Functional Activity of DNA and DNA Polymerase During Thymine Starvation of Escherichia coli 15T⁻

George E. Schaiberger, Joseph Giegel
and Bennett Sallman
Department of Microbiology
University of Miami School of Medicine
Coral Gables, Florida

Received May 23, 1967

Thymine deprivation of the thymineless auxotroph Escherichia coli 15T⁻ in an otherwise complete medium results in the loss of its ability to divide, a phenomenon which has been termed "thymineless death" (Barner and Cohen, 1955). Menningmann (1964) and also Sicard et al. (1967) conclude that thymineless death results from two processes a) the induction of an episome by thymine starvation and subsequent readdition of thymine and b) a lethal effect of thymine starvation which is independent of episome induction. Using a strain of E. coli 15T⁻ which was neither lysogenic nor colicinogenic Sicard and Simonnet (1967) observed a 50% reduction in viability in 2 hours of thymineless incubation. There are indirect indications that DNA may be altered under thymineless conditions, including a decrease in messenger RNA synthesis (McFall and Magasanik, 1962), loss of transforming activity of DNA from thymineless Bacillus subtilis (Sicard and Anagnostopoulos, 1964), difficulties of DNA extraction from thymineless cells (McFall and Magasanik, 1962) and the decrease in priming activity for RNA polymerase by DNA from thymineless cells (Luzzati, 1966). The observed loss of division ability under thymineless conditions suggested to us that an altered DNA polymerase activity or an altered DNA template activity may, in part, be involved. In this investigation DNA from cells subjected to thymineless conditions was compared to DNA from control cells as to its

FUNCTIONAL ABILITY TO SERVE AS TEMPLATE FOR DNA POLYMERASE AND AS SUBSTRATE FOR HYDROLYSIS BY AN ENDO AND EXODEOXYRIBONUCLEASE. THE ACTIVITY OF DNA POLYMERASE FROM THESE CELLS WAS ALSO COMPARED USING CALF THYMUS DNA AS PRIMER. OUR RESULTS INDICATE THAT DNA POLYMERASE ACTIVITY REMAINS UNALTERED UNDER THYMINELESS CONDITIONS. CRUDE DNA FROM THYMINE STARVED CELLS HAD A DIMINISHED TEMPLATE ACTIVITY FOR DNA POLYMERASE BUT PURIFIED DNA SHOWED NORMAL TEMPLATE ABILITY. HOWEVER, PURIFIED DNA FROM THYMINE DEPRIVED CELLS SHOWED INCREASED RESISTANCE TO NUCLEOLYTIC ENZYMES.

MATERIALS AND METHODS: THE MEDIUM AND GENERAL METHODS FOR CULTURE MAINTENANCE, GROWTH, THYMINE STARVATION AND HARVEST ARE THE SAME AS PREVIOUSLY DESCRIBED (SCHAIBERGER, ET AL. 1965). CHEMICAL DETERMINATIONS FOR TOTAL PROTEIN AND DNA WERE PERFORMED BY THE METHOD OF LOWRY ET AL. (1961) AND BURTON (1956) RESPECTIVELY. CRUDE EXTRACTS WERE PREPARED FROM CONTROL AND THYMINE STARVED CELLS BY SONIC DISRUPTION IN A BRANSON S-75 SONIFIER AT MAXIMUM POWER FOR 45 SECONDS AT 4° C. THESE SONICATES WERE CENTRIFUGED AT 10,000G FOR 20 MINUTES TO REMOVE CELL DEBRIS. CRUDE DNA WAS PREPARED BY HEATING THE ABOVE EXTRACTS FOR 20 MINUTES AT 100° C, FOLLOWED BY CENTRIFUGATION AT 10,000G FOR 20 MINUTES. THIS PROCEDURE PRODUCED GREATER THAN 75% YIELD OF DNA. PURIFIED DNA WAS ISOLATED FROM THE CRUDE EXTRACTS BY THE METHOD OF MARMUR (1961) (YIELD APPROXIMATELY 40%). CRUDE DNA POLYMERASE WAS PREPARED FROM THE CRUDE EXTRACTS BY THE AUTOLYTIC REMOVAL OF DNA (RICHARDSON ET AL. 1964). PURIFIED DNA POLYMERASE WAS OBTAINED FROM BIOPOLYMERS, INC. THE IN VITRO SYNTHESIS OF DNA WAS CARRIED OUT ACCORDING TO RICHARDSON ET AL. (1964) AND ASSAYED BY MEASURING THE CONVERSION OF C^{14}-THYMIDINE TRIPHOSPHATE INTO ACID-INSOLUBLE PRODUCT AT 30° C WHICH WAS COLLECTED AND WASHED BY FILTRATION ON 0.45μ MILLIPORE FILTERS. RADIOACTIVE DNA WAS PREPARED BY GROWING THE CELLS IN TRITIATED THYMINE (1.6 MILLUCURIES THYMINE-METHYL-H^3 PER LITER OF CULTURE MEDIUM) AND ISOLATING THE LABLED DNA (SPECIFIC ACTIVITY - 30 MICROCURIES/MICROMOLE) BY THE METHOD OF MARMUR (1961). DNAASE ACTIVITIES WERE DETERMINED BY MEASURING THE DECREASE

