KOREAN JOURNAL OF ZOOLOGY
Vol. 19, No. 3, 113-122 (1976)

Methyl Methanesulfonate Stimulated ^{3}H-Thymidine Uptake in Synchronized HeLa S_3 Suspension Cells Pretreated with Thymidine Analogs

Sang Dai Park
(Dept. of Zoology, Seoul National University)

Thymidine 相似體를 前處理한 同時化시킨 Hela S_3 細胞에 있어 Methyl Methnaesulfonte에 의한 ^{3}H-Thymidine Uptake

朴 相 大
(서울大 自然大 動物學科)
(Received September 20, 1976)

摘 要

非同時化된 HeLa S_3 細胞에 있어 MMS에 의한 ^{3}H-thymidine uptake 率은 MMS의 濃度증가에 따라 직선적으로 감소하며, BUdR 혹은 IUdR과 二重處理할 경우 그 감소율은 더욱 증가한다.

MMS에 의한 ^{3}H-thymidine uptake은 thymidine 二重 處理후 얻은 同時化된 HeLa S_3 細胞의 G_2 時期에서 檢出된다. BUdR과 IUdR은 MMS에 의한 ^{3}H-thymidine uptake率을 더욱 증가시키며 IUdR은 BUdR에 비해 더효과적인 感受性 物質로 판명되었다.

INTRODUCTION

Methyl methanesulfonate (MMS) has been appeared to be a typical ionizing-type chemical mutagen in terms of DNA repair processes of induced DNA damage (Clarkson and Evans, 1972). An incorporation of thymidine analogs into DNA of mammalian cells has been demonstrated to increase the primary lesions in DNA or to influence the repair processes of damaged DNA in irradiated cells (Smets and Cornelis, 1971; Lohman *et al.*, 1972; Scott *et al.*, 1974). Park and Um (1975), recently reported that these base analogs were found to be potent chemical sensitizers enhancing MMS-induced DNA repair synthesis in HeLa S_3 cells.

Two main techniques have been utilized for the detection of DNA repair

This investigation was supported, in part, by the Korean Trades Scholarship Foundation.

synthesis in mammalian system, *i.e.*, unscheduled DNA synthesis and ^{3}H-thymidine uptake (Cleaver, 1974). The ^{3}H-thymidine uptake method was originally developed by Evan and Norman (1968) in x-irradiated human lymphocytes. Only two exceptions have been so far reported in non-lymphocytes, in fibroblast cultures of Xeroderma pigmentosum patients (Parrington *et al.*, 1971) and chick skeletal muscle cells (Stockdale and O'Neil, 1972) following irradiation with UV-light.

The reason why this technique has been exclusively utilized in lymphocyte cultures is that all most all of the cells (99%) are in the G_0 or G_1 stage, and that the small amount of semiconservative replication occurring can be further suppressed by the addition of hydroxyurea (Cleaver, 1974). This method has been considered as the most convenient technique and has an advantage of obtaining more easily a rapid accumulation of kinetic data (Cleaver, 1974). However, no one has yet attempted the use of ^{3}H-thymidine uptake for the detection of DNA repair synthesis in synchronized mammalian cell lines.

The present studies were therefore undertaken with an attempt to establish the ^{3}H-thymidine uptake technique using synchronized HeLa S_3 suspension cells and to confirm the previous findings which showed that thymidine analogs enhanced MMS-induced unscheduled DNA synthesis.

MATERIALS AND METHODS

1. Cell Culture

An established human cell line, HeLa S_3 was used throughout this investigation. Suspension cultures of this cell line were grown in spinner culture bottles (Bellco Glass Inc.) at 37°C. The medium used was Joklik-modified minimum essential medium (Gibco) for spinners, supplemented with 5% fetal calf serum, 1% L-glutamine and antibiotics (penicillin G, 100 units/m*l*; streptomycin, 100 μg/m*l*).

2. Synchronization

Suspension cultures of this cell line were synchronized with a silght modification of the method developed by Volpe and Eremenko (1973) using 2.0 mM thymidine double shock. The cell suspension (5.0×10^5 cells/m*l*) were first treated with 2.0 mM thymidine in the growth medium for 20 hours. After the cells were washed twice with spinner salt solution (Gibco), they were returned to fresh medium and incubated for 8 hours. The second thymidine shock similar to the first lasted for 16 hours. The thymidine shock was terminated by washing the cells which were then returned to fresh medium without thymidine. This was designated as time zero.

