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SURVIVAL CHARACTERISTICS OF MAMMALIAN CELL LINES AFTER SINGLE OR MULTIPLE EXPOSURES TO ROENTGEN RADIATION UNDER OXIC OR ANOXIC CONDITIONS

by

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Much information has accumulated during the past decade on the radiation survival of a great variety of mammalian cell lines with the clonogenic ability of the cells as an index (Elkind & Whitmore 1967). Most of the observations concern the survival characteristics after irradiation under aerobic conditions. Few investigations have treated the problem of the dose-effect relationship with irradiation in hypoxia or in a total absence of oxygen. The data in the relatively few publications have often been inconsistent. Attempts to resolve the contradictions (e.g. at the 1st Gray Memorial Meeting in London 1967) have met with only partial success. The differences in the biologic materials combined with the differences in the experimental procedures, especially those that concern control of hypoxic conditions, present serious obstacles to a conjoint interpretation of the available data.

The investigations to be described were conducted with the aim of comparing the reactions of different cell lines to oxic and anoxic roentgen radiation under

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identical experimental conditions. In view of recent observations (CHAPMAN et coll. 1968, LITTBRAND & RÉVÉSZ 1969) particular attention was paid to the control of the oxygen concentration at a level previously thought to be of minor importance in radiobiologic experiments. The design of the experiments received special attention to ensure that the data should be suitable for statistical analysis. A closer knowledge of the cellular reactions to hypoxic or anoxic radiations may be both of theoretical and practical interest. It may help to throw further light on the mechanism of the biologic actions of radiations and to develop more effective plans for the treatment of neoplasms by more effectively controlling their portions with a deficient oxygen supply.

Materials and Methods

Cell cultures. Cultures of Chinese hamster cells (V 79-379-A), an Ehrlich ascites tumor (ELD), and three substrains of the latter, denoted ELT, SELD clone-a and SELD clone-g, were used. The isolation of the clones and their characteristics have already been desribed (HAUSCHKA et coll. 1957, RÉVÉSZ, GLAS & HILDING 1963b). The ELT substrain has a duplicated, i.e. hypertetraploid, chromosome set in relation to ELD and is also characterized by an increased radioresistance under certain in vivo conditions, as compared to the original cell strain (GLAS & RÉVÉSZ 1963). The cells in clone-a and clone-g have chromosome sets in the same poidy region as ELD but differ in their sensitivity to radiation in vivo, clone-a being as sensitive as ELD, and clone-g less sensitive (RÉVÉSZ et coll. 1963b).

The Chinese hamster cells were received from Dr Elkind of Bethesda in 1964 and were already adapted to growth in culture. The cultures of ELD and its substrains were established from ascites material grown in vivo. Ascites of a particular strain freshly withdrawn, was dispensed in amounts of 0.1 ml in stoppered Pyrex milk dilution bottles of 120 ml volume. The culture medium consisted of Eagle's medium in Earle's solution (EAGLE 1959) supplemented with 15 % fetal calf serum and antibiotics. After a period varying from 7 to 18 days, multiple foci of vigorously growing colonies became visible, firmly attached to the bottom of the culture flasks. The colonies were detached and dispersed by gentle trypsin treatment, the cells being cultivated in serial passages by transfer of 10^4 cells at weekly intervals, and by medium changes every second or third day. In order to protect against development of new substrains, prone to occur in prolonged serial cultures in vitro (CAILLEAU & COSTA 1961, NIELSÉN 1967), the chromosomal constitution of the cell population was consequently examined at intervals of about ten successive passages as described previously (RÉVÉSZ et coll. 1963b) and whenever changes were noted the propagation of

the strain was discontinued. New cultures were established from earlier samples, which were preserved frozen in a tumor bank.

