# Progress in NUCLEIC ACID RESEARCH

Edited by
J. N. Davidson
Waldo E. Cohn

VOLUME 1

# PROGRESS IN
# Nucleic Acid Research

edited by

J. N. DAVIDSON
*Department of Biochemistry*
*The University*
*Glasgow, Scotland*

•

WALDO E. COHN
*Biology Division*
*Oak Ridge National Laboratory*
*Oak Ridge, Tennessee*

Volume 1

1963

ACADEMIC PRESS
New York and London

ACADEMIC PRESS INC.
111 Fifth Avenue, New York 3, New York

*United Kingdom Edition published by*
ACADEMIC PRESS INC. (LONDON) LTD.
Berkeley Square House, London W.1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 63-15847

PRINTED IN THE UNITED STATES OF AMERICA

# Contents

## Polynucleotide Phosphorylase

M. GRUNBERG-MANAGO



## Messenger Ribonucleic Acid

FRITZ LIPMANN



## The Recent Excitement in the Coding Problem

F. H. C. CRICK



## Some Thoughts on the Double-Stranded Model of Deoxyribonucleic Acid

AARON BENDICH AND HERBERT S. ROSENKRANZ

## Denaturation and Renaturation of Deoxyribonucleic Acid

J. MARMUR, R. ROWND, AND C. L. SCHILDKRAUT



## Some Problems Concerning the Macromolecular Structure of Ribonucleic Acids

A. S. SPIRIN



## The Structure of DNA as Determined by X-ray Scattering Techniques

VITTORIO LUZZATI



## Molecular Mechanisms of Radiation Effects

A. WACKER

# "Primer" in DNA Polymerase Reactions

F. J. BOLLUM
*Biology Division*
*Oak Ridge National Laboratory*[1]
*Oak Ridge, Tennessee*



## I. Introduction

Recognition of the naturally occurring nucleic acids as nucleotide polymers implied the widespread existence of the nucleotidyl polymerases. The first demonstration of DNA nucleotidyl transferases (here referred to as DNA polymerases) in extracts of *Escherichia coli* (Kornberg *et al.*, 1956a,b) permitted the study of DNA synthesis *in vitro*.[2] Definition of

[1] Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

[2] In the years following the discovery and characterization of *E. coli* DNA polymerase a number of enzymes from a variety of sources have been demonstrated. Sources include regenerating liver (Bollum and Potter, 1957; Bollum, 1958a; Mant-

the activated forms and multiple requirements for the deoxyribonucleotide substrates was, at least in retrospect, not unexpected, although it added considerable complexity as well as interest to the study of the polymerase. But the observation that a "primer" is of rigid necessity was truly a remarkable discovery.

Perhaps more than any other facet of the enzymatic synthesis of DNA, the role, function, and character of primer seem to lie at the very heart of the matter, demanding definition, characterization, and, finally, exploitation. Unquestionably the most complex part of a complex reaction, the primer aspect of DNA synthesis has been revealed more slowly than others. An assessment of the nature of primer, as a prelude to elucidation of its function and ultimate exploitation, is the subject of this paper.

## II. Definitions and Polymer Principles

"Primer" was originally defined, operationally, as "a heat-stable DNA fragment(s)" required for the enzymatic synthesis of DNA. "'Primer' for the *crude E. coli* enzyme preparation was obtained (1) by the action of crystalline pancreatic DNase on *E. coli* DNA or (2) on thymus DNA, or (3) by an *E. coli* enzyme fraction (streptomycin precipitate) acting on the DNA contained in it . . . 'Primer' for the *purified* enzyme fraction was obtained only with method (3) . . . The chemical properties of the unpurified 'primer' resemble those of a partial digest of DNA" (Kornberg *et al.*, 1956a). The primer was subsequently resolved (Kornberg, 1957a) into a mononucleotide component, clarifying the requirement for all four triphosphates, and a polydeoxyribonucleotide whose function remains incompletely understood. At that time (Kornberg, 1957a) the *E. coli* system was termed "a moderately purified protein fraction which appears to increase the size of a DNA chain."

