Proc. Nat. Acad. Sci. USA
Vol. 71, No. 7, pp. 2651–2654, July 1974

Role of ATP in Excision Repair of Ultraviolet Radiation Damage in *Escherichia coli*

(permeable cells/incision step of DNA repair/ATP dependent endonuclease)

E. A. WALDSTEIN*, R. SHARON, AND R. BEN-ISHAI†

Department of Biology, Israel Institute of Technology, Haifa, Israel

Communicated by R. B. Setlow, April 18, 1974

ABSTRACT **The effect of ATP on the first step of excision repair of ultraviolet damage in DNA has been studied using toluene-treated *E. coli*. During postirradiation incubation, five to six times more single-strand breaks are formed in DNA in the presence of exogenous ATP than in its absence. The ATP-dependent as well as the ATP-independent endonucleolytic activities appear to be catalyzed by the same enzyme since both activities are almost completely absent in *uvrA* and *uvrB* mutants. An ATP-dependent endonucleolytic activity has been detected in nonirradiated toluene-treated *E. coli*. It is concluded that ATP is required *in vivo* for either the incision step of repair or an enzymatic reaction preceding it.**

Excision repair of UV (ultraviolet) damage in DNA is generally believed to proceed by means of an enzyme complex in which recognition of damage and chain incision, excision of damaged regions, repair replication, and rejoining of gaps occur together or in rapid succession (1, 2).

Recently cells made permeable to deoxynucleoside triphosphates and other low-molecular-weight substances by treatment with toluene (3) have been used to study this repair process (4, 5). These studies have shown that in DNA of irradiated toluene-treated *Escherichia coli*, dose-dependent strand incisions, rejoining of strand breaks, and repair replication can be observed. Several lines of evidence indicate that ATP may play a role in excision repair. Thus, Masker and Hanawalt (4) demonstrated that UV-stimulated repair synthesis is ATP dependent in toluenized *polA* mutants of *E. coli*. Hadden *et al.* (6), using *Bacillus subtilis* made permeable to dNTP (deoxynucleoside triphosphates) by freezing obtained ATP dependent repair synthesis after UV irradiation in a *pol*$^+$ strain.

During our studies on repair of UV damage in toluene-treated cells, it was observed that ATP stimulates an early step in excision repair in *pol*$^+$ as well as in *polA* mutants of *E. coli*. In this investigation an attempt was made to identify the step in repair that involves participation of ATP. Our results show that ATP is required for performance of the first step in excision repair.

METHODS AND MATERIALS

E. coli 1100 (*endoI*$^-$) and H514 (*uvrA*$^-$ *endoI*$^-$ *arg*$^-$ *thy*$^-$) were obtained from Dr. Hoffmann-Berling. *E. coli* KMBL 1790 (*endoI*$^-$ *thy*$^-$ *arg*$^-$ *biotin*$^-$ *pheA*$^-$), KMBL 1054 (*uvrB*$^-$ *endoI*$^-$ *thy*$^-$), KMBL 1481 (*recC*$^-$ *endoI*$^-$ *thy*$^-$ *meth*$^-$ *biotin*$^-$ *pheA*$^-$), KMBL 1479 (*recB*$^-$ *endoI*$^-$ *thy*$^-$ *meth*$^-$ *biotin*$^-$ *pheA*$^-$), KMBL 1482 and KMBL 1480 the recombination proficient isogenic partners of KMBL 1481 and KMBL 1479, respectively, were provided by Drs. A. Rörsch and I. Mattern.

Cultures were grown in M-9 medium, supplemented with 0.1% glucose, casamino acids (2.5 mg/ml), and thymidine (5 μg/ml). Amino acids and biotin, when required, were added at 50 μg/ml and 1 μg/ml, respectively. To prelabel the bacterial chromosome, [^{3}H]thymidine (5 μCi/ml, 5Ci/mmole) was added to the medium for three generations.

