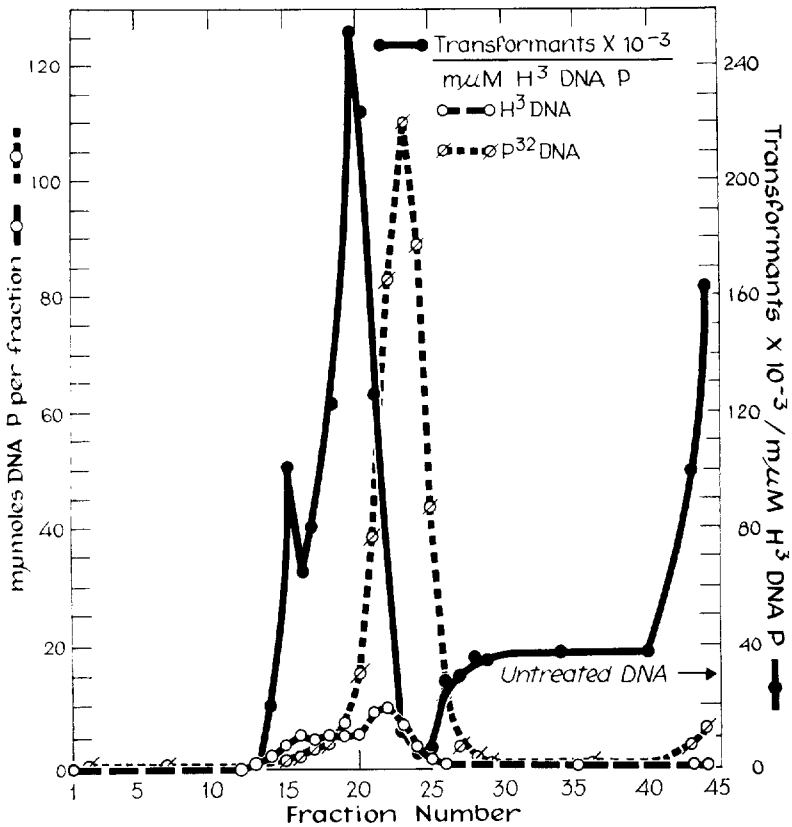

FIG. 7. Density gradient fractionation of a 5-fold replication of *B. subtilis* DNA with *E. coli* polymerase. The primer DNA contained N^{15}, H^2, and H^3; the triphosphates used for the newly synthesized DNA contained P^{32}. The product was dissolved in CsCl and centrifuged in the Spinco Model L swinging bucket rotor. A hole pierced in the bottom of the tube permitted the collection of 0.1 ml fractions. Radioactivity due to P^{32} and H^3 was determined on 10 λ aliquots using the Packard Tri-Carb Liquid Scintillation Counter.

ity as measured by transformation of the *try*$^+_2$ (indole) marker. The product mixture was fractionated as shown in Fig. 7. The distribution of H^3 and P^{32} indicates that more than half of the heavy primer is associated with the light product, and this accounts for the density shift

described above. The ratio of P^{32}/H^3 shows a continuous increase from heavy to light fractions indicating the formation of DNA complexes having a continuous range of composition from excess of primer to small amounts of primer in predominantly new material. The specific genetic activity (colonies of try^+_2 transformants per H^3) has a peak activity in the hybrid region some 2.5 times that in the heavy peak, and about 8 times the specific activity of the original primer DNA. There is a small but significant amount of activity in the lighter fractions.

The high specific activity in the hybrid region may have at least five contributions:

(a) Activity in newly synthesized DNA.

(b) Twofold increase expected on hybridization of an active primer strand with an inactive product strand, assuming that both strands of the primer are potentially active.

(c) Restoration of inactive primer strands in the formation of hybrid double helices (this would be particularly pertinent if the extraction of the DNA results in some unavoidable denaturation or other structural damage, other than damage to the nucleotide sequence, in the segment involved in the genetic test).

(d) Selective replication of some biologically inactive primer, leaving a higher concentration of biologically active material behind in the hybrid and primer regions.

(e) Density gradient fractionation of activity, as can be demonstrated directly in the primer DNA (3).

The specific genetic activity relative to H^3 in the peak light region is lower than the control, and thus gives no evidence of genetic activity of newly synthesized material. Some trivial contributions to this result include:

(a) Inhibition of transformation by product DNA molecules.

(b) Structural inhibition of primer activity when attached to product strands.

(c) Preferential replication of biologically inactive DNA strands.

Only the first of these possibilities has been ruled out by reconstruction experiments.

The analysis of the fractionation of the net synthesis product emphasizes the difficulties inherent in deciding whether synthesized DNA has biological activity. Preliminary studies (5) with heated DNA as a primer showed large increases in biological activity concomitant with enzyme synthesis. These results have been confirmed more recently using the purified polymerase. However, the final level of activity obtained is always much less than that of the unheated control and is most probably associated with hybrid molecules. Even a substantial increase

in total activity, by analogy with the high specific activity observed in the hybrid density fractions of the previous experiments, could not be taken as rigorous evidence for the replication of genetically meaningful sequences in view of the probable operation of the restoring mechanisms discussed above. In the present experiments, despite the encouraging result of the preservation of input activity in the total product and the very high specific activity of material with hybrid density, the physical separation of product molecules showed these to have negligible genetic activity, and none that cannot be accounted for in terms of tritium-labeled primer atoms. Heat denaturation of the product obtained using native heavy DNA as primer and subsequent fractionation on a preparative CsCl density gradient yields, as expected from the results described above, a net synthesis product with no more than 1% contamination by primer. This has a specific transforming activity of about 10^{-4} that of the original primer.

The basis for the lack of genetic activity in the separated fraction of the new DNA synthesized in these *in vitro* experiments is under study.

Summary

1. *E. coli* polymerase apparently homogeneous by ultracentrifugal measurements yields a very small additional component on hydroxylapatite chromatography. This component increases the priming activity of native DNA by 2- to 10-fold.

2. Endonucleases (*E. coli*, pancreatic) which yield 3′-hydroxyl groups increase the priming activity of DNA whereas endonucleases (micrococcal and splenic) which yield 3′-phosphoryl groups decrease the priming activity of DNA.

3. The new component has been shown to be a phosphatase which splits 3′-phosphoryl esters at the ends of DNA chains and thereby increases the priming activity of native DNA or DNA inactivated by micrococcal endonuclease.

4. The fate of the primer in the course of replication has been followed using N^{15}-H^{2}-H^{3}-*B. subtilis* DNA, P^{32}-labeled "light" substrates and CsCl density gradient centrifugation. There is a progressive conversion of the primer band to a hybrid of intermediate buoyant density. After 2 replications all the primer is incorporated into one major peak with a buoyant density anticipated from the composition of light and heavy atoms. The linkage of primer to product DNA does not appear to be covalent since the two are clearly separated by heating followed by fast cooling, and by alkaline titration.

5. Upon a 5-fold replication of transforming *B. subtilis* DNA labeled

with H^3 and heavy isotopes, there was a net decrease of 30% in genetic activity. Preparative density gradient centrifugation of the product mixture showed that most of the genetic activity was found in primer and predominantly hybrid material. There was no genetic activity in newly synthesized material which could not be accounted for by associated primer atoms.

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