Now if DNA polymerase enzymes are primarily involved in elon-

---

savinos and Canellakis, 1958, 1959a), a variety of rat tissues (Bollum and Potter, 1958) and tumors (Bollum and Potter, 1958; Mantsavinos and Canellakis, 1959b; Furlong, 1960; Davidson *et al.*, 1958; Smellie *et al.*, 1959), calf thymus gland (Bollum, 1958b; Harford and Kornberg, 1958), tissue culture cells (Green, 1962; Bach, 1962), and fertilized sea urchin eggs (Hinegardner and Masia, 1962). All of these crude preparations appear to have similar monomer and metal ion requirements. It is regrettably true, as is detailed later in this chapter, that primer characterization in crude enzyme systems (Sarkar, 1961; Keir *et al.*, 1962) is not very meaningful, and, as a matter of fact, proceeds with considerable uncertainty even with the more highly purified enzymes. It is for this reason that the subject matter is drawn largely from the published work on the partially purified enzymes from *E. coli*, and the phage-induced *E. coli* enzyme, and from published and unpublished experiments on the calf thymus polymerase.

gating DNA chains, then the primer is playing the role of an "initiator" or polymerization center and the analogy of DNA synthesis to linear condensation polymerization (Flory, 1953) is complete. However, subsequent investigation indicates that the replication of DNA chains may be more complex and that pre-existing chains are used as patterns for the synthesis of a complementary chain as directed by hydrogen-bonding. (The direct inclusion of primer strands in product remains to be demonstrated, however, in DNA polymerase reactions.) There appears to be no simple polymer analogy for replicative DNA synthesis, and thus even a clear definition of "primer" in the replicative reaction is not possible. The mechanism of oligodeoxyribonucleotide priming (Bollum, 1962), in which the reaction is entirely one of chain elongation, fits the definition of an initiator in linear condensation polymers, and one may assume that the statistics of linear condensation (cf. Flory, 1953) should apply as a first approximation in this special case of nucleoside triphosphate polymerization.

The analysis of high-polymer products to permit comparison of enzymatic product with primer material depends heavily upon physical methods, which in turn are based on a statistical approach. There are two points worth mentioning in this regard, the first being that sedimentation constant ($S_{20,w}$) and intrinsic viscosity [$\eta$] are not *linear* functions of molecular weight. Sedimentation constants of 20 and 23, for example, seem quite close, but since sedimentation is related to molecular weight as the 0.37 power (Doty *et al.*, 1958) the corresponding molecular weights are really quite different. The second point concerns the nature of the molecular weight obtained. Molecular weight from sedimentation and viscosity is commonly a weight average molecular weight, defined as

$$\bar{M}_w = \frac{\Sigma N_j M_j^2}{\Sigma N_j M_j}$$

where $N_j$ is the number of molecules of weight $M_j$, and such determinations are strongly biased in favor of the larger molecules in the population.

This cursory introduction to the polymer chemistry involved in DNA synthesis is intended simply to point out that the nature of the primer, in the case of replicative synthesis, is not readily defined by analogy. This difficulty arises largely from a lack of experimental information about the detailed function of the primer component, and to some degree from the problems involved in a detailed comparison of product with primer.

## III. Priming Activity as a Function of Molecular Weight and State

### A. High-Polymer DNA—MW ~ $10^6$

#### 1. Native and Denatured DNA

Lacking an *a priori* definition of "primer" for the replicative DNA polymerase reaction, it may be useful to examine some experiments on primer in the hope of devising an improvement over the original operational definition. Such experiments begin with native DNA because of its existence and availability. But when native DNA is placed in a polymerase reaction mixture and at some later time a reaction is observed, it is by no means certain that native DNA is the primer. Such a conclusion would depend upon a knowledge of mechanism, which is not at hand. The *E. coli* polymerase described by Lehman *et al.* (1958a) utilizes either native or denatured DNA, but an increased priming activity is produced by treating native DNA with minute amounts of pancreatic DNase I (Bessman *et al.*, 1958) or by heat denaturation (Lehman, 1959). Both crude and partially purified regenerating rat liver enzyme (Bollum, 1958a) utilize either native or denatured high-polymer DNA.