The degree of synchronization achieved was determined by the density of

the cell cultures at zero hour and by the rate of ^{3}H-thymidine incorporation.

For cell counting, an aliquot of 1 m*l* cell suspension was withdrawn and placed in a tube containing 1 m*l* of 0.4% trypan blue at pH 7.2. After mixing for 10 minutes, the cell density and viability of the cultures were determined by counting the cell using a hemacytometer (Phillips, 1973).

For ^{3}H-thymidine incorporation, an aliquot of 5 m*l* cell suspension (3.0×10^{5} cells/m*l*) was withdrawn at hourly or half hourly intervals beginning at zero hour up to 12 hours. These samples were pulse labeled with ^{3}H-thymidine (Amersham/Searle) for 20 minutes at a final concentration of 5 μCi/m*l* (specific activity, 20Ci/mM) at 37°C. After labeling the radioactive medium was discarded, and harvested cells were washed twice with cold spinner salt solution containing unlabeled thymidine (100 μg/m*l*). Then they were treated with ice cold 5% trichloroacetic acid (TCA) and left in ice for 20 minutes. After mixing gently, the lysed sample of 2 m*l* was transferred into counting vial and to which were added 10 m*l* of the scintillation fluid which consisted of 42 m*l* of liquifluor (New England Nuclear) per liter of toluene based scintillation cocktail. The radioactivity of the incorporated ^{3}H-thymidine was determined using a Packard Tri-carb scintillation spectrometer (Packard Instrument) with 30% usual efficiency.

3. Incorporation of Thymidine Analogs

5-bromodeoxyuridine (BUdR) and 5-iododeoxyuridine (IUdR) (Sigma) were prepared as 1 M stock solution in the growth medium and further diluted to the working concentration immediately prior to use. Synchronized cultures of suspension cells (1.0×10^{6} cells/m*l*) were treated to BUdR or IUdR in two different stages of the cell cycle. For one experiment, the late S-stage (1.5 hours) and early G_2-stage (1.5 hours) were exposed for 3 hours to 0.2 mM BUdR or IUdR. For the second experiment, the same concentration of BUdR or IUdR was used for 6 hours during the S-stage.

4. Methyl Methanesulfonate Treatment

Methyl methanesulfonate (Kodak, Ltd.) was dissolved in phosphate buffered saline (PBS) as 1 M stock solution and further diluted to the various working concentrations in the growth medium without serum 10~15 minutes prior to treatment. The suspension cultures (1.0×10^{6} cells/m*l*) of asynchronous and synchronized HeLa S_3 cells were treated to MMS from 0 to 4.0 mM for an hour at 37°C. After treatment with MMS, the cells were washed twice with medium and the second washing was replaced with the fresh growth medium and incubated at 37°C.

5. ^{3}H-thymidine Uptake Procedures

For the detection of MMS-stimulated ^{3}H-thymidine uptake in synchronized

suspension cultures of HeLa S_3 cells, the MMS treated cells were then pulse labeled with ^{3}H-thymidine at a final concentration of 10 μCi/ml in the growth medium which contained 3.0 mM hydroxyurea. The labeling was for a period of 2 hours and procedures were as follows; for one experiment cultures were labeled during mid G_2-stage covering the period between 10~12 hours, for the other experiment cultures were labeled during the late G_2-stage covering the 7~9 hours period. The incorporation of ^{3}H-thymidine was terminated by washing the cells as described previously. The cells were then suspended in 1 ml of distilled water and then lysed in 1 ml of a solution of SDS-EDTA (0.01 M NaCl, 0.01 M tris chloride, pH 7.0, 1% sodium dodecylsulfate and 0.05 M EDTA). To the samples TCA was added to a concentration of 7% and left in ice for 20 minutes. After mixing gently, 2 ml of lysed sample were transferred into a vial and 10 ml of scintillation fluid were added for the determination of ^{3}H-thymidine uptake with a liquid scintillation spectrometer (Clarkson and Evans, 1972; Volpe and Eremenko, 1973; Cleaver and Painter, 1975).