Irradiation. Radiation was generated by a Siemens roentgen unit at 190 kV and 15 mA; the radiation was filtered by 1.5 mm Al inherent filtration and a 1.0 mm Al additional filter; half-value layer 0.95 mm Cu. The dose rate was 235 R/minute at the bottom of the culture dishes which were always placed at a distance of 43 cm from focus. The dose delivered was measured by a Philips integrating dosimeter. Pyrex petri dishes were used in all the tests. The correction factor for back scatter established from survival measurements of Chinese hamster cells while irradiated on glass versus plastic was 1.35 ± 0.05.

Experimental procedure. Suspensions of monodispersed cells were prepared from 6 to 8 days old cultures growing in a non-confluent monolayer on the surface of stoppered milk dilution bottles. The cells were readily dissociated by treatment with a 0.5 % trypsin solution at a temperature of 37 °C for 10 minutes. After establishing the cell concentration in a haemocytometer, an appropriate number of cells was explanted in pyrex dishes of 6 cm diameter containing 4 to 6 ml of nutrient medium. The number of cells was chosen so that about 100 cells were expected to survive exposure to a particular roentgen dose. The dishes were incubated for 3 to 4 hours at 37 °C in a moist atmosphere with 5 % carbon dioxide in air to maintain a pH of 7.0 to 7.2. After incubation, the cells were irradiated and reincubated for 10 days with two medium changes, the first taking place 2 to 3 and the second 6 to 7 days after irradiation. The clones that developed were fixed and stained in situ. Those that comprised more than 50 cells were counted and taken to represent the surviving fraction of the explanted population. Mean values were routinely calculated from three (irradiated cultures) or six (unirradiated controls) replicate dishes. The fraction of cells surviving irradiation was expressed as the percentage of the unirradiated controls in the same experiment. The plating efficiency of unirradiated controls was defined as the percentage of 100 to 200 cells that grew into macroscopic colonies in 10 days.

Roentgen irradiation was administered at a temperature of about 27 °C in an air-tight plastic chamber of 2-liter volume. The chamber had two orifices of 6 mm diameter for the in- and outlet of gases. The lids of the culture dishes were removed, and the nutrient medium withdrawn to such an extent that no more than 1 ml fluid to cover the cells was left. The chamber was filled with either oxygen or argon, both supplemented with 1.25 % carbon dioxide, the flow rate being adjusted to 6 liter/minute. The gases passed through pre-equilibrated water warmed to a temperature of 38 °C. Argon with an oxygen contamination less than 2 ppm was used and led to the chamber through a system of stainless

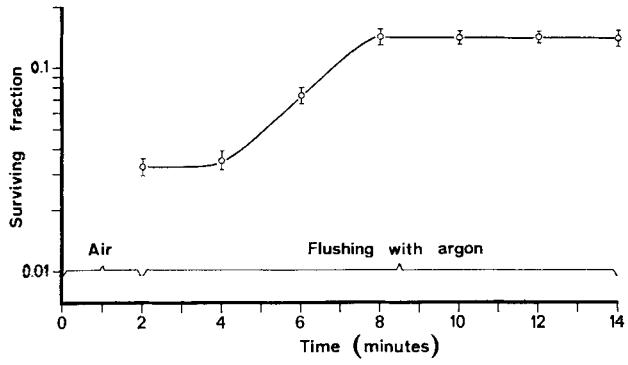

Fig. 1. Survival of Chinese hamster cells exposed to 500 R during different periods in the course of a 12-min period of flushing of the irradiation chamber with argon. The results are from 6 repeat tests. The points with S.E. stand at the end of the 2-min irradiation period. The gas flow was 6 liter/minute and the chamber had a 2 liter-volume.

steel and glass tubes with joints sealed impermeably to oxygen from the atmosphere. The gas outflowing from the radiation chamber passed a water lock and was routinely monitored by an Elcoflux oxygen analyser (Dr Thiedig & Co KG, Berlin).

The flushing period of the chamber was 20 minutes in the early tests, and 14 minutes in the final tests. The irradiation period varied with different doses and was adjusted to terminate at the end of the flushing period and never started before 6 minutes of flushing.

