EFFECTS OF IONIZING RADIATION ON THE METABOLISM OF DNA IN CULTIVATED MAMMALIAN CELLS

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Voor mijn ouders

CONTENTS

CHAPTER I

INTRODUCTION

The total amount of energy absorbed by a cell after a lethal dose of ionizing radiation is small in comparison to the marked effects that are produced. This together with the lack of extensive chemical changes in vitro with doses of a few hundred roentgens, - large enough to kill most mammalian cells -, has led radiobiologists to the tentative conclusion that changes in specific key molecules in the cell are responsible for radiation-induced lethal effects. Much work has therefore been done by numerous investigators on the effects of ionizing radiation on enzymes and other proteins and on nucleic acids. Particularly, effects on DNA, structures containing it and the metabolism of DNA, have also attracted much attention of workers interested in genetics because of the mutagenic effects of radiation. Many papers deal with attempts to correlate the effects of irradiation with DNA e.g. its base composition (1), the chromosome number and the state of ploidy (2, 3, 4, 5), nuclear volume and cellular DNA-content (6,7). Also there are many reports concerning the effects of irradiation on the metabolism of DNA which, shortly after irradiation, appeared to be strongly affected in many systems (as will be surveyed in Chapter IV).

In spite of the great amount of work done, the knowledge of the interaction between ionizing radiation and the metabolism of DNA has not progressed very far. The fields in which investigations have been carried out by the various authors can be grossly classified as a). cytochemical and radioautographic studies; b). biochemical research; c). studies on dose-relationships and d). studies with compounds externally introduced to the irradiated cells. In addition a great variety of cell types and cell systems has been used in this research.

It can be argued that the slow progress in understanding the correlation between DNA synthesis and radiation in the past years can be partly explained by the small exchange of ideas between workers using different approaches to the problem.

The aim of this investigation is to study early effects of roentgen rays on the metabolism of DNA in one particular cell system. The purpose of the present research has not been to introduce a new method but to develop a hypothesis sufficient for explaining various results obtained when cells are studied by use of different techniques. The

system chosen for study was an *in vitro* culture of mammalian cells. Besides having definable growth characteristics, cultures are also uniform with respect to cell type and are not subject to variations imposed *in vivo* by nerve or hormonal supply. The report is devided into: effects of irradiation on the mitotic cycle (Chapter III), effects on the incorporation of radioactive precursors into the DNA (Chapter IV) and the reduction of these effects by treatments with exogenous DNA (Chapter V). Chapter VI summarizes research designed to further elucidate the theory developed in the preceding chapters.

CHAPTER II

MATERIALS AND METHODS

a. Cell culture

The cell strain used in these investigations was established in our laboratory from fetal calf liver in 1958 (8). It is believed to be still parenchymic by nature. For maintenance the cells were cultivated as monolayers in Carrel flasks of 7 cm diameter with 5 ml of medium. Media were changed every 2-3 days and also 18 hours before the beginning of an experiment. The line was subcultured each week by trypsinizing the full grown cultures and diluting the cell suspensions with 10 volumes of fresh medium. For this purpose, 5 ml of trypsin (DIFCO) were added to the flasks as a sterile 0.25% solution in buffered physiological medium. The culture medium for the substrain Lele was always freshly prepared before use and consisted of Hanks' balanced salt solution containing 0.5% lactalbumine hydrolysate (NUTRIONAL BIOCHEMICALS CORPORATION - CLEVELAND) and 25% unheated calf serum. All salts used in the preparation of the solutions were delivered by MERCK and were of analytical grade. To 100 ml of complete medium, 5000 units of penicilline and 5 mg streptomycine were added (both delivered by MYCOFARM-DELFT).

Some preliminary experiments were performed with a subline of this culture, - the strain LeVe-f -, that was cultivated with 5% serum and fructose instead of glucose (Chapter III, figures 1 and 2). All other experiments were carried out with the substrain Lele.

For experimental series smaller vessels were used with a diameter of 5 cm, filled with 3 ml of the culture medium. These Carrel flasks were covered with loose glass plates which were uniform 1 mm thick. The plates were removable and permitted insertion and removal of the glass slides used in radioautography (see section c).

b. Irradiation procedures

Irradiation was performed with an X-ray tube. For the preliminary experiments with the strain LeVe-f, irradiation was performed with a PHILIPS therapeutic instrument in the RADBOUD HOSPITAL in NIJMEGEN, operating at 250 kV with 1.5 mm Thoraeus filtration and a dose - rate of 125 roentgen/minute. All other irradiation was given in our laboratory with a MACHLET X-RAY TUBE, type OEG 60, operating at 50 kV and 30 mA. The distance from the tube to the bottom of the

vessels to be irradiated was 20 cm. During irradiation the vessels were partly submerged in a waterbath at 37°C as was done also during the administration of sham-irradiation to the controls. The transport of cultures to and from the irradiation room was accomplished in a portable incubator (INVENTUM) maintained at 37°C. Filtration of the radiation was achieved by the glass plate of the vessels only. In order to calibrate the rate of ionization, a hole was fixed in the bottom of a Carrel flask similar to that used for the cultures and a PHILIPS UNIVERSAL DOSIMETER inserted in the position normally occupied by the cells. Under the conditions described the rate was measured at 405 roentgen/minute with a standard error of $\pm$ 8.1 (about 2%). No corrections for backscattering from the bottom were made, the real doses to the cells were therefore somewhat higher than those stated. This increase however, is proportional to the reported doses. Through the inherent filtration by the X-ray tube and the additional filtration by the glass covers of the vessels, a radiation beam was obtained with a H.V.L. comparable to 1.8 mm Al.

c. Radioautography

Radioautographs were generally prepared from cells on coverglasses two days after subculturing. To collect cells for this purpose, small coverglasses (24x24 mm) were put into the culture vessels before adding the diluted cell suspension. Part of the cells attached to the surface of the coverglasses and these could be easily removed from the vessels by detachment of the loose glass plate on the top. The cells were exposed to the labelled compound by incubating the cultures in a normal medium containing the tracer. Cell labelling was accomplished in two different ways. The first, here referred to as pulse-labelling, was one in which cells were exposed to ^{3}H-TdR for a period, - usually 15 - 20 minutes -, relatively short in comparison with the DNA duplication time of 8 - 9 hours (see next Chapter III). A second labelling over a period of several hours will be designated by the term continuous-labelling.

The amount of the radioactive thymidine in the medium was at least 0.25 μ C/ml but higher amounts up to 1 μ C/ml were used as well. At the end of the incubation period, the cover glasses were taken from the culture vessels, washed in Hanks' solution and fixed for 5 minutes in absolute alcohol - acetic acid (3 : 1). The coverglasses were then stored in 80% alcohol for at least 30 minutes, washed in tap-water during 20 minutes and dried in air. Slides (76 x 26 mm) were dipped in a mixture of gelatin (1%) and chromalum (1%) at 60°C and dried. The coverslips with the attached cells were dipped in this solution after it was diluted with 1 volume of distilled water, and then mounted on a coated slide with the cells on the upper surface. The preparation was then dried in a stream of air at room temperature. The

preparations were processed according to the stripping film technique, using the photographic material KODAK AR-10. After suitable exposure, the radioautographs were developed with a solution of KODAK D19b for 5 minutes, fixed and rinsed for 30 minutes in running tap-water. Nuclei were stained through the film with acid hæmalum (M a y e r) for 20 minutes, the staining solution was washed out in running tap-water during 10 minutes and the preparations were dehydrated in 70% alcohol. All solutions used for photographic processing and subsequent staining were kept below 17°C to prevent displacement of the film. After drying in a stream of air, the preparations were mounted under a coverslip with euparal.

d. Statistical aspects

The determination of percentage labelled nuclei was based on 500-1000 total cells in each preparation by scoring all labelled and non-labelled cells in 25-40 randomly chosen microscopic fields. Most of the reported values are based on counts from 3 or 4 preparations making a total of 2000 or more cells. The total number of labelled cells scored for any value reported, was never below 600.

In general the maximum separation between extreme values based on comparable radioautographs in our series did not exceed 15% while, when contrasting dissimilar experimental groups of radioautographs, no definite conclusions were drawn unless the percentages of labelled cells to be compared were not at least 20% different.

In deciding wether cells were labelled, exposure times were chosen to give at least 20 grains per labelled nucleus in the experimental preparations while the corresponding thymidineless blanks showed a mean background of 1.5, and no more than 4 grains per nucleus. With a minimum difference of 16 grains between labelled and non-labelled cells, positive ones were easily recognized.

e. Isotopes and measurement of radioactivity

^{3}H-TdR - Thymidine-deoxyribonucleoside with a tritiated methyl group. Specific radioactivity 3.0 C/mMol (SCHWARTZ, N.Y.)

^{14}C-TdR - The same compound labelled at the 2nd C-atom of the pyrimidine ring. Specific radioactivity 30.0 mC/mMol (PHILIPS-DUPHAR, AMSTERDAM)

^{32}P - Carrier-free as KH_2PO_4 (PHILIPS-DUPHAR, AMSTERDAM)

Tritium radioactivities were measured with a window-less gasflow counter (K.LANGE, HEIDELBERG), operating at 4300 V with methane (commercial grade). ^{14}C- and ^{32}P-radioactivities were counted with a G.M.-tube (PHILIPS end-window tube, type 18505) at 525 V. Samples

were brought in liquid form to aluminium planchets (diameter 2.5 cm) and dried by evaporation. Their activities were compared by making 10,000 counts with the help of the "10,000 preset" switch and recording the corresponding times.

Chemical procedures are mentioned in the text of the corresponding experiments.

f. Abbreviations

DNA	- Deoxyribonucleic acid
RNA	- Ribonucleic acid
TMP, TDP, TTP	- Thymidine deoxy - ribonucleoside mono-, di- and triphosphate
P_i	- Inorganic phosphate
PCA	- Perchloric acid
TCA	- Trichloracetic acid

CHAPTER III

EFFECTS OF IRRADIATION ON THE MITOTIC CYCLE

§ 1. INTRODUCTION

In animal cells the synthesis of DNA is restricted to a period of limited duration in the mitotic cycle (9, 10, 11). Thus, if an exponentially growing culture is irradiated, only a fraction of the irradiated cells is engaged in the synthesis of DNA. Any disturbance in the normal execution of the mitotic cycle would be expected to give marked alterations with time in the fraction of cells synthesizing DNA. If, for example, the only effect of irradiation were a blockage of cells in a phase preceding that of DNA synthesis, radioautography performed some hours after irradiation would reveal a lower percentage of positive nuclei. The result then seemingly indicates an effect on the DNA synthesis itself, although this synthesis in the individual synthesizing cell is not influenced at all.

These effects on the population of cells are likely to occur after irradiation and have to be well understood to avoid misinterpretations of biochemical data. In this chapter experiments will be described that deal with the normal processes in the mitotic cycle and with alterations induced by irradiation, - especially with regard to processes concerned with the synthesis of DNA.

The knowledge of the mitotic cycle has greatly increased since the use of radioactive isotopes, - particularly of labelled organic compounds -, in biological research. Among these, labelled thymidine has found widespread application because it is specifically incorporated into DNA only. After its incorporation, the thymidine can be readily demonstrated in the cells by radioautography. Incubations of log-phase cells from an asynchronous culture in ^{3}H-TdR for 15 minutes, yields only a limited number of positive nuclei. This suggests that DNA synthesis is limited to a relative short time interval between two mitoses.

By means of radioautography, the duration of the various phases of the cycle can be analysed and this timing has been done for different materials (e.g. 9, 10, 11).

§ 2. RESULTS

a. Changes in the mitotic cycle

The mitotic cycle of the substrain LeVe-f of our liver cells has been

previously analysed (12) and is graphically summarized in Fig.1. The different parts of the cycle and their corresponding mean durations in hours are shown.

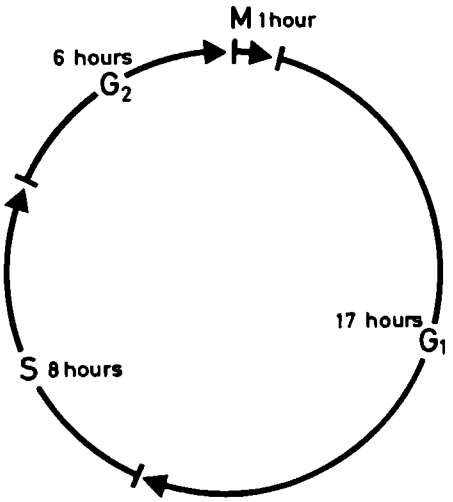

Fig. 1. The mitotic cycle of the LeVe-f strain with its different phases and the mean duration in hours of each phase (after ref. 12).

G_1 and G_2 are phases in which no DNA is synthesized, in S the DNA-content of the cells is doubled in preparation of mitosis M. The entire division cycle of the substrain Lele, which was not analysed in our laboratory in detail, requires about 34 hours for completion (personal communication by A.C.M. Pieck). In this asynchronous, exponentially growing culture, the percentage of cells in each phase of the division cycle remained essentially constant. For example, the percentage of cells in the S-Phase remained constant (32-34%) while the fraction of cells in mitosis in all cases was between 2.4 - 3.0%.

That irradiation affects the asynchrony of log-phase LeVe-f cells in such a manner that the percentage of cells in the S-phase becomes inconstant, is shown in the following experiment.

> Two days after subculturing, Carrel flasks containing cells on coverslips were irradiated (X-ray; 100 r; 250 kV) and at intervals following irradiation cultures were pulse-labelled with ^{3}H-TdR in a concentration of 0.25 μ C/ml. After 4 weeks of exposure, the radioautographs were developed, stained and the percentages of labelled nuclei tested.

In samples pulse-labelled few hours after irradiation, the percent positive nuclei was significantly higher than in the controls while sam-

ples taken at later intervals gradually returned to normal (see Fig.2).

Because of this clear effect, in all subsequent experiments label was given within 6 hours after irradiation. These experiments were all carried out with the substrain Lele. Very few degenerative cells were seen after irradiation within this short period, indicating that extensive death of cells had not occured (less than 1%).

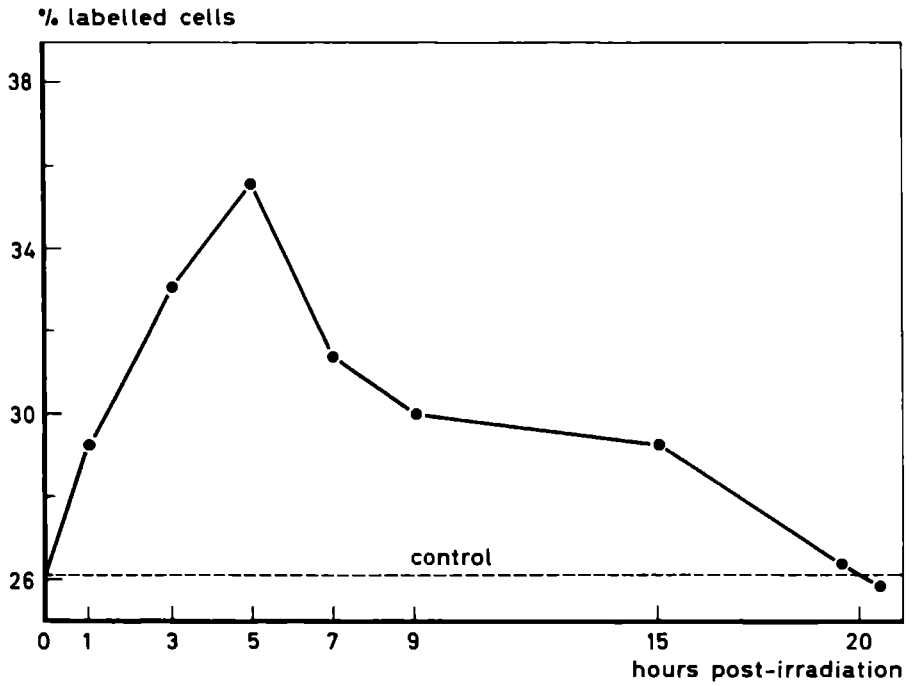

Fig. 2. The changes of the fraction of cells in S-Phase in the substrain LeVe-f, after a dose of 100 r. The mean values of 3 determinations for each point are plotted.

Mitosis was also affected by irradiation. By plotting the percent of dividing cells against the time elapsing subsequent to administration of X-ray (100 and 1,000 r of 50 kV), it is seen that the treatment induces a reduction in frequency of observed dividing cells.

However, the occurence of a similar depression in mitotic activity in sham-irradiated cultures suggests that factors other than irradiation were responsible as well (see Fig.3).

This welknown mitotic delay, caused by a block in G_2-phase (14, 27, 28), could eventually give an inhibition of growth great enough to give alterations in the relative number of cells in S-Phase. If mitosis were the only stage blocked, the fraction of cells found in the S-Phase sub-

sequent to treatment might be expected to increase asymptotically in compensation for the absence of newly-divided cells which normally "dilute" the whole population in non-irradiated controls.

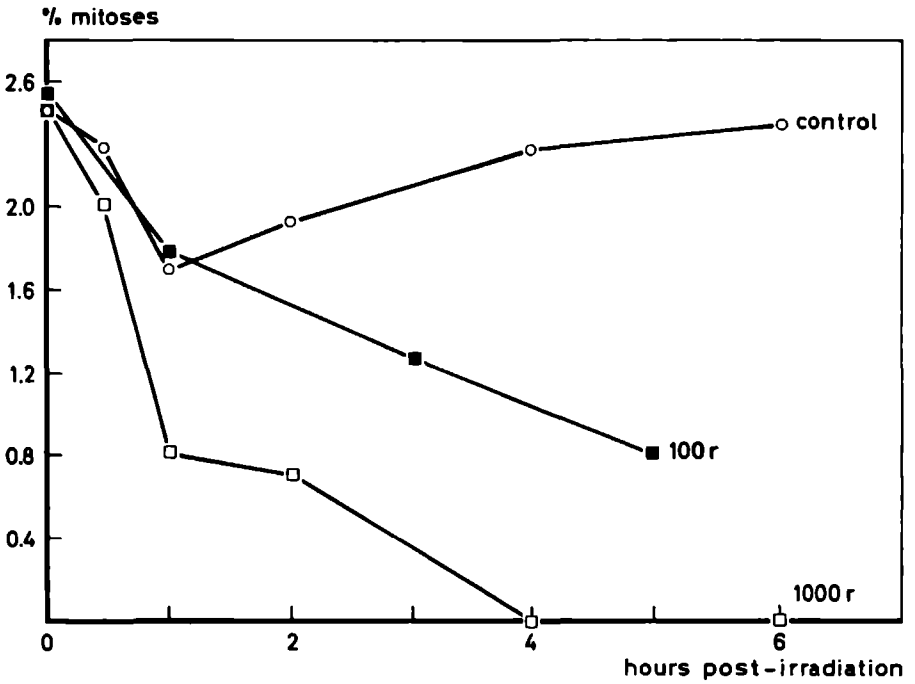

Fig. 3. Mitotic indices in cultures plotted against time elapsing subsequent to irradiation. Percentages were calculated from 3 preparations for each point of irradiated and control series. In each slide 1,000 cells were counted.

However, a net increase in the total number of cells in the sham-irradiated cultures was not observed during 6 hours, a result probably attributable to a washing effect contingent on a depression of mitotic activity similar to that found in X-irradiated cultures (see Fig.3). For example, the total number of cells in cultures sampled by trypsinizing in 0.9% NaCl 6 hours after irradiation (5000 r), was not significantly different from counts made from sham-irradiated controls (standard deviation = 4%).

Also colchicine (0.05 g/l for 6 hours, inducing simulation of irradiation effects on mitosis, failed to reveal a percentage of labelling different from the controls. So the growth-inhibiting effects of irradiation in this short time (6 hours) cannot account for changes in the fraction of labelled cells similar to the changes reported in Fig.2.

b. Changes in the fraction of cells synthesizing DNA

The observed increase in the fraction of cells in S-Phase, as was reported in Fig.2, has been restudied with the substrain Lele. This study was restricted to periods not exceeding a time of 6 hours after irradiation to prevent complications from celldeath and inhibition of growth. From the foregoing (§ 2, a) it was concluded that these factors don't interfere with the fractions of labelled cells to a measurable degree within this period of time after irradiation with doses up to 5000 r.

First the increase of cells in S-Phase with time was measured after some doses of irradiation.