The calf thymus polymerase (Bollum, 1959, 1960b) was the first purified preparation for which an absolute requirement for denatured DNA was demonstrated (Fig. 1). This observation has been extended to a wide variety of native (double-stranded) DNA's, from a variety of sources, and over a wide range of base compositions—and the only exception so far is $\phi$X174 DNA in which the natural form appears to be single-stranded (Sinsheimer, 1960). The bacteriophage-induced polymerase of *E. coli* (Kornberg *et al.*, 1959) purified by Aposhian and Kornberg (1962) appears to be quite similar to the calf thymus enzyme in its requirement for denatured DNA primer. The absolute requirement for denatured DNA, while providing some clarification of test tube reactions, poses some difficult questions concerning how "primer" DNA is formed under physiological conditions (see Section V).

An assessment of the true operational condition of the high-polymer DNA, with regard to its "strandedness" and degree of polymerization, requires (1) knowledge of the preparation and handling of the DNA, and (2) reasonable assurance that the polymerase preparation does not contain endonucleases, exonucleases, or phosphodiesterases that change the nature of the primer during the test period. A detailed account of the effect of enzymes on primer activity will be given in Section IV,D. It is clear, however, from all studies on the DNA-dependent synthesis of DNA by enzymes, that high-polymer DNA, in some form and from a

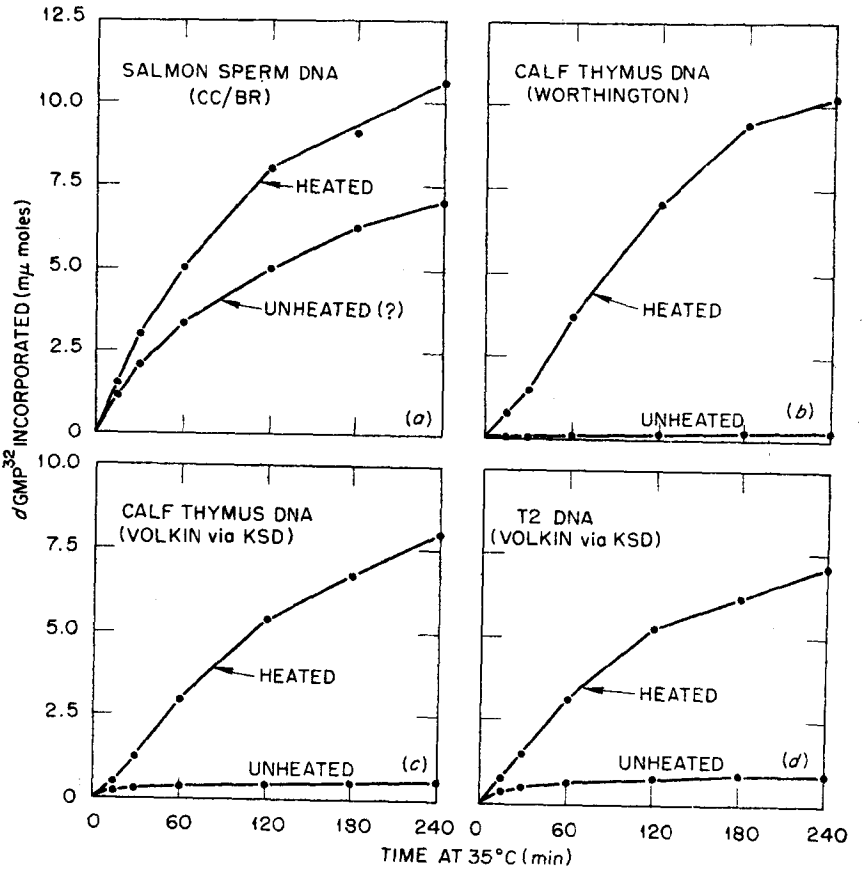


FIG. 1. Thermal conversion of nonpriming DNA to primer for the calf thymus DNA polymerase: (*a*) a partially denatured commercial preparation; (*b*) a native commercial preparation; (*c*) and (*d*) DNA's prepared by a detergent method (Bollum, 1959).

variety of sources, serves as a "primer." Thus the DNA polymerase enzymes are nonspecific with regard to the source of primer material.