Exponentially growing cells at a density of 0.5 to 1 $\times$ 10^9 cells per ml were harvested, resuspended at 5 $\times$ 10^9 cells per ml in 0.05 M potassium phosphate buffer (pH 7.4) and treated with toluene as described by Moses and Richardson (3). For each strain, duration of treatment with toluene which resulted in maximum stimulation of ATP-dependent DNA synthesis was employed. After toluenization, 2 $\times$ 10^9 cells per ml in 0.05 M potassium phosphate buffer (pH 7.4) were exposed to UV light (Mineralight lamp model no. R-51, maximum emission at 254 nm) at 15 ergs mm^{-2} sec^{-1} at room temperature.

Incubation mixtures (0.3 ml) contained 70 mM potassium phosphate buffer (pH 7.4), 13 mM Mg^{++}, 5 mM NMN (nicotinamide mononucleotide), 2 $\times$ 10^8 irradiated or nonirradiated toluenized cells and ATP as indicated in the text. After incubation for 30 min at 37° in the dark, the reaction was terminated in the cold by addition of 1 ml of 6 mM EDTA (ethylenediaminetetraacetate)-0.05 M potassium phosphate buffer (pH 7.4). Lysis of cells and sedimentation analysis in alkaline sucrose gradients were performed as described by Rupp and Howard-Flanders (7) with the exception that gradients were spun at 30,000 rpm for 120 min or 8,500 rpm for 18 hr at 4°. The number average molecular weight (M_n) was calculated by computer from the distribution of radioactivity in each gradient, omitting only the top and bottom fractions which were clearly separated from the main radioactive peak. Phage T4 DNA (single-strand molecular weight of 55 $\times$ 10^6) was used as marker. It was assumed that the empirical equation of Studier (8) which relates molecular weight to sedimentation constant holds for these gradients and that the molecular weight is proportional to $(s^0_{20,w})^{2.5}$.

Degradation of DNA was determined after 0, 15, and 30 min of postirradiation incubation. The reaction was terminated by addition of ice cold 10% trichloroacetic acid–0.1 M PP_i and the trichloroacetic acid insoluble precipitate was filtered onto Whatman GF/C glass filters, washed three times with 3 ml of cold trichloroacetic acid-PP_i, twice with 3 ml of

Abbreviations: UV, ultraviolet, NMN, nicotinamide mononucleotide; dNTP, deoxynucleoside triphosphates; EDTA, ethylenediaminetetraacetate; rNTP, ribonucleoside triphosphates. All centrifugations were done in a Beckman L2 centrifuge with Rotor No. SW50.1.

* Present address: Department of Botany, Tel-Aviv University, Tel-Aviv, Israel.

† Author to whom reprint requests should be addressed.

