ELIMINATION OF SULFUR MUSTARD-INDUCED PRODUCTS FROM DNA OF ESCHERICHIA COLI*

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CERTAIN BACTERIAL STRAINS ARE CAPABLE OF ELIMINATING ULTRAVIOLET LIGHT-INDUCED THYMINE DIMERS FROM DNA, A PROCESS WHICH MAY HAVE GREAT SIGNIFICANCE IN THE RECOVERY OF CELLS FROM THE EFFECTS OF THE RADIATION (SETLOW AND CARRIER 1964, BOYCE AND HOWARD-FLANDERS 1964). IN VIEW OF THE PRONOUNCED SIMILARITIES OF THE BIOLOGICAL ACTIONS OF ULTRAVIOLET LIGHT AND RADIOMIMETIC ALKYLATING AGENTS (E.G. INHIBITION OF CELLULAR DNA SYNTHESIS, INITIATION OF POST-TREATMENT DEGRADATION OF DNA, FILAMENT FORMATION, CROSS-RESISTANCE OR CROSS-SENSITIVITY OF SOME MUTANT STRAINS, ETC.) IT SEEMS REASONABLE TO SUPPOSE THAT OTHER TYPES OF DNA DAMAGE MIGHT ALSO BE ELIMINATED. IF TRUE, THE EXCISION PHENOMENON MIGHT BE CONSIDERED AS PART OF A GENERAL MECHANISM FOR REPAIRING GENETIC DEFECTS.

IN THE PRESENT COMMUNICATION WE PRESENT EVIDENCE THAT: (1) SULFUR MUSTARD-TREATED E. COLI $15T^{-}A^{-}U^{-}$ DEPOLYMERIZE A SIGNIFICANT PORTION OF THEIR DNA DURING POST-TREATMENT METABOLISM; (2) THE RATE OF LOSS OF SULFUR MUSTARD PRODUCTS INITIALLY BOUND TO DNA IS MORE RAPID THAN THE RATE OF OVERALL DNA DEGRADATION; (3) SEVERAL SULFUR MUSTARD-DNA ALKYLATION PRODUCTS (INCLUDING MONOFUNCTIONALLY AND BIFUNCTIONALLY ALKYLATED GUANINE) ARE ELIMINATED TO EQUAL EXTENTS; AND (4) SULFUR MUSTARD-TREATED BACTERIA AFTER A LAG RECOVER THE CAPACITY TO SYNTHESIZE DNA, SUGGESTIVE OF A CAUSATIVE RELATIONSHIP BETWEEN THE EXCISION OF ALKYLATION PRODUCTS FROM DNA AND RESUMPTION OF DNA REPLICATION.

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MATERIAL AND METHODS

The bacterial strain used: Escherichia coli 15T⁻A⁻U⁻ (thymine, arginine and uracil requiring) was a gift from Professor S. S. Cohen, University of Pennsylvania. Bacteria were grown in the glucose-ammonia-salts medium of Hershey (1960) which was supplemented as required with 3μg thymidine, 10μg uracil and 20μg L-arginine per ml. [^{3}H]- thymidine (Schwartz Bio Research, Inc.) was added to the medium (5μc/ml) for studies of DNA synthesis and DNA breakdown. The generation time in a fully supplemented medium was 60 minutes. Viability was determined from colony counts following overnight incubation on nutrient agar plates. Medium shifts were rapidly achieved by filtration on millipore filters and resuspension of bacteria in the appropriate, prewarmed medium. The composition of media with respect to required nutrients is designated by the convention established by Maaløe and Hanawalt (1961). For example, if *E. coli* 15T⁻A⁻U⁻ bacteria were cultured in a medium containing thymidine, but lacking arginine and uracil, the designation is (+T,-AU).