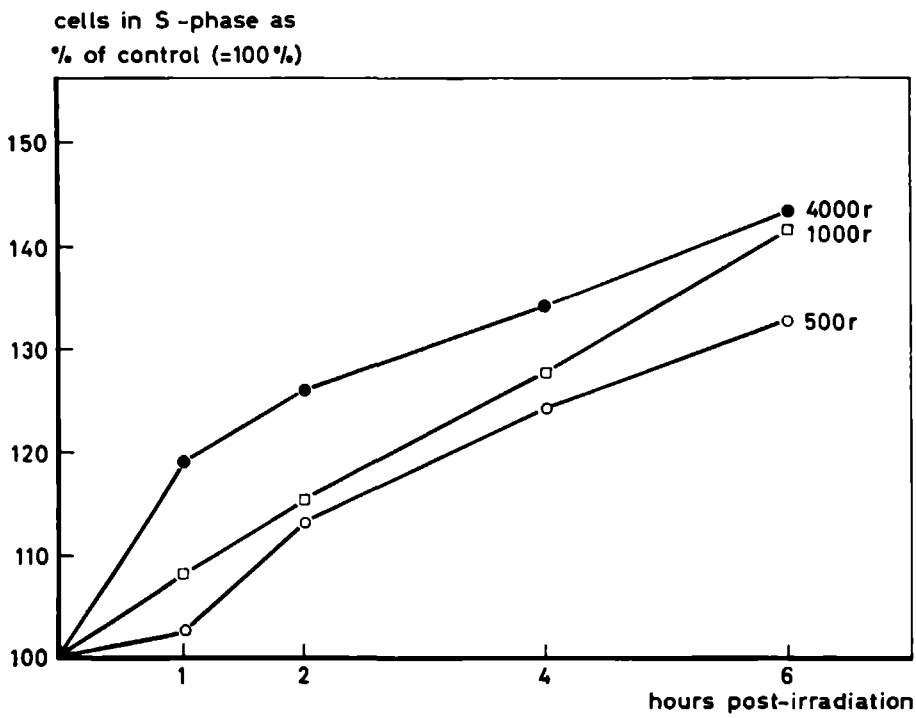

Fig. 4. The radiation-induced increase in the fractions of cells synthesizing DNA, expressed as percentages of control values (32.6% of all cells), the latter being set at 100%. Pulse-labelling was performed at different times after irradiation on 3 cultures from each control and irradiated group. Irradiation doses are represented in the graph.

2 Days after subculturing, culture vessels with coverslips were irradiated in groups of 12 with 500, 1000 and 4000 r, while controls were sham-irradiated. At different times after irradiation, 3 cultures from

each group were used for pulse-labelling of 15 - 20 minutes in a medium containing ^{3}H-TdR in a concentration of 0,5 μ C/ml. After 4 weeks of exposure, the radioautographs were developed and prepared for microscopic analysis as described above. The fractions of labelled cells were scored and the mean values from 3 separate determinations were expressed as percentages of the corresponding controls, the percent value of the latter being set at 100. Values are reproduced graphically in Fig.4.

From this experiment it appears that there is a rise in the fraction of cells synthesizing DNA. This increase depends on the dose applied and the time elapsed after irradiation. As will be discussed later on, this phenomenon has been observed by many authors in other cell systems as well, but there exists no consistent explanation for the effects studied.

Theoretically there are three principal explanations:

1. The increase is only relative because the S-population still increases for some time after cell division has stopped.
2. In the irradiated cultures, more cells enter the S-Phase from the G_1-Phase.
3. After irradiation cells in S-Phase continue with the synthesis of DNA for a longer time. As the fraction of time spent in synthesis increases, so will the fraction of cells in that synthetic phase. In this case the transition of cells into G_2-Phase will be decreased.

The possibility mentioned under 1. must be of minor importance during the timespan used in our experiments as mentioned already (§ 2, a).

To check if irradiation induces an increase in the number of cells initiating synthesis of DNA, experiments were performed in which cell cultures were irradiated, directly covered with ^{3}H-TdR medium and kept there, at the same time with controls, continuously untill sacrified for radioautography.

2 Days after subculturing, a number of cultures with cells on coverslips were irradiated. Immediately after the end of the irradiation, the ordinary medium was replaced with one containing 0,25 μ C/ml ^{3}H-TdR.

For determination of % labelling, 3 cultures from each dosage group were sampled at spaced intervals and prepared for radioautographic analysis.

Radioautographs were developed after an exposure time of 3 weeks. Mean value and irradiation doses are reproduced in Fig.5.

The increase with time in the fraction of labelled cells during a continuous-labelling, represents the rate at which cells enter the S-Phase. In the dose-range from 0 - 4000 r, no difference between control and

irradiated cultures was observable within a period of 6 hours following irradiation.

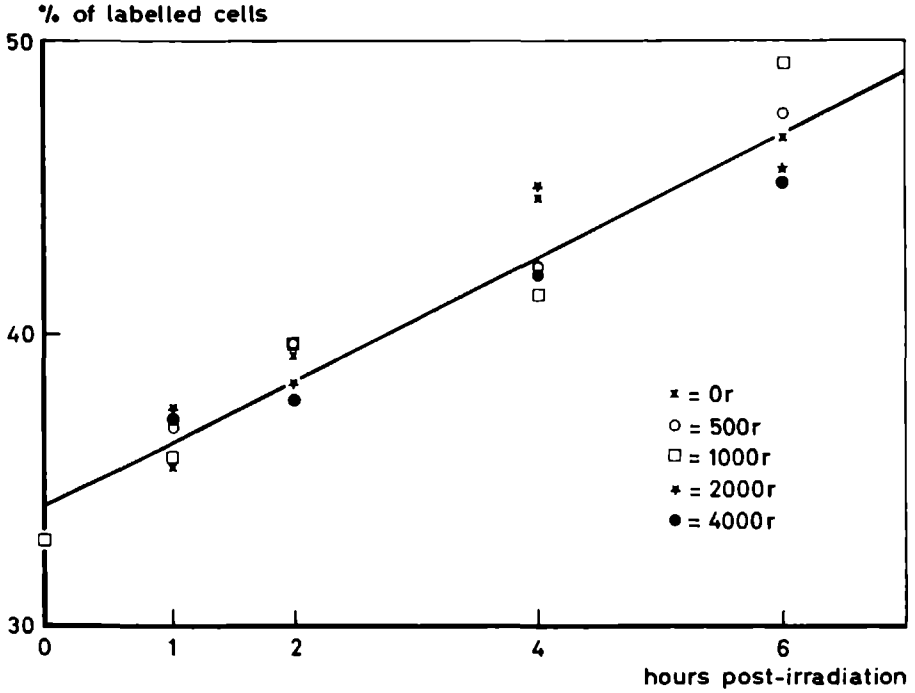

Fig. 5. The percentage of labelled cells in control and irradiated cultures incubated immediately after irradiation in medium containing radioactive thymidine. The mean values of three determinations are plotted in this graph.

It may therefore be deduced that in our material the effect of irradiation is a lengthening of the S-Phase and through this an increase of the number of cells in that phase. It also implies that after irradiation cells might be entering the G_2-Phase at a reduced rate. This phenomenon can be measured by a rather simple method, based upon the following principle:

> When a culture is continuously labelled with ^{3}H-TdR for a definite period of time, all cells that are in, have been in or come into S-Phase during this period will be recognizable in radioautographs. The fraction of cells continuous-labelled during t hours will be designated as C_t. By pulse-labelling with ^{3}H-TdR a similarly treated culture for 15 minutes at t hours after the beginning of the experiment, P_t is obtained, i.e P_t = % of cells actually in the S-Phase at that time. Consequently, the difference C_t - P_t will represent the percentage of cells that left

the S-Phase by entering the G_2-Phase. In Fig.6 this calculation is schematically illustrated.

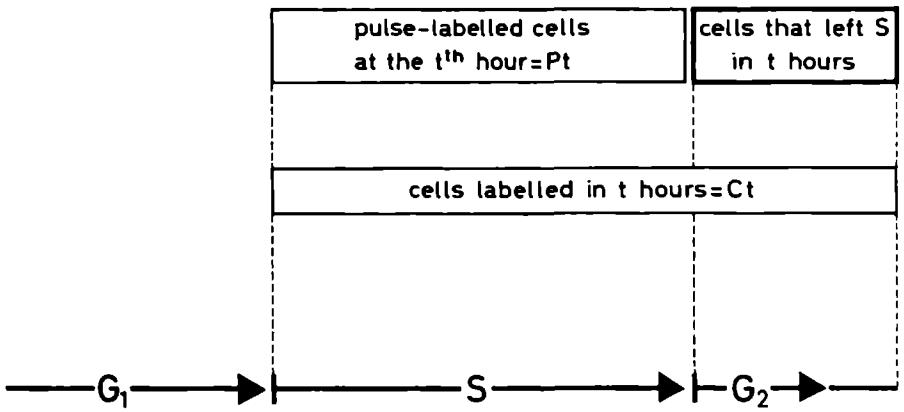

Fig. 6. Diagram demonstrating that the number of cells entering the G_2-Phase in t hours can be calculated from the difference C_t - P_t.

After 2 days of growth, groups of 8 cultures with cover glasses were irradiated with 250, 500, 1000, 2000, and 4000 r and also 8 cultures were sham-irradiated for controls. 4 Cultures of each group were allowed to incubate for 6 hours after which they were pulse-labelled in medium containing ^{3}H-TdR (0.5 μC/ml).

The other 4 cultures were continuously labelled during the entire 6-hour period following exposure to X-ray. Radioautographs were exposed for 5 weeks and the mean values C_6 (fraction of cells labelled after continuous exposure to ^{3}H-TdR for 6 hours) and P_6 (fraction labelled following a pulse-labelling given 6 hours after irradiation) were scored. These values and the difference C_6 - P_6 are reproduced in Fig.7.

As can be seen in Fig.7, the mean values of C_6 are the same for control and irradiated cultures, again stressing the fact that irradiation does not accelerate the transition of cells into the S-Phase (see also Fig.5). The fraction of cells, however, actually in this phase at the end of the experiment (= P_6) increases with increasing doses of X-ray, thus specifying a dose-dependent decrease in the fraction of cells that enters the G_2-Phase during the first 6 hours following irradiation. (= C_6-P_6).

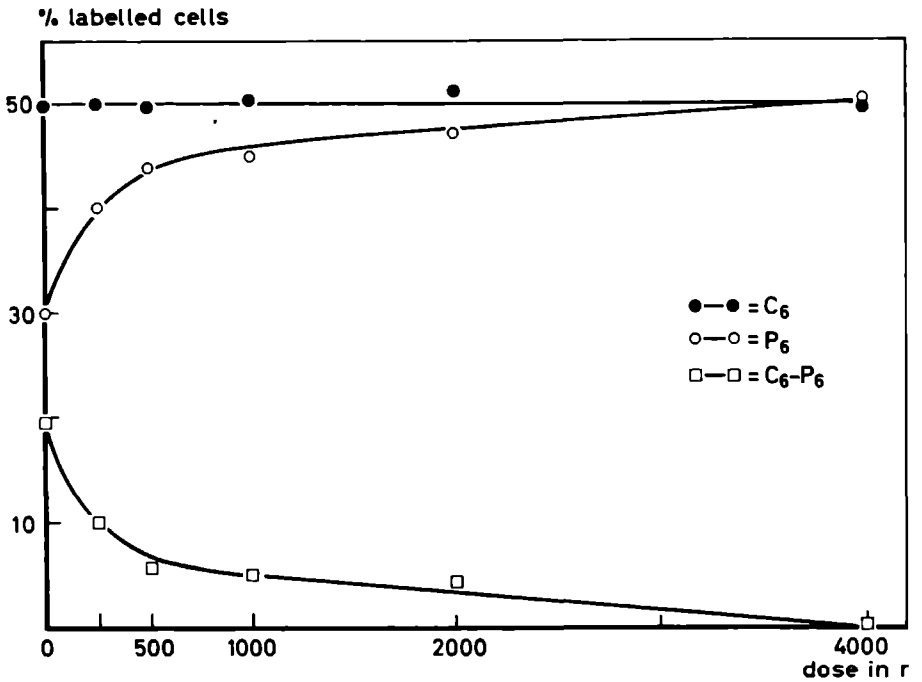

Fig. 7. C_6, P_6 and C_6 - P_6 in relation to the dose of irradiation. C_6 - P_6 represents the fraction of cells which enter the G_2-Phase during the 6-hour period subsequent to irradiation. Details in text.

c. Changes in the intensity of labelling

It was observed in all radioautographs, comparable with regard to the concentration of ^{3}H-TdR used for pulse-labelling and with equal exposure times before development of the film, that irradiation reduces the number of silvergrains over the nuclei of labelled cells. This indicates that the irradiated cells do not incorporate as much of the radioactive compound into their newly synthesized DNA per unit time as do unirradiated cells. It has been observed that the impairment of tritium uptake is more pronounced after exposure to higher doses and also, that there is less label in nuclei from samples taken long after exposure to X-ray than in those pulse-labelled with ^{3}H-TdR immediately after irradiation.

Because of the low energy of the electrons emitted by the tritium isotope (0.017 MeV; mean range in water : 0.001 mm), radioautography involving grain counts in nuclei labelled with ^{3}H-TdR probably does not give a true index of DNA radioactivity. Any change in the shape of structures containing DNA, such as swelling, clumping or the changes

that accompany the proces of mitosis, can produce variation in the amount of tritium-produced radiation absorbed by the cell and consequently in the grain count of this cell without any alteration of the total radioactivity present in the DNA. Therefore, the measurement of the rate of incorporation of radioactive precursors into the DNA will be done according to procedures to be described in the following chapters.

As mentioned in Chapter II, § 2, d., the observed reduction in the incorporation of thymidine from the medium was taken into account when preparing radioautographs. Such exposure times as well as concentrations of labelled thymidine were always chosen for obtaining an accurate discrimination between labelled and unlabelled cells.

§ 3. DISCUSSION

In the literature nearly all possible changes have been described concerning the effects of irradiation on the transition of cells through the S-Phase. No specific changes at all were noted by Whitmore et al. (13) in strain L mouse cells after doses up to 5000 r. Variations in the number of cells synthesizing DNA could be attributed to a block in the G_2-Phase. There was no accumulation of cells in S-Phase, only a relative increase in the number of cells synthesizing DNA. Howard and Pelc (14) and Lajtha et al. (15) produced a block in the G_1-Phase of cells from bean root meristem resp. bone marrow by irradiation, which resulted in a delayed entry into the S-Phase, - a result not in agreement with those reported by Kuyper et al (16, 17) and by Das and Alfert (18). These authors concluded that the observed increase in number of cells synthesizing DNA after irradiation was the result of an accelerated G_1 to S transition. Painter (19, 20) attributed a similar increase of positively labelled HeLa cells to a prolonged S-Phase. He named this irradiation-produced prolongation the "S-retention effect". Using a subline of strain L mouse cells, Mak and Till (21) found reduced rates of S to G_2 as well as G_1 to S shifts after irradiation, but reported different degrees of radiosensitivity for S to G_2 and G_1 to S transitions. Because of the differences in the radiation-induced pertubations in proliferating cell populations, Davies and Wimber (22) concluded that there exists a dose-, species- and probably a tissue-specificity in sensitivity to qualitative and quantitative changes induced by irradiation.

It is clear that the effect of irradiation on our cell line can be explained also as a "S-retention effect" without a reduced or increased transition of cells from G_1 into S. This statement, however, is only valid within a dose-range from 0 - 4000 r and for relative short periods

after irradiation (up to 6 hours). Under these circumstances no secondary changes can play a role as a result of the block in G_2 or of cell-death.

Since, at the present, precise quantitative data concerning rates of DNA synthesis in individual irradiated cells are not available, little can be concluded concerning the reason for prolongation of the S-Phase after irradiation. The rate of synthesis seems to be depressed as seen from the reduced incorporation of tritiated thymidine in the radioautographs. Therefore, the lengthening of the S-Phase might be ascribed at least to the potential of (even lethally) irradiated cells to synthesize premitotic levels of DNA, however at a reduced rate, according to Kelly (23), Casperson (24) and Whitfield (25).

The observed S-retention effect has implications for our further work. In determining the extent of radiation-induced changes in the rate of DNA synthesis, it seems appropriate to distinguish between the amount of DNA synthesized in a certain time by the whole population and by the individual cell in S-Phase - i.e. the rate of synthesis in a single cell in S-Phase would be expected to be lower than inferred from the measured synthetic rate of the whole population. This complication has not been often considered in literature - particularly where the DNA synthesis of whole organs is studied after irradiation (e.g. 26).

CHAPTER IV

THE INCORPORATION INTO THE DNA OF IRRADIATED CELLS OF RADIOACTIVE PRECURSORS

§ 1. DOSE-EFFECT RELATIONSHIPS

a. Introduction

The rate at which radioactive precursors are incorporated into the DNA of different cell systems after exposure to ionizing irradiation has been investigated by many authors; the compounds studied include, for example, formate -1-^{14}C (29), 5-iododeoxyuridine with ^{131}I (30) or ^{32}P (14, 31, 32 and 33) and thymidine as ^{3}H- or ^{14}C-TdR (e.g. 16, 34, 35 and 36). In these experiments, incorporation was measured as radioactivity in the cell fraction insoluble in cold acid (37), as specific activity of extracted DNA (38) or by means of quantitative radioautography (i.e. by counting silvergrains over nuclei labelled with ^{3}H-TdR - see, e.g. 36 and 39). It has been tacitly assumed by most authors that, in their experiments, the rate of label uptake is directly correlated with the rate of DNA synthesis in irradiated and control cells. In spite of differences in the radiosensitivity of the various cell systems studied, reports of curves relating dose to observed effect are in fair agreement with each other (29, 31, 37, 38, 40, 41, 42, 43, 44 and 45) - that is, reduction in the rate at which DNA takes up precursors following irradiation is an inverse diphasic function of increasing dose. On the basis of the uniformity in the reported results, Looney et al. (40) have concluded that radiation-induced impairment of DNA synthesis is fundamentally equivalent in all cell types studied. A generalized semi-logarithmic plot of this dose-response relationship is shown in Fig.8.

To simplify all subsequent discussion in the present report, the following terminology will be used uniformly throughout. The "S-effect" will be defined as the reduction in the rate of precursor incorporation into DNA which is induced by irradiation during synthesis, whereas the "G_1-effect" is a similar retardation of incorporation rate induced, however, by irradiation during the G_1-Phase (even though measured in the S-Phase immediately following - see ref. 14). The two arms on either side of the point of inflection in the semi-logarithmic plot of the "S-effect" dose-response curve are termed the S_1- and S_2-components. Lajtha et al. (29) have proposed that these two components of the curve represent impairment of two different mechanisms involved in the synthesis of DNA, each with its own characteristic sensitivity to

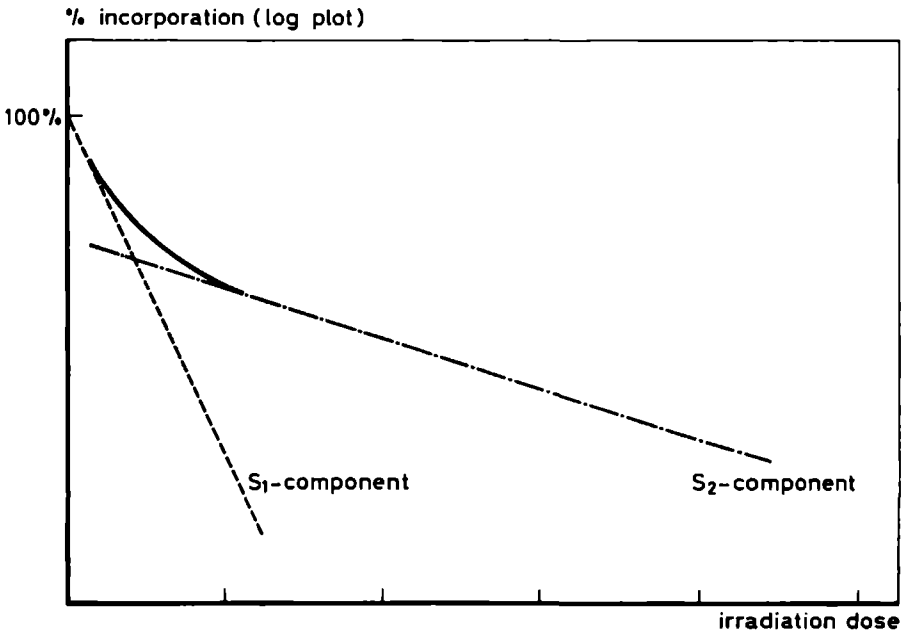

Fig. 8. Schematic representation of a dose-effect curve in semi-logarithmic plot, showing the extrapolated S_1- and S_2-components.

irradiation - the irradiation-induced modifications on the two mechanisms will be termed the S_1- and S_2-effects respectively.