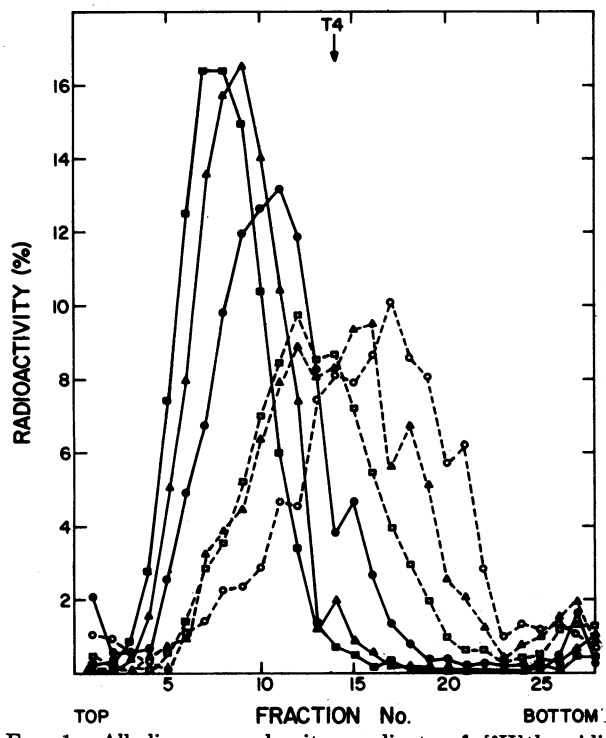

FIG. 1. Alkali sucrose density gradients of [^{3}H]thymidine labeled DNA. Toluene-treated *E. coli* KMBL 1482 were irradiated and incubated for 30 min with NMN (5 mM) in the presence (*solid line*) and in the absence (*broken line*) of ATP (0.66 mM). O,Δ, and □ denote UV doses of 300, 600 and 900 ergs mm^{-2}, respectively. Conditions of centrifugation were 30,000 rpm for 120 min at 4°.

cold 0.01 M HCl and once with 3 ml of alcohol. The filters were dried and counted in a toluene-based scintillation fluid.

RESULTS

To test for the first step of excision repair independently of subsequent repair processes, toluene-treated *E. coli* (*endoI*$^-$ strains) were irradiated and incubated in the absence of deoxynucleoside triphosphates and ATP and in the presence of NMN which inhibits rejoining of breaks (9). From the sedimentation pattern of DNA in alkaline sucrose gradients a dose-dependent decrease in the molecular weight of single-stranded DNA is evident (Fig. 1). Since no similar decrease in the sedimentation of DNA is obtained by irradiation alone, the decrease in molecular weight during postirradiation incubation is interpreted to be a result of the formation of single-strand breaks in irradiated DNA (step 1 of the repair process). Also tested in the experiments described in Fig. 1 is the effect of ATP on this incision step. It is seen that significantly more dose-dependent single-strand breaks are produced during postirradiation incubation in the presence of exogenous ATP than in its absence.

Fig. 2 shows results from a representative experiment performed to test if, in the presence of ATP, single-strand breaks are introduced into DNA of nonirradiated cells. Toluene treated *E. coli* were incubated in the presence of ATP and in its absence (control), and analysis in alkaline sucrose gradients was performed. Under the usual conditions of high-speed centrifugation, control DNA showed an abnormal sedimentation distribution in as much as a large percentage of DNA was present in a high-molecular-weight tail. To overcome

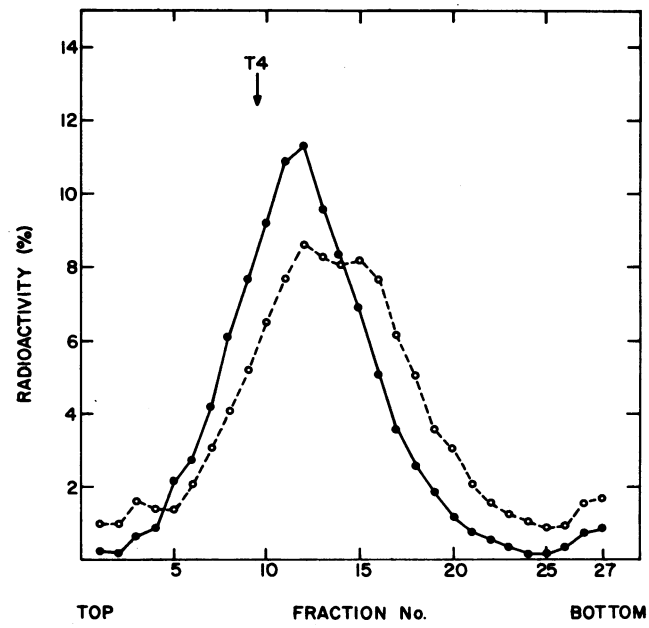

FIG. 2. Sedimentation profile of nonirradiated DNA. After toluene treatment *E. coli* KMBL 1482 were incubated for 30 min with NMN (5 mM) in the presence (*solid line*) and in the absence (*broken line*) of ATP (0.66 mM). Conditions of centrifugation were 8500 rpm for 18 hr at 4°.

possible anomalous effects of high-centrifugation force on control DNA, sedimentation at low rotor speeds was employed (10, 11). Under these conditions, a random molecular weight distribution was obtained (Fig. 2) and the number average molecular weight (M_n) of control DNA was determined to be in the range of 0.8 to 1.2 × 10^8. From the differences between $1/M_n$ values of DNA after incubation in the presence of ATP and of DNA from controls, we estimated that in nonirradiated cells ATP induces formation of 0.4–1.6 strand breaks per 10^8 daltons.