Sulfur mustard (bisβchloroethyl sulfide) was obtained from Edgewood Arsenal, Md., and [^{35}S]-sulfur mustard (30 mc/mmole) was purchased from Nuclear Chicago Corporation. Mustard treatments were performed by suspending bacteria in 5×10^{-3}M phosphate buffer - 0.15M NaCl, pH 7.65 and adding an appropriate quantity of a freshly prepared solution (in the same buffer) of sulfur mustard. Following exposure (1 hour at room temperature) cells were millipore-filtered, washed with unsupplemented medium and assayed for viable cells or, in other experiments, cultured in the desired post-treatment medium. For determination of sulfur mustard bound to DNA, bacteria previously labeled in their DNA with [^{3}H]-thymidine were exposed to 2×10^{-4}M [^{35}S] sulfur mustard, millipore-filtered to eliminate radioactive hydrolysis products, resuspended in a (-T,-AU) medium and timed aliquots were analyzed for TCA-insoluble, DNAase-digestible (10μg/ml) radioactivity. Control experiments showed that sulfur mustard-DNA reaction products (^{35}S radioactivity) remained bound to DNA during precipitation

WITH COLD TCA AND WERE QUANTITATIVELY CONVERTED TO ACID-SOLUBLE PRODUCTS BY DNAASE, WHICH DID NOT, HOWEVER, RELEASE RADIOACTIVITY FROM ALKYLATED RNA OR PROTEIN. PAPER CHROMATOGRAPHIC ANALYSIS OF ALKYLATED DNA PRODUCTS WAS PERFORMED AFTER ACID HYDROLYSIS OF THE DNA ASE DIGEST ACCORDING TO THE METHOD OF BROOKES AND LAWLEY (1961), RADIOACTIVE ZONES BEING IDENTIFIED WITH THE AID OF A THIN WINDOW TRACERLAB STRIP COUNTER. OTHER RADIOACTIVITY MEASUREMENTS WERE MADE IN A PACKARD SCINTILLATION SPECTROMETER.

RESULTS AND DISCUSSION

EFFECTS OF SULFUR MUSTARD ON VIABILITY.

FIGURE 1 ILLUSTRATES THE HIGH TOXICITY OF SULFUR MUSTARD FOR EXPONENTIALLY GROWING E. COLI 15T⁻A⁻U⁻. HOWEVER, THE SURVIVAL OF MUSTARD-TREATED CULTURES DEPENDED TO A LARGE DEGREE ON POST-TREATMENT GROWTH

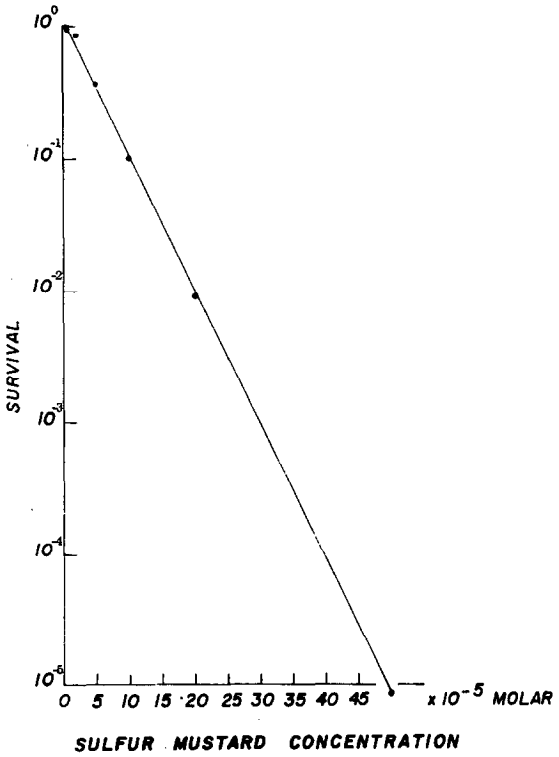

FIG 1. LOSS OF VIABILITY DUE TO EXPOSURE OF EXPONENTIALLY GROWING E. COLI 15T⁻A⁻U⁻ TO SULFUR MUSTARD.