The purpose of the present research was to examine the effect of increasing doses of irradiation on ^{14}C-TdR uptake into DNA of the Lele cell line. Specifically investigated were:

1) the shape of the dose-effect curve;
2) possible change in the shape of this curve with time elapsing after irradiation and:
3) distortions in the curve obtained when dose-effect data are plotted from irradiated populations of the type described in the previous chapter.

b. Results

Cells in Carrel flasks were irradiated according to the procedures outlined in chapter II. Normal culture fluid was replaced with prewarmed (37°C) ^{14}C-TdR-containing medium (10^{-6}M thymidine, labeled to give 0.03 μC/ml with a specific activity of 30 mC/mMol). Replacement of the medium was conducted in a walk-in incubator held at 37°C. At the end of the incubation period, the ^{14}C-TdR medium was decanted and the cells were trypsinized and centrifuged. They were then washed consecutively in 5 ml of each of the following solutions

(5 minutes per wash): 96% alc., 70% alc. and two times in 96% alc.-ether (3 : 1). RNA was removed by incubation for 18 hours in 0.5 N perchloric acid (= PCA) at 4°C, the resulting suspension was centrifuged and the pellet washed with cold 0.5 N PCA.

Extraction of DNA was finally accomplished by allowing the cell suspension to stand for 45 min. in 2 ml of 1.0 N PCA at 70°C. After cooling, 2 ml of 1 N KOH were added to the suspension and the resulting mixture allowed to stand for 30 min. in the cold. After this, cell debris and insoluble potassium perchlorate were spun off, 2 ml aliquots were removed from the supernatant for estimation of DNA (Burton's modification of the diphenyl-amine test was used - see ref.46) and 0.5 ml amounts were pipetted in duplicate on to aluminium planchets for counting the ^{14}C. The specific activities were calculated as counts per minute/mg DNA (= c.p.m./mg DNA). Since self-absorption should be the same in experimentals as in controls, these calculations were used directly without corrections.

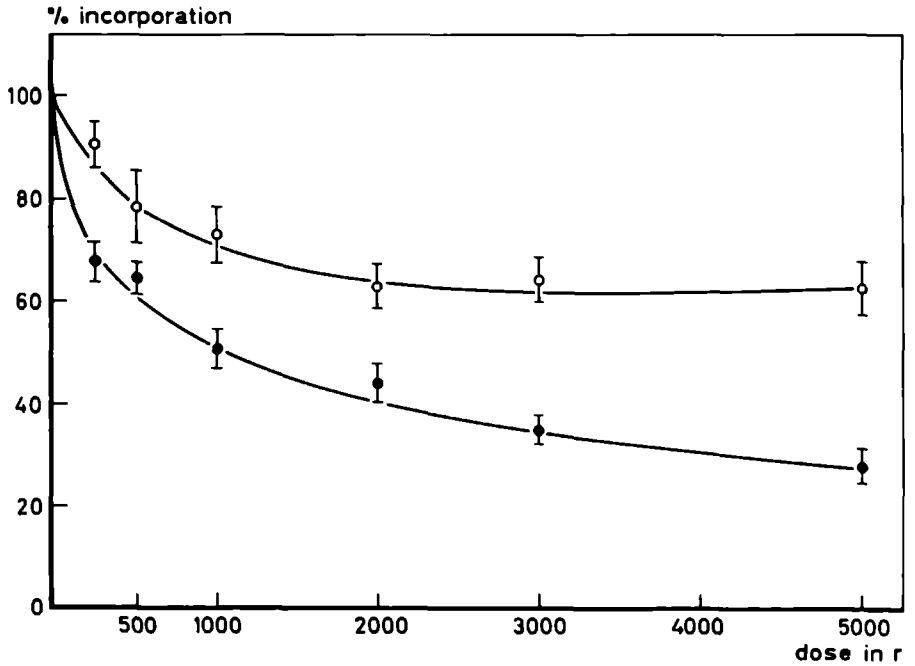

Fig. 9. The rate of ^{14}C-TdR incorporation into DNA expressed as the specific activity of the DNA extract made from cells incubated in labelled medium for one hour. Cells were incubated in tagged medium for a one-hour period beginning either 30 min. (= o——o) or 3.5 hours subsequent to irradiation (= ● —— ●). Each point and its accompanying standard deviation is based on 6 independent determinations and is given as percent of the activity found in sham-irradiated controls.

Using the above described recipe, the specific ^{14}C-TdR activity was measured in DNA extracts derived from Lele cells which had received

varying doses of X-ray and subsequent had been incubated for one of two intervals (two one-hour intervals were tested - one beginning 30 min. and the other 3.5 hrs subsequent to X-ray exposure). The results of these experiments are plotted in Fig.9.

The retardation in rate of ^{14}C-TdR incorporation was inversely proportional to the dose applied and was more evident, after all dosages given, in DNA extracts from cultures labelled 4 hours after exposure to X-ray than in those receiving label in the one-hour interval elapsing between 30 and 90 minutes post-irradiation. It should also be emphasized that the curves in Fig.9 do not represent decline in ^{14}C-TdR incorporation at the level of individual cells, but rather reflect the impaired uptake in the culture as a whole; for example, since irradiation exposure results in an *increase* in the number of cells actively synthesizing DNA (see Chapter III), the decrease of radioactivity in single cells in S-Phase would be more pronounced than inferred from the curve in Fig.9. In order to discover the rate of thymidine incorporation *per cell* it was therefore necessary to repeat the above-described labeling procedures for both intervals of incubation, using ^{3}H- TdR instead of ^{14}C-TdR and making radioautographs.

In this experiment the cultures were pulse-labelled during 20 minutes at the 1st and the 4th hour post-irradiation for determination of the mean number of cells synthesizing DNA during the intervals of incubation in the foregoing experiment (see Fig.9) after the same doses of X-ray. Results are summarized in Table I.

TABLE I

Dose in r	Percentages of pulse-labelled cells:	
	1 hour post-irrad.	4 hours post-irrad.
0	32.4 %	32.6 %
500	33.6 %	40.7 %
1000	34.1 %	41.2 %
2000	36.4 %	43.9 %
3000	35.4 %	45.2 %
5000	36.4 %	47.9 %

The fractions of cells synthesizing DNA at the 1st and the 4th hour following exposure to different doses of X-ray. Recorded values are based on analysis of radioautographs from 2-day old cultures, pulse-labelled with ^{3}H-TdR at the stated times post-irradiation.

The rate of thymidine incorporation per synthesizing cell is reported as specific radioactivity (from Fig.9) divided by the fraction of labelled cells (from Table I) for each dosage and period of incubation. These data are represented graphically in Figs. 10a and 10b:

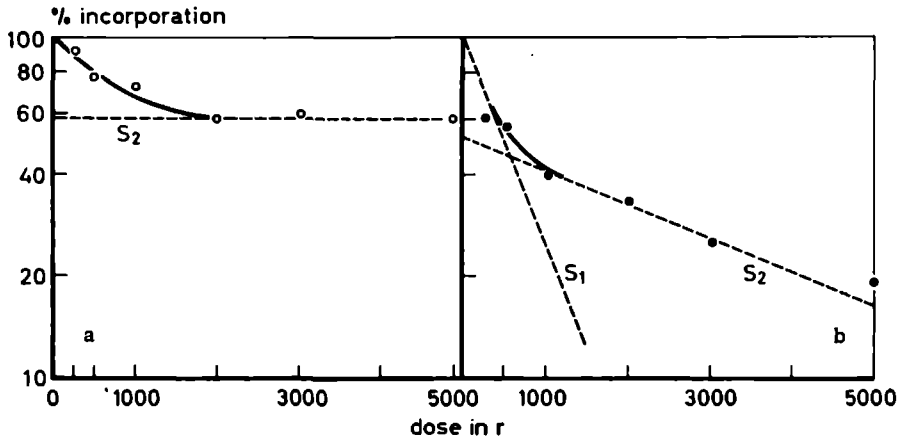

Fig. 10a. The rate of ^{14}C-TdR incorporation per cell calculated from the specific radioactivity of the DNA extract and the mean number of synthesizing cells for the incubation interval between 30 and 90 minutes after exposure to different doses of X-ray.

Fig. 10b. A curve corresponding to the one in 10a except that this one is based on cultures which received label (^{14}C-TdR) between 3.5 and 4.5 hours after irradiation. Mean number of synthesizing cells was scored from cultures pulse-labelled at the 4th hour post-irradiation with ^{3}H-TdR. The dashed lines in both figures indicate hypothesized S-components.

c. Conclusions

The curves shown in Figs. 10a and 10b suggest the following conclusions:

a) The dose-effect curve based on labelling shortly after irradiation (30 to 90 minutes) exhibits only an S_1-component with saturation characteristics - i.e. the slope of the S_2-component is zero.
b) A few hours after irradiation the dose-effect curve is evidently diphasic, showing both an S_1- and S_2-component.
c) The magnitude of the S_1-component increases with time intervening between irradiation and labelling; the S_2-component is detectable only in cultures receiving label some time (here from 3.5 - 4.5 hours) after exposure to X-ray.

Many workers (e.g. Lajtha et al., ref.29) have interpreted their dose-response curves - similar to ours - as diphasic. We hesitate to

draw such conclusion from our data or to assume that the existence of distinct S_1- and S_2-effects can be deduced from our Figs. 10a and 10b with certainty. Therefore the experiments which follow were designed to ascertain whether the so-called S_1- and S_2-effects actually exist and, if so, possibly to study their nature.

§ 2. ON THE MECHANISM OF X-RAY INDUCED DECREASE IN THYMIDINE INCORPORATION

a. Introduction

In spite of the many reports which clearly establish a relationship between irradiation and retardation in uptake of exogenous thymidine, very little is presently known about the mechanism responsible for this inhibition. In this section an attempt will be made to more accurately define this mechanism and, for example, to establish whether the resulting inhibition is competitive in nature. If the hypothesis is valid that a simple relation exists between intra- and extracellular

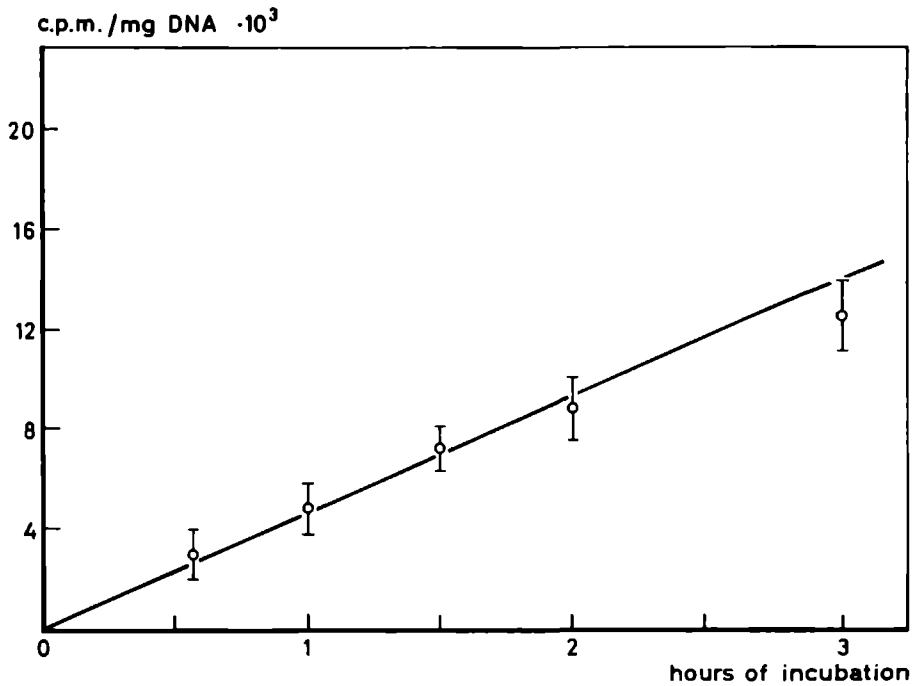

Fig. 11. The increase in the specific activity of DNA in cells cultivated for different times in 10^{-6} M ^{14}C-TdR. Each point together with its standard deviation is based on 6 separate determinations.

concentrations of exogenous (radioactive) thymidine, the appropriate experiment for elucidating the aformentioned mechanism of inhibition might be one in which varying concentrations of labelled compound were added to the culture medium. The validity of this hypothesis is suggested by the observations of Jacquez (47) and of Crathorn and Shooter (48) and, furthermore, is discussed more completely in § 4 of this chapter.

b. Results

The following experiment was designed as a partial check of the above-hypothesised simple ratio and is based on the reasoning that if exogenous thymidine equilibrates with the intracellular pool and is quickly incorporated into the DNA, a linear increase in the specific radioactivity of the DNA can be expected during incubation of cells with ^{14}C-TdR, provided the supply of radioactive substrate is not depleted.

A few 6-day old cultures were labelled with ^{14}C-TdR by replacing the normal medium with a fresh one containing the radioactive compound (10^{-6}M). Specific activity of DNA in the cultures was determined after 0.5, 1, 1.5, 2 and 3 hours incubation in label. The mean and standard deviation associated with each incubation time are plotted in Fig.11 with each point being based on 6 separate determinations.

In this plot the observed linear increase in the specific activity of the DNA during the first two hours of incubation in ^{14}C-TdR excludes the possibility that radioactive substrate is depleted and simultaneously suggests a confirmation of the original hypothesis that a simple partition exists between intra- and extra-cellular concentrations of exogenous radioactive thymidine.

Making use of the above results, an experiment was carried out to determine the nature of the S_1 post-irradiation effect. In enzymology, the inhibitory effects are studied by measuring enzyme activities at different substrate concentrations in the presence of the inhibiting compound. Because X-rays act as an inhibitor on thymidine incorporation, the nature of this inhibition can be studied by incubating control and irradiated cells in graded concentrations of substrate (viz. labelled thymidine).

Different specific radioactivities of exogenous ^{14}C-TdR were prepared by adding known amounts of cold thymidine to culture medium already containing 10^{-6}M radioactive thymidine (0.03 μC/ml). After two hours

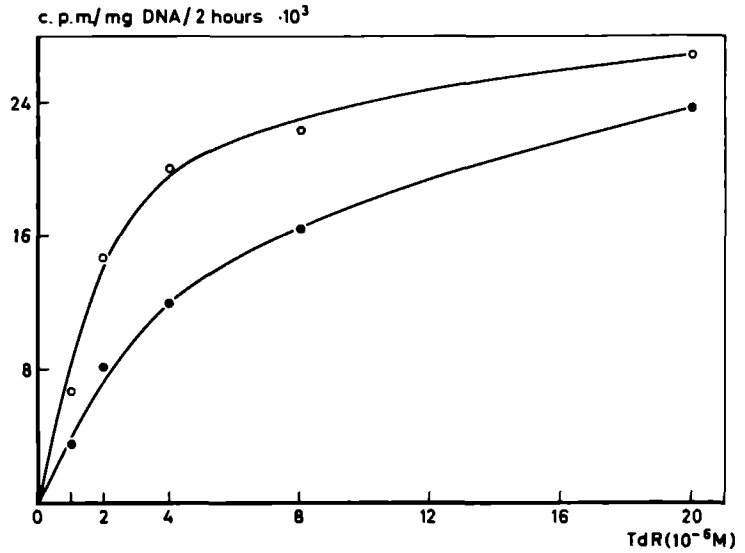

Fig. 12. The rate of ^{14}C-TdR incorporation into the DNA at different concentrations of thymidine in control (▪ o——o) and irradiated cultures (500 r = ●——●).

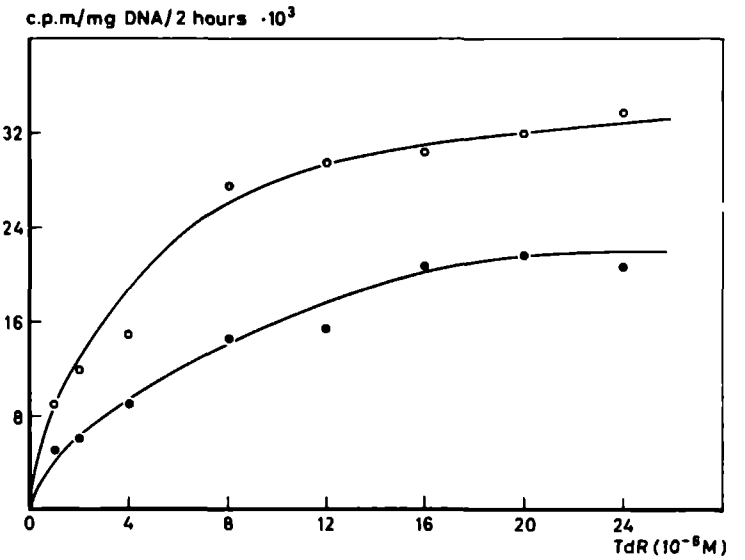

Fig. 13. The same as fig.12, but with an irradiation dose of 1000 r (= ●——●). (o——o = control).

incubation, the specific activity of extracted DNA was determined as usual and the resulting values were then corrected by multiplying the observed decay rates by the dilution factor of the tracer compound. The concentrations tested were 1, 2, 4, 8 and 20.10-6M with each reported point being based on 3-5 cultures. The irradiation dose was 500 r (see Fig.12).

Another experiment similar to the foregoing was performed with an X-ray dose of 1000 r and thymidine concentrations of 1, 2, 4, 8, 12, 16, 20 and 24. 10-6M. Three separate cultures were used for each point (Fig.13).

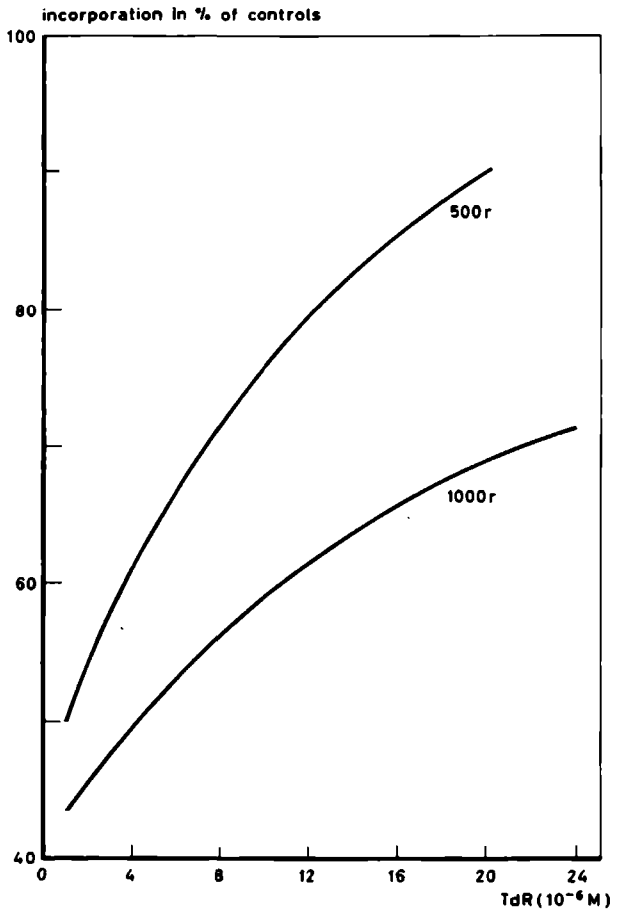

Fig. 14. The rate of ^{14}C-TdR uptake into DNA measured at different concentrations of thymidine in the medium and expressed as percentages of the corresponding control rates. The curves are based on values directly taken from figs. 12 and 13.

As expected, exposure to X-ray results in a decrease of thymidine incorporation from the medium, but in figs. 12 and 13 it is also evident that this decrease is progressively less pronounced in series of media with increasing concentrations of exogenous thymidine. In order to emphasize this progressive reversal in inhibition of uptake, mediated by increasing concentrations of added thymidine, the incorporations as graphed in Figs. 12 and 13 were converted into percents of the corresponding rates in the controls and plotted in Fig. 14 against thymidine concentrations.

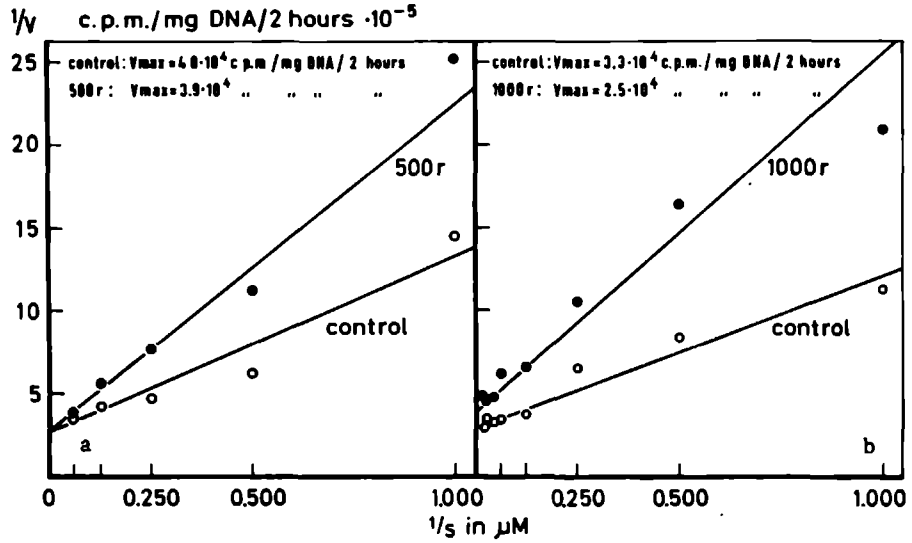

Fig. 15a. The values from fig.12 in a Lineweaver-Burk plot, indicating that exposure to 500 r results in a competitive inhibition of thymidine incorporation from the medium.
Fig. 15b. As fig.15a except that the values used were taken from fig.13.