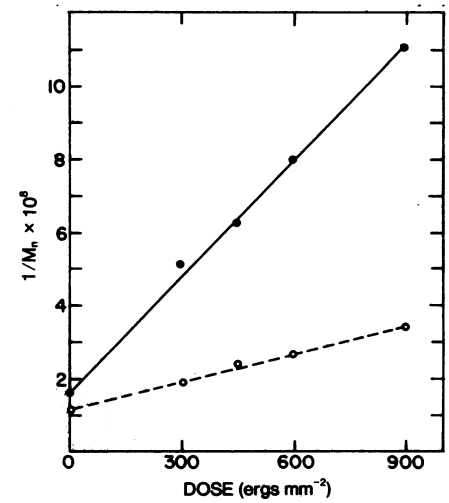

FIG. 3. Reciprocal of the number average molecular weight (M_n) plotted against UV dose. Toluene treated *E. coli* KMBL 1482, nonirradiated and after exposure to various doses of UV light were incubated for 30 min with NMN (5 mM) in the presence (*solid line*) and in the absence (*broken line*) of ATP (0.66 mM) and sedimented in alkaline sucrose. Conditions of centrifugation were: nonirradiated DNA 8500 rpm for 18 hr at 4°, irradiated DNA 30,000 rpm for 120 min at 4°.

In irradiated DNA, the ATP-dependent breaks which were observed (Fig. 1) are formed in addition to those induced in nonirradiated DNA. It is seen in Fig. 3 that a linear relationship exists between the postirradiation induction of strand breaks (expressed in terms of $1/M_n$) and radiation dose. From the slopes and the intercepts with the ordinate, a fluence of 100 ergs mm^{-2} was calculated to produce 6 and 27 breaks per chromosome (2.5×10^9 daltons) in the absence and in the presence of exogenous ATP (0.66 mM), respectively. Similar results were also obtained using various other *E. coli* uvr^+ *endoI*$^-$ strains (Table 1). During postirradiation incubation in the presence or in the absence of ATP, DNA degradation to acid soluble products did not exceed 5% in any of the *E. coli* strains.

The effect of ATP concentration on endonucleolytic cleavage of DNA in irradiated and nonirradiated cells is shown in Fig. 4. Maximum stimulation of strand breakage is observed at ATP concentrations which are apparently lower for nonirradiated DNA than for irradiated DNA. At similar concentrations, none of the other ribo- or deoxynucleoside triphosphates tested (GTP, UTP, dATP) could replace ATP effectively in either reaction, (shown for GTP in Fig. 4).

Further evidence for the role of ATP in the first step of repair was obtained using *E. coli* strains having mutations in the *uvrA* or *uvrB* genes, which are involved in control of the incision step (12). As shown in Table 1 the appearance of

TABLE 1. *Effect of ATP on strand incisions in DNA of irradiated E. coli*

Strains	Breaks per chromosome per 100 ergs mm^{-2}* ATP (mM)		
	0	0.66	1.35
W1100	5	20	
	7	27	
KMBL 1790	5	17	31
	7	16	29
KMBL 1482†	7	30	
	6	18	32
	6	27	
	6	23	36
KMBL 1481 (*recC*)	7		34
	6		26
	6	23	
KMBL 1480†	6	40	
KMBL 1479 (*recB*)	6	33	
KMBL 1054 (*uvrB*)‡	2	4	
	2	5	
H514 (*uvrA*)‡	2	4	
	2	4	4

Toluene-treated cells were irradiated with doses of 0, 300, 600, and 900 ergs mm^{-2} and incubated in the presence of NMN with or without ATP. Nonirradiated and irradiated DNA were sedimented in parallel at 8500 rpm for 18 hr and 30,000 rpm for 2 hr, respectively at 4°. In each experiment samples (0.01 ml, 4×10^6 cells) containing 3000–4000 acid insoluble cpm were applied to the gradient. In nonirradiated cells of all the above strains, the absolute number of ATP-dependent breaks per chromosome ranged from 10 to 40.