CONDITIONS (FIG 2). THUS IN A FULLY SUPPLEMENTED MEDIUM (+T,+AU) SURVIVORS DID NOT IMMEDIATELY RESUME MULTIPLICATION AT THE RATE NOTED FOR UNTREATED CONTROLS, SUGGESTING THE PRESENCE OF REVERSIBLE DAMAGE SINCE THESE BACTERIA EVENTUALLY DID RECOVER AND PRODUCED NORMALLY APPEARING COLONIES ON PLATES FOLLOWING OVERNIGHT INCUBATION. CONTRARIWISE, DAMAGED SURVIVORS NOT ONLY FAILED TO RECOVER IN NUTRITIONALLY DEFICIENT MEDIA, BUT INDEED UNDERWENT EXTENSIVE LOSSES OF VIABILITY (LATENT KILLING). THE MOST EXTENSIVE LATENT KILLING TOOK PLACE IN A (-T,-AU) MEDIUM WHICH COMPLETELY BLOCKED ANY RESIDUAL DNA SYNTHESIS AND EVEN CAUSED UNTREATED BACTERIA TO SUCCUMB

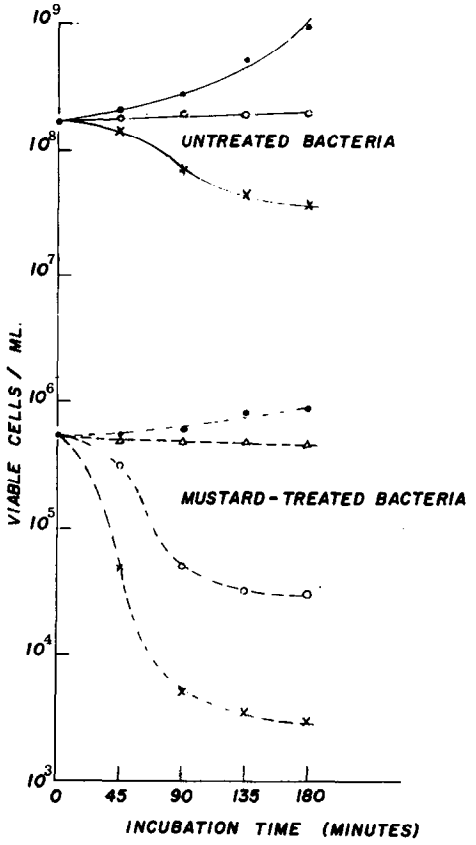

FIG 2. EFFECTS OF NUTRITIONALLY SELECTED POST-TREATMENT GROWTH CONDITIONS ON SURVIVAL OF SULFUR MUSTARD-TREATED *E. COLI* $15T^-A^-U^-$.

● (+T,+AU); ○ (+T,-AU); ✕ (-T,-AU); △ (MINUS GLUCOSE).

SULFUR MUSTARD CONCENTRATION WAS 1.5×10^{-4} M.

DUE TO THYMINELESS DEATH. LATENT KILLING WAS ALSO OBSERVED IN A (+T,-AU) POST-TREATMENT MEDIUM IN WHICH UNTREATED BACTERIA REMAINED VIABLE, WERE ABLE TO COMPLETE THEIR ALREADY INITIATED DNA SYNTHESIS, BUT FAILED TO INITIATE A NEW ROUND OF DNA REPLICATION (MAALØE AND HANAWALT 1961). THESE RESULTS SUGGEST THAT RESUMPTION OF DNA SYNTHESIS IS A NECESSARY, BUT NOT SUFFICIENT, REQUIREMENT FOR THE RECOVERY OF MUSTARD-DAMAGED BACTERIA.