RESULTS

The rate of ^{3}H-thymidine uptake in asynchronous cultures is shown in Fig. 1. In the control the rate of ^{3}H-thymidine incorporation (cpm) was rated as 100%. The uptake rate in the MMS treated group (96.8%) decreased as dose increased. The decreasing rates were marked in BUdR-MMS (90.8%) and IUdR-MMS (88.4%) groups. These results indicated that MMS treatment in asynchronous cultures reduces the normal DNA synthesis, and that the combined

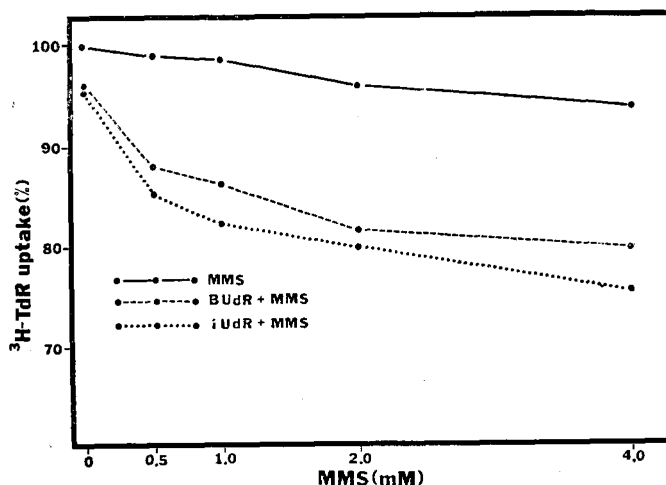

Fig. 1. Sensitization effect of BUdR or IUdR on MMS-stimulated incorporation of ^{3}H-thymidine in asynchronous cultures of HeLa S_3 suspension cells.

treatment enhances the reduction of the normal DNA synthesis.

The variation of cell density during the synchronization procedure by the thymidine double shock is presented in Table 1. Under these conditions, 95% of the cells were synchronized at the end of thymidine double shock, and 7.3% of the cells (12 hours later) were thought to be cells out of the G_2 stage, possibly S-stage cells.

Table 1. Variations in cell density of HeLa S_3 suspension cultures during synchronization produced by the thymidine double shock.

Treatment	Hours treated (hrs)	Hours accumulated (hrs)	Expected[a] value (cells/ml)	Experimental[b] value (cells/ml)	Remarks[c]
1st TdR (2.0 mM)	20	20	4.16×10^5	4.16×10^5	Initial density
Fresh medium	8	28	3.66×10^5	4.00×10^5	+ 9.3%
2nd TdR (2.0 mM)	16	44	3.50×10^5	4.50×10^5	+28.6%
Fresh medium	0	44	4.00×10^5	4.20×10^5	+ 5.0%
Fresh medium	12	56	3.70×10^5	4.00×10^5	+ 7.3%

a: Cell dnesity minus the loss from each transfer procedure during centrifugation (5.0×10^4).
b: Each value represents an average of triplicate counts.
c: Percentage increase over the expected value.

The rates of ^{3}H-thymidine incorporation in synchronized cells from the time zero up to 12 hours after treatment with the second-thymidine shock followed by ^{3}H-thymidine labeling for 20 minutes can be followed in Fig. 2. The maximum peak of ^{3}H-thymidine incorporation occurred at 7.5 hours. Thus, it was assumed that the duration of the S-stage of this cell line in suspension

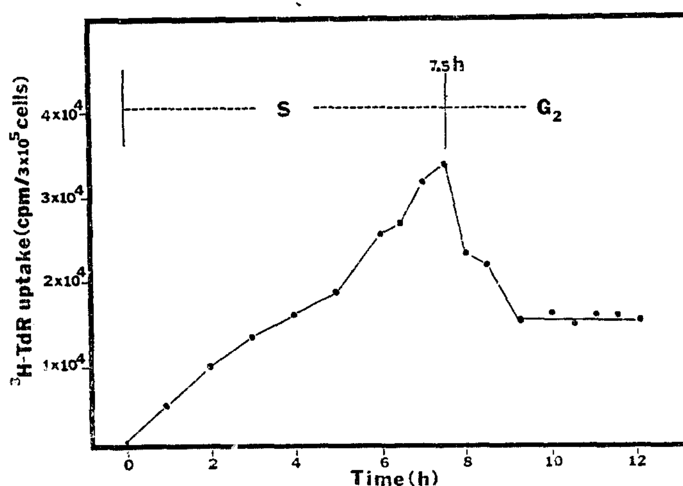

Fig. 2 Cell cycle analysis of HeLa S_3 suspension cells synchronized by the thymidine double shock.

cultures was for about 7.5 hours, and that the G_2 stage followed this period. However, a large amount of ^{3}H-thymidine labeling was still shown to be occurring in the cells at 8.5 hours. The data indicated that the S-stage lasted for about 1.5 hours and the rate of ^{3}H-thymidine incorporation was 2.4 fold higher than those of Volpe and Eremenko (1973). From the data in Table 1 and Fig. 2, it may be suggested that 100% synchronization was not achieved and that S-stage cells were mixed with G_2 stage cells under these experimental conditions.

