Nucleic Acids Research

Inhibitors of repair DNA synthesis

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ABSTRACT

We have measured repair DNA synthesis in UV-irradiated normal human fibroblasts, grown to a defined state of quiescence in order to avoid the problem of discriminating repair from replicative DNA synthesis. We have assessed the effects of various DNA synthesis inhibitors on repair. Inhibition of repair synthesis by hydroxyurea, 1-β-D-arabinofuranosylcytosine and aphidicolin is associated with the ability to accumulate DNA breaks due to enzymic incision at DNA damage sites; the inhibition by novobiocin is in accord with its known ability to block incision.

INTRODUCTION

Repair DNA synthesis in UV-irradiated mammalian cells may be detected by autoradiography or scintillation counting following incubation of cells with radioactive DNA precursor. In the former procedure cells engaged in repair are discriminated visually from those also engaged in replicative DNA synthesis, and quantitation of repair synthesis is possible, though tedious and prone to variability [1]. For scintillation counting, there are various ways of distinguishing repair from replicative synthesis, involving physical separation (e.g. density labelling and CsCl gradient sedimentation, or chromatography on benzoylated naphthoylated DEAE cellulose), or the supposedly specific suppression of replicative DNA synthesis with inhibitors such as hydroxyurea (HU), or a combination of these approaches [2 - 4].

We have developed an alternative method for measuring repair DNA synthesis in normal human diploid cells by scintillation counting of incorporated radioactive precursor; it requires no physical separation or chemical suppression of replicating DNA. Cells must simply be held in a quiescent state, i.e. with minimal S phase contamination, and we describe a procedure for monitoring the attainment of this rigorously defined state.

The method has been used to assess the effects of the DNA synthesis inhibitors HU and 1-β-D-arabinofuranosylcytosine (ara C) on repair, and

we are able to confirm that the now well-established disruption of excision
repair by these drugs (reviewed in ref. 5) is reflected in a reduction of
repair synthesis. We also present data relating to aphidicolin and novo-
biocin, both only recently implicated in the inhibition of repair.

MATERIALS AND METHODS

Cell culture.   Normal human diploid fibroblasts, BCL-D1 (Gibco Europe) and
Flow 2000 (Flow Laboratories) were grown routinely in Eagle's minimal
essential medium supplemented with 10 - 15% foetal calf serum. For most
experiments, cells in this medium were distributed equally into 35 mm dishes
(Gibco Europe) at a 1 in 3 split ratio from confluent culture, and grown to
confluence with regular medium changes (with medium supplemented with 10%
foetal calf serum) as described in Results. Cells used were between
passage 12 and 22.

Inhibitors.   Stock solutions of HU (Sigma), ara C (Sigma) and novobiocin
(Boehringer Mannheim) were prepared in phosphate-buffered saline [6] at 10 x
to 100 x the final concentration. Aphidicolin (a gift from Dr. A.H. Todd of
I.C.I.) was dissolved in dimethylsulphoxide at 1 - 5 mg/ml and diluted with
phosphate-buffered saline to prepare stock solutions of 10 x to 100 x the
final concentration. Inhibitors were added to cells 30 min before UV
irradiation and/or incubation with [^{3}H]thymidine, and fresh inhibitors were
provided at the start of the incubation with [^{3}H]thymidine.

Assay for repair and replicative DNA synthesis.   After removing medium from
dishes, cells were irradiated (or mock-irradiated) with a germicical tube
emitting at 254 nm at a dose rate of 0.4 - 1 $Jm^{-2}s^{-1}$. Medium with 10%
serum and containing [Me^{3}H]thymidine (1 μCi/ml, 48 Ci/mmole, Radiochemical
Centre, Amersham) was added (together with inhibitors as appropriate) and
cells were incubated for 2 h at 37°C. They were then rinsed with cold saline
(0.9% NaCl) and lysed in 1 ml of 0.5M NaOH. Macromolecules were precipitated
with trichloroacetic acid and - after chilling for 30 min - filtered onto
GF/C glass fibre filters (Whatman) for scintillation counting of ^{3}H
incorporated into DNA.