THE RECOVERY AND LATENT KILLING EFFECTS OBSERVED WITH MUSTARD-DAMAGED BACTERIA PROMPTED US TO LOOK NEXT FOR CONCOMITANT CHANGES OF CELLULAR DNA SYNTHESIS AND DNA STABILITY SINCE DNA IS PRESUMED TO BE THE SENSITIVE TARGET. WE VISUALIZED THAT RECOVERY RESULTS FROM REMOVAL OR CIRCUMVENTION OF INTERFERING ALKYLATION PRODUCTS IN DNA, ANALOGOUS TO THE RESTORATION OF DNA SYNTHESIS FOLLOWING EXCISION OF ULTRAVIOLET LIGHT-INDUCED THYMINE DIMERS (SETLOW ET AL 1963). ON THE OTHER HAND, LATENT KILLING MAY BE THE CONSEQUENCE OF DEFECTIVE OR INEFFICIENT REPLACEMENT DNA SYNTHESIS, WHICH WOULD TEND TO AMPLIFY THE MUSTARD-INDUCED DNA DAMAGE.

RELATIONSHIP BETWEEN SYNTHESIS AND BREAKDOWN OF DNA IN MUSTARD-TREATED BACTERIA

SULFUR MUSTARD STRONGLY INHIBITED BACTERIAL DNA SYNTHESIS (FIG 3). HOWEVER, WHEN POST-TREATMENT INCUBATION WAS CARRIED OUT IN A FULLY SUPPLEMENTED MEDIUM (+T,+AU), DNA SYNTHESIS RECOVERED AFTER A LAG OF APPROXIMATELY 45 MINUTES. SUCH RECOVERY OF DNA SYNTHESIS IS CONSISTENT WITH THE PREVIOUS FINDING (FIG 2) THAT IN A COMPLETE MEDIUM MUSTARD-DAMAGED SURVIVORS RETAINED THEIR CAPACITY FOR REPRODUCTION. ON THE OTHER HAND, IN A NUTRITIONALLY DEFICIENT MEDIUM (+T,-AU), IN WHICH EXTENSIVE LATENT KILLING WAS NOTED, ONLY A LIMITED AMOUNT OF DNA SYNTHESIS TOOK PLACE. DNA SYNTHESIS ALSO FAILED TO RECOVER IN THE PRESENCE OF CHLORAMPHENICOL. THESE RESULTS ARE COMPATIBLE WITH THE VIEW THAT MUSTARD-TREATED BACTERIA FAIL TO COMPLETE THE ALREADY-INITIATED DNA SYNTHESIS (PRESUMABLY BY VIRTUE OF INTERFERING INTERSTRAND CROSSLINKS) AND THAT CULTURAL CONDITIONS WHICH FAVOR THE INITIATION OF A NEW ROUND OF DNA REPLICATION ARE REQUIRED

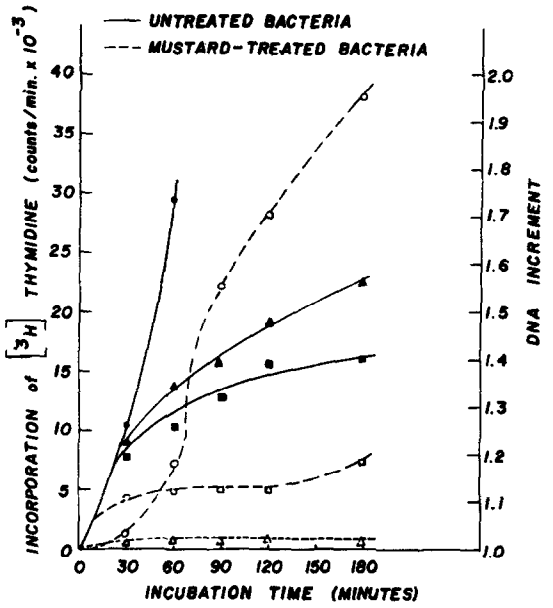

FIG 3. INHIBITION AND RECOVERY OF DNA SYNTHESIS BY SULFUR MUSTARD-TREATED E. COLI 15T⁻A⁻U⁻. ●,○ (+T,+AU); ▲,△ (+T,+AU,+CM); ■,□ (+T,-AU). SULFUR MUSTARD CONCENTRATION WAS 2×10^{-4} M. CHLORAMPHENICOL (CM) WAS 25 μG/ML.

