MOLECULAR MECHANISM OF THYMINE-LESS DEATH*

Horst-Dieter Mennigmann** and Waclaw Szybalski

McArdle Memorial Laboratory
University of Wisconsin
Madison 6, Wisconsin

Received October 17, 1962

The aim of the present study was to elucidate the molecular mechanism of "thymine-less death", the progressive loss of cell viability, resulting from a genetic or biochemically-induced (Cohen and Barner, 1954; Cohen et al, 1958) selective block in thymidylate synthesis. The earlier experiments of Mennigmann and Szybalski (1962) indicated that structural changes in DNA might be implicated in thymine-less death, since loss of cell viability was accompanied by reduction in the transforming activity of the extracted DNA, and by loss of its fibrous structure as evidenced by the floculent nature of the precipitate formed upon addition of ethanol to the deproteinized DNA solution.

EXPERIMENTAL To a vigorously shaken culture of Bacillus subtilis wild-type strain 168 (Szybalski et al., 1960), growing exponentially in enriched minimal medium ($MgSO_4 \cdot 7H_2O$- 0.4 g; citric acid$\cdot H_2O$ - 4 g; K_2HPO_4 - 20 g; $NaNH_4HPO_4 \cdot 4H_2O$ - 7 g; Difco Casamino Acids - 15 g; glucose - 5 g; water up to 1000 ml.), 15 µg/ml. of 5-fluorodeoxyuridine (FUdR), as selective inhibitor of thymidylate synthesis, was added when the cell concentration had reached approximately 5×10^7 cells/ml. At selected times thereafter aliquots of the culture were removed for various estimations. FUdR causes

*Supported by grant CY-5215 from the National Cancer Institute.

**Fellow of the Rockefeller Foundation; present address: Institute of Microbiology, University of Frankfurt, Frankfurt/Main, Germany.

immediate inhibition of DNA synthesis and cell division, followed by progressive loss of colony-forming capacity, beginning after approximately 1 hr. of the thymine-less state (Fig. 1; curves: DNA and VIABLE COUNT).

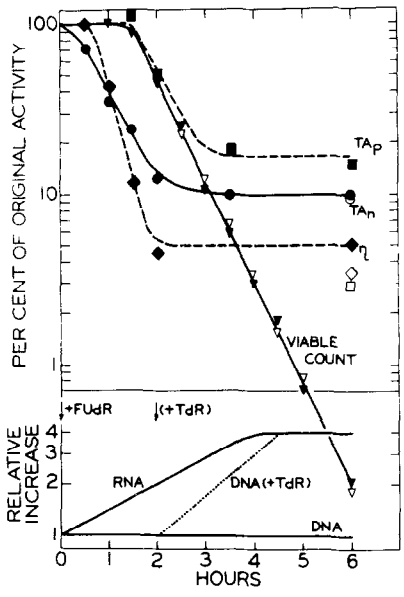

Figure 1. Changes in cell viability (VIABLE COUNT), macromolecular synthesis (DNA, RNA), transforming activity (TA), and reduced specific viscosity (η) of extracted DNA during FUdR-effected inhibition of thymidylate synthesis lasting either for 6 hrs. (solid symbols), or for 2 hrs. followed by thymidine addition (open symbols; +TdR). For determination of transforming activity either a non-deproteinized, raw cell lysate (TA_n: ●, ○) or deproteinized, RNase-treated DNA (TA_p: ■, □) were used; the latter served also for viscosity measurements (η: ◆, ◇).

For determination of transforming activity, the washed cell pellet was resuspended in 0.15M NaCl + 0.015M Na_3·citrate (SSC) and lysed by addition of 100 μg lysozyme per ml. followed by 1% sodium lauryl sulfate (Duponol). The specific transforming activity of this raw cell lysate (3×10^6 ind^+ transformants/μg DNA), diluted at least 500-fold to eliminate the lytic effect of Duponol on the receptor cells (indole-requiring strain 21), began to decrease almost immediately and leveled off at 10% of the original value within 3 to 4 hours after FUdR addition (Fig. 1; curve TA_n). The specific transforming activity of the DNA (5 hrs. of FUdR inhibition) deproteinized by repeated vigorous shaking with chloroform and butanol (4:1) decreased to 1/30 - 1/60 of the original value (not included in Fig. 1). When very gentle phenol deproteinization was employed to minimize shear, and was followed by RNase treatment, further phenol deproteinization, and prolonged dialysis in the cold, the curve marked TA_p (Fig. 1) was obtained. Viscosity measurements were performed on the latter phenol-deproteinized

DNA preparation (2 μg DNA/ml.) using a joint effluent time for the three top bulbs of an Ubelohde-type low-shear capillary (2000 x 1 mm) viscometer (Eigner, 1960). The drop in the reduced specific viscosity from 60 dl/g, measured at an average shear rate of approximately 80 sec^{-1}, paralleled roughly the loss of transforming activity, and leveled off at 5% of the original value after approximately 2 hrs. of the thymine-less state (Fig. 1; curve η).