The survival of Chinese hamster cells treated with 500 R at different times in the course of a 12 min-flushing of the irradiation chamber with argon is illustrated in Fig. 1. For flushing times of 4 minutes or longer the survival was always similar, indicating that equilibration between the gas phase and the liquid phase that surrounded the cells was complete within 4 minutes. This finding is in agreement with theoretical calculations discussed elsewhere (LITTBRAND & RÉVÉSZ 1969).

After radiation exposure the dishes were removed from the chamber, refilled with 5 ml fresh medium and incubated in air and carbon dioxide. The exposure procedure with multiple irradiations was repeated after various time intervals. Unirradiated control cultures were sham-irradiated under the same conditions as the others. The cultures which received a single exposure dose and belonged to the same experimental series in which the effect of the same dose split into fractions was studied were also sham-irradiated at appropriate intervals. The simi-

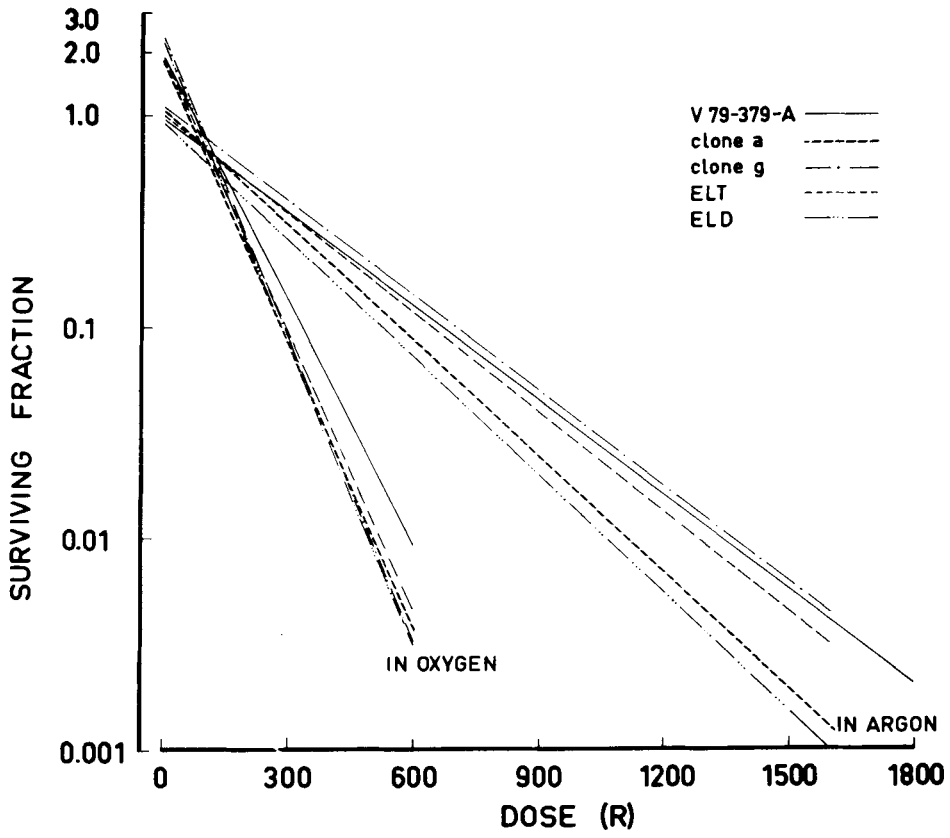

Fig. 2. Survival regression of the cell lines after exposure to roentgen radiation in argon or oxygen. The numerical value of the regression parameters and their S.E. is presented in Table 1.

larity between the plating efficiency of the cells that were exposed in either argon or oxygen indicates that the difference in the gas phase does not affect the survival of unirradiated cells to any appreciable extent under the conditions used (cf. Tables 3, 4 and 6).