FOR THE RECOVERY OF DNA SYNTHESIS (REPLACEMENT SYNTHESIS FOLLOWING EXCISION OF LETHAL PRODUCTS?).

SULFUR MUSTARD INITIATED THE POST-TREATMENT DEPOLYMERIZATION OF BACTERIAL DNA (FIG 4). THE EXTENT OF DNA BREAKDOWN WAS LARGELY DEPENDENT ON THE PRESENCE OF GLUCOSE IN THE MEDIUM AND VARIED SIGNIFICANTLY WITH CULTURAL (NUTRITIONAL) CONDITIONS. RELATIVELY SMALL AMOUNTS OF DNA WERE DEGRADED IN A COMPLETE (+T,+AU) MEDIUM, IN WHICH THE LARGEST RECOVERY OF DNA SYNTHESIS TOOK PLACE, WHEREAS THE MOST EXTENSIVE BREAKDOWN WAS OBSERVED IN THE PRESENCE OF CHLORAMPHENICOL, WHICH PREVENTED RECOVERY OF DNA SYNTHESIS. WE SUGGEST THAT DNA DEGRADATION AND DNA SYNTHESIS ARE COMPETING REACTIONS, WITH THE FORMER PROCESS BEING FAVORED WHEN THE LATTER IS INHIBITED. AN INTERESTING OBSERVATION WAS THAT DNA DEGRADATION BEGAN IMMEDIATELY IN THE POST-TREATMENT MEDIUM AND THUS PRECEDED THE RECOVERY OF DNA SYNTHESIS,

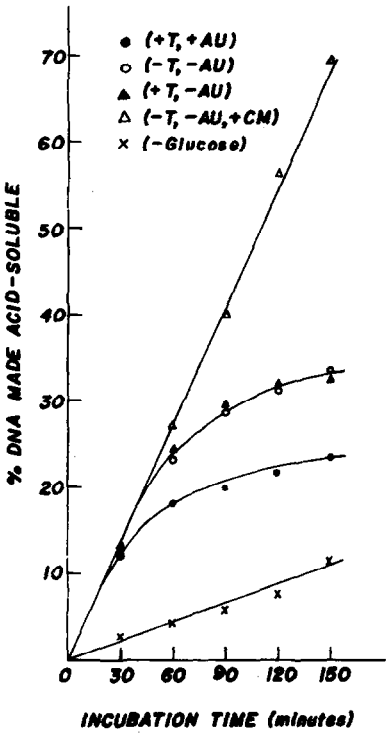

FIG 4. INFLUENCE OF POST-TREATMENT GROWTH CONDITIONS ON DEPOLYMERIZATION OF DNA BY SULFUR MUSTARD-TREATED E. COLI 15T⁻A⁻U⁻. SULFUR MUSTARD CONCENTRATION WAS 2×10^{-4} M. CHLORAMPHENICOL (CM) WAS 25 μG/ML.

SUGGESTING THAT RECOVERY CAN OCCUR ONLY AFTER INTERFERING SUBSTANCES HAVE BEEN ELIMINATED OR CIRCUMVENTED.

ELIMINATION OF SULFUR MUSTARD-INDUCED ALKYLATION PRODUCTS FROM DNA.