Autoradiography.   Cells in 35 mm dishes, irradiated and incubated with
[^{3}H]thymidine as above, were rinsed with cold saline and fixed with methanol:
glacial acetic acid (3:1). After repeated extraction of acid-soluble
material with cold 5% (w/v) trichloroacetic acid, and a final rinse with
distilled water, the dishes were coated at 47°C with G5 emulsion (Ilford)
diluted 1:2 with 2% glycerol in water; emulsion was poured onto the dish

and immediately drained off, leaving a thin layer. Autoradiographs were developed after incubation for 1 week at 4°C, and stained with toluidine blue.

Assay for DNA breakage. Cells were grown on eight-chamber tissue culture slides (Lab-Tek Division, Miles Laboratories). 3×10^4 cells per chamber were inoculated in 0.3 ml of medium with 10% foetal calf serum and [^{3}H]thymidine (0.2 μCi/ml, 55 Ci/mmole). They were incubated for 2 days in order to label the DNA, and the medium was then changed. Cells were pre-incubated with inhibitor at different concentrations for 30 min, and then irradiated with a range of ultraviolet doses as described [7]. They were incubated with inhibitor for a further 20 min, rinsed with cold saline, and lysed in situ with 50 μl of alkaline sucrose solution (5% w/v sucrose, 0.3M NaOH, 0.5M NaCl) per chamber for 15 min at 4°C. The alkali was neutralised with 15 μl 2M acetic acid, and the DNA transferred to nitrocellulose filters (Schleicher & Schuell) for digestion with single-strand specific S1 nuclease (prepared from α amylase (Sigma) by the method of Sutton [8]) as described [7]. The % digestion of DNA (i.e. the % DNA unwound during alkaline treatment) relative to unirradiated cells reflects the frequency of DNA breaks.

RESULTS

In preliminary experiments with cell cultures which had reached confluence, the level of incorporation of [^{3}H]thymidine into DNA during a brief pulse varied widely, and the effect of UV irradiation on this incorporation was sometimes a reduction, sometimes a stimulation. Even in a confluent culture, it appears that a significant proportion of cells may be in S phase, so that repair synthesis tends to be masked by incorporation due to replication. Inhibition of predominant replicative synthesis accounts for the reduction of incorporation by UV irradiation; whereas in cultures with fewer cells in S phase, the stimulation of incorporation by UV represents repair DNA synthesis. It was clearly necessary to devise a protocol for reaching a defined state of culture - quiescence - in which so few cells are in S phase that repair DNA synthesis can be detected and measured reliably. Fig. 1 depicts an experiment in which [^{3}H]thymidine incorporation was measured in UV-irradiated or unirradiated samples of two normal cell lines at different times during several weeks of culture. Cultures appeared (by microscopic examination) to be confluent and non-dividing at the time of the first sample; but incorporation of thymidine was substantial, and was reduced by UV irradiation in one case and only
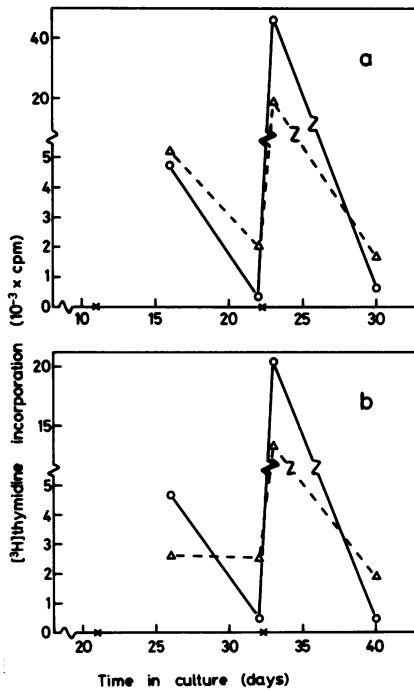
Fig. 1.   [^{3}H]thymidine incorporation by cells approaching quiescence. (a) BCL-D1; (b) Flow 2000. Sample cultures irradiated with UV (10 Jm^{-2}) (▲), or unirradiated (O), were pulsed with [^{3}H]thymidine at intervals after setting up on day 0. ✗ on axis indicates change of medium.