In Figures 12 and 13, the observation that incorporation increases asymptotically with higher concentration of thymidine suggests that the S_1-effect (see Fig.10a) is the consequence of competitive inhibition - that is, competition between exogenous thymidine and an unknown compound for synthetic sites in newly forming DNA strands. If the value for the specific radioactivity of the DNA in Figs. 12 and 13 are taken as indices of ^{14}C-TdR incorporation rates and represented in a Lineweaver-Burk plot, no difference can be observed between the "V_{max}" of controls and of cultures receiving 500 r whereas only a 25% decrease in "V_{max}" was observed after exposure to 1000 r (Figs.15a and 15b).

Painter and Rasmussen (45) attributed a similar thymidine dependent reversal of the S_1-effect to the influence of high thymidine concentrations on enzymes involved in DNA synthesis as earlier noted by Morris et al. (49). Therefore the following experiment was designed to determine if thymidine in the concentrations used here influences the rate with which DNA incorporates ^{32}P in the presence of different concentrations of unlabelled thymidine.

A group of 6-day old cultures was incubated for 2 hours in media containing 0.5 μC/ml inorganic $^{32}P(0.95.10^{-3}M)$ with concentrations of unlabelled thymidine which varies between 0 and $30.10^{-6}M$. After the incubation, DNA was extracted and its specific activity measured as usual. DNA in three separate cultures was assayed for each experimental point (Fig.16).

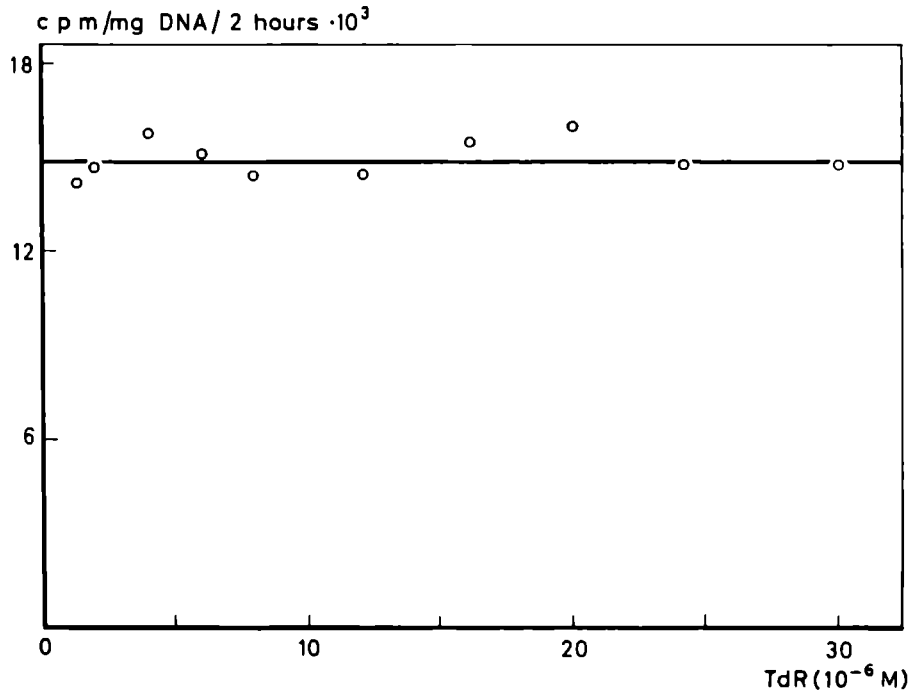

Fig. 16. The specific radioactivity in DNA extracted from cells cultured 2 hours in medium containing inorganic ^{32}P (0.5 μC/ml; 0.95 . $10^{-3}M$) and different concentrations of cold thymidine. Each point represents the mean value of three determinations.

The results of this assay suggest that exogenous thymidine in the concentrations used here and for the experiments presented in Figs.

12 and 13 did not interfere with the rate of DNA synthesis during a 2-hour period of incubation. Therefore in our cell strain, it is concluded that the suppression of the S_1-effect observed in high thymidine concentrations is not traceable to direct influence of the labelled compound on DNA synthesis as proposed by Painter and Rasmussen.

c. Conclusions

Within 2 hours after exposure to X-ray up to 1000 r, the S_1-effect represents the only type of decrease in rate with which DNA incorporates exogenous ^{14}C-TdR (compare Figs. 10a and 10b). The effect of irradiation under these circumstances seems to be mainly competitive inhibition in the uptake of exogenous thymidine into DNA.

§ 3. ON THE INCORPORATION OF PHOSPHATE AFTER IRRADIATION

a. Introduction

Since many reports on the rate of DNA synthesis after irradiation have made use of $^{32}P_i$ incorporation, it might be of interest to study the possible influence of irradiation on uptake of ^{32}P by DNA and to determine whether, as in the case of ^{14}C-TdR, the magnitude of this influence depends on the extracellular concentration of inorganic phosphate. In the use of ^{32}P, the advantage that high concentrations of P_i can be employed under physiological conditions renders possible the direct measurement of V_{max} for the rate of isotope incorporation without recourse to extrapolation. On the other hand, measurements of phosphate incorporation are complicated by the inexistence of a simple relation between intra- and extracellular P_i concentrations. De Hevesy (50), for example, has demonstrated that mammalian cells, *in vitro* rapidly accumulate radioactive phosphate against the diffusion gradient. Therefore variations in the concentration of extracellular phosphate might not necessarily lead to corresponding changes in the cell. In addition, one may expect that the P_i pool in the cell is much larger than that of deoxyribonucleotides and therefore the quantitative experiments involving graded concentrations of extracellular ^{32}phosphate in this paragraph must be interpreted with reservations.

b. Results

The DNA uptake of ^{32}P was measured as the specific activity of extracted DNA. A preliminary experiment was first performed to determine whether tracer is taken up in DNA during a period of time which is short in relation to the intervals of incubation used in subsequent experiments.

A group of cultures was incubated in medium with 0.95 . 10^{-3}M ^{32}P (0.5 μC/ml of final culture fluid) - the figure 0.95 representing the average between the 0.765 . 10^{-3}M phosphate found in the Hank's solution and lactalbumine together and the 1.5 . 10^{-3}M in the serum component (25% of complete medium). After each of several different incubation times, DNA was extracted from 6 cultures and used for separate assay of specific radioactivity (for extraction and counting procedures used, see the section on ^{14}C-TdR determinations). A comparison of the specific activities in hot acid extracted DNA and in DNA digested by DNA-ase I (Worthington, DN-100) precluded the possibility that part of the radioactivity in the acid extract originated from other sources than DNA. The results of this experiment are shown in Fig.17.

The linear increase of the specific activity with incubation time (Fig. 17) suggests that the exogenous ^{32}P readily mixes with the intracellular P_i-pool.

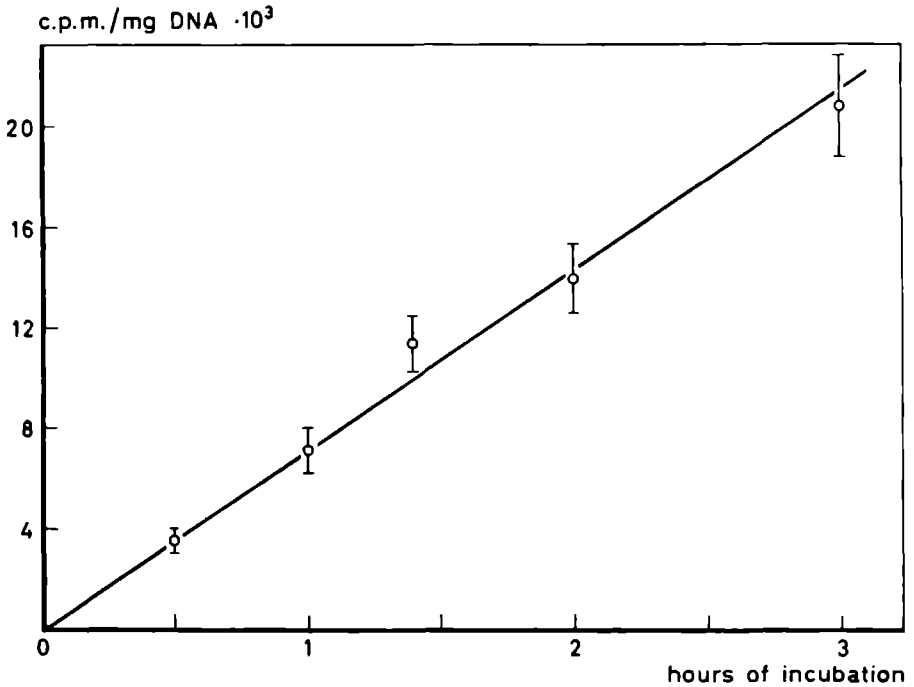

Fig.17. The increase in the specific activity of DNA after different periods of incubation in medium containing $^{32}P_i$ (0.5 μ C/ml; 0.95 . 10^{-3}M). Each point and its standard deviation is based on 6 determinations.

Prior to testing the capacity of high phosphate concentrations for reversing inhibition of ^{32}P incorporation after irradiation, it was neces-

sary to pretreat cells in such a way as to reduce the intracellular P_i level. That is, if irradiation releases a small amount of competing molecules, the change of its being detected - by competitively inhibiting ^{32}P incorporation -, might be better if the pre-irradiation pool is as small as possible. Two kinds of pretreatments were applied in an attempt to obtain low P_i concentrations in cells before being used in studies of post-irradiation phosphate incorporation. These will be henceforth called pretreatments A and B.

Pretreatment A - Cultures were incubated for two hours in Hank's solution without phosphate. Furthermore the solution was changed two times during incubation. By this method it was hoped that intracellular concentrations might be lowered by washing phosphate out of the cells as well as by incorporation into the macromolecular pool.

Pretreatment B - As in pretreatment A, cells were washed in Hank's solution without phosphate but glucose was omitted as well. In addition to the depletion mechanisms mentioned above, this treatment might also effect a decrease of intracellular P_i concentrations by inducing phosphorylation of glycogen stores (instead of exogenous sugar) in the manufacture of glucose-1-phosphate.

The effectiveness of both pretreatments in lowering the P_i-content was determined by measuring phosphate after completion of incubation. For this purpose, 40 cm petri dishes were each filled with 40 ml of normal medium with suspended cells and allowed to grow until filled with cells. From these, duplicate dishes with about 10^8 cells each, were then incubated for two hours at 37°C in each of the following solutions:
a. normal medium
b. Hank's solution without phosphate and
c. Hank's without phosphate and without glucose.

During incubation all media were renewed two times. After incubation, all media were discarded and the P_i extracted for ten minutes in the cold room (4°C) with 15 ml ice-cold trichloracetic acetic acid (9%) in each petri dish. A subsequent extraction under the same conditions yielded less than 10% of the first extraction and therefore was omitted in all further experiments. The TCA was removed from duplicate 5 ml samples of the first extract by means of 5 partitions against equal amounts of ether and the P_i quantitatively determined according to the technique of Wollenberger (51). In order to express P_i in units per gram of protein, cells were scraped off the petri dishes, centrifuged, weighed in the wet state and their protein was assayed by the method of Lowry (52). The results of two separate experiments carried out as outlined above, are shown in Table II.

From Table II it is evident that both kinds of pretreatment lower the intracellular P_i concentration to about one-third of the control value. The intracellular concentration in the control cultures was calculated to be 3 mMoles P_i per 1000 g wet cells or about 3 times higher than that of the medium (viz.-$0.95.10^{-3}$M). It is also apparent that pretreatments

TABLE II

Medium used for incubation (3 times changed within 2 hours)	P_i-content in mMol P_i/g protein	
	Exp.I	Exp.II
A. Normal medium	0.103 (=100%)	0.086 (=100%)
B. Hanks' solution without phosphate	0.030 (29%)	0.023 (27%)
C. Hanks' solution without phosphate and glucose	0.038 (37%)	0.025 (29%)

Intracellular P_i-concentrations after 2 hours' incubation in the media described in the text.

A and B are no different with respect to their efficiency in reducing the P_i concentration. Therefore post-irradiation incubation of cells treated according to method B were carried out at lowered glucose content of the medium containing ^{32}P. It was hoped that under these circumstances the rate of ^{32}P uptake from the medium would be reduced and therefore the effect of possible competing compounds - released by irradiation - more pronounced. The rate of incorporation of ^{32}P into DNA after irradiation was studied (using the outlined methods of pre-incubation of cells) at different extracellular concentrations of ^{32}P.

A number of cultures were kept at 37°C in phosphate deficient media according to the methods A and B, after which half of the ones from each pretreatment were given 500 r X-ray and then incubated in Hank's solution with ^{32}P (0.765 to 765.10^{-6}M; 0.5 μC/ml). During this step, cells which had been pretreated according to method A were incubated in medium with a normal (=0.1%) glucose concentration whereas those with pretreatment B were incubated in 0.01% glucose. After incubation, DNA was extracted and its specific radioactivity determined as usual. Rates of ^{32}P incorporation are given in Table III with each reported activity being the average of measurements made from 7 or 8 cultures.

From the results summarized in Table III, it is concluded that X-ray retards the uptake of exogenous phosphate into the DNA in a manner comparable to the radiation-induced reduction of ^{14}C-TdR incorporation. After pretreatment B (without glucose) and subsequent studying of incorporation in glucose concentrations of 0.01 instead of 0.1%, the irradiation induced inhibition was more exaggerated. In media with weak

TABLE III

Concentration of P_i in 10^{-6}M (^{32}P = 0.5 μC/ml)	Pretreated according to A; incubated in 0.1% glucose				Pretreated according to B; incubated in 0.01% glucose			
	c.p.m./mg DNA/2 hours		incorp. in % of contr.	P-value (t-test)	c.p.m./mg DNA/2 hours		incorp. in % of contr.	P-value (t-test)
	contr.	500 r			contr.	500 r		
765	1,178 ± 71	1,203 ± 94	102	not sign.	1,451 ± 112	1,483 ± 112	102	not sign.
76.5	4,051 ± 92	4,130 ± 133	102	not sign.	---	---	-	---
7.65	76,012 ± 3,481	70,406 ± 3,462	93	$p < 0.01$	11,018 ± 872	9,075 ± 563	82	$p < 0.001$
0.765	91,980 ± 4,131	83,294 ± 3,377	90	$p < 0.002$	13,346 ± 973	9,608 ± 867	72	$p < 0.001$

The effect of irradiation (= 500 r) on DNA uptake of ^{32}P in media containing different concentrations of inorganic phosphate (at constant $^{32}P_i$-concentration of 0.5 μ C/ml). Cells were pretreated either according to method A or B as described in text. The mean and the standard deviation of each activity given, is based on DNA measurement from 7 - 8 cultures. For procedures see text.

P_i concentrations the rate of incorporation was about 8 times lower if cells were incubated in 0.01% glucose (after treatment B) than in 0.1% glucose (subsequent to treatment A). Since the intracellular P_i concentrations were similar after both kinds of pretreatment (see Table II), it seems likely that the difference in level of inhibition is exclusively attributable to the two concentrations of glucose employed during post-irradiation incubation in ^{32}P.

It may be useful to point out that P_i concentrations in normal media (i.e. $0.95 . 10^{-3}M$) are higher than those needed in the present experiment (i.e. $0.765 . 10^{-3}M$) for completely suppressing the influence of X-ray on ^{32}P incorporation. Therefore the S_1-effect of irradiation (viz. the main effect in the first two hours subsequent to irradiation with doses beneath 1000 r - see Figs. 10a and 10b) will be absent from dose-effect curves based on ^{32}P uptake in normal medium, and any inhibition observed might best be regarded as a result of the S_2-effect. This dose-effect curve was made in the following way:

> Six-day old cultures were exposed in quadruplicate to each of several doses of X-ray and subsequently incubated for 3.5 hours at 37°C. After this, they were labelled for one hour in normal medium with ^{32}P (0.5 μC/ml). DNA was extracted and the specific activity measured as usual.
>
> A parallel experiment was carried out to determine as accurately as possible the fraction of cells synthesizing DNA during the ^{32}P-labelling period of the experiment described above. Six-days old cultures with coverslips were exposed to X-ray as in the series above, pulse-labelled with ^{3}H-TdR (1.0 μC/ml) 4 hours after exposure and six slides from each dosage group were prepared for microradioautography. The rate of incorporation as well as the mean percent cells synthesizing DNA in this period are summarized in Table IV.

Even though, p e r c u l t u r e, the rate of phosphate incorporation into DNA is unaffected by doses up to 5000 r, the concommittant increase in number of cells synthesizing DNA following irradiation provides for a net retardation in the uptake p e r c e l l. This net retardation, here assumed to be the result of an S_2-effect, may be calculated as follows:

$$\frac{^{32}P/DNA\ (irradiated)}{\%\text{ - lab.cells (irrad.)}} \Big/ \frac{^{32}P/DNA\ (control)}{\%\text{ - lab.cells (control)}} \times 100\%$$

... and, since the specific radioactivity was constant at all doses, the calculation may be simplified in the form:

$$\text{Rate of DNA synthesis p e r c e l l in percent of control} = \frac{\%\text{-labelled cells (control)}}{\%\text{-labelled cells (irradiated)}} \times 100\%$$

TABLE IV

Dose in r	Rate of ^{32}P incorporation: c.p.m./mgDNA/1 hour (mean from 4 determ.)	Percent labelled cells at 4th hour post-irradiation
0	6,842	32.9 ± 1.9
500	6,651	36.0 ± 1.2
1000	6,782	37.6 ± 2.1
2000	6,905	41.1 ± 3.2
3000	6,882	44.6 ± 2.2
5000	6,741	49.3 ± 3.6

The rate with which DNA takes up ^{32}P in the one-hour interval beginning 3.5 hours subsequent to irradiation and the mean percent cells synthesizing DNA in the middle (i.e. 4th hour post-irradiation) of this interval.

Fig.18 shows a superimposition of a plot (semi-logarithmic) from the above-calculated effect (values from Table IV) on the curve from Fig. 10b - the latter represented the effect of irradiation in the rate of thymidine incorporation per cell in the same incubation period used here). The dosage-incorporation curve as measured in terms of ^{32}P incorporation per cell, may be drawn as a straight line which reasonably coincides with the S_2-component of Fig.10b - that is, it has the same slope but does not intercep the Y-axis at 100% incorporation.

On the basis of similarities between the S_2-component of fig.10b (—·—·—·—) and the dose-effect curve per cell using ^{32}P as tracer (---------), it is concluded that the high concentrations of labelled inorganic phosphate employed in DNA tagging experiments using the normal medium, restrict obtainable results to those caused by an S_2-effect on the rate of DNA synthesis after X-ray.

§ 4. DISCUSSION

There are two primary hypotheses advanced in attempting to explain the diphasic effect of increasing doses of irradiation on the rate with which DNA incorporates exogenous precursors. The first (see Painter, 38) was that the S_1- and S_2-effects may be attributed to a differential synthetic behavior of two distinct cell populations, each, in vi-

tro , with its own unique response to irradiation. According to the hypothesis, the S-Phase cells would be more sensitive and the G_1 cells more resistant to irradiation. The second hypothesis (Lajtha and co-workers, ref.29) is more biochemical in nature and has found wider acceptance. Its essential feature is that DNA synthesis occurs in two steps each of which possesses its own characteristic sensitivity to irradiation. The first and most sensitive step (whose modification resulted in the S_1-effect) was thought to involve mechanisms necessary for synthesis of precursors, for example phosphorylation of nucleotides, whereas the S_2-effect was conceived as the consequence of direct damage to the DNA matrix.