WE NEXT INQUIRED IF MUSTARD-INDUCED ALKYLATION PRODUCTS ARE ELIMINATED FROM DNA DURING THE POST-TREATMENT DEPOLYMERIZATION PHASE, AND IF THEY ARE ELIMINATED, AT WHAT RATE RELATIVE TO THAT OF DNA DEGRADATION? A DOUBLE LABEL EXPERIMENT PROVIDED ANSWERS TO BOTH QUESTIONS. CELLS, PREVIOUSLY LABELED IN THEIR DNA WITH [^{3}H]-THYMIDINE, WERE EXPOSED TO 2×10^{-4} M [^{35}S]-SULFUR MUSTARD AND THE SPECIFIC ACTIVITY OF ALKYLATION PRODUCTS REMAINING IN DNA ($\frac{^{35}S}{^{3}H}$) AFTER VARIOUS TIMES IN A (-T,-AU) MEDIUM WAS DETERMINED (SEE METHODS). AS WILL BE SEEN IN FIG 5, ^{35}S - CONTAINING ALKYLATION PRODUCTS ARE ELIMINATED FROM DNA AS A FUNCTION OF TIME IN THE POST-TREATMENT INCUBATION MEDIUM, THE RATE AND EXTENT OF ELIMINATION EXCEEDING THE RATE

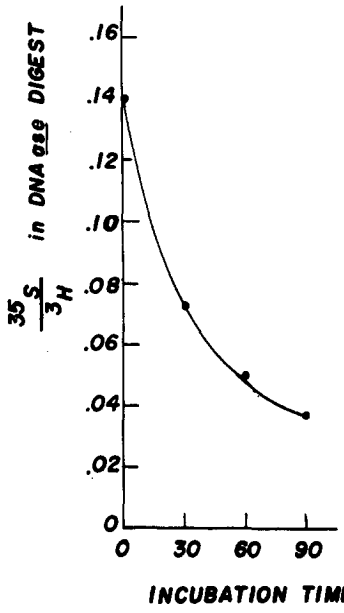

FIG 5. FRACTION OF [^{35}S] RADIOACTIVITY REMAINING IN THE TCA-INSOLUBLE, DNAASE - SENSITIVE FRACTION AT VARIOUS TIMES AFTER EXPOSURE OF E. COLI 15T⁻A⁻U⁻ TO [^{35}S]-SULFUR MUSTARD. SULFUR MUSTARD CONCENTRATION WAS 2×10^{-4}M.

AND EXTENT OF OVERALL DNA DEGRADATION. THE PREFERENTIAL ELIMINATION OF ALKYLATED PRODUCTS SUGGESTS THAT DNA DEGRADATION WAS INITIATED AT OR IN THE VICINITY OF ALKYLATED SITES. ALTERNATIVELY, DNA DEGRADATION AND ELIMINATION OF ALKYLATION PRODUCTS ARE TWO INDEPENDENT PROCESSES PROCEEDING AT CHARACTERISTICALLY DIFFERENT RATES. HOWEVER, THE FORMER INTERPRETATION IS MORE CONSISTENT WITH OUR FINDING (PAPIRMEISTER ET AL 1964) THAT E. COLI BACTERIA POSESS AN EXONUCLEASE WHICH PREFERENTIALLY DEGRADES SULFUR MUSTARD-TREATED DNA. THE RATE AT WHICH ALKYLATION PRODUCTS ARE ELIMINATED FROM DNA, AS WAS ALSO TRUE OF GENERAL DNA BREAKDOWN, WAS ENHANCED WHEN GLUCOSE WAS INCLUDED IN THE MEDIUM (UNPUBLISHED DATA).

FIG. 6 SHOWS THAT POST-TREATMENT INCUBATION FOR 120 MINUTES RESULTED IN ESSENTIALLY COMPLETE ELIMINATION OF AT LEAST 3 DIFFERENT ALKYLATION PRODUCTS FROM DNA: (1) BIFUNCTIONALLY ALKYLATED GUANINE DIMERS (PEAK I), (2) MONOFUNCTIONALLY ALKYLATED GUANINE (PEAK II) AND (3) AN UNIDENTIFIED