IN ACID-INSOLUBLE RADIOACTIVITY. ALL RADIOACTIVE SAMPLES WERE COUNTED IN A LIQUID SCINTILLATION SPECTROMETER. RADIOACTIVE NUCLEOSIDE TRIPHOSPHATE AND H^3-THYMINE WERE OBTAINED FROM SCHWARZ BIORESEARCH, INC. THE NUCLEOLYTIC ENZYMES, SNAKE VENOM PHOSPHODIESTERASE (EC 3.1.4.1), AND ENDONUCLEASE II (BOVINE SPLEEN EC 3.1.4.6), WERE PURCHASED FROM WORTHINGTON BIOCHEMICAL CORPORATION. CALF THYMUS DNA WAS OBTAINED FROM SIGMA CHEMICAL COMPANY.

RESULTS AND DISCUSSION: THE ABILITIES OF AN EXONUCLEASE (SVP) AND ENDONUCLEASE II (ENDO II) TO DEGRADE PURIFIED DNA FROM CONTROL AND THYMINE-STARVED CELLS ARE COMPARED IN TABLE 1. SVP INITIATES HYDROLYSIS OF SINGLE STRANDED DNA AT THE 3'-OH END PRODUCING 5' MONONUCLEOTIDES IN A STEPWISE FASHION (FELIX ET AL. 1960). TABLE 1 SHOWS THAT SVP ATTACKED CONTROL DNA AT FOUR TIMES THE RATE THAT IT HYDROLYZED DNA FROM THYMINE-STARVED CELLS. ENDO II ATTACKS DNA IN A MORE RANDOM FASHION LIBERATING OLIGONUCLEOTIDES. AS SHOWN IN TABLE 1 ENDO II ATTACKED CONTROL DNA AT A MUCH GREATER RATE THAN DNA FROM THYMINE-DEPRIVED CELLS. MOREOVER, DNA FROM THYMINE-STARVED CELLS APPEARED TO HAVE A RESISTANT CORE WHICH WAS NOT SUSCEPTIBLE TO HYDROLYSIS BY THIS ENZYME.

TABLE 1. DNA FROM CONTROL AND THYMINELESS CELLS AS SUBSTRATE FOR SNAKE VENOM PHOSPHODIESTERASE AND ENDONUCLEASE II

PER CENT DNA HYDROLYSIS

MINUTES HYDROLYSIS	SNAKE VENOM PHOSPHODIESTERASE		ENDONUCLEASE II	
	CONTROL	4 HR THYMINELESS	CONTROL	4 HR THYMINELESS
0	0.0	0.0	0.0	0.0
10	12.9	3.0	30.8	11.7
20	24.5	6.2	57.6	17.1
40	29.0	12.9	87.6	18.3

USING SVP, THE REACTION MIXTURES (2.0 ML) WERE INCUBATED AT 30° C AND CONTAINED 0.5 MG ENZYME, 13.5μ MOLES OF MG ACETATE, AND 30 mμ MOLES DNA-H^3 IN PHOSPHATE BUFFER, PH 7.0. USING ENDO II, THE REACTION MIXTURES (2.0 ML) WERE INCUBATED AT 25° C AND CONTAINED 150 μG ENZYME, 160 μ MOLES NA-ACETATE BUFFER, PH 4.6, AND 30 mμ MOLES DNA-H^3. SAMPLES (0.2ML) WERE REMOVED AT ABOVE SHOWN INTERVALS FOR RADIOACTIVE ASSAY.