The above changes were all measured during 6 hrs. of continuous FUdR inhibition. In variants of this experiment, when the block in thymidylate synthesis was reversed within less than 1 hr. by addition of thymidine (TdR), cell viability was retained; but later TdR addition (10 μg/ml.), e.g. at the 2nd hr., as represented by the arrow (+TdR) in Fig. 1, restored only DNA synthesis (dotted line, +TdR), without modifying the loss of cell viability (open triangles) and transforming activity of the raw lysate (open circles). Indeed the losses in transforming activity and the viscosity of the gently deproteinized and RNA-free DNA, measured 4 hrs. after TdR addition (+TdR), were even more pronounced (open squares and diamonds). The course of RNA synthesis was not modified by TdR reversal (Fig. 1, curve RNA).

All the above described changes measured 6 hrs. after FUdR addition, without (-TdR) or with TdR reversal at the 2nd hr. (+TdR), are summarized in Table 1.

The sedimentation constant (35,000 rpm; 10 μg DNA/ml of 0.15M NaCl), $S_{20,W}$, and the buoyant density in the CsCl gradient (47,770 rpm, 25° C.) were also determined and remained essentially unchanged throughout the experiment, with the exception of a decrease in the $S_{20,W}$ in the case of TdR reversal.

Forcing the diluted cell lysate (5×10^{-3} μg DNA/ml.) once through a 27 gauge syringe needle reduced the transforming activity of the DNA isolated from 6 hr. TdR-starved cells by a factor greater than 100, while similar shear resulted only in a 3-fold reduction in transforming activity for the lysate of normal cells (Table 1).

Table 1.

RELATIVE CHANGES IN THE BIOLOGICAL AND MOLECULAR PROPERTIES OF DNA AND CELL SURVIVAL DURING THE THYMINE-LESS STATE

	FUdR	TdR	Viscosity (η)	Sedimentation ($S_{20,w}$)	Buoyant density (g/cm^3)	Spec. transforming activity			Cell survival (viable count)
						TA_p	TA_n	after shear*	
	hrs.	hrs.	Purified DNA				Raw lysate		
1.	-	-	100%	28	1.703	100%	100%	30%	100%
2.	0-6	-	5%	28	1.703	16%	10%	< 0.1%	< 0.2%
3.	0-6	2-6	3.5%	22	1.703	3%	10%	< 0.1%	< 0.2%

Line 1. corresponds to 0 time, line 2. to 6 hrs. (filled symbols) and line 3. to 6 hrs. (open symbols) in Fig. 1.

*In relation to TA_n measured at 0 time.

Heat denaturation of normal DNA resulted in an increase in its buoyant density in the CsCl gradient by 0.015 g/cm^3 and in only minimal spreading of the band; the band width increased very strongly in the denatured DNA isolated from thymine-starved, non-viable bacteria.

DISCUSSION The experimental results indicate that "thymine-less" death is accompanied or even preceded by some damage to the molecular structure of the DNA, as evidenced by the loss of its viscosity and transforming activity, and by sharply increased sensitivity to shear. On the other hand, when shear during purification is eliminated, the sedimentation coefficient of the DNA is not affected. These data are most compatible with a model which postulates single-strand breaks ("nicks") in the DNA helices with resulting partial collapse of its rigid structure, as presented in Fig. 2. The postulated "nicks", which would appear and be rendered irreversible during thymine starvation, might be related to some prerequisite enzymatic steps for the process of DNA replication, which cannot reach fulfillment in the

absence of thymidylates. Several other predictions based on this model are compatible with the experimental observations:

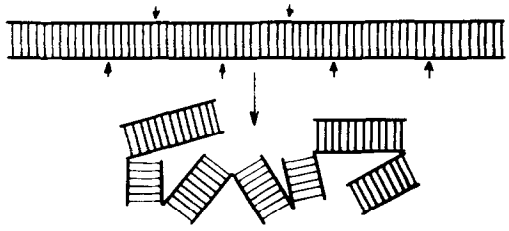

Figure 2. Diagrammatic representation of the postulated structural changes in native DNA during the thymineless state: single-strand breaks at points indicated by arrows, followed by partial collapse of the DNA molecules during isolation.

(1) The sedimentation behavior of the collapsed molecules should not be grossly affected, since the size of the molecules would remain essentially constant and the occasional breaks would be balanced out by the more compact DNA structure.

(2) The buoyant density in the CsCl gradient and the melting temperature of the DNA should likewise remain unaltered, as also reported by others (Nakada, 1962; Luzzati and Revel, 1962), since the molecules remain double-stranded.