slightly increased in the other. Six days later (with no change of medium), incorporation in unirradiated cells was at a very low level, and irradiation stimulated incorporation about five-fold. Other samples given a medium change and incubated with [^{3}H]thymidine the next day showed greatly increased incorporation and an inhibitory effect of UV (i.e. some cells had re-entered the division cycle as a result of the medium change), but one week later incorporation was again low, and stimulated by UV. We define the state of quiescence as one in which (under these labelling and culture conditions) the incorporation of [^{3}H]thymidine due to replication does not exceed 500 cpm, and (more important) in which UV irradiation stimulates incorporation by at least two-fold.

Even in the defined quiescent state there is a tiny fraction of cells in S phase, and because (in terms of incorporation per cell) replicative DNA synthesis is so much greater than repair synthesis, the incorporation by

these few cells should not be ignored. To assess the effect of UV
irradiation on this residual S phase synthesis, we examined a culture of
proliferating cells; with sufficient cells of a population in S phase,
repair synthesis is negligible compared with replication. A dose of 2 Jm^{-2}
reduced [^{3}H]thymidine incorporation to 81% of the level in unirradiated
cells, and 10 Jm^{-2} reduced it to 43%.

We applied the technique to an examination of effects of various DNA
synthesis inhibitors on repair. Table 1 gives the results of treating
quiescent cells with HU and ara C after UV irradiation (or mock irradiation).
The inhibitory effects of HU or ara C on incorporation of [^{3}H]thymidine due
to repair in irradiated cells are less than the effects on incorporation in
unirradiated cells; given together, these inhibitors suppress repair
incorporation almost as severely as S phase incorporation. Fig. 2 shows
autoradiographs obtained in a parallel experiment. The density of grains
over nuclei of UV-irradiated cells is 70.7 $\pm$ 2.9 without inhibitors, and
23.5 $\pm$ 0.9 after incubation with HU and ara C (mean values $\pm$ S.E.; 50 cells

Table 1. Effects of inhibitors on incorporation of [^{3}H]thymidine into DNA
in unirradiated and irradiated cells.

UV dose (Jm^{-2})	% Incorporation of [^{3}H]thymidine into DNA			
	No addition	+ HU (10^{-2}M)	+ ara C (10^{-4}M)	+ HU (10^{-2}M) + ara C (10^{-4}M)
0	100	9	20	12
2	100 (2.14 x)*	-	-	36
10	100 (3.69 x)*	85	45	16

*Factors in parentheses indicate the stimulation of total incorporation
as a result of UV irradiation.
    The % incorporation in the case of unirradiated cells is calculated
simply from the relative values of cpm.
    After irradiation, the incorporation (without inhibitors) represents
partly repair synthesis and partly residual S phase synthesis. The latter
component, estimated from the incorporation in unirradiated cells adjusted
for the inhibitory effect of UV on replication (see text), was subtracted
to give a value for repair incorporation, presented as 100%.
    From the cpm incorporated in the presence of inhibitor(s), residual
S phase incorporation (i.e. incorporation in unirradiated cells with
inhibitors, adjusted for the additional inhibitory effect of UV) was
subtracted; the resultant figure is expressed as a percentage of the
normalised incorporation in cells without inhibitors.
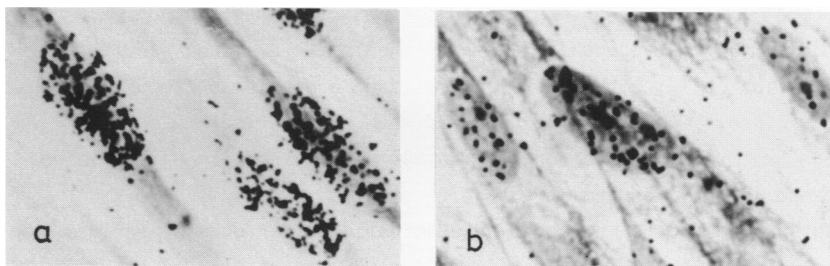
Fig. 2. Unscheduled DNA synthesis; inhibition by HU and ara C. Autoradio-graphs of BCL-D1 cells incubated with [^{3}H]thymidine (5 µCi/ml; 55 Ci/mmole) after UV irradiation (10 Jm^{-2}). (a) Control; (b) with HU (10^{-2}M) and ara C (10^{-4}M).

scored).