* Number of breaks calculated from slopes of the plot $1/M_n$ versus dose (see Fig. 3).

† Isogenic partner of strain listed below.

‡ Number of breaks after a dose of 150 ergs mm^{-2}.

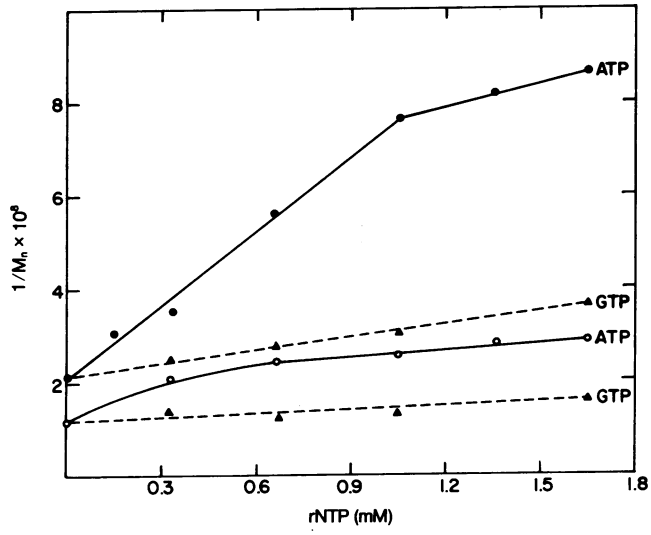

FIG. 4. Influence of rNTP concentration on strand incisions in DNA. Toluene treated *E. coli* KMBL 1482 nonirradiated (*open symbols*) or exposed to 450 ergs mm^{-2} (*solid symbols*) were incubated for 30 min in a reaction mixture containing NMN (5 mM) and increasing concentrations of ATP or GTP. Nonirradiated DNA was sedimented at 8500 rpm for 17 hr at 4° and irradiated DNA at 30,000 rpm for 120 min at 4°, respectively. *rNTP* is ribonucleoside triphosphate.

postirradiation breaks in the absence as well as in the presence of ATP is drastically reduced in these mutants. Furthermore, the small number of breaks observed seem to be induced after exposure to low UV doses only. At doses higher than 150 ergs mm^{-2} additional break formation in DNA is almost completely eliminated. The mutations in the *uvrA* or *uvrB*