Statistical considerations. A main consideration in the design of the experiments concerned the suitability of the data expected for a statistical evaluation of their significance. In determining the variance of the slope constant ($1/D_0$) and the extrapolation number (n), the statistical requirement for independent observations is satisfied if several repeat tests of the survival regression are performed; then the $1/D_0$ and n of each survival curve may be regarded as a single observation. The statistical degrees of freedom are determined solely by the number of

Table 1

Regression parameters of the radiation survival of different cell lines as established by least square analysis of data in repeated tests — Two to four different roentgen doses were used to establish the survival in each test

Cell line	Gas phase during radiation exposure	Plating efficieny Mean ± S. E. (%)	Number repeat tests	Total number surviving fractions determined*	Slope constant of the survival regression Mean ± S. E. (^{e}log. units/100 R)	D_0 (R)**	Extrapolation number***	
							Mean ± S. E. (^{e}log. units)	Arithmetic value of mean
ELD	Argon	76 ± 5	17	36	−0.429 ± 0.014	233	−0.020 ± 0.143	0.98
	Oxygen	83 ± 6	7	20	−1.062 ± 0.031	94	0.692 ± 0.158	2.00
ELT	Argon	35 ± 2	18	52	−0.362 ± 0.009	276	0.021 ± 0.080	1.02
	Oxygen	41 ± 6	7	23	−1.025 ± 0.032	98	0.780 ± 0.124	2.18
SELD clone-a	Argon	41 ± 3	20	54	−0.427 ± 0.011	234	0.227 ± 0.170	1.26
	Oxygen	41 ± 2	16	48	−1.048 ± 0.033	95	0.740 ± 0.078	2.10
SELD clone-g	Argon	48 ± 4	24	68	−0.340 ± 0.016	294	0.030 ± 0.225	1.03
	Oxygen	31 ± 1	13	38	−1.087 ± 0.026	92	0.833 ± 0.110	2.30
Chinese hamster cells	Argon	76 ± 4	16	59	−0.354 ± 0.009	282	0.037 ± 0.083	1.04
	Oxygen	64 ± 9	6	18	−0.885 ± 0.020	113	0.632 ± 0.076	1.88

*The surviving fraction was determined from the mean value in three irradiated and six control replicate cultures.

**Reciprocal of the slope constant indicating the increment of radiation dose which reduces the surviving fraction to 37 %.

***The intersection of the statistical regression line with the ordinate at zero dose.

separate survival curves, regardless of the number of survival determinations which build up any of the curves. The survival determinations pertaining to one individual survival curve are dependent upon a common control, the same particular conditions regarding the quality of the medium, pH and temperature variations which may bias all the observations within a test and do not satisfy, therefore, the requirement for independent data.

These considerations led us to perform a series of at least five repeat tests for determining the parameters of the survival regression of a particular cell line after irradiation. In each test, the survival is in general established after exposure of cells in replicate dishes to two or three different roentgen doses. These are of such a magnitude that the dose-effect relationship can be assumed

Table 2

The ratio and its standard error between the slope constant of the oxic and anoxic survival regressions of the five cell lines tested — Data presented in table 1 were used for calculations — S. E. was approximated by formula (1) given in the text

Cell line	Ratio between the slope constants of the oxic and anoxic survival regressions. Mean ± S. E.
ELD and substrains	
ELD	2.48 ± 0.11
ELT	2.83 ± 0.12
SELD clone-a	2.45 ± 0.07
SELD clone-g	3.20 ± 0.12
Chinese hamster cells	2.50 ± 0.09

to be exponential. The survival data even after such a few exposure doses are obviously sufficient for the statistical determination of a linear survival regression by least square analysis. Since the confidence limit of the regression line is inversely related to the difference between the exposure doses (PORTER 1963), one of the doses is chosen, as a routine, closest to a possible 'shoulder region' of the expected survival curve, and another dose to give the minimum survival (in the range 10^{-3}) still detectable by our method with sufficient accuracy. A third dose with a magnitude between the two extremes is also given to permit the control of the linearity of the survival regression. The series of slope constants and extrapolation numbers obtained in repeat tests are then used for the calculation of the mean and the variance of these survival parameters and for the evaluation of their statistical significance.