THE CAPACITY OF EXTRACTS FROM THYMINE DEPRIVED CELLS FOR IN VITRO SYNTHESIS OF DNA WAS EXAMINED. CRUDE EXTRACTS OF CULTURES DEPRIVED OF THYMINE FOR 1 OR 2 HOURS SHOW A DECREASED CAPACITY FOR INCORPORATION OF C^{14}-TTP INTO DNA (FIG 1). IF THYMINE IS ADDED TO THE CULTURE AFTER 2 HOURS OF DEPRIVATION, THE ABILITY OF CRUDE EXTRACTS OF THIS CULTURE TO INCORPORATE C^{14}-TTP INTO DNA REMAINS IMPAIRED (FIG 1). COHEN AND BARNER (1955) HAVE DEMONSTRATED THAT DURING THE PERIOD AFTER THYMINE READDITION, A 50% INCREASE IN DNA OCCURS, INDICATING THAT THESE CELLS ARE CAPABLE OF DNA SYNTHESIS IN VIVO. THE DNA SYNTHESIZED DURING THIS PERIOD MAY WELL REPRESENT DNA OF THE DEFECTIVE PROPHAGE. THESE OBSERVED DECREASES IN THE RATE OF IN VITRO DNA SYNTHESIS MAY BE DUE TO (1) DECREASED LEVELS OF DNA POLYMERASE, (2) PRODUCTION OF AN INHIBITOR OF THE REACTION OR OF AN ALTERATION OF THE DNA ITSELF OR (3) INCREASED LEVELS OF NUCLEASE ACTIVITY DURING THYMINE STARVATION. THE DATA PRESENTED BELOW APPEAR TO SUPPORT THE SECOND HYPOTHESIS.

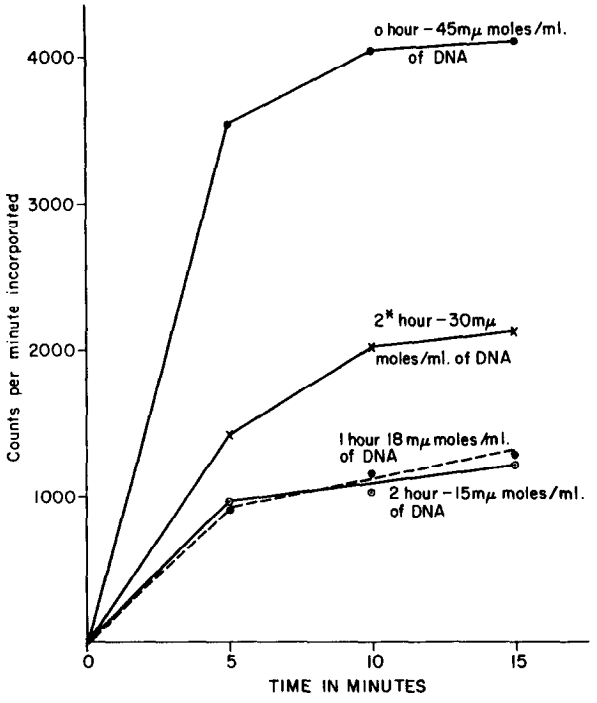

FIG. 1 - ABILITY OF CRUDE EXTRACTS TO SYNTHESIZE DNA. ASSAY MIXTURES CONTAINED PER ML.- 80 GAMMA'S PROTEIN, dATP, dGTP, dCTP - 10 mμ MOLES EACH C^{14}-dTTP- 5 mμ MOLES SPEC. ACT. 11 MC/MMOLE $MgCl_2$ - 2 μ MOLES; KPO_4-20 μ MOLES 2-MERCAPTOETHANOL - 0.3 μ MOLES FINAL PH 7.1 - NO EXOGENOUS DNA OR DNA POLYMERASE ADDED

2*HOUR - CULTURES STARVED FOR THYMINE FOR 2 HOURS, THEN GROWN IN THE PRESENCE OF THYMINE FOR 2 ADDITIONAL HOURS.

THE LEVEL OF DNA POLYMERASE WAS MEASURED IN CRUDE EXTRACTS OF THYMINE DEPRIVED CELLS USING CALF THYMUS DNA AS PRIMER (FIG 2). DNA POLYMERASE CONTINUES TO BE SYNTHESIZED DURING THYMINE DEPRIVATION IN PARALLEL WITH NET PROTEIN SYNTHESIS. NO SIGNIFICANT CHANGE IN SPECIFIC ACTIVITY OCCURS FOR AT LEAST 4 HOURS OF THYMINE DEPRIVATION OR UPON THE READDITION OF THYMINE THEREAFTER EVEN THOUGH THERE IS A 50% INCREASE IN DNA FOLLOWING SUCH ADDITION. THUS, IT APPEARS THAT LACK OF SUFFICIENT POLYMERASE IS NOT RESPONSIBLE FOR THE OBSERVED DECREASE IN SYNTHETIC ABILITY OF THESE CRUDE EXTRACTS. NUCLEASE ACTIVITY UNDER THE CONDITIONS OF THE DNA POLYMERASE ASSAY WAS EXAMINED AND FOUND TO BE LOW AND APPROXIMATELY THE SAME IN ALL THE CRUDE EXTRACTS.