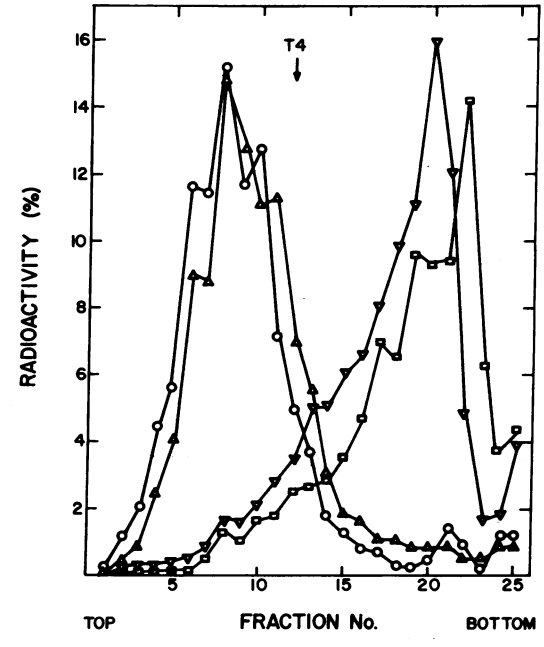

FIG. 5. Rejoining of ATP-dependent breaks in irradiated DNA. Washed toluene-treated *E. coli* KMBL 1482 were irradiated (450 ergs mm^{-2}) and incubated for 30 min in the presence of ATP (1.35 mM) with the following additions: ○, none; △, NAD (2 mM); ▽, NAD (2 mM) and 33 μM of each dATP, dGTP, dCTP, dTTP. □, sedimentation profile of DNA immediately after irradiation. Conditions of centrifugation were 30,000 rpm for 120 min at 4°.

genes do not affect the ATP-dependent endonucleolytic activity which is observed in nonirradiated cells.

Experiments similar to those described in Figs. 1 and 2 were performed with *recB* and *recC* mutants of *E. coli* in order to determine if the *recBC* DNase, which possesses an ATP stimulated endonucleolytic activity (13), participates in the induction of strand breaks in irradiated or nonirradiated DNA. Since ATP-dependent endonucleolytic cleavage in nonirradiated and irradiated DNA of these mutants is similar in extent to that observed in their isogenic partners (Table 1), it may be concluded that the *recBC* nuclease is not involved in these reactions.

In order to determine if some of the ATP-dependent breaks in irradiated DNA result from reverse ligase action and whether such breaks can be rejoined during postirradiation incubation, toluene-treated *E. coli* were washed with buffer prior to irradiation in order to remove residual amounts of endogenous NAD and dNTP and incubation was conducted in the absence of NMN. Fig. 5 shows that ATP-dependent breaks appear in the presence of a concentration of NAD that inhibits the reverse ligase reaction (24) and that although the breaks are not rejoined in the presence of NAD, the restoration of high-molecular-weight DNA does take place in the presence of the four dNTP as well as NAD.

DISCUSSION

The results reported here show that in toluene-treated UV-irradiated *E. coli*, five to six times more single-strand breaks are produced in DNA in the presence of exogenous ATP than in its absence. This stimulation by ATP is observed in all the uvr^+ strains as well as in *recB* and *recC* mutants.

Under our experimental conditions a fluence of 100 ergs mm^{-2} converted 0.036% of the radioactive thymine in DNA to thymine-containing dimers. Since this corresponds to 335 dimers per *E. coli* chromosome (14), it seems that in the presence of optimal concentrations of ATP, incision occurs at 10% of the estimated dimer sites.

The possibility that the observed ATP-dependent and ATP-independent UV-specific endonucleolytic activities are due to two different enzymes seems unlikely since both activities are drastically reduced in *uvrA* and *uvrB* mutants. These findings raise the question of whether low endogenous concentrations of ATP which remain in *E. coli* after toluene treatment may elicit the apparent ATP-independent UV-specific endonucleolytic reaction. Preliminary experiments indicate that this indeed may be the case since about 10% of the amount of ATP present in intact *E. coli* can be detected in toluene-treated cells. It has been observed that in intact cells starvation or metabolic inhibitors such as KCN inhibit incision (15). Our results support the suggestion (15) that this inhibition may be due to a depletion of intracellular ATP caused by the above conditions.

UV-specific endonucleases have been isolated from *M. luteus* (16–20), T4 (21, 22) and *E. coli* (A. Braun and L. Grossman, personal communication). *In vitro*, none of these endonucleases seem to require ATP for their action when isolated irradiated native DNA serves as substrate. *In vivo*, however, the substrate for the enzyme is the irradiated bacterial chromosome which exists in a highly-folded conformation (23). The difference in the tertiary structure of the DNA *in vivo* and *in vitro* may perhaps account for the difference in ATP requirements observed in these two systems. Experiments are presently in progress to test if UV-specific endonucleases exhibit ATP dependence when DNA in a form more similar to its "native" state is used as substrate. If *in vivo* ATP is not required for the action of the UV-specific endonuclease *per se* it may be needed for an enzymatic reaction preceding incision. A likely candidate for such a function is the ATP-dependent endonucleolytic activity which we detected in nonirradiated, toluene-treated cells. The latter is present in all the *E. coli* strains tested and it produces between 10 to 40 single-strand breaks per chromosome. It is possible that this activity accounts for the ATP requirement observed for DNA replication in *E. coli* made permeable (3) and that its role is to make the bacterial chromosome accessible to the replication as well as to the repair enzyme complexes. The ATP requirement shown here for the incision step in repair may be the reason for its requirement for repair replication in UV irradiated, toluenized cells (4, 6).

