Volume 5 Number 11 November 1978 Nucleic Acids Research

DNA ligase activity in UV-irradiated monkey kidney cells

M.Mezzina and S.Nocentini

Fondation Curie, Section de Biologie, 26 Rue d'Ulm, 75231 Paris Cedex 05, France

Received 18 August 1978

ABSTRACT

The DNA ligase activity of monkey kidney CV-1 cells has been measured at different stages of culture growth and after different time intervals following ultraviolet irradiation. Results indicate that:
- The level of enzyme activity is about twice higher in non synchronous, rapidly dividing cells than in confluent cultures.
- UV-irradiation of cells induces a "de novo" synthesis of DNA ligase.
- This induction is dose dependent in its extent and kinetics, and may lead to a DNA ligase level in UV-irradiated stationary cultures of the same order as observed in unirradiated exponentially growing cells.
- This induction seems to be independent of semiconservative DNA synthesis since it is not affected by fluorodeoxyuridine.

INTRODUCTION

DNA ligase activity in mammalian cells increases in response to a variety of proliferative stimuli and parallels the increase of DNA synthesis rate (1-5). This supports the idea that in mammals, as it has been satisfactorily proved in T_4 phage and in E.coli (6-8), DNA ligase plays a role in DNA replication by joining Okazaki-type fragments to produce the mature DNA strand of nascent chromosome.

In the scheme of the excision repair mechanism operating in UV-irradiated cells, the DNA ligase accomplishes the step of sealing the gaps and, consequently, of leading the repair process to completion (9). The participation of DNA ligase to such a process, occurring in absence of semiconservative DNA synthesis, is established in E.coli (8) but not yet in mammalian cells essentially because of the unavailability of conditional lethal mutant cell lines defective in ligating activity.

In an attempt to provide some insight into the possible involvement of DNA ligase in the excision repair process in mammalian systems, we have studied how DNA ligase activity is affected by UV-irradiation and subsequent DNA repair in stationary or rapidly dividing CV-1 cells, cultured in the presence or in the absence of inhibitors of DNA and protein synthesis. These

monkey kidney cells are able to repair UV-damage as shown by the recovery, in condition of dark repair, of some cellular functions indicative of the physiological integrity of cellular DNA, such as RNA transcription (10) or Herpes Virus production capacity (11), previously impaired by UV-irradiation.

We report that DNA ligase activity increases in UV-irradiated cells during post-exposure incubation in a dose dependent manner and independently of semiconservative DNA synthesis.

MATERIALS AND METHODS

Enzymes

Purified T_4-infected *E.coli* DNA ligase, polynucleotide kinase and exonuclease III were purchased from Miles (U.S.A.) and Boehringer (West Germany), *E.coli* alkaline phosphatase (type III and III-S) from Sigma (U.S.A.). α-DNA polymerase, partially purified from CV-1 cells, was kindly provided by Dr R.Wicker.

Chemicals

γ-^{32}P-Adenosine triphosphate (ATP; 20 and 3,000 Ci/mmol) was obtained from New England Nuclear Corporation (U.S.A.), ^{3}H-Thymidine (10 Ci/mmol) from C.E.A. (France), oligodeoxythymidilate (12-18 nucleotides long) and polydeoxyadenilate (about 1,100 and 250 nucleotides long) from Miles and Collaborative Research (U.S.A.) respectively.

Cells

African green monkey kidney cells, CV-1, checked for the absence of mycoplasma, were cultured at 37°C in modified Eagle's medium supplemented with 3% tryptose phosphate broth and 5% heat inactivated new born calf serum. For the experiments, 7 days old cells were plated in new dishes at 1/5 of their saturation density. Under such conditions, cultures are confluent after about 5 days.

Irradiation procedure

CV-1 cells grown in 14.5 cm plastic Petri dishes (Greiner - Germany) were irradiated, after removal of the culture medium, with a single dose of UV-irradiation at different time intervals before the preparation of cellular crude extracts for DNA ligase assay (time zero). A Philips germicidal tube was used with a maximal emission at 254 nm. The dose rate was 0.5 watt/m^2, as measured by a Latarjet dosimeter (12). After irradiation, the medium previously removed was added back to the cultures which were incubated at 37°C in the dark until time 0.