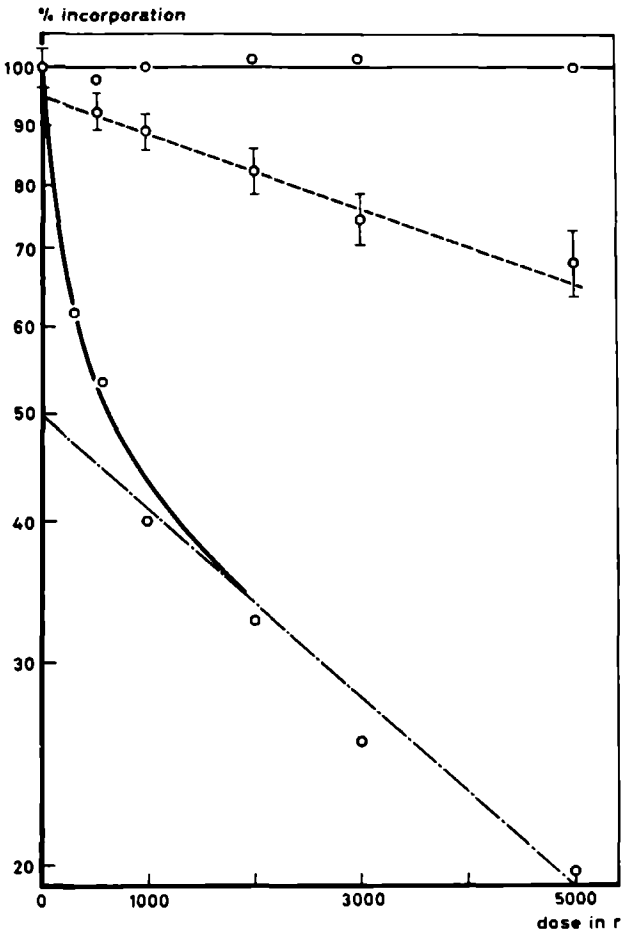

Fig. 18. The $^{32}P_i$-uptake into DNA as it occurs in the rates of uptake per culture (= o——o) and per cell (= o-------o) after X-ray exposure as described in the text. The lower curve is re-drawn from fig.10b.

Hypotheses founded on the existence of two cell classes with different radiosensitivities cannot account for our findings. That is, the highest doses of irradiation might be expected to block synthesis in the most sensitive class giving a decrease in the number of synthesizing cells - a result directly opposite to the increase in S-Phase cells found in our experiments (Tables I and IV). Equally inconsistent with the Painter hypothesis are the reports of Looney (36) on regenerating rat liver and of Smith (39) on tissue cultures in which they found that irradiation impairment of DNA synthesis was the same in all cells.

On the other hand, the explanation of Lajtha et al. that the dose-response curve is the result of two sequential steps in DNA synthesis, also seems inadequate in view of the necessity for the corollary that DNA synthesis should be limited by the most sensitive of the two steps. That is, at higher doses, the superimposition of the S_2-effect on an already existing S_1-effect should produce additional inhibition of incorporation and thus make for an increased steepness of the curve instead of the observed flattening.

In addition, Lajtha et al. concluded that development of insufficiencies in the system responsible for DNA synthesis occured in a period immediately following irradiation - a contention hardly supported by the experimental research of many authors. For example, when thymidine-kinase and DNA-polymerase were both isolated from regenerating livers of irradiated rats and tested in vitro (Bollum et al., ref. 53), it was found that the activities of these enzymes first began to decline about 6 hours after irradiation. Therefore these authors concluded that irradiation did not directly affect the activities of enzymes but produced damage resulting in inhibition of their biosyntheses. Comparable results have been obtained by other workers using the same objects (54, 55). Also pertinent to the conclusions of Lajtha and co-workers are the results of Walwick and Main (56) who, in working with regenerating rat livers, were unable to demonstrate post-irradiation effects (250 - 10,000 r given in vitro) on either the activity of isolated DNA synthesizing enzymes or on primer activity of purified DNA. Furthermore the Walwick and Main data are in agreement with earlier findings of Wheeler and Okada (57) who discovered that radiation damage to DNA as primer for its own synthesis cannot be related to reduction in the incorporation of precursors in vivo. Finally, Crathorn and Shooter (48) found that irradiation of ascites cells caused a retardation in the uptake of labelled thymidine into DNA but did not discover changes in activities of enzymes which phosphorylate thymidine or in DNA-polymerase. Our observations also failed to demonstrate a post-irradiation impairment of the system for DNA synthesis - that is, the S_1-effect on the incorporation rates of both thymidine and phosphate showed an unaltered "V_{max}". Likewise, our results concerning ^{32}P incorporation at high but normal P_i con-

centrations suggest that the S_1-effect is without any effect on the rate of DNA synthesis.

This paradoxical situation (viz. an unchanged rate of DNA synthesis associated with the competitive inhibition in uptake of exogenous precursors), seems to be explainable only by hypothesizing that the endogenous (and thus non-radioactive) pools of thymidine and phosphate, which compete with labelled substrates for synthetic sites on DNA molecules, are augmented after irradiation. Such an irradiation-induced change of endogenous metabolic pools (henceforth referred to as "pool-changes") could be manifested as a specific activity in DNA lower than found in non-irradiated controls without changes in the rate of DNA synthesis.

The possible existence of pool changes has been reviewed by Lajtha (58) who observed that many cells in irradiated animals undergo necrotic autolysis; therefore we propose that breakdown products from DNA may be responsible for the observed lowering of specific activity in DNA molecules which incorporate ^{14}C-TdR and ^{32}P subsequent to irradiation.

Hell et al. (35) noted that the diminished incorporation of thymidine following irradiation was dependent on the concentration of added ^{3}H-TdR after 2000 r; incorporation was retarded by a factor of 47-50% in media with $1.4 \cdot 10^{-6}$M thymidine but only 12% in concentrations of $140 \cdot 10^{-6}$M. These authors attributed their results to a dilution of radioactive thymidine by (endogenous) products of DNA-breakdown. A similar case was found by Painter and Rasmussen (45) in cultures of Hela and Chineze hamster cells. Here the rate of thymidine incorporation following the exposure to 300 r was depressed 39% in media with $0.15 \cdot 10^{-6}$M labelled thymidine but was reduced only 17% in $10 \cdot 10^{-6}$M TdR. These authors, however, interpreted their findings as the effects of high exogenous concentrations of the nucleoside on the rate of DNA synthesis and not as a pool-effect. This possibility was investigated in our cell line by incubating in thymidine concentrations as high as $30 \cdot 10^{-6}$M, but no inhibition of ^{32}P uptake into DNA was observed (see Fig.16).

In another publication on HeLa cells, Painter and Rasmussen (44) obtained results apparently conflicting with their other findings (45). As before, radiation caused a reduced incorporation of thymidine, but, in these experiments, no influence of tracer concentration on the magnitude of the effect was observed. This contradiction is probably traceable to the high thymidine concentrations used in this second study (between 15 and $40.000 \cdot 10^{-6}$M). For example, Marin and Bender (59) found that the rate of exogenous ^{3}H-TdR incorporation in HeLa

cells was a monotonic increasing function of substrate concentration approaching a maximum asymptote at thymidine concentrations above 3.10^{-6}M (a result qualitatively similar to the one reported here, see Figs. 12 and 13). A comparison with this (already maximal) 3.10^{-6}M concentration with the lowest one employed by Painter and Rasmussen (15.10^{-6}M) makes it evident that these authors' results do not exclude the possibility of an intracellular pool-effect, functioning in dilution of exogenous thymidine - that is, if there is a limiting factor in the rate of thymidine uptake, a dependence of post-irradiation effect on thymidine concentration would not be observed at concentrations of 15.10^{-6}M and higher. The existence of a factor limiting the rate rate of thymidine incorporation is suggested by the work of Jacquez (47). This research concluded that in media with low nucleoside concentration, equilibration between intra- and extracellular nucleosides was not dependent on mere diffusion but apparently mediated by transport, possibly a sugar transport mechanism. If this transport mechanism increased to maximum efficiency as a given substrate concentration was approached (e.g. 3.10^{-6}M), pool-dilution effects would not be demonstrable in extracellular concentrations above that values. A similar glucose transport mechanism involved in thymidine uptake may be deduced from data of Hilz et al. (42). These authors demonstrated that the rate of thymidine incorporation in ascites cells maintained in vitro without sugar was about three times higher than in media containing 50.10^{-6}M glucose. It is evident from the foregoing that the possibility of demonstrating a post-irradiation pool-effect by varying extracellular concentrations of labelled thymidine, depends on the characteristics of a (incompletely defined) nucleoside transport mechanism. The dependence of this mechanism on the cell line used is already demonstrated - maximal incorporation in HeLa cells is approached in media with 3.10^{-6}M thymidine whereas, in our cell line, the corresponding value is about 20.10^{-6}M.

In summation, an attempt will be made to formulate a hypothesis concerning effects of irradiation on the metabolism of DNA in our cell line during the first hours after irradiation. The S_1-effect is ascribed to a decrease in the rate of exogenous substrate incorporation and does not result from an impairment of DNA synthesis. In other words, there is no manifest impairment of DNA synthesis following exposure to X-ray and, as a result, the total incorporation of thymidine and phosphate remains unchanged with only the uptake of (labelled) precursors from the medium being depressed. It is hypothesized that endogenous concentrations of free thymidine and phosphate are augmented by irradiation-induced autolysis of DNA, and that by accumulating in the cellular phase, these breakdown products could compete with tracer for sites in the synthesizing DNA matrix or possibly impede the entry

of labelled compounds into the cell. On the other hand, the S_2-effect is best observed after higher doses of irradiation and is hardly detectable in the first two hours following X-ray exposure. It is concluded that the S_2-effect represents a true retardation in the rate of DNA synthesis by undefined mechanisms. In the present research, in fact, only the prolongation of the S-Phase (= S-retention effect - see chapter III) derived from this S_2-induced retardation was detected and not the S_2-effect itself (see Fig.18). Even though the S_2-effect is here shown to be distinct from the S_1-effect, its real nature remains unclear and difficult to elucidate because of its complexity and association with all radiation damages to the cell which reduce the rate of DNA synthesis.

Among the questions remaining open concerning this hypothesis are:

a. does the deduced pool-effect of tracers studied occur in the medium by autolysis of dying cells or is it the result of intracellular hydrolysis;
b. could breakdown of DNA give nucleosides and orthophosphate as the only substances responsible for pool-dilution;
c. is there a causal relation between the S_1-and the S_2-effect or is the latter brought about by radiation damage other than that responsible for the S_1-effect? Investigations of these questions are reported in the next chapters.

CHAPTER V

THE PROTECTION EFFECTS OF EXOGENOUS DNA ON IRRADIATED CELLS

§ 1. EFFECTS ON IRRADIATED CELLS

a. Introduction

Even though not yet cited in "Radiation Protection and Recovery" (60), recent literature on protection against ionizing radiation has frequently involved treatment with exogenous DNA. For example, Kanazir (61) obtained an enhanced survival in irradiated rats by injection of DNA solutions and even provided a slight protection by similar treatment with RNA. After irradiation with ultra-violet light, bacterial cultures incubated with homologous DNA showed a clear survival advantage over those in plain media (62). In rats, irradiation-induced depression of mitotic activity was counteracted by injection of either heterologous or homologous DNA solutions (63). Similarly, Savkovic (64, 65) observed that protection against reduced survival and fertility in irradiated rats was provided by injection of homologous DNA. Miletic et al. (66) reported that isologous and heterologous DNA's were equally effective in restoring reproductive potential in irradiated cultures of mouse L cells. Since the protection of mouse L cells occured irrespective of the origin of the DNA used, it was concluded that a "nutritive mechanism" was operating - i.e. the added DNA re-supplied substrates whose biosynthesis had been hindered by irradiation.

The only existing hypothesis accounting for the protection provided by DNA is that of Miletic co-workers. Even though their results suggested that exogenous DNA might be, in some way, responsible for prevention or repair of irradiation-induced disturbances in metabolism of endogenous DNA, compelling evidence in favor of such a "nutritive mechanism" appears to be lacking. For example, there are no data showing that exogenous DNA facilitates the synthesis of endogenous DNA after irradiation and, in addition, there are no indications that the immediate cause of death in irradiated cultures (namely the effect actually counteracted by DNA treatments) is related to a defect in DNA metabolism.

If, in irradiated cultures receiving DNA treatments, there is a specific mechanism responsible for sustaining normal endogenous DNA synthesis, its identification might help elucidate the means by which irradiation interferes with DNA metabolism. In the present research,

therefore, endogenous synthesis is studied in irradiated and control cultures maintained in media with added DNA.

b. Results

As shown in chapter IV, retardation in cellular DNA synthesis following irradiation is traceable to an effect called the S_2-effect, whereas an S_1-effect of irradiation acts on tracer uptake only. Of these, only the first one is used here for testing the protection imparted by exogenous DNA, because, as reported in § 2. of this chapter, an S_1-like effect is induced by DNA added to non-irradiated cultures of our cell line. The S_2 post-irradiation effect provides a means for quantitation of DNA-protection by its correlation with increase in number of S-Phase nuclei in asynchronous cultures. This quantitation is obtained by letting P_C be the percent control cells taking up label during the 6th hour after sham-irradiation and letting the corresponding values be P_p and P_x for irradiated cultures incubated with and without DNA respectively (measurements were performed by scoring radioautographs made from cells receiving ^{3}H-TdR for 20 minutes after 6 hours of incubation with and without DNA subsequent to irradiation). For every combination of irradiation dose and DNA concentration tested, the protective effect may be expressed as the percentage of protection (= % protection) and calculated with the help of:

$$\frac{P_x - P_p}{P_x - P_c} \times 100\% = \% \text{ protection.}$$

This equation provides a relatively simple quantitative index for assaying the protective action afforded by exogenous DNA. For example, in case of no protective effect at all, P_p and P_x are equal and "% protection" will become zero, whereas, when $P_p = P_c$, the calculated protection is 100%.

The protective effect given by different concentrations of homologous DNA to irradiated cells in vitro, was investigated by using the following schedule:

> Fifteen calf liver cell cultures were incubated 2 days on coverslips after which they were divided into 5 groups. The culture medium in each group was replaced with similar fluids containing, however, respectively 0 (= 2 groups), 50, 150 or 300 μg/ml homologous (calf thymus) DNA supplied as the sodium salt by British Drug House. Fifteen minutes before irradiation, the medium was exchanged in a temperature room at 37°C.
>
> X-ray (= 500 r) was administered to all cultures except the sham-irradiated controls; 6 hours later the fluid in all cultures was again exchanged with medium containing ^{3}H-TdR (0.5 μ C/ml) and allowed to incubate for 15-20 minutes. Coverslips were then removed from the

culture vessels, placed in fixative and further prepared for radioautography. Mean values of percent labelled cells for each set of three determinations are plotted in Fig.19 and, to provide an example of the calculation, are summarized in Table V.

TABLE V

Dose in r	Amount of DNA: µg/ml	Percent cells in S at the 6th post-irradiation hour	% protection: $\frac{P_x - P_p}{P_x - P_c} \times 100\%$
0	0	33.5 (= P_c)	-
500	0	48.8 (= P_x)	0
500	50	43.9 (= P_p)	32
500	150	41.1 (= P_p)	50
500	300	38.9 (= P_p)	65

The percentage of protection afforded by exogenous DNA against the radiation-induced increase of the fraction of cells synthesizing DNA; details in text.

From these results it is concluded that the presence of exogenous DNA in the medium during exposure to X-ray and subsequent incubation, retards the build-up of cells synthesizing DNA in the 6 hours period after irradiation.

The mechanism responsible for counteracting radiation damage might be better understood if the relationship between the degree of protection and the time when DNA is added - relative to the moment of irradiation - were known. Therefore an experiment was performed in which DNA was added to the medium at intervals before and after irradiation with a fixed dose.

After incubating cells for 2 days on coverslips, the fluid covering them was replaced with 3 ml of fresh medium. Sterile DNA solution (0.3 ml of 2200 µg/ml) was then aseptically added to give final amounts of 200 µg/ml. This DNA-treatment was given to quadruplicate samples at each of the following times: 2 hours (that is immediately after renewal of the medium) and 0.5 hours before irradiation (= 500 r) and 0.5 and 3 hours afterwards. Irradiation and DNA blanks were also prepared in quadruplicate - i.e. 4 cultures were irradiated without receiving DNA whereas 0.3 ml DNA was also added to 4 cultures which received only a sham exposure to X-ray. Except for administration of X-ray, all manipulations were performed in a temperature room at 37°C and pulse-labelling was accomplished with ^{3}H-TdR (0.5 µC/ml) after 6 hours subsequent to irradiation. The results, expressed as %

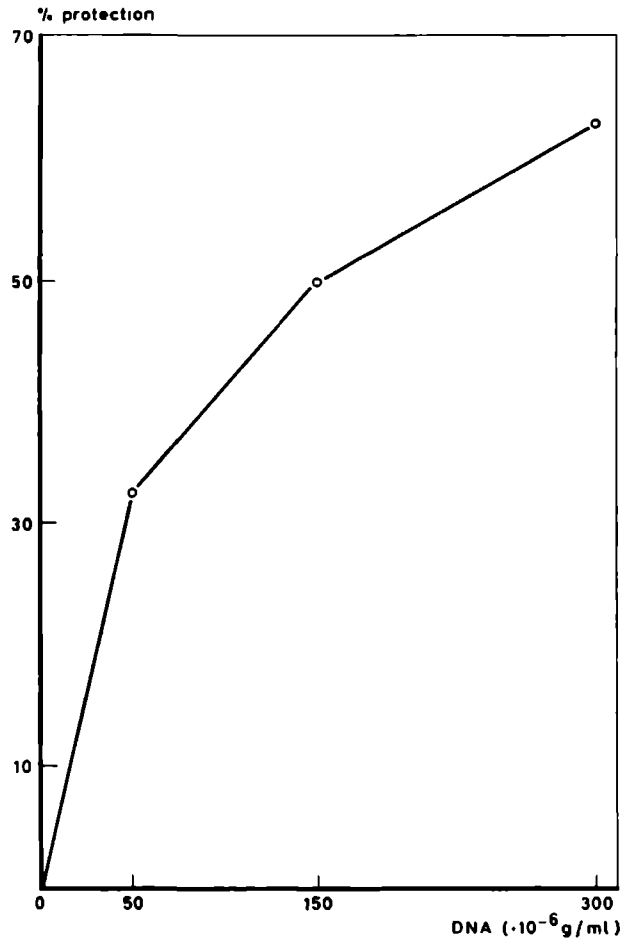

Fig.19. The values "% protection" as calculated in Table V, plotted against the corresponding amount of DNA.

protection, are plotted in Fig.20. The irradiated but unprotected cultures are plotted at the 6 hours point in the graph.

In view of the hypothesis of Miletic et al. (66), mentioned in the introduction of this section, it seems appropriate to determine whether protection conferred by DNA is attributable to its breakdown and subsequent uptake by endogenous DNA or whether the molecule added functions as a whole unmodified unit. To investigate this question, the protective effect of commercial DNA was tested after submitting fixed amounts of it to various degrees of hydrolysis. The resulting mixtures

of polymerized and hydrolysed DNA were then added to cultures to be irradiated for measurements of % protection.

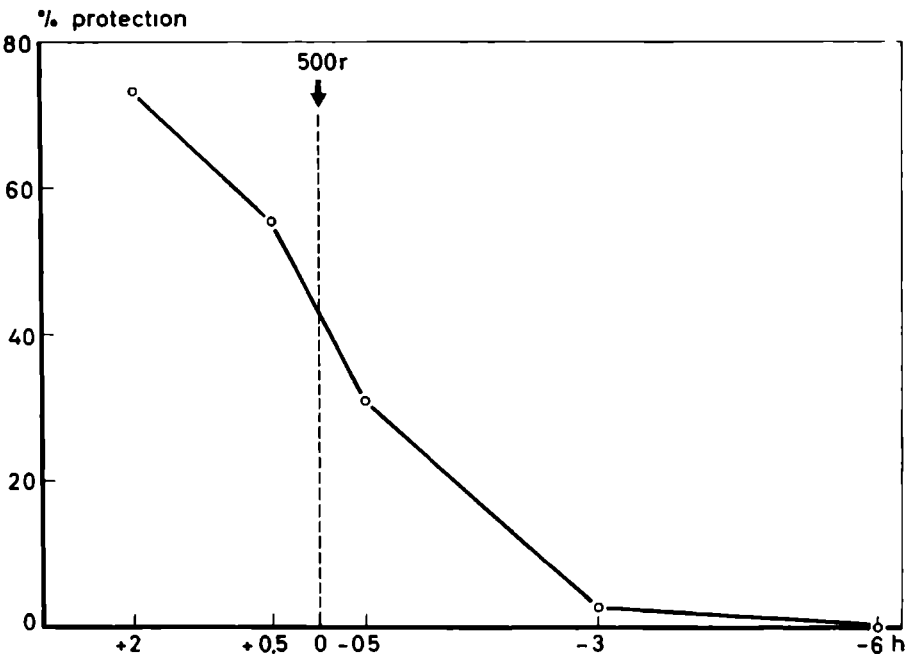

Fig.20. The extent of protection by exogenous DNA (200 μg/ml of culture medium) - proportional to a decrease in number of cells synthesizing DNA after a dose of 500 r - when added at spaced intervals before and after exposure to X-rays. For procedure and explanation see text.