For two other inhibitors of DNA synthesis, we compared effects on repair and replicative DNA synthesis. The concentration response curves for novobiocin are shown in Fig. 3a; they are quite similar for the two modes of DNA synthesis, and virtually complete inhibition occurs at 10^{-3}M. Aphi-dicolin also inhibits [^{3}H]thymidine incorporation due to repair as well as replication (Fig. 3b), though the concentration response curves indicate that inhibition may be incomplete. The inhibitory effect is further illus-trated in the autoradiographs of Fig. 4; aphidicolin reduces the nuclear grain density in UV-irradiated cells, from 33.8 ± 3.6 to 6.8 ± 0.4 (mean values ± S.E.; 50 cells scored).

Inhibitors of DNA synthesis such as HU and ara C cause the accumulation of DNA breaks in UV-irradiated cells, as a result of enzymic incision/excision at damage sites with incomplete resynthesis and therefore blocked ligation [5]. To determine whether aphidicolin has a similar effect, we examined the UV-dependent formation of DNA breaks in proliferating normal human cells incubated with a range of concentrations of aphidicolin (Fig. 5). The assay for DNA breakage depends on the increased rate of unwinding of nicked DNA in alkali, and the susceptibility of unwound DNA to digestion by S1 nuclease. Incubation of cells with aphidicolin following irradiation does cause accumulation of breaks, in a concentration-dependent way, and the presence of HU at 10^{-2}M potentiates the effect. The maximum extent of digestion of DNA by S1 nuclease in this assay corresponds well with the level of digestion seen in these cells irradiated with the same dose and incubated with HU (10^{-2}M) and ara C (10^{-4}M) [7].
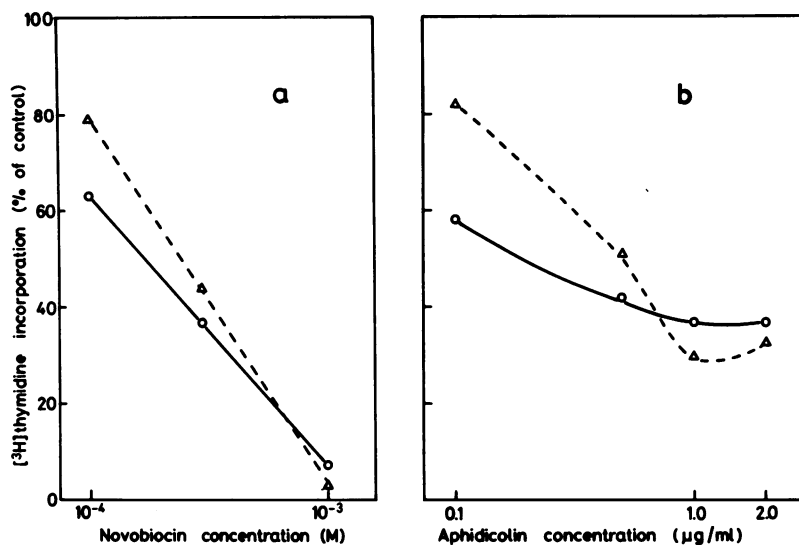
Fig. 3. Replicative and repair DNA synthesis; inhibition by novobiocin or aphidicolin. Unirradiated and irradiated (10 Jm^{-2}) quiescent cultures of Flow 2000 were incubated with [^{3}H]thymidine in the presence of various concentrations of novobiocin (a) or aphidicolin (b). Data for replicative DNA synthesis (O) come from unirradiated cells, representing the very small number of dividing cells in the population; incorporation of [^{3}H]thymidine is expressed relative to that in unirradiated cells incubated with no inhibitor. [^{3}H]thymidine incorporation due to repair (△) is calculated for irradiated cells as in Table 1 and related to that in irradiated cells without inhibitor. Results are calculated from means of duplicate samples. The stimulation by UV of overall incorporation in cells not treated with inhibitor was between 2.7 x and 3.9 x in different experiments. In a control experiment (not shown), addition of 0.2% dimethylsulphoxide (equivalent to the amount present at the highest concentration of aphidicolin) produced slight increases (10 - 20%) in incorporation with or without irradiation.