DNA synthesis

DNA synthesis was measured in terms of incorporation of ^{3}H -

Thymidine into acid-precipitable material. Cell cultures were labelled at different growth stages with ^{3}H-Thymidine (2 µCi/ml) at 37°C for 3h, then washed three times with cold phosphate-buffered saline and rapidly dissociated with trypsin (0.25%)- E.D.T.A. (0.5 mM). The number of cells per Petri dish was determined. Aliquots were made 10% (v/v) trichloroacetic acid (TCA) and held in ice for 30 min. The precipitates were collected on glass fiber filters, washed with cold TCA 5%, dried and counted for incorporated ^{3}H-radioactivity.

Cellular crude extracts

Cells were scraped from Petri dishes with a glass spatula and washed twice with phosphate-buffered saline. Cells were then suspended (3×10^{7} cells/ml) in 0.5 M KCl, 20 mM Tris(hydromethyl)-amino methane-HCl pH 7.5,2 mM dithioerythritol(DTE), 0.5% Triton X-100, and lysed by sonication in ice for 2 x 15 sec with 1 min cooling interval in between. The crude lysate was centrifuged at 105,000 x g for 1 hour at 2°C and immediately assayed for DNA ligase activity. Proteins and DNA contents in the crude extracts were determined according to the methods of Lowry (13) and Burton (14) respectively.

Preparation of 5'-^{32}P-oligo dT

Olivera's method (15) was used with slight modifications: 0.3 µ-mol/ml of oligo dT_{12-18} were incubated with alkaline phosphatase (type III, 5 units) at 37°C for 30 min. The reaction mixture was treated twice with the same volume of water-saturated phenol, centrifuged at 2,000 x g for 10 min. The water phase was collected and treated twice with the same volume of ethyl ether.

Dephosphorylated oligo dT (50 pmols) was incubated at 37°C with polynucleotide kinase (4 units) and 15 pmols of γ-^{32}P-ATP (3×10^{4} cpm/pmol) in the presence of 60 mM TRIS-HCl pH 7.5, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol. Every 15 minutes, fresh polynucleotide kinase was added until the plateau of ^{32}-P incorporation was reached, as determined by measuring acid precipitable ^{32}P- radioactivity, The reaction was then stopped by adding phenol. Specific activity of 5'-^{32}P-oligo dT prepared in this way was 2 to 4 x 10^{6} cpm/nmol.

DNA ligase assay

The standard assay mixture contained: 20 mM TRIS- HCl pH 7.5, 5 mM $MgCl_2$ 1 mM ATP, 2 mM DTE, 220 µg/ml bovine serum albumin, 5 µM poly dA, 5 µM 5'-^{32}P-oligo dT (1 to 3 x 10^{5} cpm/nmol) and 200 µg/ml proteins of cellular crude extracts. The reaction was carried out at 37°C in a final

volume of 0.25 ml. At times 0, 3, 6, 9 and 15 minutes, aliquots of 45 µl were removed, boiled 2 min and chilled rapidly in ice. Each sample was then treated with 0.1 unit of alkaline phosphatase (type III-S) at 85°C. After 15 min, fresh alkaline phosphatase was added and the incubation continued for another 15 min. The reaction was stopped by cooling and by adding 0.2 ml of carrier DNA (200 µg/ml) and 0.2 ml of 20 % TCA. The precipitate was retained on a 0.45 µ nitrocellulose filter (Millipore, France), dried and assayed for radioactivity in a PPO-POPOP-toluene scintillation mixture. The enzymatic activity in the different samples was determined taking into account the linear portion of kinetics. Under the standard condition, 1 unit of DNA ligase converts 1 nmol of 5'-^{32}P dT residues into alkaline phosphatase resistant form in 1 hour at 37°C. Each preparation of poly dA- ^{32}P-oligo dT substrate was preliminary tested in the standard assay with purified T_4-infected E.coli DNA ligase (0.05 units/ml). After 30 min of incubation at 37°C, 100% of the 5'-^{32}P-dT residues were converted to an alkaline phosphatase-resistant form.