Results

Single exposures. In a series of repeat tests, cultures of Chinese hamster cells, ELD and its derivatives ELT, SELD clone-a and clone-g were exposed to radiation in the dose range 200—600 R in oxygen or 800—1 800 R in argon.

Assuming an exponential decrease of the survival in the dose ranges used (ELKIND & WHITMORE 1967), the survival regression was determined by least square analysis in each test, and the mean regression of the different test groups calculated. The results are illustrated in Fig. 2. The numerical value of the regression parameters are summarized in Table 1.

The reciprocal of the slope constant of the survival regressions (D_0) indicating the dose that reduces survival to 37 per cent gave values between 92 to 98 R

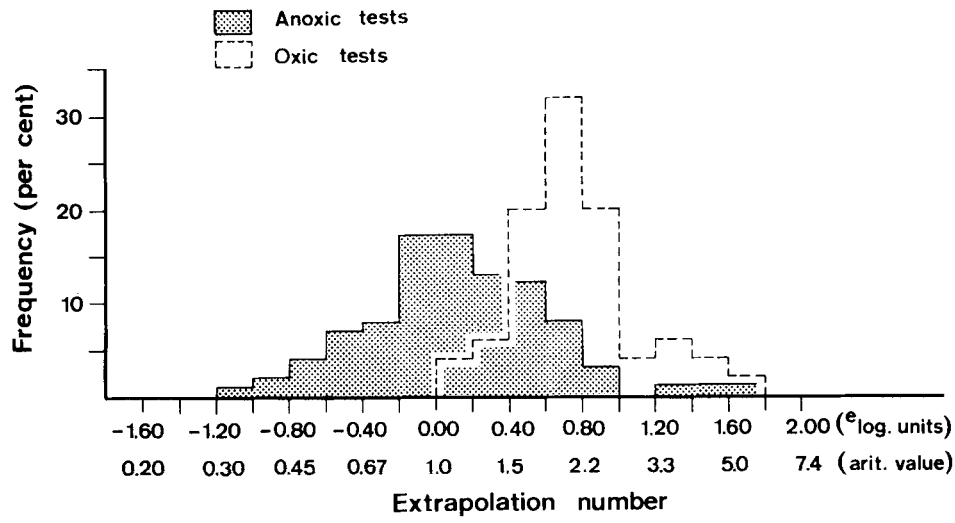

Fig. 3. Frequency distribution of the extrapolation number of the survival regressions calculated for each of the 49 oxic and 95 anoxic tests presented in Table 1.

when irradiation of ELD and its substrains was made in oxygen, and between 233 to 294 R when these cell strains were exposed to radiation in argon. The differences between the slope constants of the oxic cells were, as found by t-test analysis, not significant. When treated in argon, the slope constant of ELD and clone-a differed significantly from ELT and clone-g ($p < 0.001$), but the difference between ELD and clone-a or ELT and clone-g was not significant. The D_0 for Chinese hamster cells was 113 R in oxygen, and 282 R in argon.

The intersection of the regression line of the individual tests with the ordinate at zero dose gave extrapolation numbers the log frequency distribution of which did not deviate from a normal distribution (cf. Fig. 3). The means were in the region of 2 after oxic treatments, and close to unity after the anoxic treatments. When tests performed with the same cell strain are considered, the difference between the mean extrapolation number in oxygen and in argon is significant ($p < 0.001$ or < 0.02).