This work was started in cooperation with N. Segev who performed some of the preliminary experiments. We thank Drs. A. Rörsch, I. Mattern, and H. Hoffmann-Berling for their generosity in supplying bacterial strains and are indebted to Dr. H. Gershon for her help in the preparation of the manuscript.

1. Setlow, R. B. & Carrier, W. L. (1964) *Proc. Nat. Acad. Sci. USA* **51**, 226–231.
2. Boyce, R. P. & Howard-Flanders, P. (1964) *Proc. Nat. Acad. Sci. USA* **51**, 293–300.
3. Moses, R. E. & Richardson, C. C. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 674–681.
4. Masker, W. E. & Hanawalt, P. C. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 129–133.
5. Segev, M., Miller, C., Sharon, R. & Ben-Ishai, R. (1973) *Biochem. Biophys. Res. Commun.* **52**, 1241–1245.
6. Hadden, C. T., Billen, D. & Corrigan, K. (1973) *Biochim. Biophys. Acta* **324**, 461–471.
7. Rupp, W. D. & Howard-Flanders, P. (1968) *J. Mol. Biol.* **31**, 291–304.
8. Studier, F. W. (1965) *J. Mol. Biol.* **11**, 373–390.
9. Campbell, J. L. R., Fleischman, A. & Richardson, C. C. (1971) *Fed. Proc.* **30**, 1313.
10. Myers, D. K., Johnson, L. D. & Chetty, K. G. (1973) *Can. J. Biochem.* **51**, 397–406.
11. Kavenoff, R. (1972) *J. Mol. Biol.* **72**, 801–806.
12. Taketo, A., Yasuda, S. & Sekiguchi, M. (1972) *J. Mol. Biol.* **70**, 1–14.
13. Goldmark, P. J. & Linn, S. (1972) *J. Biol. Chem.* **247**, 1849–1860.
14. Boyle, J. M. & Setlow, R. B. (1970) *J. Mol. Biol.* **51**, 131–144.
15. Setlow, R. B. & Carrier, W. L. (1968) in *Replication and Recombination of Genetic Material*, eds. Peacock, W. J. & Brock, R. D. (Australian Academy of Science, Canberra).
16. Rörsch, A., Kamp, C. V. D. & Adema, J. (1964) *Biochim. Biophys. Acta* **80**, 346–348.
17. Carrier, W. L. & Setlow, R. B. (1970) *J. Bacteriol.* **102**, 178–186.
18. Kaplan, J. C., Kushner, S. R. & Grossman, L. (1971) *Biochemistry* **10**, 3315–3324.
19. Nakayama, H., Okubo, S. & Takagi, Y. (1971) *Biochim. Biophys. Acta* **228**, 67–82.
20. Waldstein, E. A. & Tomilin, N. V. (1971) *Proc. Acad. Sci. USSR* **200**, 1459–1462.
21. Yasuda, S. & Sekiguchi, M. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1839–1845.
22. Friedberg, E. C. & King, J. J. (1971) *J. Bacteriol.* **106**, 500–507.
23. Worcel, A. & Burgi, E. (1972) *J. Mol. Biol.* **71**, 127–147.
24. Modrich, P., Lehman, I. R. & Wang, J. C. (1972) *J. Biol. Chem.* **247**, 6370–6372.