## 2. PRIMER-PRODUCT RELATIONSHIPS

The products formed in *E. coli* DNA polymerase reactions primed by high-molecular weight primers are themselves of high molecular weight. In addition, they appear to have the hydrogen-bonded secondary structure of native DNA (Schachman *et al.*, 1958). However, it should not be concluded that the products formed are of chain length identical to the primer chain length. Published sedimentation values (Lehman, 1959;

Adler *et al.*, 1958) are close to but not identical with primer values, and the distribution of sedimentation coefficients appears broadened (Schachman *et al.*, 1958). Viscosimetry confirms the high-polymer, H-bonded nature of the product of *E. coli* polymerase. It would be desirable, however, to have physical data on products formed in polymerase reactions that do not have endogenous nuclease activity and in which primer requirement is clearly defined.

The recent availability of a calf thymus polymerase fraction devoid of endonuclease (tested with native $P^{32}$-DNA or *Hemophilus influenzae* transforming activity as substrate) and essentially free of exonuclease or phosphodiesterase (tested with heated $P^{32}$-DNA or denatured *H. influenzae* transforming activity as substrate) may provide a product suitable for more exact physical characterization. The nuclease-free enzyme catalyzes the exact doubling of high-molecular weight primers such as $\phi$X174 and denatured calf thymus DNA (cf. Fig. 4, below). The products band in CsCl at a lower density than the primer DNA, indicative of the high molecular weight and native configuration of the product. Perhaps the best illustration of the nature of the calf thymus polymerase product, at least in a relative sense, is its chromatographic isolation in the position of native DNA on methylated serum albumin columns (Lerman, 1955; Mandell and Hershey, 1960; Sueoka and Cheng, 1962). The most useful property of this absorbent in the present situation is its strong binding of denatured DNA (i.e., primer), which is not eluted by gradients up to 1 *M* NaCl, whereas native DNA, up to MW $130 \times 10^6$, is eluted below 1 *M* NaCl (Mandell and Hershey, 1960). The column

TABLE I

CHROMATOGRAPHY OF NATIVE DNA AND VARIOUS CALF THYMUS POLYMERASE PRODUCTS ON METHYLATED ALBUMIN

| Preparation chromatographed[a] | Mid-point of elution[b] (*M* NaCl) |
|---|---|
| DNA, native | 0.60–0.62 |
| DNA, denatured | >1.0 |
| Polymerase product; no sonication, heated primer | 0.60 |
| Polymerase product; 1-minute sonication, heated primer | 0.44 |
| Polymerase product; 10-minute sonication, heated primer | 0.39 |
| Polymerase product; 60-minute sonication, heated primer | 0.39 |
| Same as above, heat-denatured | 0.55 |

[a] All preparations from same *H. influenzae* DNA

[b] Samples chromatographed on 1 × 2 cm methylated albumin columns (Sueoka and Cheng, 1962) using a linear gradient from 0.1 to 1.0 *M* NaCl at pH 6.7. Polymerase products synthesized with various *H. influenzae* primers and calf thymus polymerase Fraction E (Bollum, 1961, unpublished).

thus provides an analysis of the elution point of a polymerase product with respect to that of the *native* primer, prior to its denaturation to form primer. Any polymerase product appearing in the gradient elution can be tested for its secondary structure by rechromatography after heating. Product peaks can also be analyzed directly for buoyant density in CsCl and enough material for sedimentation and light-scattering may be obtained from preparative columns. The results to date (Table I) indicate the similarity of elution points of product and native DNA.

### 3. Nearest-Neighbor Experiments

The incorporation of a single $P^{32}$-deoxyribonucleoside 5′-triphosphate in the presence of the three nonradioactive triphosphates, followed by degradation of DNA polymerase product to 3′-mononucleotides, provides the "nearest-neighbor" identification. This type of experiment, an extension of the "base incorporation ratio" studies (Lehman *et al.*, 1958b), repeated with all four combinations of one substrate labeled with $P^{32}$ and three unlabeled, yields the data for computing dinucleotide frequencies and for assessing the polarity of the strands of product DNA. The analyses of Josse *et al.* (1961) and Swartz *et al.* (1962) on *E. coli* polymerase products demonstrate the complementary and the antiparallel nature of the newly synthesized strands. The results also indicate that DNA's of similar base composition may have different dinucleotide frequencies. The complementarity observed is the most convincing evidence that the polymerase product base order is specified by the base order of the primer DNA.