DISCUSSION

The method we describe here for measuring [^{3}H]thymidine incorporation associated with DNA repair avoids the problem of isolating repaired DNA simply by testing cells in a state of quiescence, in which S phase cells are so rare that repair synthesis accounts for the majority of the [^{3}H]thymidine incorporation occurring after UV irradiation. The method is most readily applied to certain cell lines, notably non-transformed cells which are able to enter a G$_0$ state. We suggest a protocol similar to that represented in the experiments of Fig. 1, the state of quiescence being monitored by the relative incorporation of [^{3}H]thymidine in irradiated and unirradiated samples.
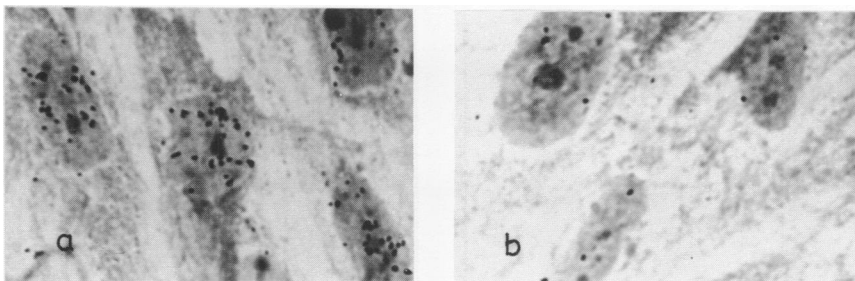
Fig. 4. Unscheduled DNA synthesis; inhibition by aphidicolin. Autoradio-
graphs of BCL-D1 cells incubated with [^{3}H]thymidine (1 µCi/ml, 55 Ci/mmole)
after UV irradiation (10 Jm^{-2}). (a) Control; (b) with aphidicolin
(1 µg/ml).

    The calculation of incorporation due to repair requires a simple

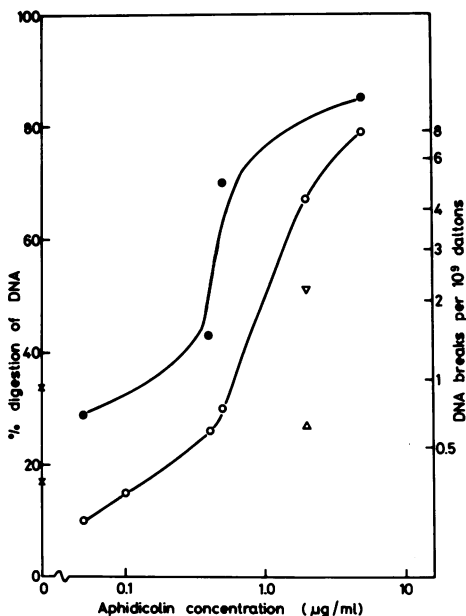correction, subtracting that part of the total incorporation which is due

Fig. 5. DNA break accumulation in UV-irradiated cells incubated with aph-
idicolin. Flow 2000 cells grown on chamber slides and prelabelled with
[^{3}H]thymidine were irradiated (2 Jm^{-2}) and incubated with aphidicolin (o ;
concentrations as shown) or with HU (✗ ; 10^{-2}M) or with aphidicolin and HU
(●). Data for cells irradiated with 0.5 Jm^{-2} (▲) or 1 Jm^{-2} (▼) and
incubated with 2 µg/ml aphidicolin are also shown. A scale of estimated DNA
break frequency is given, based on the calibration described in ref. 7.

to residual S phase synthesis; this is estimated from the amount of incorporation in unirradiated dishes and the extent of inhibition of S phase synthesis by UV. When studying effects of inhibitors, the calculation (as in Table 1) depends on the assumption that the S phase synthesis resistant to inhibitors is normally susceptible to inhibition by UV. The correction, however, is of minor importance, since repair synthesis is the predominant component of the incorporation.