As described in § 2 of this chapter, the serum used in ordinary culture media exhibits weak DNase activity. Therefore different degrees of hydrolysis were obtained by incubating five, 20 ml lots of medium - each containing 200 μg/ml DNA and kept under sterile conditions at 37°C - for 0, 8, 16, 24 and 48 hours respectively. These were subsequently frozen and stored until use. At the beginning of the experiment all five lots of media were quickly warmed to 37°C and 3 ml of each used for replacing culture fluid in each of 4 vessels containing cells on cover glasses. After incubating in the various DNA solutions for 15 minutes, all cultures were given a 1000 r dose of X-ray and kept at 37°C for 6 hours before being pulse-labelled with tritiated thymidine and prepared for radioautography. Controls were sham-irradiated and incubated in a medium without DNA, that was pre-incubated for 48 hours at 37°C. The same medium was used for incubating irradiated but unprotected controls.

Also, aliquots from the media were taken for assay of DNA hydro-

lysates. The DNA and the protein in each aliquot was precipitated with an equal volume of ice-cold 1.o N perchloric acid and, after centrifugation, the amounts of diphenylamine-positive material present in the respective supernatants were compared with DNA assays of precipitate in control medium (= 200 μg/ml without incubation at 37°C). The degree of hydrolysis in various media was expressed as the percent of DNA converted into acid-soluble form, and the results of duplicate determinations are given in Table VI.

TABLE VI

Time of incubation of DNA in medium (37°C)	Percentage of all DNA (200 μg/ml) converted into a cold acid-soluble fraction
0 hours	0
8	55
16	58
24	69
48	79

The production of acid-soluble (= cold 0.5 N perchloric acid) hydrolysate by incubating calf thymus DNA (200 μg/ml) for different periods of time in culture medium at 37°C.

From the curve in Fig.21, it is evident that the protective effect of DNA diminishes with increasing degree of degradation - a result not expected if DNA-hydrolysates (and not polymerized DNA) were utilized as substrates for recovery from X-ray damage. Therefore, instead of suggesting that a nutritive mechanism is responsible for recovery from the S_2-effect, the results obtained here lead to the conclusion that macromolecular DNA is directly responsible for the observed protection.

The last experiment in this paragraph is designed to determine whether protective effects of macromolecular DNA are attributable to its non-specific poly-anionic proporties (see below). For example, Yost et al. (71) observed that irradiation of whole rats gave depressed rates of phosphorylation in particulate fractions of cells from various organs. Furthermore, this depression of phosphorylation was reversed by intraperitoneal injection of DNA. These authors, having reference to observations of polyanions such as RNA and polyethylene sulfonate acting as accelerators of phosphorylation, attributed their results to the polyanionic nature of DNA.

In the following experiment, the protective effects of RNA were therefore studied.

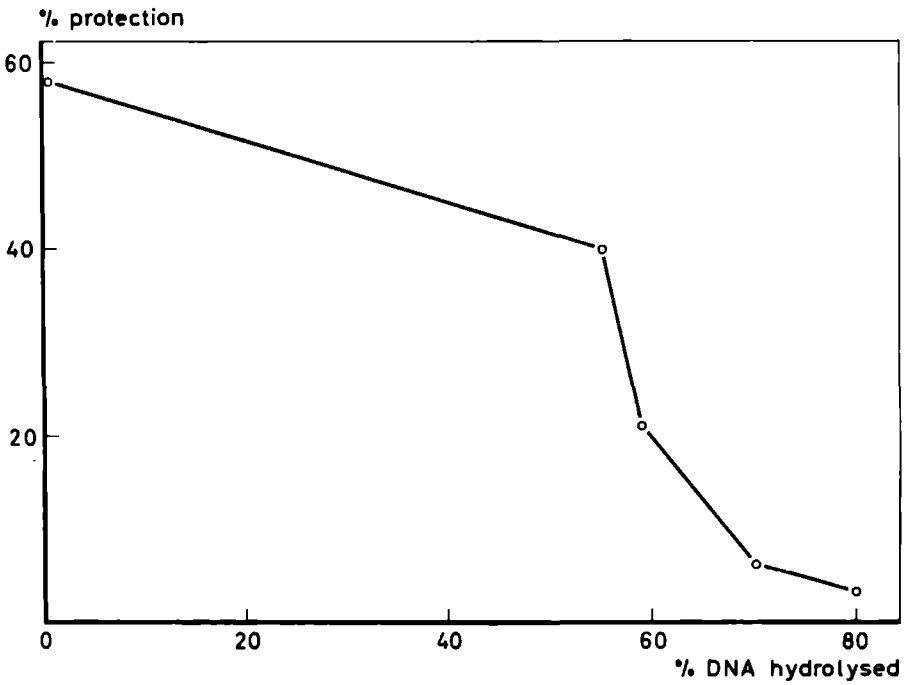

Fig.21. Changes induced by hydrolysis of the DNA used for protection on cells against the post-irradiation S_2-effect. Five batches of media with DNA (200 μ g/ml) - each in a different state of hydrolysis - were added 15 minutes before the cells were exposed to 1000 r. Details in text.

Cells cultured 2 days on coverslips were placed in media containing yeast RNA (Boehringer) of two different concentrations (500 and 2000 μ g/ml). After 2 hours incubation, half of the cultures were irradiated with 500 r and pulse-labelled with ^{3}H-TdR for radioautographic scoring of the percent of S-Phase cells in irradiated, RNA-protected and control cultures (see experiments on protective action of DNA). Similarly, the rate of ^{14}C-TdR incorporation was measured in irradiated and sham-irradiated cultures (6-days old) with and without added RNA. Results of both experiments are summarized in Table VII.

From the results summarized in this Table, it is concluded that RNA does not, as DNA, protect cells against radiation damage - either by counteracting the reduction in rate of ^{14}C-TdR incorporation or by reversing increases in percentages of cells synthesizing DNA.

TABLE VII

Dose in r	Amounts of RNA: µg/ml	14C-TdR incorporation: c.p.m./mg DNA/2 hours	% of control	Percent pulse-labelled cells 6 hours post-irradiation
0	0	8,415	-	32.5 ± 2.3
0	500	8,508	-	-
0	2000	8,508	-	33.6 ± 1.8
500	0	6,388	76	44,3 ± 1.8
500	500	6,720	79	42.6 ± 3.1
500	2000	6,535	77	42.0 ± 2.5

The inexistence of a protective effect of RNA on the rate of thymidine incorporation into DNA (mean of 3 determinations) and on the fraction of cells synthesizing DNA at the 6th post-irradiation hour. Each value and its standard deviation is based on 6 determinations in this series. X-ray dose in all cases: 500 r.

c. Conclusions

The addition of homologous DNA to cell cultures before or shortly after exposure to X-ray, results in protection against the irradiation--induced S-retention effect. The progressive decrease in protection with increasing degree of exogenous DNA hydrolysis, suggests that protection is somehow related to DNA as a macromolecule. Furthermore, the failure of comparable treatments with exogenous RNA, indicates that the observed protection is ascribable to the specific nature of DNA and is not related to its non-specific polyanionic properties.

§ 2. EFFECTS ON NON-IRRADIATED CELLS

a. Introduction

In order to elucidate the mechanism by which exogenous DNA protects against X-ray-induced impairment of endogenous DNA synthesis, experiments in this section are designed to test for intervention of exogenous DNA in metabolic and physiological processes of our cell line. Although much recent research has dealt with the influence of DNA-solutions on cells and whole organisms, only a minor part of it has been aimed at explaining the radioprotective action of this compound. That is, the major research effort has been devoted to pro-

duction of genetic transformation in higher organisms and in mammalian cell cultures. For example, Borenfreund and Bendich (67) in a radioautographic study on HeLa cell cultures, found that isologous and heterologous DNA's were selectively taken up into nuclei. Therefore, these authors concluded that the added DNA had been assimilated into the normal DNA fraction of the cell nuclei. Furthermore, they observed that 24 hours after starting the incubation of cells in medium containing about 25 μg/ml of labelled DNA, the incorporation of this DNA amounted at least 10% of the total DNA in each cell. No information was given concerning whether the labelled exogenous DNA was incorporated in macromolecular form or as degradation products.

In another report it has been shown that in vitro incorporation of ^{32}P-labelled lymphocytic DNA into nuclei of mouse bone marrow cells is not accompanied by simultaneous uptake of exogenous $^{32}P_i$, and therefore, it was suggested that exogenous DNA was assimilated without being degraded (see Hill, ref.68). Comparable results were obtained by Popovic et al. (69) with labelled homologous DNA injected into irradiated rats. Also in this case, the label, as demonstrated radioautographically in various tissues, was predominantly localized in the nucleus. Since it was observed, for example, that nuclei in cell types normally unable to synthesize DNA, still accumulate label, it was inferred that DNA was incorporated as the molecule. Rabotti (70) was able to prepare two kinds of base labelling in DNA used for incorporation experiments, - one type from rat ascites cells labelled in vivo with ^{14}C -formate and the other from a similar in vitro system. These in vivo and in vitro methods resulted in two kinds of DNA, respectively with different ratios of specific radioactivity between adenine and thymine. Since these two different ratios were not altered after uptake of DNA of either kind by ascites cells in vitro it was concluded that the DNA was incorporated without losing its structural integrity.

In other investigations, attention has been focussed on the fate of exogenous DNA after its incorporation into cells. For example, Tsumita and Iwanaga (72) reported that labelled homologous DNA found in the blood of rats after intravenous injection, became rapidly converted in a form soluble in cold perchloric acid. Likewise it was discovered that label incorporated in many other tissues slowly disappeared with only the liver permanently retaining a significant level of labelled DNA. Since DNase activity of the rat serum is too low to account for the observed rate of hydrolysis, these authors concluded that exogenous DNA was incorporated intracellularly in unaltered form, split, and subsequently released into extracellular fluid as small acid-soluble units. On the basis of the same data it was furthermore concluded that the injected DNA was never actually assimilated into endogenous, chromo-

somal DNA. Hill (68) also observed that, after incorporation, exogenous DNA disappeared from bone marrow cells within 48 hours.

On the basis of the literature reviewed, it may be hypothesized that different types of cells incorporate DNA in polymerized form, degrade it, and then release the split products in acid-soluble form. The purpose of the following experiments was therefore to test the relevance of this hypothesis in our cell line. In addition to uptake and intracellular breakdown studies, the influences of exogenous DNA on cell growth and on endogenous DNA synthesis are investigated.

b. Results

The purpose of the first experiment was to examine the effects of exogenous DNA on mitosis and on the percent cells in S-Phase.

Media in separate 2-day old cultures were aseptically replaced (in temperature room at 37°C) with homologous DNA concentrations - respectively 0, 25, 125, and 200 μg/ml culture fluid. Cultures were allowed to incubate for 6 hours and, at the end of that time, 3 coverslips from each of the 4 DNA-concentrations were fixed in alcohol: glacial acetic acid (3 : 1) and stained with hematoxylin for determination of mitotic index. Assay of DNA influence on the S-Phase was accomplished by: (1) pulse-labelling for 15 minutes in 0.5 μC ^{3}H-TdR/ml after 6 hours incubation and (2) by continuous-labelling with the same ^{3}H-TdR concentration during the entire incubation period in DNA of 6 hours. (amount of DNA: 200 μg/ml). 3 Coverslips were used for each determination of percent labelled cells. The results are reported in Table VIII.

TABLE VIII

Concentration of DNA in μg/ml	Mitotic index in percent of all cells	Percent pulse-labelled cells	Percent continuous--labelled cells
0	3.6	33.0	49.3
25	3.8	30.6	-
100	3.2	33.0	-
200	3.7	32.7	50.0

Mean mitotic indices and percent cells continuous- and pulse-labelled with ^{3}H-TdR after 6 hours' incubation in various concentrations of exogenous DNA.

The results reported in Table VIII, fail to demonstrate an effect of exogenous DNA on the mitotic cycle - i.e. mitotic index as well as pulse- and continuous-labelling are not changed by incubation of cells for 6 hours in media with added DNA.

In the following experiment, the rate of ^{14}C-TdR incorporation was studied in media with different amounts of added DNA.

Six-days old cultures were divided into 6 lots, each receiving fresh medium with a different amount of DNA (viz. 0, 10, 25, 50, 100 and 200 μg DNA/ml). Each of these 6 lots was subdivided into two groups - the first being incubated in 10^{-6}M ^{14}C-TdR (0.03 μ C/ml) for the first 2 hours subsequent to renewal of the medium and the other being similarly labelled in the 2-hour period elapsing from the 3rd untill the 5th hour after renewal, by dissolving the necessary ^{14}C-TdR in 0.1 ml culture medium and pipetting it into each of the respective cultures. For the 0 - 2 hour group, the ^{14}C-TdR was already predissolved in the renewal medium. At the end of the two respective labelling periods, the DNA was extracted as usual and the specific radioactivities, relative to controls, plotted against concentrations of added DNA (as the mean of three determinations (Fig.22).

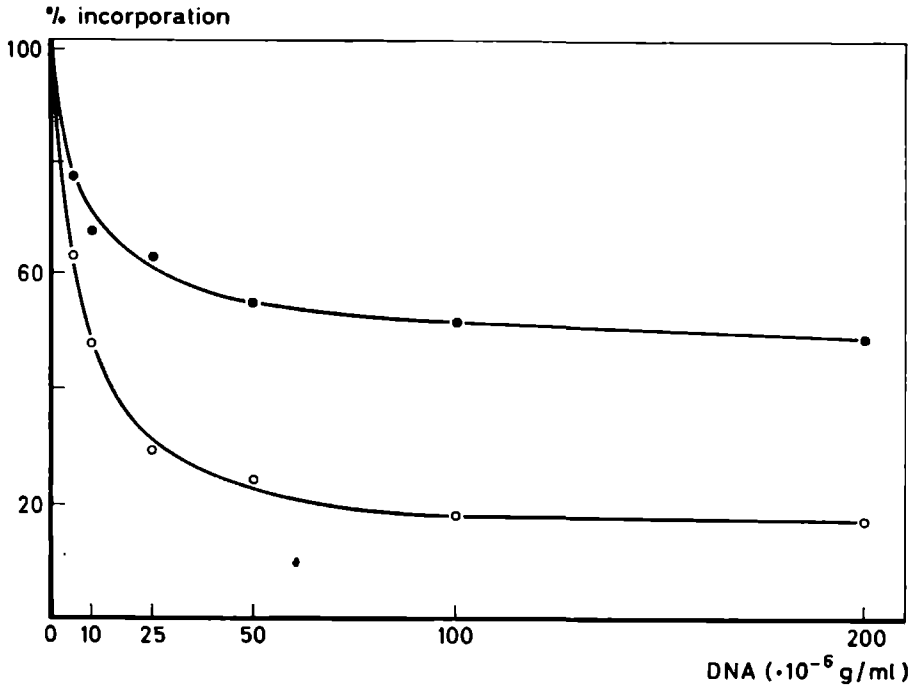

Fig. 22. The influence of different exogenous DNA concentrations on the rate of ^{14}C-TdR incorporation (10^{-6}M). Incubations were performed from 0 - 2 (= ● —— ●) and from 3 - 5 (= o —— o) hours after addition of homologous DNA to the medium.

The data in this experiment demonstrate decreased rates of ^{14}C-TdR incorporation in media with exogenous DNA. The decrease is more pronounced in cells incubated with ^{14}C-TdR in the 3-5 than in the 0-2 hour interval and is similarly more exaggerated in increasing DNA-concentrations. On the other hand, in the mitotic cycle, the rate of DNA synthesis does not appear to be altered by exogenous DNA (Table VIII), thus suggesting that the decreases observed here are attributable to dilution of specific ^{14}C-TdR radioactivity by products from the breakdown of the exogenous DNA. This dilution gives an S_1-like effect - a reasonable result in view of the evidence for a similar dilution produced by X-ray-induced autolysis of endogenous DNA.

If the net uptake of ^{14}C-TdR is reduced by competition with degradation products of exogenous DNA, there should be relatively less reduction in media with high concentrations of the label and perhaps no measurable reduction at all in $^{32}P_i$-uptake at normal physiological concentrations of phosphate ($0.95 \cdot 10^{-3}$M). The incorporation in endogenous DNA in the presence of homologous DNA was therefore investigated in the following experiment.

Six-day old cultures were divided in 6 lots (each consisting of 6 cultures) to study tracer incorporation with and without added DNA (100 μg/ml). Incorporation was studied in the following media:

a) 10^{-6}M ^{14}C-TdR (0.03 μC/ml with a specific radioactivity of 30 mC/mMol),

b) $30 \cdot 10^{-6}$M ^{14}C-TdR (0.3 μC/ml, specific radioactivity 10 mC/mMol and

c) $0.95 \cdot 10^{-3}$M $^{32}PO_4$ (0.5 μC/ml).

DNA was extracted after 2 hours incubation and its specific radioactivity taken as an index of tracer incorporation. The mean and standard deviation for each of 6 determinations are summarized in Table IX.

These results are in agreement with the above conclusion that exogenous DNA does not effect cellular DNA-synthesis but that its decomposition products merely dilute the specific radioactivity of incorporated precursors. Thus enzymatic splitting of exogenous DNA as well as scission of endogenous DNA after irradiation both give products effective in retarding the rate of labelled thymidine incorporation. The S_1-effect of irradiation is thus mimicked by added DNA.

Since the above experiments do not reveal whether exogenous DNA is broken down extra- or intracellularly, the following experiment was carried out: uptake of radioactive thymidine was measured according to routine procedures in media that were freshly supplemented with exogenous DNA and in media incubated for some hours at 37°C before (that is, the effect on thymidine incorporation might be increased if hydrolysis of exogenous DNA occurs in the medium). Also incorpora-

TABLE IX

Type and concentration of labelled compound	Rate of ^{14}C-TdR incorporation in c.p.m./mg DNA/2 hours		Incorporation in percent of controls
	Control	+DNA (100 µg/ml)	
A. ^{14}C-TdR; 10^{-6}M, 0.03 µC/ml. Spec.act. 30 mC/mMol.	7,500 ± 283	4,151 ± 210	55 ± 8.7
B. ^{14}C-TdR; 30.10^{-6}M, 0.3 µC/ml. Spec.act. 10 mC/mMol.	12,151 ± 495	11,356 ± 465	93 ± 8.1
C. $^{32}P_i$; $0.95.10^{-3}$M, 0.5 µC/ml.	16,655 ± 781	16,326 ± 618	98 ± 8.5

The influence of labelled precursor concentration on the rate of uptake into endogenous DNA in media with added homologous DNA (100 µg/ml) or without it (= controls). For procedure see text.

TABLE X

Medium used for incubation with ^{14}C-TdR(10^{-6}M) DNA: 100 µg/ml medium	Incorporation of ^{14}C-TdR in percentages of controls (= without exogenous DNA)
A. Normal medium; DNA dissolved just before incubation with cells	53 ± 3.1
B. Medium with DNA pre-incubated for 2 hours at 37°C before the addition of ^{14}C-TdR and the incubation with cells	29 ± 1.6
C. Medium heated at 60°C for one hour before dissolving DNA and subsequent incubation with cells	70 ± 5.2

Effect of pre-incubation of exogenous DNA in culture medium and of heating the medium before measurement of ^{14}C-TdR incorporation as depressed by the presence of added DNA. Cells were incubated for 2 hours in labelled thymidine with and without exogenous DNA.

tion was studied in media prepared from normal and from pre-heated serum (1 hour at 60°C) - that is, inactivation of thermolabile DNases might be able to reverse inhibition of thymidine incorporation if DNA breakdown occurs extracellularly. The results are summarized in Table X.

It is concluded from these data that the breakdown of exogenous DNA to give compounds diluting the ^{14}C-TdR tracer occurs at least partly in the culture fluid. Therefore the last experiment of this paragraph was undertaken to determine whether the added DNA is taken up into cells and, if so, to study the possibility of an additional intracellular hydrolysis.

Tritium-labelled isologous DNA was prepared by cultivating cells in a medium containing ^{3}H-TdR (0.2 μC/ml). Mass-cultures, maintained in flattened flasks, each containing 50 ml of medium, were cultured for 48 hours in tracer subsequent to 2 day's incubation after subculturing. After this, the cells were washed in Hank's solution and maintained for an additional 24 hours without thymidine. Cells were trypsinized and the DNA was extracted from the nucleoprotein (isolated according to Cole and Ellis, see ref. 81) by the method of Kirby, modificated by Hagen (83). Aliquots of the resulting extract were taken for measurement of specific radioactivity (found to be 23,480 c.p.m./mg DNA) and the remainder diluted to a concentration of 100 μg/ml medium. The medium used for incubation was heat-treated at 80°C for 7 minutes to reduce the DNase activity of the serum component (longer heating denaturated the proteins). The medium was then supplemented with non-radioactive thymidine (final concentration 2.10^{-3}M) in order to: (1) block endogenous DNA synthesis - see refs. 28 and 49, and (2) to lower the specific activity of hydrolysates resulting from exogenous DNA to a level that it would not significantly label neo-synthesized DNA.