The ratios between the slope constants of the survival regressions after oxic and anoxic irradiation are presented in Table 2, the data given in Table 1 being used for the calculation. The S.E. of the ratios were approximated by the following formula:

$$S_R = \sqrt{\frac{R^2_0}{R^2_A}\left(\frac{S^2_0}{R^2_0} + \frac{S^2_A}{R^2_A}\right)\left(1 + \frac{9S^2_A}{R^2_A}\right)} \qquad (1)$$

Table 3

Survival of one hundred Chinese hamster cells after exposure to roentgen doses in the range 50—150 R in either the presence or absence of oxygen — The mean plating efficiency was 96 ±3 and 95±2 per cent in the tests with argon and oxygen, respectively

Number of repeat tests:		10				19				
Exposure dose: (R)		50		75		100		125		150	
Gas phase during radiation exposure:		Ar	0_2	Ar	0_2	Ar	0_2	Ar	0_2	Ar	0_2
Surviving fraction (%)	Arithmetic value of mean:	93.8	92.6	90.6	88.4	87.0	83.6	80.3	80.8	79.1	73.1
	Mean±S.E.: (^{e}log. units)	4.541 ± 0.025	4.529 ± 0.033	4.506 ± 0.033	4.482 ± 0.023	4.466 ± 0.015	4.426 ± 0.021	4.386 ± 0.028	4.392 ± 0.022	4.371 ± 0.019	4.293 ± 0.021
Ratio between surviving fractions in argon and oxygen Mean±S.E.: (^{e}log. units)		0.012* ± 0.041		0.024* ± 0.042		0.040* ± 0.023		—0.007* ± 0.031		0.078** ± 0.024	

*P (probability of no difference from zero) > 0.05.
**P<0.01.

where S_R is the S.E. of the ratio between the respective slope constants, R_0 and R_A are the means of the slope constant of the survival regression obtained in repeat tests in oxygen and argon, and S_0 and S_A are the S.E. of R_0 and R_A, respectively.

In comparing the ratio between the oxic and anoxic slope constants of ELD with the ratios of its substrains, the t-test analysis will indicate that ELD or clone-a has a ratio with a significantly smaller value than ELT or clone-g ($p < 0.001$ or < 0.05). The value for ELT differs significantly from that of clone-g ($p < 0.05$) but the difference between the ratio of ELD and clone-a is not significant.

Effect of doses below 150 R. The difference between the extrapolation numbers after oxic and anoxic irradiation implies a crossing of the regression lines. As can be seen from Fig. 2, the crossing occurs in a dose range around 120 R for each cell strain. In this dose range, therefore, oxygen may not enhance the

Table 4

Survival of Chinese hamster cells after exposure in argon or oxygen to either a single dose of 300 R or to the same dose divided into three equal fractions and separated by a time interval of three hours — During the intervals the cells were incubated in air and carbon dioxide — The data are from 13 repeat tests

Exposure dose (R)	Treatment in oxygen			
	Cells exposed	Plating efficiency Mean ± S. E. (%)	Surviving fraction	
			Mean ± S. E. (^{e}log. units)	Arithmetic value of mean
3 × 100	300	98 ± 3	3.529 ± 0.041	34.1
300	500		2.955 ± 0.048	19.2

radiosensitivity of the cells. After larger doses on the other hand the oxygen enhancement ratio defined by the ratio between the dose in oxygen and in anoxia which produces identical survival will obviously increase (cf. Fig. 2). It can be calculated (RÉVÉSZ & LITTBRAND 1967a) that the maximum oxygen enhancement ratio will be defined by the ratio between the slope constant of the oxic and anoxic survival regressions (cf. Table 2).

The effect of oxygen was examined in two series of experiments on the survival around and below the dose range where the oxic and anoxic regression lines cross. Chinese hamster cells in one series were exposed to a single radiation dose in the range 50 to 150 R in the presence or absence of oxygen. The results are summarized in Table 3. The presence of oxygen does not seem to exert any major influence on the surviving fraction after exposure to a dose of 125 R or less. The differences between the oxic and the anoxic groups are statistically insignificant. On the other hand, the surviving fraction is significantly smaller ($p < 0.01$) after exposure to 150 R in oxygen than after treatment with the same dose in argon.