Fig. 3 shows the effects of BUdR or IUdR on MMS-stimulated ^{3}H-thymidine uptake in synchronized cultures of HeLa S_3 suspension cells. BUdR or IUdR was introduced for 3 hours covering the late S and G_2 stages (between 6~9 hours), followed by MMS (between 9~10 hours) and ^{3}H-thymidine labeling (between 10~12 hours). To suppress the normal DNA synthesis of S-stage cells

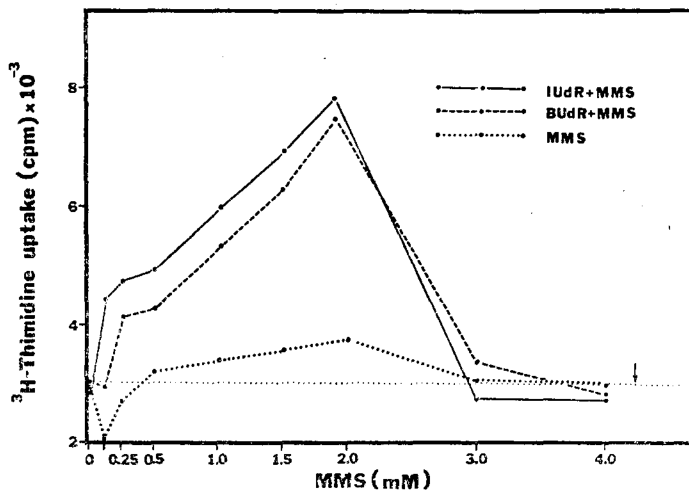

Fig. 3 sensitization effect of BUdR or IUdR on MMS-stimulated incorporation of ^{3}H-thymidine in G_2 stage cells of synchronized Hela S_3 suspension cultures.

mixed in G_2-stage, 3.0 mM hydroxyurea was added during the ^{3}H-thymidine treatment. The incorporation of ^{3}H-thymidine stimulated by MMS (unscheduled DNA synthesis) was detected in G_2-stage cells at dose levels between 0.5~3.0 mM MMS. In lower and higher dose ranges the uptake rates were lower than the control. This result might be due to failure in stimulating the DNA repair synthesis (at lower dose) and inhibiting the DNA repair synthesis (at higher dose). The maximum and minimum effective doses stimulating DNA repair

synthesis was found in 2.0 and 3.0 mM MMS treated cultures, which represented 23.1% and 0.6% above the conteol value, respectively. The single treatment with BUdR or IUdR showed almost the same value of ^{3}H-thymidine uptake as in the control. These results suggest that BUdR or IUdR alone may not induce DNA repair synthesis. The combined treatment with BUdR or IUdR and MMS, however, considerably increased the rate of ^{3}H-thymidine uptake. In the BUdR-MMS group, the ^{3}H-thymidine uptake rate above the control appeared at dose levels of MMS between 0.25 and 3.0 mM. The maximum and minimum rates were found at 2.0 and 3.0 mM which were about 6.5 and 21 times greater than the corresponding single treatment with MMS. In IUdR-MMS, the uptake rates above the control were found to be at dose levels between 0.1~2.0 mM, with the maximum and minimum rates at 2.0 and 0.1 mM MMS, respectively. The average rates of ^{3}H-thymidine uptake which exceeded the control value in MMS, BUdR-MMS and IUdR-MMS treated groups were 23.1%, 71.3% and 97.0%, respectively. From Fig. 3, it may be concluded that the combined treatment with BUdR or IUdR and MMS greatly enhances ^{3}H-thymidine uptake.

The dose response of MMS in synchronized HeLa S_3 suspension cultures substituted with BUdR or IUdR is depicted in Fig. 4. HeLa S_3 suspension cells