RESULTS

First, we determined under different experimental conditions the growth of CV-1 cells and their DNA synthesis rate, as estimated by ^{3}H-Thymidine incorporation into acid-precipitable material.

Seven days old cells in confluent monolayers were trypsinized and seeded in 3 cm plastic Petri dishes with fresh medium at 1/5 of saturating density. Cultures were counted and ^{3}H-Thymidine incorporation was determined at 24 h intervals over a period of 7 days. Fig. 1A shows that, after a lag period of 24 h CV-1 cells enter into a period of exponential growth for about two days and finally settle slowly in a stationary phase on about the fifth day. The rate of DNA synthesis is maximal 24 hours after seeding (Fig.1B), probably because of a cell synchronization effect of trypsinization, and decreases later, its evolution preceding that of cell division rate by 24 h.

In the same experiments a portion of cultures was irradiated with three different doses on day 1, and tested afterwards for cell number and DNA synthesis as unirradiated controls.

As expected, UV-irradiation of one day old cultures, despite the induction of an unscheduled DNA synthesis (9), reduces drastically ^{3}H- Thymidine incorporation and thus, all the more, semiconservative DNA synthesis. Cell division is consequently reduced or totally inhibited

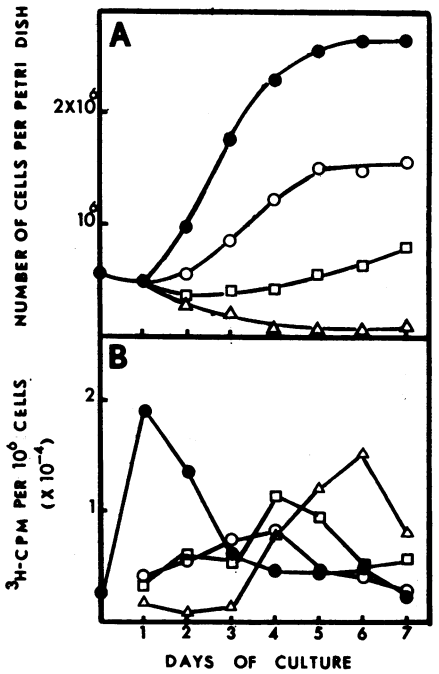

FIG. 1(A) Growth of CV-1 cells and (B) incorporation of ^{3}H-Thymidine
The values are the means of two separated experiments in which each point corresponds to three different cultures. Control cells (●——●), cells irradiated on day 1 with 9 J/m^2 (○——○), 17.5 J/m^2 (□——□) and 35 J/m^2 (△——△).

depending on the UV dose. Some cells are killed and detach from the growth surface: after exposure ≥ to 17.5 J/m^2, a decrease in the average number of cells per Petri dish occurs as soon as one day after irradiation. At later times both ^{3}H-Thymidine incorporation and cell numbers per Petri dish, at least for the doses ≤ 17.5 J/m^2, increase as surviving cells actively metabolize and divide.

In parallel experiments the levels of DNA ligase activity corresponding to a given stage of cell growth were measured. Cells were seeded as previously indicated, daily harvested and frozen (-70°C) before all samples were simultaneously assayed for DNA ligase. Fig.2 illustrates that the specific activity (s.a;) of the enzyme,which is about 5 units/mg of proteins in the extracts of 7 days old confluent cultures before subcultivation, enhances more than twice during the exponential phase of growth with a peak on the 2nd-3rd day of culture and sinks down back to its former level when the stationary state is reached.