(3) Upon strand separation, the size of the "nicked" DNA molecules, as measured by their sedimentation pattern in the CsCl gradient, should decrease precipitously.

(4) "Nicked" molecules should be very sensitive to shear.

(5) The shift in the decay curve for gently deproteinized DNA (TP_p) as compared with the raw lysate (TP_n) could be caused by selective loss of the most fragile DNA molecules during the purification process.

(6) The further accentuation of the decreases in the viscosity, sedimentation constant and transforming activity upon TdR rescue would indicate the irreparability and perpetuation of the original DNA damage, aggravated by further breaks during the resumed replication of the damaged molecules.

All the altered DNA participates in the replication process, since substitution of 5-bromodeoxyuridine (BUdR) for TdR after 2 hrs. of FUdR inhibition results in complete disappearance of BUdR-free DNA and the appearance of

approximately equal amounts of unifilarly ("hybrid") and bifilarly BUdR-labeled DNA, as evidenced by the analytical CsCl gradient centrifugation procedure. The observation of these two rounds of semiconservative DNA replication agrees well with the chemically determined four-fold increase in total DNA within 3 hrs. after TdR reversal [Fig. 1; curve DNA (+TdR)].

(7) The chromosome fragmentation subsequent to exposure of Vicia faba cells to FUdR (Taylor et al., 1962), the mutagenicity of the thymine-less state (Coughlin and Adelberg, 1956), and its additive effect on UV damage (Gallant and Suskind, 1961) could all reflect the lability of the nicked DNA to various stresses.

(8) The changes observed during FUdR inhibition could all be ascribed to the thymine-less state and not to secondary interference with RNA synthesis, since low concentrations of uracil, which should have at least partially protected the cell RNA from the eventual incorporation of 5-fluorouracil (derived from FUdR), actually accentuated the thymine-less death, and since even more pronounced loss in DNA viscosity, to less than 1% of the original value, was observed with a thymine-less strain of Escherichia coli, 15T$^-$ after 2 hrs. of thymine starvation (0.3% survival).

(9) One could suspect that the observed molecular changes during the thymineless state would result in overlapping breaks in the DNA molecules and thus would favor genetic recombinations or the induction of lysogenic phages; the literature provides examples of both types of phenomena (Melechen and Skaar, 1962).

(10) The early portion of the molecular damage to the DNA seems to be reversible, since it precedes the irreversible thymine-less death. This system would thus permit studying the mechanism of the "healing" of single-strand "nicks" in DNA. It is difficult, however, to determine from the present experiments whether the irreversible molecular damage (thymine-less death) reflects only the large accumulation of "nicks" or also some secondary damage, e.g., exonucleolytic attack at the points of the breaks.

If these notions are correct, procedures which produce immunity to thymine-less death, including simultaneous inhibition of protein and/or RNA synthesis (Maaløe and Hanawalt, 1961) should also eliminate the irreversible molecular alteration of the DNA. Experiments along these lines are in progress.

In summary, one might conclude that thymine-less cell death is preceded by single-strand breaks in the DNA molecules, as evidenced by their increased sensitivity to shear, decrease in viscosity, and partial loss of transforming activity. This sequence of changes ("irreparable mistakes in attempted DNA synthesis" - Maaløe and Hanawalt, 1961) is similar to that reported during the action of exonuclease 2 on native transforming DNA.

ACKNOWLEDGEMENT The untiring assistance of Mrs. Gisela Mennigmann is gratefully acknowledged.

REFERENCES

Cohen, S. S., and Barner, H. D., Proc. Natl. Acad. Sci., 40, 885 (1954).
Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., and Lichtenstein, J., Proc. Natl. Acad. Sci., 44, 1004 (1958).
Coughlin, C. A., and Adelberg, E. A., Nature, 118, 529 (1956).
Eigner, J., Ph.D. Thesis, Harvard University (1960).
Gallant, J., and Suskind, R., J. Bacteriol., 82, 187 (1961).
Luzzati, D., and Revel, C., Biochim. Biophys. Acta. 61, 305 (1962).
Maaløe, O., and Hanawalt, P. C., J. Mol. Biol., 3, 144 (1961).
Melechen, N. E., and Skaar, P. D., Virology, 16, 21 (1962).
Mennigmann, H. D., and Szybalski, W., Bacteriol. Proc., p. 43 (1962).
Nakada, D., Biochim. Biophys. Acta., 55, 505 (1962).
Szybalski, W., Opara-Kubinska, Z., Lorkiewicz, Z., Ephrati-Elizur, E., and Zamenhof, S., Nature, 188, 743 (1960).
Taylor, J. H., Haut, W. F., and Tung, J., Proc. Natl. Acad. Sci., 48, 190 (1962).