Other assays for repair DNA synthesis in which [3H]thymidine incorporation is measured directly rely on suppressing replication with HU [3, 9, 10]. HU does inhibit repair incorporation, though less severely than replication, as we show here, and it seems unwise to base a quantitative assay on this procedure. HU acts by blocking DNA precursor production [11]. Repair synthesis, which makes a much smaller demand on the precursor pool than does replication, is apparently able to continue for some time, or at a reduced rate, using the existing precursor pool which remains in the presence of HU (hence the partial nature of the inhibition); but the residual pool size may well depend on culture conditions, and so the extent of inhibition of repair synthesis by HU is unlikely to be constant. Such an effect may account for the variability in the level of HU-resistant [3H]thymidine incorporation found by Lehmann and Stevens [3].

In any cellular assay for repair DNA synthesis involving incubation with labelled precursor, an endogenous precursor pool will influence the specific activity of the label incorporated. Comparisons of incorporation levels in different cells are therefore meaningful only if they are in identical states of growth and culture conditions, and the use of the quiescent state as defined in this paper goes some way to meeting those requirements.

The inhibition of repair incorporation by novobiocin is an expected consequence of its ability to block enzymic incision at DNA damage sites [12]. Novobiocin has a significant effect on both repair and replicative DNA synthesis at 10^{-4}M, and the concentration-dependence of inhibition of incision is very similar [12]. Novobiocin has a direct inhibitory effect on DNA polymerase α, at considerably higher concentration [13]; but it remains likely that the primary effect on repair is the prevention of incision. Mattern and Scudiero [14] also report the inhibition of repair DNA synthesis by novobiocin, suggesting the involvement of a DNA topoisomerase-mediated step in repair.

Aphidicolin is a specific inhibitor of DNA polymerase α [15], and it has been used in several attempts to resolve the question of whether

polymerase α is responsible for repair DNA synthesis after UV irradiation. Berger et al.[16] and Hanaoka et al.[17], studying permeabilised human lymphocytes and isolated HeLa cell nuclei respectively, found that UV-stimulated incorporation of [^{3}H]TTP is blocked by aphidicolin. Snyder and Regan[18] and Waters[19] have shown that aphidicolin prevents rejoining of breaks introduced into DNA during excision repair. Our observations that repair incorporation in whole cells is reduced by aphidicolin and that excision-related DNA breaks accumulate in a concentration-dependent way add to the body of evidence that polymerase α is involved in repair synthesis. In a conflicting report[20], there is merely a qualitative demonstration that some repair synthesis is resistant to aphidicolin, with no information on the control level of repair synthesis without the inhibitor. Inhibition of DNA synthesis by aphidicolin is competitive with all four deoxyribonucleoside triphosphates[21]; thus the extent of inhibition of repair synthesis by aphidicolin may vary with the size of the cellular DNA precursor pool. The greater accumulation of DNA breaks seen when HU is present with aphidicolin (Fig. 5) presumably reflects depletion of this pool by HU.

The main aim of this report is to show that the inhibition of repair is reflected in a reduced level of incorporation of [^{3}H]thymidine into DNA. This finding is, on the face of it, unsurprising; but there are numerous reports that HU and ara C have little effect on repair incorporation or may even increase it[2, 22 - 26]. In these reports, HU and/or ara C were examined individually rather than in combination, and it is clear from Table 1 that these inhibitors have a synergistic effect. Dunn and Regan[27] found that ara C at 10^{-5}M reduced repair synthesis by 56% - but only with 2 mM HU also present. We observe a potentiation of the inhibitory ara C effect by HU, but not an absolute dependence.

The ability of HU and ara C to cause accumulation of DNA breaks in UV-irradiated cells is the basis of an increasingly popular assay for cellular repair[5, 28]. It is assumed that DNA breaks made by repair endonuclease persist in the presence of inhibitors because repair synthesis and ligation are blocked. The demonstration in this paper that HU and ara C reduce incorporation of [^{3}H]thymidine due to repair lends strong support to this interpretation.