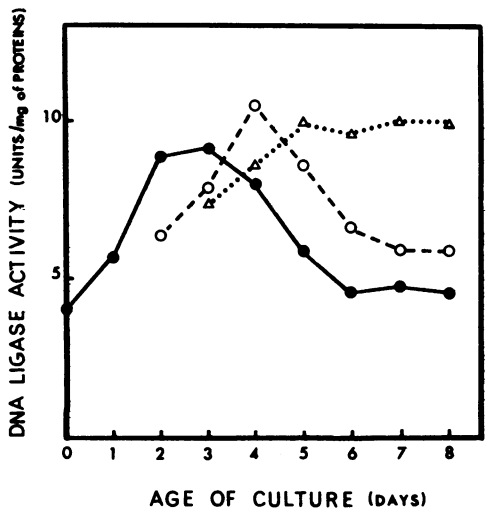

FIG. 2 DNA ligase activity as function of age of culture in control cells (●——●) and in cells UV-irradiated at 17.5 J/m^2 24 h (○----○) or 48 h (△·····△) before harvesting.

In order to study the effects of UV-irradiation, DNA ligase activity as a function of age of culture was also measured on extracts from cells which have been exposed to a single dose of UV of 17.5 J/m^2 24 h or 48 h before harvesting (fig.2). If cells are irradiated during the rapidly dividing phase, until the 3rd day, when the ligase activity is increasing, the treatment leads to a slight reduction of the level of the enzyme if compared with that in control cells of the same age. However, the ligase level in irradiated cells of a given age is higher than that in unirradiated cells at the time of UV-exposure. This observation indicates that, despite the inhibition of cell division and of ^{3}H-Thymidine incorporation, irradiated cells seem to present an appreciable synthesis of DNA ligase. This phenomenon is particularly clear when the irradiation takes place on confluent cultures with low and relatively constant DNA ligase levels. In fig. 2 it can be seen that the s.a. of ligase increases twice in cells which have been exposed to UV-irradiation 48 h before, and equals the levels of ligase observed in actively dividing cells.

Limiting our study to the period of "plateau", we have subsequently searched for a possible dose-dependence of ligase activity change in irradiated cells. Different confluent monolayers were irradiated with three different UV doses (9, 17.5 and 35 J/m^2) 48, 36, 24, 12 and 0h before the preparation of cell extracts. The results are reported in fig.3.

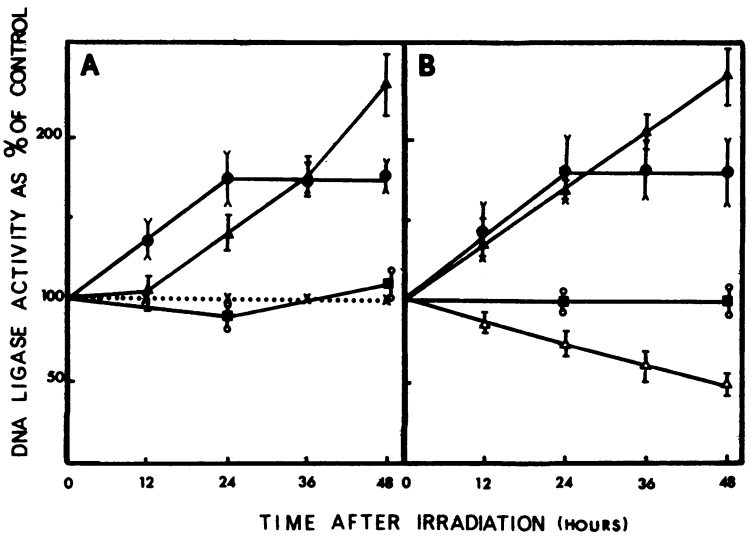

FIG.3 A) DNA ligase activity as a function of the time of post exposure incubation in cultures irradiated with a dose of 9 J/m^2 (●——●), 17.5 J/m^2 (▲——▲) and 35 J/m^2 (■——■). Each point represents the mean of at least 8 experiments. Dotted line indicates the constant level of ligase in unirradiated cultures during the whole interval of the experiment. B) Effects of DNA and proteins synthesis inhibitors. Drugs were added to all cultures at time - 49 h and maintened until the harvesting of cells; full symbols indicate FudR (10^{-3}M) treated cells; open symbols indicate cycloheximide (5 µg/ml) treated cells. Data are mean values of at least 4 experiments. Bars indicate the standard error of the mean.