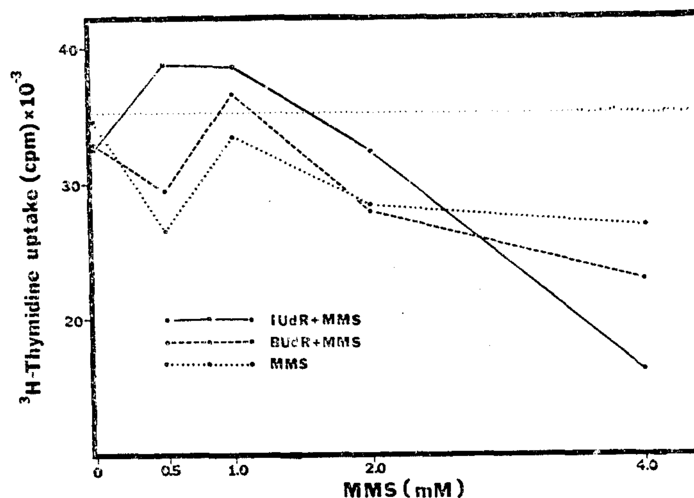

Fig. 4 Sensitization effect of BUdR or IUdR on MMS-stimulated incorporation of ^{3}H-thymidine in late S-early G_2 stage cells of synchronized HeLa S_3 suspension cultures.

were exposed to BUdR or IUdR for 6 hours in the S-stage followed by MMS in the S-stage and ^{3}H-thymidine treatment in the late S—early G_2 stages (between 7~9 hours). In the control, the rate of ^{3}H-thymidine uptake was much higher as compared to the previous experiment. This might be due to the S-stage cells. In the MMS group, ^{3}H-thymidine uptake at all concentrations were below the control level. The highest uptake rate was found in cells treated with 1.0 mM followed by 2.0 mM treated cells. In BUdR-MMS, the uptake rate above control levels appeared in 0.1 mM MMS treated cells, whereas in IUdR-MMS (0.5 and 1.0 mM) treated cells the highest value above the control appeared in the 0.5 MMS treated cells. From Fig. 4, it is assumed that the effect of sensitizers on MMS-stimulated ^{3}H-thymidine uptake varies with different stages of the cell cycle. The shift of the most effective dose from 2.0 mM to 1.0 mM or 0.5 mM may be influenced by longer treatment with these sensitizers as well as the different stages of the cell cycle.

The overall results clearly indicate that ^{3}H-thymidine uptake technique can detect the unscheduled DNA synthesis in synchronized cultures. This is enhanced considerably by pretreatment with thymidine analogs. More defined experiments including cell synchronization and pretreatment with chemical sensitizer may provide an excellent assay technique for the detection of DNA repair synthesis.

DISCUSSION

MMS-induced DNA repair synthesis has been reported by several workers (Hahn *et al.*, 1968; Fox and Fox, 1973) by means of autoradiography. All published data indicate that MMS-induced unscheduled DNA synthesis is dose dependent up to 2.0~3.0 mM. Clarkson and Evans (1972) showed that incorporation of ^{3}H-thymidine in human lymphocytes after different dose levels of MMS was found to increase with increasing dosage up to 3.0 mM and then it declined at higher concentrations. These results are in good agreement with that of the present investigation.

It has been postulated that an incorporation of base analogs into DNA in place of thymine enhances an increase of the primary lesions in DNA leading to single strand breaks attributed to a random increase in energy absoption of the BU-containing DNA strand after irradiation, which can be repaired by repair replication or other repair processes of damaged DNA (Lohman *et al.*, 1972; Sawada and Okada, 1972). A similar result was recently reported by using of ionizing-type chemical mutagen, MMS, which showed that thymidine analogs enhanced MMS-induced DNA repair synthesis (Park and Um, 1975). The present results obtained from the ^{3}H-thymideine uptake technique in

synchronized cultures confirmed the previous findings. However, it should be explained how these two different chemicals act on DNA molecules as mutagen to enhance the DNA repair syuthesis.

There are two assay techniques for the detection of DNA repair synthesis, autoradiography and ^{3}H-thymidine uptake. The former has been used in asynchronous cultures and the latter has been exclusively used with non-proliferative lymphocytes. The ^{3}H-thymidine uptake method has been known as the most convenient and rapid technique for the detection of DNA repair synthesis. (Spiegler and Norman, 1969; Regan and Setlow, 1974; Cleaver, 1974).

Although the unscheduled DNA synthesis is equated with repair replication, none of the methods for repair replication can be used for the detection of the sensitizing effect of thymidine analogs on DNA repair synthesis, since methods for repair replication require a pretreatment with BUdR or BU in order to make heavy strands or to sensitize the DNA strands (Cleaver, 1974). Thus, the present investigation was to device a technique for the detection of DNA repair synthesis in exponentially growing mammalian cell line by means of ^{3}H-thymidine uptake and cell synchronization.