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REFERENCES

1. Cleaver, J.E. and Thomas, G.H. (1981) in DNA Repair: A Laboratory Manual of Research Procedures, Friedberg, E.C. and Hanawalt, P.C. Eds., Vol. 1B, pp. 277 - 287, Marcel Dekker, New York.
2. Smith, C.A. and Hanawalt, P.C. (1976) Biochim. Biophys. Acta 432, 336 - 347.
3. Lehmann, A.R. and Stevens, S. (1980) Mutat. Res. 69, 177 - 190.
4. Strauss, B.S. (1981) in DNA Repair: A Laboratory Manual of Research Procedures, Friedberg, E.C. and Hanawalt, P.C. Eds., Vol. 1B, pp. 319 - 339, Marcel Dekker, New York.
5. Collins, A.R.S. and Johnson, R.T. (1981) in DNA Repair: A Laboratory Manual of Research Procedures, Friedberg, E.C. and Hanawalt, P.C. Eds., Vol. 1B, pp. 341 - 360, Marcel Dekker, New York.
6. Dulbecco, R. and Vogt, M. (1954) J. Exp. Med. 99, 167 - 199.
7. Collins, A., Jones, C. and Waldren, C. (submitted).
8. Sutton, W.D. (1971) Biochim. Biophys. Acta 240, 522 - 531.
9. Trosko, J.E. and Yager, J.D. (1974) Exp. Cell Res. 88, 47 - 55.
10. Stefanini, M., Dalpra, L, Zei, G., Giorgi, R., Falaschi, A. and Nuzzo, F. (1976) Mutat. Res. 34, 313 - 326.
11. Reichard, P. (1972) in Advances in Enzyme Regulation, Weber, G. Ed., Vol. 10, pp. 3 - 16, Pergamon Press, Oxford.
12. Collins, A. and Johnson, R (1979) Nucleic Acids Res. 7, 1311 - 1320.
13. Burke, J.F., Duff, P.M. and Pearson, C.K. (1979) Biochem. J. 178, 621 - 626.
14. Mattern, M.R. and Scudiero, D.A. (1981) Biochim. Biophys. Acta 653, 248 - 258.
15. Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978) Nature 275, 458 - 460.
16. Berger, N.A., Kurohara, K.K., Petzold, S.J. and Sikorski, G.W. (1979) Biochem. Biophys. Res. Commun. 89, 218 - 225.
17. Hanaoka, F., Kato, H., Ikegami, S., Ohashi, M. and Yamada, M.-a. (1979) Biochem. Biophys. Res. Commun. 87, 575 - 580.
18. Snyder, R.D. and Regan, J.D. (1981) Biochem. Biophys. Res. Commun. 99, 1088 - 1094.
19. Waters, R. (1981) Carcinogenesis 2, 795 - 797.
20. Pedrali-Noy, G. and Spadari, S. (1980) Mutat. Res. 70, 389 - 394.
21. Dicioccio, R.A., Chadha, K. and Srivastava, B.I.S. (1980) Biochim. Biophys. Acta 609, 224 - 231.
22. Cleaver, J.E. (1969) Radiat. Res. 37, 334 - 348.
23. Brandt, W.N., Flamm, W.G. and Bernheim, N.J. (1972) Chem.-Biol. Interact. 5, 327 - 339.
24. Schor, S.L., Johnson, R.T. and Waldren, C.A. (1975) J. Cell Sci. 17, 539 - 565.
25. Clarkson, J.M. (1978) Mutat. Res. 52, 273 - 284.
26. Collins, A.R.S. and Johnson, R.T. (1979) J. Cell. Physiol. 99, 125 - 138.
27. Dunn, W.C. and Regan, J.D. (1979) Mol. Pharmacol. 15, 367 - 374.
28. Snyder, R.D., Carrier, W.L. and Regan, J.D. (1981) Biophys. J. 35, 339 - 350.