## B. Sonicated DNA—MW ~ $10^5$

Physical studies by Doty *et al.* (1958) demonstrated that DNA degraded by sonic oscillation reaches a limiting molecular weight of about $3 \times 10^5$. The fragments thus produced have a double helical configuration like the native high-molecular weight material, indicating that fragmentation is produced by double-chain scission. That sonic fragments are capable of primer function was implicit in the earliest papers on the *E. coli* polymerase, since the crude sonic extracts of *E. coli* employed contained primer (Kornberg *et al.*, 1956a).

In an attempt to derive a more concise definition of the molecular weight requirement for the partially purified calf thymus polymerase, a series of sonicated preparations were examined for primer activity. The DNA fragments produced by sonic oscillation do not serve as primer for this enzyme but the heat-denatured fragments exhibit priming activity (Bollum, 1960a). These results thus not only confirm the native configuration of the sonic fragments, as first demonstrated by Doty *et al.*

(1958), but also indicate that length is not a critical requirement in primer for polydeoxyribonucleotide synthesis. While these experiments did not define the minimum length for priming, several other interesting results were obtained. The first is that most native DNA's subjected to a 10-minute sonication followed by heat denaturation exhibit a 2- to 5-fold increase in initial rate of synthesis (Table II). The second result, shown

TABLE II
EFFECT OF SONICATION ON INITIAL VELOCITY

| DNA preparation | Rate increase[a] |
|---|---|
| *H. influenzae* | 2.2, 3.0 |
| Calf thymus | 2.5 |
| *E. coli* | 5.0 |
| *Micrococcus lysodeikticus* | 5.2 |

[a] Expressed as

$$\frac{\text{moles dXMP}^{32}\text{/15 minutes with 10-minute sonicated primer}}{\text{moles dXMP}^{32}\text{/15 minutes with unsonicated primer}};$$

all primers heat-denatured before assay as required for the calf thymus enzyme.

in Table I, indicates that the chromatographic mobility of the products formed by the calf thymus enzyme are related to the molecular weight of primer, and that they have the "native" configuration. The interpretation of the primer "activation" (Table II) observed in these experiments is not immediately obvious since the results may arise from easier or more complete denaturation, or from changed kinetic properties of the fragments produced.

A more detailed analysis of the apparent "activation" of sonicated primer is afforded by "primer" saturation curves obtained for a series of sonicated (then denatured) *H. influenzae* DNA samples. A cursory analysis of the curves (Fig. 2) suggests that sonicates are better primers than full length DNA, as noted in the previous paragraph. A more careful analysis, however, leads to a somewhat different conclusion. Reciprocal plots ($1/V$ vs. $1/S$) give a single $V_{max}$ for all sonicates and show that the most marked effect of sonication is on $K_m$. In this analysis, however, substrate concentration is plotted as DNA phosphorous and, since there are more polymer molecules in the sonicated samples, $K_m$ appears to be directly related to the number of primer molecules per mole of DNA-P. The result is, therefore, much as anticipated (Table III) since $K_m$ decreases upon sonication by a factor of twelve and reaches a limiting value in much the manner of the molecular weight decrease observed upon sonication (Doty *et al.*, 1958).

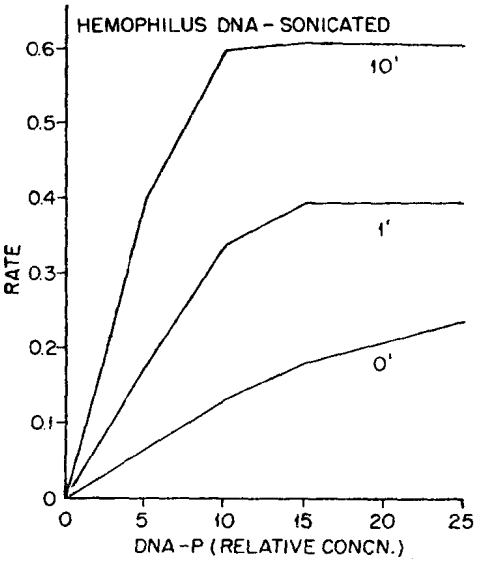


FIG. 2. Substrate saturation curves for normal and sonicated *H. influenzae* DNA. 0′ is normal and 1′ and 10′ refer to the minutes of sonic degradation. A 60-minute sample curve was identical to the 10-minute sample. 10 on the abscissa corresponds to 7.32 mμmoles DNA-P.