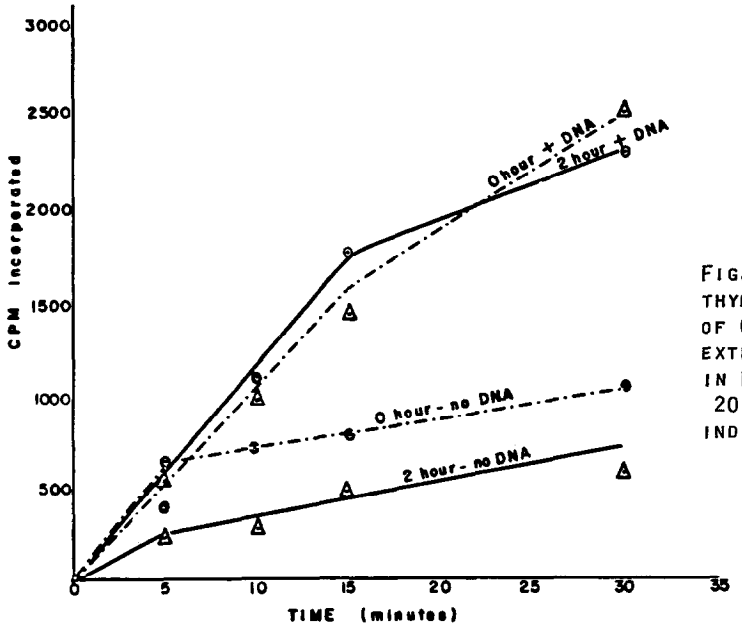

FIG. 2 - EFFECT OF ADDED CALF THYMUS DNA ON SYNTHETIC ABILITY OF 0 HOUR AND 2 HOUR CRUDE EXTRACTS. ASSAY MIXTURES AS IN FIG. 1 - CALF THYMUS DNA 20 MμMOLES/ML ADDED AS INDICATED

TO TEST THE POSSIBILITY THAT THE DNA WAS ALTERED DURING THYMINE DEPRIVATION, CRUDE DNA WAS PREPARED FROM THE ABOVE EXTRACTS. THIS DNA WAS ASSAYED FOR TEMPLATE ABILITY USING PURIFIED DNA POLYMERASE. AS SHOWN IN FIG 3, DNA FROM THYMINELESS CELLS WAS A POORER TEMPLATE FOR THE ENZYME THAN CONTROL DNA. WHEN DNA FROM THESE SAME CULTURES WAS ISOLATED AND PURIFIED BY THE PROCEDURE OF MARMUR (1961), NO DIFFERENCE IN THEIR ABILITY TO SERVE AS

TEMPLATE WAS OBSERVED (FIG 3) WHEN ASSAYED WITH PURIFIED POLYMERASE.