The data presented here indicate that MMS-induced DNA repair synthesis in HeLa S_3 suspension cells can be detected by ^{3}H-thymidine uptake technique. They also confirm the previous results which showed that BUdR and IUdR enhances MMS-induced DNA repair synthesis. As clearly shown, pretreatment with BUdR or IUdR in synchronized cells increased about 4~4.5 fold of DNA repair synthesis as compared with the single treatment of MMS. Therefore, the proposed technique can be used for the detection of DNA repair synthesis in synchronized cultures of mammalian cells, especially by treatment with either of these thymidine anlogs like the methods for the detection of repair replication.

SUMMARY

The rate of ^{3}H-thymidine uptake induced by MMS in asynchronous HeLa S_3 suspension cells was decreased in direct proportion to dose increase. The combined treatment of BUdR or IUdR with MMS was more effective in reducing the rate of ^{3}H-thymidine uptake.

MMS-stimulated ^{3}H-thymidine uptake was detected in synchronized G_2 stage cells of HeLa S_3 suspension cultures following treatment with thymidine double shock. BUdR and IUdR greatly enhanced MMS-stimulated ^{3}H-thymidine uptake. IUdR was found to be a more effective sensitizer than BUdR in this respect.

REFERENCES

Clarkson, J.M. and H.J. Evans, 1972. Unscheduled DNA synthesis in human lymphocytes after exposure to UV-light, γ-rays and chemical mutagens. *Mutation Res.* **14** : 413—430.

Cleaver, J.E., 1974. Repair processes of photochemical damages in mammalian cells. *In:* Advances in Radiation Biology (J.T. Lett, H. Adler and M. Zelle, editors). Academic Press Inc., New York **4** : 20—56.

Cleaver, J.E. and R.B. Painter, 1975. Absence of specificity in inhibition of DNA repair replication by DNA-binding agents, carcinogens, and steroids in human cells. *Cancer Research* **35** : 1773—1778.

Evans, R.G. and A. Norman, 1968 Radiation stimulated incorporation of thymidine into the DNA of human lymphocytes. *Nature* **217** : 455—456.

Fox, M. and B.W. Fox, 1973. Repair replication and unscheduled DNA synthesis in mammalian cell lines showing different sensitivity to alkylating agents. *Mutation Res.* **19** : 119—128.

Hahn, G.M., S.J. Yang and V. Parker, 1968. Repair of sublethal damage and unscheduled DNA synthesis in mammalian cells treated with monofunctional alkylating agents. *Nature* **220** : 1142—1144.

Lohman, P.H.M., D. Bootsma and A.H. Hey, 1972. The influence of 5-bromodeoxyuridine on the induction of breaks in the deoxyribonucleic acid of cultivated human cells by x-irradiation and ultraviolet light. *Radiation Res.* **52** : 627—641.

Park, S.D. and K.I. Um, 1975. Sensitization effects of thymidine analogs on methyl methanesulfonate induced DNA repair synthesis. *Korean J. Zool.* **3** : 131—140.

Parrington, J.M., J.D.A. Delhanty and H.P Baden, 1971. Unscheduled DNA synthesis, UV-induced chromosome aberrations and SV_{40} transformation in cultured cells from xeroderma pigmentosum. *Ann. Human Genetics* **35** : 149—160.

Phillips, H.J., 1973. Dye exclusion tests for cell viability. *In:* Tissue Culture Methods and Application (P.F. Kruse, Jr. and M.K. Patterson, Jr., editors). Academic Press Inc., New York, pp. 406—408.

Regan, J.D. and R.B. Setlow, 1974. Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens. *Cancer Research* **34** : 3318—3325.

Sawada, S. and S. Okada, 1972. Effects of BUdR-labelling on radiation-induced DNA breakage and subsequent rejoining in cultured mammalian cells. *Intern. J. Radict. Biol.* **21** : 599—602.

Scott, D., M. Fox and B.W. Fox, 1974. The relationship between chromosomal aberrations, survival and DNA repair in tumer cell lines of differential sensivity to x-rays and sulphur mustard. *Mutation Res.* **22** : 207—221.

Smets, L.A., and J.J. Cornelis, 1971. Repairable and irrepairable damage in 5-bromouracil-substituted DNA exposed to ultraviolet radiation. *Intern. J. Radiat. Biol.* **19** : 445—457.

Spiegler, P. and A. Norman, 1969. Kinetics of unscheduled DNA synthesis induced by ionizing radiation in human lymphocytes. *Radiation Res.* **43** : 187—195.

Voloe, P. and T. Eremenko, 1973. A method for measuring cell cycle phases in suspension cultures. *In*: Methods in Cell Biology (D.M. Prescott, editor). Academic Press Inc.,**4** : 113—126.