Several 5-day old cultures, containing approximately the same number of cells, were incubated different periods of time in the above-prepared medium. The DNA in each of three cultures (from each incubation period) was then extracted and its total radioactivity measured. An estimate of the amount of DNA incorporated was obtained by dividing the total radioactivities per culture by the specific radioactivity of the DNA used for incubation as recorded above. A second series of cultures were incubated, exactly as above in labelled DNA-thymidine medium for 24 hours, washed thoroughly in Hank's solution and then re-incubated respectively for different periods in medium containing non-radioactive thymidine (2.10^{-3}M) only. Three cultures from each re-incubation were used for assay of remaining tritium radioactivity in the cells after trypsinization and 2-times washing with a mixture of alcohol 50% and 0.5 N PCA (1 : 1). In addition, in all cultures, the total amount of DNA was measured; the values varied randomly between 139 and 176 μg DNA per culture.

It may be concluded from the results (Fig.23a) that blockage of intracellular DNA synthesis does not prevent assimilation of exogenous (labelled) DNA. Since the neo-synthesis of DNA was blocked in the cell by the addition of excess thymidine and, also, degradation of exogenous DNA blocked in the medium by heat inactivation of serum DNA-ase, it seems likely that the DNA was incorporated in macromolecular form (possibly by pinocytosis). Adsorption of labelled DNA at the cell surface cannot be adduced from the plot in Fig.23a.

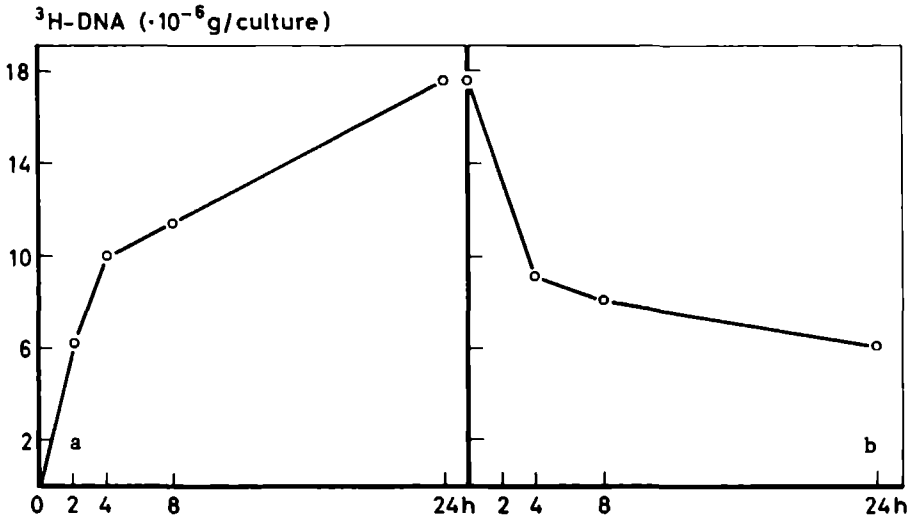

Fig.23a. The incorporation of labelled, isologous DNA by cells incubated in a medium containing labelled DNA (100 μg/ml) prepared as described in text.

Fig.23b. Loss of labelled DNA after its incorporation from the medium during incubation of cells in the presence of excess thymidine.

Fig.23b shows that incorporated DNA gradually disappears from cells or becomes converted into units soluble in the alcohol-perchloric acid mixture used for washing. In contrast with chromosomal DNA, therefore, it appears that the incorporated exogenous DNA is catabolyzed by cellular enzymes.

§ 3. DISCUSSION

The results of the first paragraph of this chapter suggest that exogenous DNA protects cells against X-ray induced retardation of DNA synthesis (i.e. the S_2-effect). Two difficulties are encountered in the interpretation of this result - the first being that the quantitative data

reported are based on the degree of repression in the S_2-effect. That is, the S_2-effect, as already discussed earlier (see chapter IV, § 4) might be non-specific and therefore its repression by exogenous DNA needs not reflect mechanisms specifically involved in synthesis of DNA.

The second problem is involved with whether the action of exogenous DNA against ionizing radiation is not more accurately termed an induced "recovery" than it is a "protection". Normally, in radiobiology, reference to protective compounds suggests preventing changes in physicochemical properties of biomolecules or cell structures. For example, well known protective compounds such as cysteine or other thiols, bind injurious radicals generated by irradiation - a mechanism which, due to the short half-life of the radicals formed, becomes inoperative shortly after irradiation. Since, however, DNA operates even when added after cells are exposed to X-ray (see Fig. 20), the word "recovery" seems more suitable than "protection". Nevertheless the tentative hypothesis developed in this paragraph is based on a protective action of exogenous DNA against irradiation damage. In order to explain a protective mechanism which can remain operative after irradiation, the proposed hypothesis involves the protection against injurious compounds generated after irradiation with longer half-life than radiation-induced radicals.

The hypothesis proposed here is that the whole exogenous DNA molecules hinder injurious enzymatic hydrolysis of endogenous DNA by capturing the enzymes long enough to permit re-establishment of homeostasis. This hypothesis is based on the following points:

1. Since added DNA did not create disturbances in the cell cycle as assayed in the percent of dividing cells or of those in the S-Phase, the retardation it produces in radioactive thymidine incorporation (see Fig.22) was attributed to a pool-dilution effect.
2. It is evident (from Figs. 23a and 23b) that exogenous DNA undergoes continuous intracellular degradation (i.e. the hydrolytic removal of DNA as shown in Fig.23b would be, as indicated in Fig.23a, constantly replaced by DNA from the extracellular pool).
3. The continuous degradation of DNA probably takes place in the nucleus as in several cell systems it becomes localised there after incorporation (67, 68 and 69).

If, as these 3 points suggest, enzymes are continuously occupied with the hydrolysis of exogenous DNA in nuclei of cells during and immediately subsequent to irradiation, they might be not available for hydrolysis of endogenous, chromosomal DNA (see chapter IV for data showing that the S_1-effect results from an irradiation-induced enzymatic lysis of DNA). This protection may be described as bio-

logical because its prevents damage to the genetic system of irradiated cells.

A confirmed corollary of this hypothesis is that exogenous DNA would lose its protective capacity if enzymatically hydrolysed (see Fig.21), whereas the possibilities of ascribing protection to a nutritive mechanism or to polyanionic properties of DNA were also excluded (Fig.21 and Table VII).

The proposed hypothesis would have been more convincing if a direct connection between exogenous DNA and nullification of the S_1 post-irradiation effect could have been established. However, the radiomimetic properties of added DNA limited our experimental measurements to those involving the S_2-effect (or better, the S-Retention effect, see chapter III). If, on the other hand, proof of a cause and effect relationship between the S_1- and the S_2-effect (i.e. enzymatic lysis of endogenous DNA after irradiation causes a reduction of its own synthesis) could be furnished, it would constitute a substantial confirmation of the "enzyme capture" hypothesis presented here. The possibility of such a cause-effect relationship will be investigated in the next chapter.

CHAPTER VI

ON THE NATURE OF AND THE RELATION BETWEEN THE S_1- AND THE S_2-EFFECT

§ 1. RADIATION-INDUCED BREAKDOWN OF DNA

a. Introduction

In spite of the observations of Daoust et al. (73), Malkin (74) and of Pelc (75), it is generally accepted that in normal cells there is no turnover of DNA. Our results suggest (chapter IV) or warrant the supposition (chapter V) that X-ray induces breakdown of cellular DNA.

In micro-organisms, considerable breakdown of DNA after irradiation has been demonstrated. Stuy (76, 77) concluded that the radiation-induced breakdown in E-coli was caused by activation of DNases. Kos and Draculic (78) could not demonstrate the involvement of DNA-splitting enzymes in this breakdown, but the authors observed that the metabolism of the irradiated cells was necessary for manifestation of breakdown. Also in E.freundii marked breakdown of DNA (up to 25% of all DNA) was observed after irradiation by Osterrieth (79, 80) but with no reference to celldeath.

Many research has been carried out on the DNA or the DNA-nucleoprotein in irradiated mammalian cells. An increase in the amount of "free" DNA (that is, not bound to protein and therefore soluble in 0.14 M NaCl, see Cole and Ellis, ref.81) and an increased lability of the complex between DNA and protein (see Hagen, ref.83) were observed. These observations were confirmed many times more in radiosensitive tissues, usually of rats (82, 84, 85, 86, 87, 88 and 89). In all research the occurence of a lag-phase of at least one hour, suggested the involvement of the metabolism in provoking the effects studied.

Some authors demonstrated these effects before visible histological damage (83, 87). By others (84, 89), it was concluded that DNase was the enzyme most likely to affect the DNA-protein complex.

From the literature, no evidence can be derived that the release of DNA-splitting enzymes might be considered as a primary effect of irradiation. Changes in activity and localization of DNases were often found, but the effects were only observable some hours after exposure, - see the review of Goutier (90). In many instances DNase-release

appeared to be correlated with celldeath (e.g. 91, 92, 93) or with non-specific hormonal influences (94). If DNase plays a role in the radiation effects studied in our research, it will be the enzyme already present in the cells, possibly acting by a change in its localization.

In this paragraph, experiments will be described intended to support the hypothesis of DNA-breakdown as the cause of the S_1-effect. Furthermore it is attempted to determine if the effects, denoted as "pool-effects", occur in the intra- or the extracellular phase.

b. Results

The effect of X-ray on the amount of polydeoxyribonucleotides, extractable according to the method of Cole and Ellis (81), was studied.

> 6-Day old cultures were irradiated with 1000 r while controls were sham-irradiated. At different times after exposure, cultures were trypsinized, cells (about 10^8) were collected by centrifuging and resuspended in 2 ml ice-cold 0.14 M NaCl. The suspensions were desintegrated sonically (M.S.E. ultrasonic desintergrator, 60 Watt, 20,000 Hrz during 90 sec.) and the insoluble nucleoprotein was centrifuged in the cold (10 minutes, 4,000 r.p.m.). "Free" DNA was precipitated from the supernatant by adding cold PCA to a final concentration of 1 N. The resulting precipitate was hydrolysed in 2 ml hot 1 N PCA (45 minutes at 70°C) for the estimation of the amount of diphenylamine-positive substances. The measured amounts were compared with the amount of protein-bound DNA (determined in the acid hydrolysates of the first precipitate) and calculated as percent of total DNA. Data from different experiments are collected in Table XI.

The results, summarized in this Table, show great variability between various experiments. This might be partly the consequence of the great and inconstant time elapse (more than 1 hour) between trypsinization and the first centrifugation because samples were desintegrated in another institute. Furthermore, the 0.14 M NaCl-suspensions were not of the same concentration in each experimental series (0.05 - 0.12% with respect to all DNA). Because the separation is founded on differences in solubility, the small amount of nucleoprotein still soluble in the NaCl-solution is relatively great in the very dilute suspensions used here (compare control values and the concentration of corresponding suspensions in Table XI). However, it is difficult to obtain many cells with our method of culturing. In spite of the differences between the experimental series, our data show nevertheless in each experiment a clear increase of free DNA with time after irradiation.

Free DNA released by irradiation as well as exogenous DNA incorporated from the medium, are supposed to be broken down in the cell

TABLE XI

Number of experiment	Concentration of suspension = % total DNA in 0.14 M NaCl	Percent DNA soluble in 0.14 M NaCl but insoluble in 1 N perchloric acid				
		control	Hours after 1000 r			
			1	3	4	5
I	0.09	3.8	-	-	8.1	-
II	0.05	5.5	6.4	7.1	-	8.5
III	0.08	5.5	-	6.4	-	7.1
IV	0.12	1.8	3.5	-	3.5	-

The amounts of polydeoxyribonucleotides as percentages of total DNA in control and irradiated (= 1000 r) cells.

to give compounds lowering the specific radioactivities of labelled precursors. During this breakdown, the concentration of thymidine-5'-phosphate is not likely to increase to a measurable degree because at high $^{32}P_i$-concentrations no inhibition of label uptake into DNA was observed (that is, no partition between TdR-5'-P from breakdown and TdR-5'-^{32}P from synthesis was detectable). This implies that any phosphorylated nucleoside is rapidly hydrolysed by phosphatases or that only TdR-3'-phosphate is formed during breakdown. In fig.24 the supposed mechanism of breakdown and pool-dilution is represented schematically.

As shown in this scheme, both breakdown and synthesis require divalent cations and can therefore be inhibited by suitable concentrations of NaF. Because the S_1-effect of radiation depends on the specific radioactivity of the precursor-pool only, it is independent on the rate of synthesis but strongly dependent on the presupposed breakdown that delivers non-radioactive precursors. Consequently, the S_1-effect might be suppressed in increasing concentrations of NaF. This possibility was checked experimentally in the following way:

> 6-Day old cultures were exposed to 500 r. Immediately after exposure, the medium was changed with a medium containing ^{14}C-TdR (10^{-6}M) and graded concentrations of NaF. After 2 hours incubation, the specific radioactivity of extracted DNA was determined as usual in control (= sham-irradiated but NaF-containing cultures) and irradiated cells. Each point was determined in quadruplo and the whole experiment was repeated once. The results of both experiments are graphically represented in fig.25a. In fig.25b the incorporation rates are plotted as percentages of corresponding controls.

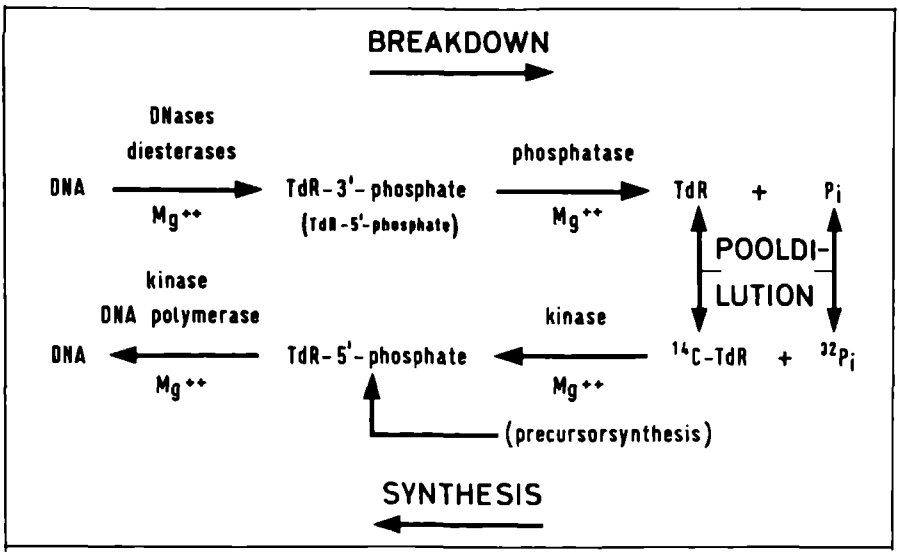

Fig.24. Schematic representation of breakdown of polydeoxyribonucleotides and the formation of products able to compete with tracers for incorporation into neo-synthesized DNA (for thymidine and inorganic phosphate only).

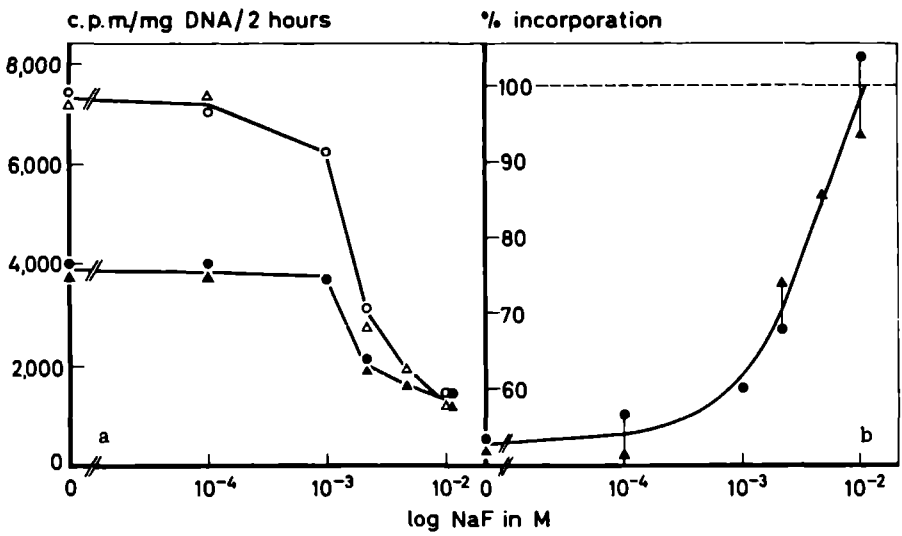

Fig.25a. Specific radioactivity of the DNA of control (= Δ, o) and irradiated (▲, ● = 500 r) cultures incubated for 2 hours in ^{14}C-TdR (10^{-6}M) and graded concentrations of NaF.

Fig.25b. Values from fig.25a but here incorporation rates of irradiated series are plotted as percentages of corresponding controls.

The incorporation of ^{14}C-TdR is reduced by NaF in control and in irradiated cells. Simultaneously the S_1-effect of irradiation is diminished and even fully suppressed at 10^{-2}M NaF. Because it was already noted in chapter IV that the radiation-induced S_1-effect on tracer incorporation was not related to impairment of the rate of DNA-synthesis, the observed suppression of this effect is atrributed to an inhibition of DNA-breakdown (see fig.24).

The reduction in the specific radioactivity of labelled precursors can take place in the intracellular phase by the breakdownproducts of DNA but also in the culture fluid by autolysis of dying cells. The latter possibility is not very likely, for marked histological damage was not observed (see chapter III). If, however, exogenous thymidine and P_i are supposed to enter the cells, "leakage" of endogenous compounds during breakdown into the culture fluid must be possible as well. Therefore, the role of the medium in the radiation-induced reduction of thymidine uptake was studied.

The medium of 6-day old cultures was replaced by a fresh one and the cultures were subsequently exposed to various doses of X-ray. At different times after exposure, culture fluids were collected, centrifuged and supplemented with ^{14}C-TdR (10^{-6}M). A number of normal cultures were incubated with these media in quadruplo for 2 hours while controls were incubated with media from sham-irradiated cultures. Specific radioactivity of extracted DNA was measured as usual. Mean values of incorporation rates are summarized in Table XII.

TABLE XII

Dose in r	Time of collection of medium (hours post-irradiation)	Incorporation of ^{14}C-TdR(10^{-6}M) in percent of controls
500	3	97
4,000	4	92
10,000	2	98
10,000	6	78

Effect of culture medium from irradiated cultures on the rate of thymidine incorporation into DNA of cells incubated for 2 hours in these media supplemented with the tracer compound.

The effects of irradiation on label incorporation were studied during the course of this research within a time smaller than 4.5 hours after doses of at most 5000 r, - see chapter IV. From the data collected in Table XII it can be derived that only a very small part of the observed effects is attributable to factors belonging to the culture fluid.

§ 2. ON THE RELATION BETWEEN THE S_1- AND THE S_2-EFFECT

a. Introduction

In the present research effects of X-ray on the metabolism of DNA were attributed to an S_1- and an S_2-effect. In this paragraph an attempt will be described for correlating both effects. The S_2-effect, - assumed to be a true decrease in rate of DNA-synthesis -, is detectable only if some time has elapsed after irradiation when a clear S_1-effect is already acting on tracer incorporation (compare figs.10a and 10b). The S_2-effect, however, needs not be the consequence of the preceeding S_1-effect because X-ray can damage the cell in many ways.

Exogenous DNA protects the cell against the S_2-effect as demonstrated in chapter V, § 1. The protection mechanism of exogenous DNA, however, was assumed to be a protection against the cause of the S_1-effect, viz. breakdown of cellular DNA. These results therefore strongly suggest DNA-breakdown to be the principal cause of both the S_1- and the S_2-effect.

The difficulty in demonstrating this relation is the impossibility to change simultaneously both effects in the same sense. Exogenous DNA protects against the S_2-effect but shows concomittant S_1-like effects (Table IX). DNA-breakdown, and therefore the S_1-effect is counteracted by NaF-treatment but the same compound reduces DNA-synthesis, thus providing for an S_2-radiomimetic effect.

By Chèvremont et al. (95, 96) it was noted that the enzymes DNase and RNase, dissolved in culture fluid, are able to attack specifically the nucleic acids of chicken fibroblasts in vitro. If DNase also attacks the DNA of the cells used in the present research, the enzyme might provide a tool for studying the relationship between S_1- and S_2-effects, that is, the breakdown of DNA might give pool-dilution as well as a build-up of cells in the S-Phase.

b. Results

The influence of DNase was studied on the rate of tracer incorporation and on the fraction of cells synthesizing DNA.