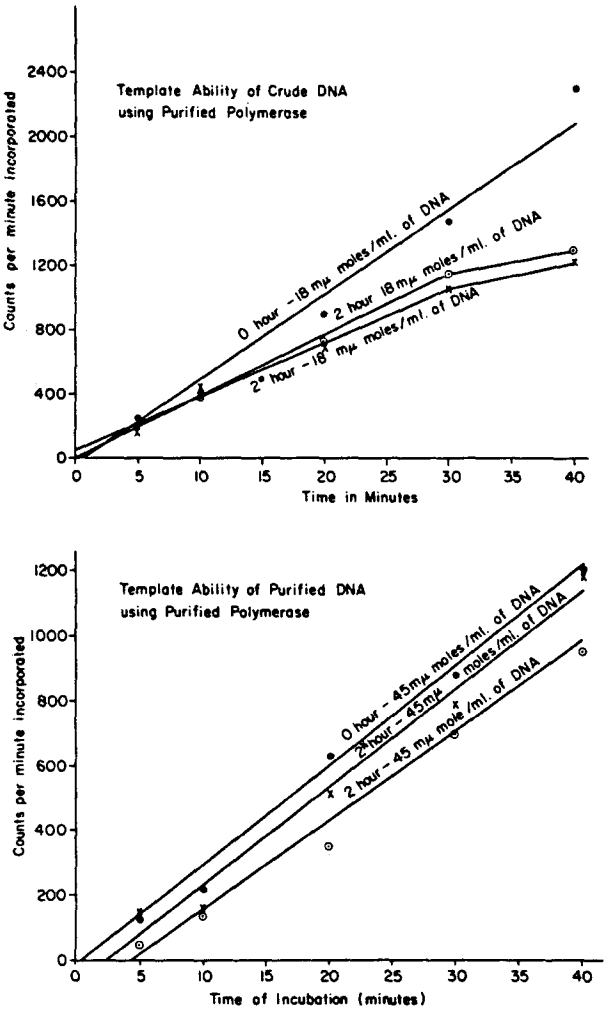

FIG. 3 - TEMPLATE ABILITY OF CRUDE AND PURIFIED DNA USING PURIFIED POLYMERASE. CRUDE DNA WAS OBTAINED FROM CRUDE EXTRACTS BY HEATING AT 100°C FOR 20 MIN (SEE TEXT). PURIFIED DNA WAS OBTAINED FROM THESE SAME EXTRACTS BY THE PROCEDURE OF MARMUR. ASSAY MIXTURES CONTAINED 0.75 UNITS/ML OF PURIFIED DNA POLYMERASE AND DNA AT THE CONCENTRATIONS INDICATED

IN OTHER EXPERIMENTS, PURIFIED DNA ISOLATED FROM CELLS DEPRIVED OF THYMINE FOR AS LONG AS SIX HOURS ALSO SHOWED NO DECREASE IN TEMPLATE ABILITY WHEN MEASURED WITH CRUDE POLYMERASE FROM CONTROL CULTURES OR WITH CRUDE POLYMERASE FROM 2 HR THYMINE DEPRIVED CULTURES. RNAASE TREATMENT OF THE CRUDE DNA PREPARATION HAD NO EFFECT ON THE OBSERVED INHIBITION.

These results lead us to conclude that a heat stable substance is produced during thymine deprivation, possibly associated with DNA, which leads to a decrease in the ability of such DNA to serve as template for DNA polymerase. Luzzati, (1966) has recently reported an impaired template ability for DNA from thymine-deprived cells for RNA polymerase. With further purification of the thymine-deprived DNA this author observed a 15-35% increase in the priming efficiency for RNA polymerase. This observation and our results, showing complete restoration of DNA template ability for DNA synthesis upon purification, suggests to us that DNA under thymineless conditions becomes associated with some constituent (perhaps a protein) which is disassociated upon purification. The possibility that our isolation procedure selects for unaltered DNA is not eliminated.

Our failure to observe reversal of increased resistance to nucleolytic action upon purification - in contrast to its restoration of DNA priming ability - may indicate a varied quantitative effect of the postulated component or that more than a single qualitative change is associated with DNA. It has been reported by Burton and Smith, (1965) and Luzzati, (1966) that no detectable changes in physical properties are observable in purified DNA during thymine deprivation even though highly purified DNA has been reported to contain 0.1 to 1.0 per cent residual protein (Leaner and Cruft, 1966, Olenick and Hahn, 1964). It is possible that such residual protein may affect enzymatic activity without altering physical characteristics.

References

K. Burton, Biochem. J. 62, 315 (1956).
H. Barner and S. S. Cohen, J. Bact. 69, 59 (1955).
F. Felix, J. L. Potter and M. Laskowski, Sr., J. Biol. Chem. 235, 1150 (1960).
J. L. Leaner and H. J. Cruft, Biochem. J. 101, 665 (1966).
O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. 193, 265 (1961).
D. Luzzati, J. Bact. 92, 1435 (1966).

J. MARMUR, J. MOL. BIOL. 3, 208 (1961).
E. MCFALL AND B. MAGASANIK, BIOCHEM. BIOPHYS. ACTA 55 (1962), 920.
H. D. MENNINGMAN, B. B. R. C. 16, 373 (1964).
D. NAKADA, BIOCHEM. BIOPHYS. ACTA. 55, 505 (1962).
J. G. OLENICK AND F. E. HAHN, BIOCHEM. BIOPHYS. ACTA, 87, 535 (1964).
C. C. RICHARDSON, C. L. SCHILDKRAUT, H. V. APOSHIAN AND A. KORNBERG, J. BIOL. CHEM. 239, 222 (1964).
G. E. SCHAIBERGER, B. SALLMAN AND J. L. GIEGEL, J. GERONT. 20, 23 (1965).
N. SICARD, G. SIMONNET AND L. ASTRACHAN, BIOCHEM. BIOPHYS. RES. COMM. 26, 532 (1967).
N. SICARD AND C. ANAGNOSTOPOULOS, C. R. ACAD. SCI. 259, 4173 (1964).
B. J. SMITH AND K. BURTON, BIOCHEM. J. 97, 240 (1965).