This analysis provides two further speculations. (1) An estimation of molecular weight may be made from the determination of $K_m$ on the basis of DNA-P. While alternative methods of molecular weight determination are available, the primer method may yield number averages instead of the weight averages obtained by the more readily available methods. (2) Most work claiming an "activation" of primer may now be interpreted on the basis of increasing the number of polymer particles

TABLE III
KINETIC CONSTANTS—SONICATED PRIMERS

| Minutes of sonication[a] | $V_{max}$ | $K_m$ (moles $\times 10^{-6}$) |
|---|---|---|
| 0 | 0.91 | 199 |
| 1 | 0.72 | 46 |
| 10 | 0.83 | 17 |
| 60 | 0.83 | 17 |

[a] *H. influenzae* DNA was degraded in a Raytheon 9 KC magnetostrictive oscillator at 4° to 6°, heat-denatured, and initial velocities measured as a function of DNA-P concentration.

per mole of DNA-P. Thus, enzymatic "activation" of primer (Bessman *et al.*, 1958, see also Fig. 3 and Section IV,D) is the early result of a

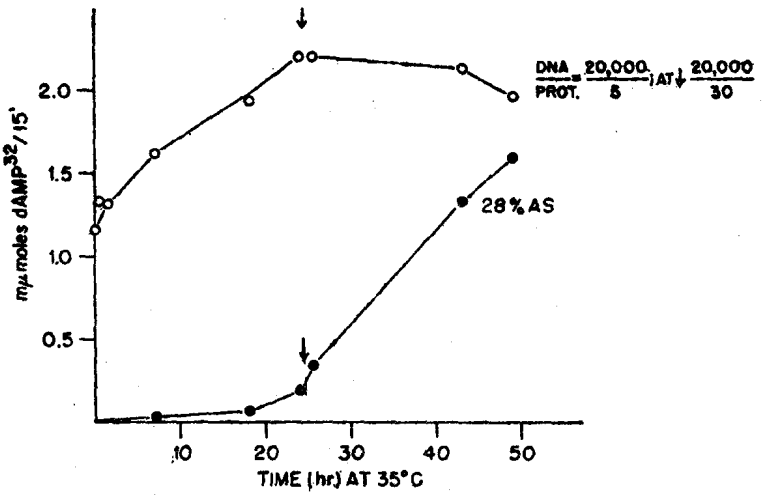


FIG. 3. Primer production by DNase II (spleen). KEY: ●, tested with the calf thymus enzyme before heat denaturation; ○, tested with the calf thymus enzyme after heat denaturation.

random degradation of polymer. Later stages of a random degradation will probably produce lower molecular weight pieces having a low affinity for the enzyme. The sonication process results in nonrandom degradation (Freifelder and Davison, 1962) in which most of the pieces formed still have a high affinity for the enzyme—largely because of the fact that even the limit pieces are much larger than the enzyme.

It is also apparent that any random degradation process (endonuclease) occurring in a DNA polymerase reaction will give false kinetics. For example, a slow stage followed by an increasing rate of DNA synthesis may well arise from the increase in number of primer molecules from degradation, resulting in more effective "saturation" of the enzyme.

## C. Degraded DNA—MW ~ $10^4$

Apurinic acid tested with *E. coli* polymerase shows no priming activity. Extensive digestion of DNA by DNase I produces material inactive as primer for the *E. coli* polymerase (Lehman *et al.*, 1958a). A limited digestion, however, activates *E. coli* DNA and calf thymus DNA for the *E. coli* polymerase.

The priming activity of fragments of calf thymus DNA, produced by slow digestion with DNase II (or DNase I), is shown in Fig. 3 and is