Unirradiated cultures checked through the 48 h of the experiments show a fairly constant level of DNA ligase activity. The irradiation, up to 35 J/m^2 at time zero, does not modify the s.a. of the enzyme if compared with unirradiated control. When the time interval between the irradiation and the harvesting of the cells increases, DNA ligase activity evolves depending on the doses (fig. 3A). For the doses of 9 and 17.5 J/m^2 the s.a. of the enzyme enhances with the time of post-exposure incubation while for 35 J/m^2 almost no change is observed during a period of 48 h. The increase of the enzyme reaches 1.7 times the level of the control 24 h after 9 J/m^2 and remains at this level during the following 24 hours. After 17.5 J/m^2 the level of DNA ligase increases linearly for 48 hours and then reaches a level equal to 2.3 times that of the control.

Further experiments have been carried out in cultures treated with Fluorodeoxyuridine (FudR), an inhibitor of semiconservative DNA synthesis

(16). Although we have employed confluent cells with low mitotic activity and UV-irradiation inhibits efficiently DNA synthesis even in non confluent cultures (see fig. 1), we have used FudR to make sure that the enhancement of ligase activity observed in irradiated cells was not linked to a revival of DNA synthesis after UV-exposure. Results reported in fig. 3B are in complete agreement with those of fig. 3A and indicate that the increase of DNA ligase activity during post-exposure incubation seems totally independent of semiconservative DNA synthesis. If cells were treated with cycloheximide (5 µg/ml), an inhibitor of protein synthesis (17), the level of DNA ligase activity decreases considerably with time of post-exposure incubation in cells irradiated with 17.5 J/m^2. This suggests that the enhancement of the enzyme activity is related to a "de novo" synthesis of proteins.

It is worth noting that the level of DNA ligase activity in non irradiated cultures treated with FudR and cycloheximide during 48 hours does not change with regard to untreated controls.

The apparent increase of DNA ligase activity could have alternative explanations like, for istance, the presence of DNA ligase inhibitors in control extracts or the presence of activators in the irradiated cells extracts. To investigate such possibilities, mixing experiments were carried out. Control and -48 h irradiated CV-1 extracts were mixed together or, separately, with purified T_4 DNA ligase at different concentration. In all cases, the enzyme activity measured was that expected by calculating the sum of the individual ligase activities of the mixed fractions. Thus it seems that the two-fold increase of the ligase activity cannot be ascribed to the action of the effectors.

In standard condition of assay, the two homopolymers constituting the substrate of DNA ligase are added so that the dA/dT ratio is 1 in order to maximize the extent of joining (19). However the hypothesis could be raised that transient "gaps" between two adjacent oligo dT in poly dA-oligo dT substrate might exist and might be filled by DNA polymerase, more or less, depending on possible different levels of this activity in the different extracts; i.e. that the substrate is modified while ligating enzyme level is unchanged in the different samples. To investigate such a possibility we have measured the ligase activity of cells irradiated at t = 48 h and control cells in the presence of an excess of partially purified α-DNA polymerase from CV-1 cells (about 20 times higher than the activity present in crude extracts). The relative ligase levels between irradiated and control cells

are not modified in these conditions. Since the substrate can be modified by degrading enzymes, we measured the levels of nucleolytic and dephosphorylating activities in the different extracts. The substrate (in which poly dA was about 250 nucleotide long) was incubated with cellular extracts at 37°C or 85°C in the conditions described in Methods. In control experiments, E.coli alkaline phosphatase (type III-S) and exonuclease III (0,2 units/ml) were used. As shown in fig. 4A, degrading activity measured at 37°C is about twice higher in irradiated cells at t = 48 h than in control cells. Such an activity was not detected in extracts incubated at 85°C. The comparison of the effects on ligase substrate at the two temperatures of exonuclease III, of alkaline phosphatase and of cellular extracts suggests that degrading activity of the last ones is due to nuclease(s). Since the degradation of ligase substrate leads to a reduction of joining rate (fig. 4B), the ligase levels, that we have determined in irradiated cells, are rather underestimated.