A number of cultures, some of them containing coverslips, were cultured for 6 days. DNase (neutral DNase, WORTHINGTON DN-100; 70,000 dornase units/mg) was dissolved in prewarmed (37°C) medium

to an amount of 0.1 mg/ml. Cultures without coverslips were incubated for 2 hours in this medium together with $^{32}P_i$ (0.5 μ C/ml) or with ^{14}C-TdR (10^{-6}M). Specific radioactivity of DNA was measured as usual after incubation in tagged media. Controls were incubated without enzyme. The cultures with coverslips were used for continuous- or pulse-labelling with ^{3}H-TdR (0.5 μ C/ml) during or after 6 hours incubation with or without DNase. All determinations of specific radioactivity and of percent labelled cells in the radioautographs made from the coverslips, were performed on 6 separate cultures for each point. Mean values and standard deviations are reproduced in Table XIII.

TABLE XIII

Time of incubation and tracer used	INCORPORATION: c.p.m./mg DNA/2 hours	
	control	+ DNase
$^{32}P_i$; $0.95 \cdot 10^{-3}$M 0.5 μC/ml.	16,379 ± 522	16,101 ± 612
^{14}C-TdR; 10^{-6}M 0.03 μC/ml.	7,987 ± 212	3,672 ± 103
	PERCENT LABELLED CELLS	
^{3}H-TdR; 0.5 μC/ml:		
- continuously for 6 hours	52.3 ± 3.2	51.8 ± 2.9
- pulse-labelling after 6 hours	33.1 ± 1.3	43.8 ± 1.7

The radiomimetic properties of DNase (0.1 mg/ml) for the incorporation rates of ^{14}C-TdR and for the fraction of pulse-labelled cells.

The effects of DNase on tracer incorporation and on the fraction of cells in S-Phase as summarized in Table XIII, demonstrate clearly the radiomimetic properties of the enzyme. With respect to ^{14}C-TdR incorporation, its effect is comparable with a dose of 1000 - 1500 r whereas its influence on the number of cells synthesizing DNA 6 hours subsequent to the addition of the enzyme, can be compared with 500 - 750 r of X-ray.

The inexistence of an effect on the rate of ^{32}P incorporation demonstrates that the observed inhibition of thymidine incorporation by the NDase treatment is - like the S_1-irradiation effect - not the consequence of a reduced rate of DNA synthesis.

§ 3. DISCUSSION

The experiments described in the foregoing paragraphs of this chapter were designed to elucidate some of the suppositions or questions mentioned in the preceeding chapters. Though far from being complete, compelling evidence was obtained that DNA-cores are splitted from the nucleoprotein in the hours subsequent to irradiation. Because of the experimental limitations, due to the difficulty of obtaining large amounts of cells, we can not decide whether any "free DNA" is present in non-irradiated cells. From the results in Table XI, however, the radiation-induced increase of these amounts can be deduced with certainty. It is a reasonable supposition that this free DNA, - as exogenous DNA incorporated from the medium -, will be hydrolysed in the cell, possibly according to the scheme proposed in Fig.24. Some additional evidence for the assumed DNA-breakdown after irradiation was obtained by the possibility to suppress the S_1-effect by NaF. The incorporation of labelled thymidine into cells incubated in media from irradiated cultures, was affected to a very small degree, thus permitting us to ascribe the radiation effects to intracellular processes. The clearly radiomimetic properties of DNase point out that the induction of DNA-breakdown might be the primary cause of changes in DNA-metabolism after irradiation and, in addition, they demonstrate that S_1- as well as S_2-like effects can result from an enzymatic attack on cellular DNA. A more fully account for the results mentioned in this chapter will be given in the general discussion in the forthcoming pages.

GENERAL DISCUSSION AND CONCLUSIONS

In the bovine type cell used here for studying the effects of irradiation on DNA synthesis, analyses were restricted to cells found in the DNA-synthetic period of interphase (≈ S-Phase) during a short interval (6 hours or less) following X-rays. Attention was focused on the S-Phase because it was judged to be the most suitable subdivision of interphase for studying the effects of irradiation on DNA-synthesis - the G_2-Phase, by definition, occurs after synthesis and the G_1-Phase, in particular the G_1 to S transition, is apparently unaffected by doses of X-ray as high as 4000 r (see chapter III). On the other hand, the post-irradiation time limit of 6 hours was imposed in order to minimize long-range shifts and variation in the relative frequencies of cells found in different phases of the synthesis-division cycle. Such variations, for example, would be brought about by the following two factors: (1) degeneration of the mitotic apparatus in dividing cells and (2) delayed initiation of mitosis in the G_2 subpopulation. This approach, although it did not terminate in a complete description of irradiation damage to DNA synthetic mechanisms, provided data from which a coherent elucidation of X-ray induced changes may be deduced. These data and derived conclusions are summarized in the following paragraphs.

Present results, based on rough counts of silvergrains over ^{3}H-TdR labelled nuclei and on the more exact technique of measuring specific radioactivity in DNA extracted from cells incubated with ^{14}C-thymidine, confirm previous reports that irradiation exposure results in lowered rates of DNA synthesis. In addition, this conclusion is based on the observation that after irradiation the S-Phase is lengthened (here called the S-retention effect - the G_1 to S transition is not affected by X-rays).

Data in chapter IV show that the specific radioactivity of DNA, extracted from post - irradiation cultures containing ^{14}C-TdR, is a decreasing diphasis function of increasing X-ray doses. These two phases were attributed to a two-fold irradiation effect on ^{14}C-TdR incorporation - respectively termed the S_1- and S_2-effect. Of these two, the S_1-effect appears earlier (within 0.5 hours) after irradiation exposure than

the S_2-effect. The observations that the S_1-effect becomes saturated at relatively low doses and that it is suppressed by high concentrations of labelled exogenous precursors (viz. ^{14}C-thymidine and $^{32}P_i$), suggest ascribing the S_1-effect to irradiation-induced release of substrates (e.g. enzymatic hydrolysis of endogenous DNA) which compete with added label for incorporation sites in neosynthesizing DNA. Specific results supporting this hypothesis are:

1. Increasing concentrations of labelled precursor in the medium suppress the S_1-effect (compare Fig.14 and Table III).
2. Intracellular degradation products of homologous DNA added to cultures, competitively inhibit uptake of DNA-precursors and thus mimic the S_1-effect of X-rays (Table IX; Fig.22).
3. The per-cell amount of free polydeoxyribonucleotides increases with time after irradiation (Table XI).
4. NaF reverses the S_1-effect by inhibiting the enzymatic breakdown of this "free" polydeoxyribonucleotide (Figs.25a and 25b).
5. Hydrolysis of endogenous DNA by added DNase produced an S_1-like effect (Table XIII).
6. Apparently all radiation effects exclusively involve intracellular changes (Table XII).

The S_2-effect, also an irradiation-induced inhibition of substrate incorporation into DNA, differs from the S_1-effect in that it develops later, does not display saturation characteristics but becomes progressively more pronounced with increasing dose of irradiation and, when quantitated and plotted against irradiation dose, gives a slope with a more gradual increase (= decrease in incorporation) than the S_1-effect. It is here attributed to a true decrease in rate of DNA synthesis (as distinct from the S_1-effect which is a true effect on tracer incorporation, but only a seeming effect on the synthetic rate). As such, the S_2-effect is more difficult to analyse than the S_1-effect. That is, the literature and to some extent the present research, suggests the occurence of damage to many processes related to DNA-synthesis. These might, for instance, include effects on synthesis and activity of deoxyribonucleotide kinases, DNA-polymerases and primer properties of DNA. Disruption of any of these biochemical processes could bring about a retardation of DNA synthesis per cell and consequently provoke a prolonged S-Phase.

The following two observations suggest that the S_1- and S_2-effects are the result of a common cause - that is, X-ray induces enzymatic decomposition of the DNA-protein complexes which, in turn, produces both the S_1- and S_2-effects.

a. Exogenous DNA counteracts the S_2-effect by preventing the enzymatic hydrolysis of endogenous DNA which is the cause of the S_1-effect

(see the "enzyme capture" hypothesis in chapter V, § 3).

b. Added DNase completely mimics the activity of X-ray in producing both S_1- and S_2-like effects (Table XIII).

In chapter III the S-retention effect was described and tentatively attributed to a decreased rate of DNA synthesis. It now appears that, by itself, this decrease is not sufficient to account for the total prolongation of the S-Phase seen in post-irradiated cultures - a conclusion which suggests that reparative synthesis of DNA may also play a role in prolonging the S-Phase. For example, after 4000 r, the measured retardation of DNA synthesis was less than 30% (measured 4 hours after X-ray exposure, see Fig.18) whereas experimental results (Fig.7) indicate that, 6 hours after the same dose, no S-Phase cells had completed synthesis. On the other hand, in controls, the corresponding percent of cells completing synthesis in a six-hour period is about 20% of all cells, that is, about 60% of the original DNA-synthetic subpopulation. As already mentioned, data of other authors (23, 24, 25) suggest that hyperploid amounts of DNA are not synthesized in post-irradiated cells but that the normal premitotic level is reached. If this holds true for our cell line as well, not only the normal diploid amount of DNA but also the amounts liberated from the DNA-protein complex by X-ray (see Table XI) might be neo-synthesized. Such "reparative" or "complementing" synthesis has also been reported after irradiation in Escherichia coli by Stuy (5,6) and in slime molds by McGrath et al. (98).

The repair of sub-lethal radiation damage during the S-Phase of mammalian cells (see Sinclair and Morton, ref.98 and Kiefer, ref.100) might also be attributable to "reparative" synthesis of DNA.*

Although the present data do not directly establish that DNA-splitting enzymes are freed by irradiation (e.g. by changing permeability of lysosomes), this is held as the most likely interpretation of the observations (see fig.26). It is therefore proposed that our results are in accordance with the "enzyme release hypothesis" of Bacq and Alexander (101).

*) Current research by the author and H. Dewaide with the same cell strain indicates that incubation with $^{32}P_i$ and colchicine of irradiated (= 400 r) cells for 20 hours, gives DNA extracts with a specific radioactivity about 20% higher than in controls. This result also suggests that reparative synthesis in the conservative DNA-strands occurs.

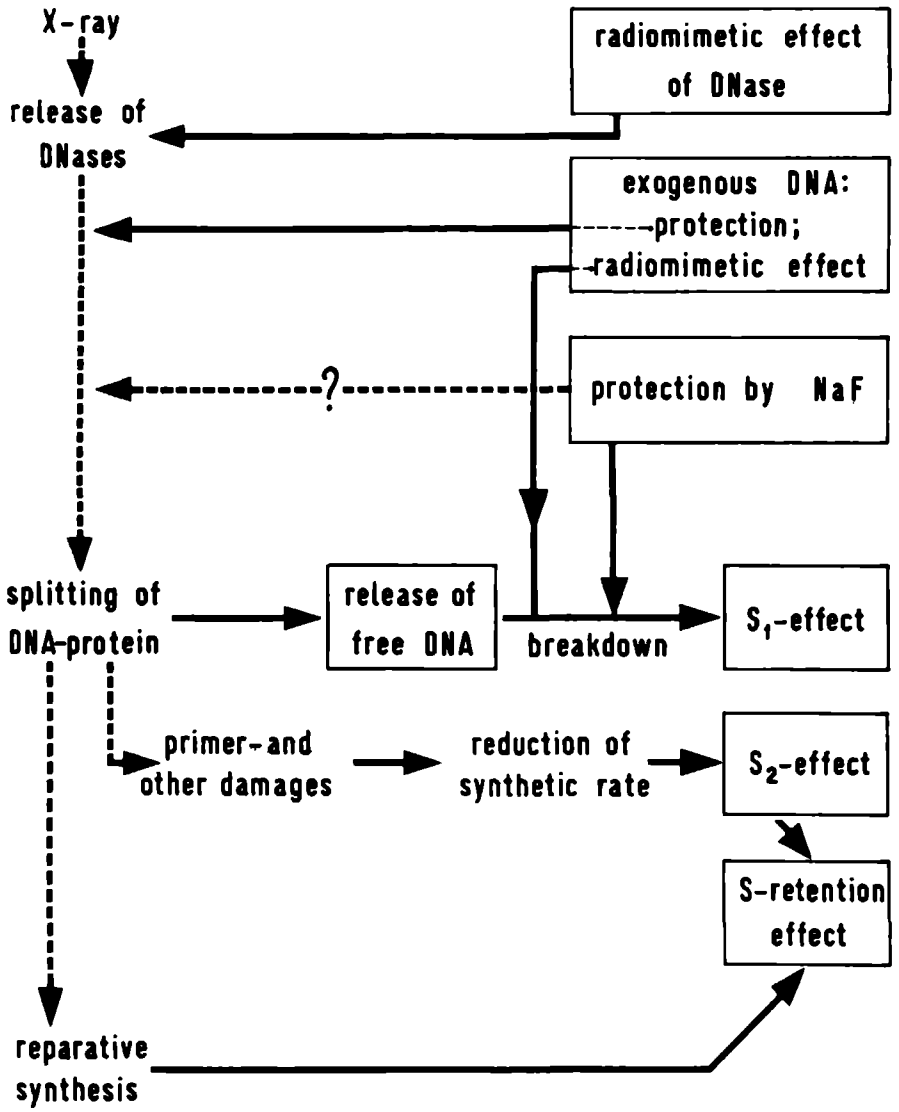

Fig.26. Schematic representation of the observed effects (enclosed at the right side) and their assumed interrelationships.

Fig.26. Schematisch overzicht van de waargenomen verschijnselen (omlijnd) en hun veronderstelde onderlinge samenhang.

SAMENVATTING

De synthese van DNA in embryonaal groeiende kalfslevercellen vindt plaats in een periode, de zg. S-fase, die door een G_1- en een G_2-fase gescheiden wordt van respectievelijk de voorafgaande dan wel de volgende celdeling, - zie hiervoor Fig.1. Omdat ioniserende straling alle deel-fasen van de delingscyclus op een verschillende wijze kan beïnvloeden, werd het onderzoek naar de DNA stofwisseling beperkt tot een periode van ten hoogste 6 uren volgende op een bestraling. Binnen dit tijdsbestek werd waargenomen dat na bestraling met doses tot 4000 r een onveranderd aantal cellen begint met de synthese van DNA maar dat het aantal cellen dat in deze periode de synthese beëindigt in afhankelijkheid van de dosis vermindert. Dit verschijnsel, aangeduid als het S-retentie effect, wijst op een vertraagde synthese van DNA zoals ook bleek uit een vermindering van het aantal zilverkorrels in de autoradiogrammen van cellen na een kortstondig verblijf in een medium dat radioactief thymidine bevatte.

Voor een nauwkeuriger meting van de snelheid van DNA synthese na bestraling, werd in hoofdstuk IV de inbouw van radioactieve bouwstenen in het DNA gemeten. In overeenstemming met de vele gegevens uit de literatuur, werd na incubatie in thymidine gemerkt met ^{14}C een verlaging van de specifieke activiteit van het DNA gemeten volgens een bifasische curve. De beide componenten van deze curve werden toegeschreven aan een S_1- en een S_2-effect. Zie hiervoor de Figuren 8, 10a en 10b.

Het S_1-effect is niet alleen meetbaar bij lagere doses dan het S_2-effect maar wordt bovendien eerder na bestraling vastgesteld. Door de remming van de inbouw van zowel radioactief thymidine als van anorganisch fosfaat na bestraling te bestuderen bij verschillende concentraties, werd vastgesteld dat deze remming competitief is en niet het gevolg van enige vertraging in de snelheid van DNA synthese. Bovendien bleken alle stralingsinvloeden die bestudeerd werden door intracellulaire factoren tot stand te komen. Aangenomen werd dat het S_1-effect het gevolg is van een enzymatische afbraak van DNA-ketens waarbij nucleosiden en anorganisch fosfaat ontstaan die de specifieke radioactiviteit van gemarkeerde verbindingen in de cel verlagen en de

opname ervan uit het medium vertragen.

Voor deze opvatting kunnen de volgende argumenten worden aangevoerd:

1. Het S_1-effect wordt onderdrukt door verhoging van de uitwendige concentraties van de gemarkeerde verbinding (nl. ^{14}C-thymidine en $^{32}P_i$). Zie hiervoor Fig.14 en Tabel III.
2. Homoloog DNA wordt in de cel afgebroken na opname in polymere vorm; door de gevormde afbraakprodukten ontstaat een effect op de opname van bouwstenen van het DNA vergelijkbaar met het S_1-effect.
3. Na bestraling is een toename van de hoeveelheid "vrij" DNA waar te nemen (Tabel XI).
4. Natriumfluoride remt de afbraak van DNA en remt tevens het S_1-effect van bestraling (Figuren 25a en 25b).
5. Het enzyme DNase veroorzaakt een effect vergelijkbaar met het S_1-effect indien gebracht in het kweekmedium van onbestraalde cultures (Tabel XIII).

Het S_2-effect treedt eerst enige tijd na bestraling op en is vooral bij hogere doses meetbaar. Het omvat alle stralingsinvloeden die leiden tot een vertraging van de snelheid waarmee DNA wordt gesynthetiseerd zoals o.a. remming van de activiteit en de biosynthese van desoxyribonucleotide kinasen en DNA-polymerase en de beschadiging van het "primer" DNA. Het S_2-effect is meetbaar als een vertraging in de DNA-synthese per cel waardoor een verlenging van de synthesesuur ontstaat. Als gemeenschappelijke oorzaak van zowel het S_1- als het S_2-effect werd de inductie van een enzymatische afbraak van een deel van het cellulaire DNA-eiwit complex aangenomen. Deze opvatting berust op de volgende waarnemingen:

a. DNA in het medium van bestraalde cultures geeft bescherming tegen het S_2-effect terwijl deze beschermende werking wordt toegeschreven aan het "afvangen" van enzymen die na bestraling het DNA-eiwit complex aantasten. Zie hiervoor hoofdstuk V, § 3.
b. DNase tast het cellulaire DNA aan waarbij dezelfde verschijnselen optreden als na een bestraling. Zowel een S_1- als een S_2-radiomimetisch effect werden na behandeling van cellen met dit enzyme waargenomen. Zie hiervoor Tabel XIII.

De gemeten remming van de snelheid van DNA synthese is niet voldoende om de verlenging van de syntheseduur te verklaren. Zoals weergegeven in Fig.18, bleek deze remming 4 uren na een dosis van 40000 r minder dan 30% te zijn, terwijl 6 uren na dezelfde dosis nog geen enkele cel de synthese van DNA had voltooid; in dezelfde periode daarentegen had ongeveer 60% van de oorspronkelijke S-fase populatie deze fase verlaten in de onbestraalde cultures (zie hiervoor Fig.7). Daarom werd de mogelijkheid naar voren gebracht dat in de bestraalde cellen

tevens een "reparatiesynthese" van DNA optreedt, waarbij de hoeveelheid afgebroken DNA nieuw wordt gesynthetiseerd.

Hoewel niet op een directe wijze werd vastgesteld dat na bestraling DNA-splitsende enzymen in de cel vrijkomen (b.v. door permeabiliteitsveranderingen in de membraan van lysosomen), wordt dit gehouden voor het meest waarschijnlijke gevolg van bestraling ter verklaring van de waargenomen verschijnselen. De resultaten van ons onderzoek en de veronderstelde onderlinge samenhang van de beschreven effecten, zijn schematisch weergegeven in Figuur 26.

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STELLINGEN

1. De door ioniserende straling veroorzaakte remming van de inbouw van radioactief thymidine berust slechts ten dele op een remming van de synthese van DNA.
   Dit proefschrift, pag.47

2. De mogelijke toepassing van DNA in de therapie van stralingsziekten verdient nadere bestudering.

3. Het verdient aanbeveling om in wetenschappelijke publicaties de concentraties van radioactieve verbindingen niet alleen in eenheden van radioactiviteit, maar ook in molaire eenheden te vermelden.

4. De experimenten van Rabotti over de opname van gemarkeerd DNA door Ascitescellen bewijzen niets.
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5. De immunologische aard van de incompatibiliteitsreactie van het gametophytische type wordt mede bevestigd door de grote overeenkomst van dit proces met de reactie van plantaardig weefsel op het binnendringen van pathogenen.

6. De niet specifieke inbouw van gemarkeerd thymidine door Riella spec. is waarschijnlijk toe te schrijven aan een afbraak van thymidine.
   Eigen waarnemingen

7. Het doorlichten van personen voor keuringsdoeleinden dient zodanig te worden georganiseerd, dat zinloze herhalingen van dit onderzoek worden voorkomen.

8. De invoering van een doctoraalexamen Lichamelijke Opvoeding is gewenst.

9. Omdat zijn specifieke vermogens ook als aanpassingen uiterst efficiënt zijn, is de mens bij uitstek het doel van de evolutie.

10. Het verdient aanbeveling dat ook de Nederlandse overheid eisen stelt aan de veiligheid van de door haar te kopen motorvoertuigen en dat aan deze eisen openbare bekendheid wordt gegeven.