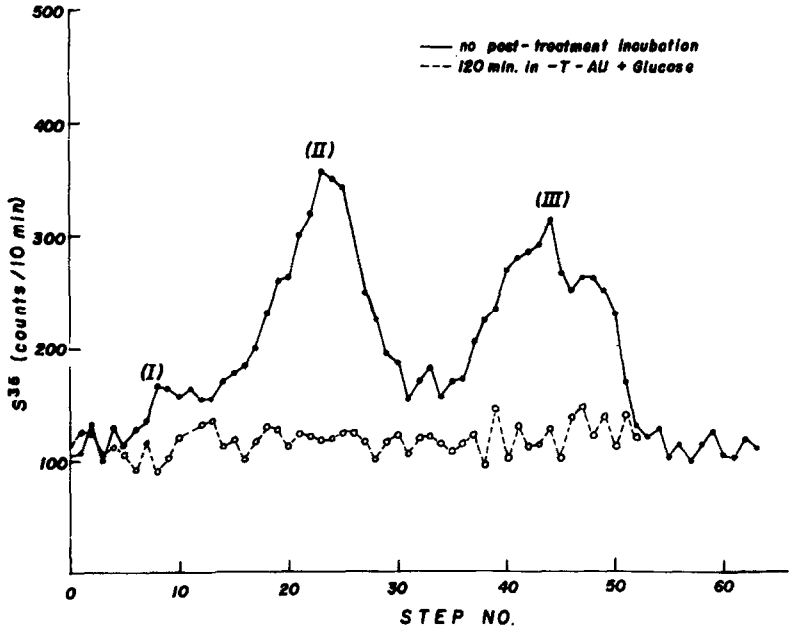

FIG 6. PAPER CHROMATOGRAPHIC ANALYSIS OF ^{35}S - CONTAINING COMPOUNDS OBTAINED BY ACID HYDROLYSIS OF THE TCA-INSOLUBLE, DNAASE - SENSITIVE FRACTION BEFORE AND AFTER 120 MINUTES INCUBATION OF SULFUR MUSTARD-TREATED E. COLI 15T⁻A⁻U⁻.

PRODUCT(S) (PEAK III) WHICH HAS AN RF VALUE SIMILAR TO THAT OBTAINED WITH PREHYDROLYZED SULFUR MUSTARD (I.E. THIODIGLYCOL) AND THUS PROBABLY REPRESENTS PRODUCTS OBTAINED FROM THE ACID HYDROLYSIS OF SULFUR MUSTARD WHICH HAD BEEN ESTERIFIED TO THE PHOSPHATE BACKBONE OF DNA. THE SIMULTANEOUS ELIMINATION FROM DNA OF SUCH CHEMICALLY DIFFERENT MOIETIES IMPLIES THAT THE EXCISION OF DNA DAMAGES IS DUE TO A RELATIVELY NON-SPECIFIC NUCLEOLYTIC PROCESS WHICH MAY MERELY REQUIRE AN INITIATION POINT ON THE DNA MOLECULE (E.G. A FREE 3'OH END). SUCH INITIATION POINTS MIGHT BE PRODUCED DIRECTLY THROUGH THE ACTION OF A VARIETY A PHYSICAL OR CHEMICAL AGENTS OR, INDIRECTLY, BY NUCLEASES WHICH MAY BECOME ACTIVATED DURING UNBALANCED GROWTH CONDITIONS (I.E., WHEN DNA SYNTHESIS IS INHIBITED).

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1. BOYCE, R.P., AND HOWARD-FLANDERS, P., PROC. NATL. ACAD. SCI. U.S., 51, 293 (1964).

2. BROOKES, P., AND LAWLEY, P.D., BIOCHEM. J., 80, 496 (1961).

3. HERSHEY, A.D., VIROLOGY 1, 108 (1955).

4. MAALOE, O., AND HANAWALT, P.C., J. MOL. BIOL., 3, 144 (1961).

5. PAPIRMEISTER, B., DAVISON, C.L., AND MCMICHAEL, P., IN PREPARATION.

6. SETLOW, R.B., AND CARRIER, W.L., PROC. NATL. ACAD. SCI. U.S., 51, 226 (1964).

7. SETLOW, R.B., SWENSON, P.A., AND CARRIER, W.L., SCIENCE, 142, 1464 (963).