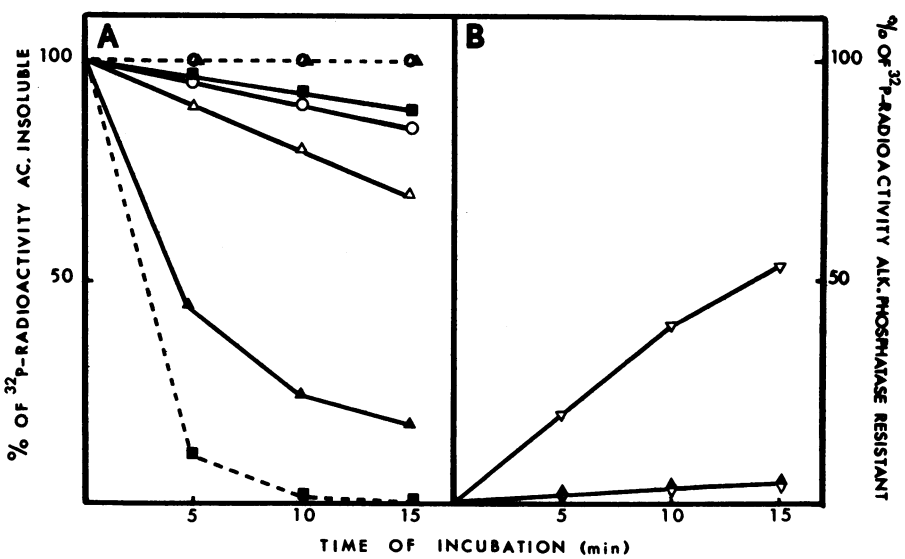

FIG. 4 (A) Percentage of acid-insoluble ^{32}P-radioactivity as a function of time of incubation with: extracts of control cells (○), extracts of irradiated cells (△), alkaline phosphatase (■) and exonuclease III (▲). Continuous and broken lines indicate 37°C and 85°C incubations respectively. (B)Percentage of acid-insoluble ^{32}P-radioactivity resistant to alkaline phosphatase, as a function of time of incubation with T_4 ligase (▽) and T_4 ligase mixed with exonuclease III (◆).

DISCUSSION

Trypsinization of stationary cells and their seeding in fresh medium causes the resumption of metabolic activities aiming the cell multiplication. Confirming previous reports on differently stimulated tissues or cellular cultures (1-5) we have observed with monkey kidney cells that the DNA ligase activity is increased in rapidly dividing cells if compared with its level in stationary cells.

Parallel studies in our laboratory have shown that α-DNA polymerase also increases in rapidly dividing CV-1 cells (R.Wicker et al. to be published), strictly connected with the increse of DNA ligase activity. A parallelism between the two activities was also reported by other authors in phytohemagglutinin-stimulated lynphocytes (1) and in virus-infected cells (3, 21, 22). The results obtained are thus in agreement with the model postulating that DNA ligase could act together with other enzymes, as DNA polymerases, thymidine kinase and RNAse H (3, 22, 18) during the phase of intense DNA synthesis.

Damage caused by UV-irradiation is repaired, in normal cells, by different mechanisms; except photoreactivation, all hypothetically implicate the action of nucleolytic, polymerizing and ligating activities. Our results also indicate that UV-irradiation of CV-1 cells may result in an important "de novo" synthesis of DNA ligase, in spite of the inhibition of semi-conservative DNA synthesis. Such an induction is dose-dependent in its extent and kinetics. These data show the existence of a good correlation between the level of DNA ligase activity following UV-exposure of cells and the occurrence, as previously demonstrated in the same cells (10, 11), of a DNA repair process which can be identified to the excision-repair mechanism. Such a correlation is strongly suggestive of a direct involvement of the ligating activity in the DNA repair process in eukaryotic systems as generally postulated. In this respect, it has been recently reported that a conditional lethal mutant of yeast, showing defects in DNA replication and an increased UV-sensitivity at non permissive temperature, has a low level of DNA ligase activity which disappears completely after a temperature upshift (24). To our knowledge, in mammalian cells, a genetic approach to this point has not yet given clear evidence of such an involvement.

Other enzymatic activities or functions, such as deoxyribonucleotide kinases (23) or the capacity to reactivate UV-irradiated viruses (24), are induced in CV-1 cells after UV-irradiation. They are considered to play possibly a role in DNA repair or to be a consequence of its occurrence. The

studies carried out in our laboratory on DNA polymerases α, β and γ in the same cells have revealed a constant level of the three activities during the 48 h following the irradiation (R.Wicker, to be published). We observe thus a complete dissociation of behaviour between DNA ligase and DNA polymerizing activities after UV-irradiation of confluent cultures; contrarily to what is observed in unirradiated, rapidly dividing cells in which they seem to be regulated in coordination.

Evidences of the existence of two different DNA ligases in mammalian cells have been reported (25-27). The preferential induction of the larger form of ligase has been observed during rat liver regeneration (4). Work is in progress in our laboratory to investigate the possibility of a specific induction of one of two forms after UV-irradiation. Similar experiments are also carried out on human cells of different origins.

ACNOWLEDGEMENTS

We are grateful to Dr G.Brun for suggestions in working out the DNA ligase assay and to Dr P.Yot for helpful discussion during the preparation of the manuscript. We thank Mrs C.Baroche for excellent technical assistance. This work was supported by grant n° 76-60 of Institut National de la Sante et de la Recherche Medicale and n° 76-7-1192 of Delegation Generale a la Recherche Scientifique et Technique

REFERENCES

1. Pedrini,A.M., Nuzzo,F., Ciarocchi,G., Dal Pra,L. and Falaschi,A. (1972) Biochem. Biophys. Res. Commun. 47, 1221
2. Tsukada,K. and Ichimura,M. (1971) Biochem. Biophys. Res. Commun. 42, 1156
3. Spadari,S. (1976) Nucleic Acid Res. 3, 2155
4. Söderhäll,S. (1976) Nature 260, 640
5. Tsukada,K., Hokari,S., Hayasaki,N. and Ito,N. (1972) Cancer Res. 32, 886
6. Fareed,G. and Richardson,C. (1967) Proc. Natl. Acad. Sci. U.S.A. 58, 667
7. Newman,J. and Hanawalt,P. (1968) J. Mol. Biol. 35, 639
8. Pauling,C. and Himm,L. (1967) Proc. Natl. Acad. Sci. U.S.A. 60, 1595
9. Cleaver,J.E. (1974) Advances in Radiation Biology (Lett,J.T., Adler,H. and Zeller,M. eds) Academic Press,New York, 1-75
10. Coppey,J. (1976) J. Gen. Virol. 32, 1
11. Nocentini,S. (1976) Biochim. Biophys. Acta 454, 114
12. Latarjet,R., Morenne,P. and Berger,R. (1953) Ann. Inst. Pasteur 85,174
13. Lowry,H.O., Rosebrough,N.J., Farr,A.L. and Randall,R. (1951) J. Biol. Chem. 193, 265
14. Burton,H. (1955) Biochem. J. 61, 473
15. Olivera,B.M. (1971) Methods in Enzymology 21, 311
16. Hartmann,K.V. and Heidelberger, C. (1962) J. Biol. Chem. 236, 3006
17. Henni,H.L. and Lubin,M. (1964) Science 146, 1474
18. Sawai,Y. and Tsukada,K. (1977) Biochim. Biophys. Acta 479, 126
19. Olivera,B.M. and Lehman,I.R. (1968) J. Mol. Biol. 36, 261
20. Sambrook,J. and Shatkin,A.J. (1969) J. Virol. 4, 719
21. Beard,P. (1972) Biochim. Biophys. Acta 269, 385

22. Nasmyth,K.A. (1977) Cell 12, 1108
23. Coppey,J.(1977) Nature 265, 260
24. Lytle,C.D. (1971) Int. J. Radiat. Biol. 19, 1138
25. Pedrali Noy,G., Spadari,S., Ciarocchi,G., Pedrini,A. and Falaschi,A. (1973) Eur. J. Biochem. 39 343
26. Teraoka,H., Shimoyachi,M. and Tsukada,K. (1975) FEBS Letters 54, 217
27. Söderhäll,S. and Lindhal,J. (1975) J. Biol. Chem. 250, 8438