The failure of oxygen to enhance cellular radiosensitivity when treatment is given with relatively small radiation doses was put to a further and more rigorous test in another series of experiments. Chinese hamster cells were exposed three times to a dose of 100 R in the presence or absence of oxygen with an interval of 3 hours between the exposures. Cultures treated under identical conditions with a single dose of 300 R and two subsequent sham-irradiations were used as particular controls. The results are presented in Table 4. The surviving fractions were of about the same magnitude after triple exposures when either oxygen or

Table 4 *(cont.)*

Treatment in argon				Ratio between survival fractions in argon and oxygen	
Cells exposed	Plating efficiency Mean ± S. E. (%)	Surviving fraction		Mean ± S. E. (^{e}log. units)	Arithmetic value of mean
		Mean ± S. E. (^{e}log. units)	Arithmetic value of mean		
300	99 ± 4	3.568 ± 0.036	35.5	0.039 ± 0.040	1.04
300		3.536 ± 0.040	34.3	0.581 ± 0.043	1.79

argon was the gas phase. On the other hand, when a single treatment was given with the same total dose, the survival was larger by a factor of 1.79 in argon than in oxygen. The survival after treatment with triple doses may also be calculated as 1.78 times larger than after treatment with a single dose in the presence of oxygen. When oxygen was absent, only an insignificant difference was noted in the effect between a single and triple radiation treatment.

Estimations of the oxygen enhancement ratio from the data presented in Table 3 offer further support to the conclusion that oxygen fails to enhance radiosensitivity in treatment with small roentgen doses. Such estimations may be made by calculating the difference between the survival after irradiation with a dose in argon and with another dose in oxygen, and the corresponding ratio between the doses. Table 5 lists the survival ratios that correspond to roentgen doses chosen in such a way that their ratios are between 1 and 2. In considering such dose combinations, the survival appears always to be better after oxic than after anoxic treatment. The differences have varying degrees of statistical significance. Since an identical survival after oxic and anoxic treatment would indicate that the oxygen enhancement ratio equals the corresponding dose ratio, it may be concluded from the data in Table 5 that the oxygen enhancement ratio in these experiments is significantly lower than 2 and probably also lower than 1.2.

Split-dose ratio under oxic and anoxic conditions. The effect of two exposures to radiation on the survival of ELD and Chinese hamster cells was investigated in relation to the effect of a single exposure to the same total dose combined with a subsequent sham-irradiation. The time interval between two treatments

Table 5

Survival ratio after oxic and anoxic exposure to roentgen doses chosen in such a way that the dose ratio was between 1 and 2 — Data in Table 3 were used for the calculations

Exposure-dose ratio: in argon/in oxygen	Survival ratio: Surviving fraction after treatment in oxygen/ surviving fraction after treatment in argon (^{e}log units)
$\frac{75}{50} = 1.5$	0.022
$\frac{100}{50} = 2.0$	0.062*
$\frac{100}{75} = 1.3$	0.015
$\frac{125}{75} = 1.7$	0.096
$\frac{150}{75} = 2.0$	0.111**
$\frac{125}{100} = 1.3$	0.041
$\frac{150}{100} = 1.5$	0.056
$\frac{150}{125} = 1.2$	0.022

*P (probability of no difference from zero) < 0.05 **P < 0.001

was 4, 12 or 18 hours. Irradiations were performed either in the presence or absence of oxygen. The results are illustrated in Fig. 4. Independently of cell strain and time interval between treatments, the surviving fraction after two exposures was of a similar magnitude as after a single exposure to the equivalent dose when oxygen was absent. Thus, the split-dose ratio defined by the ratio between the surviving fractions after a single and split doses was always close to unity. On the other hand, when irradiated in the presence of oxygen, double or treble the number of cells survived after split-dose treatment than after corresponding single-dose treatment. When the tests, performed with the same cell strain and with identical time interval between the exposures, are considered, the difference between the split-dose ratio after oxic and anoxic treatment is significant ($p < 0.001$ or < 0.005).

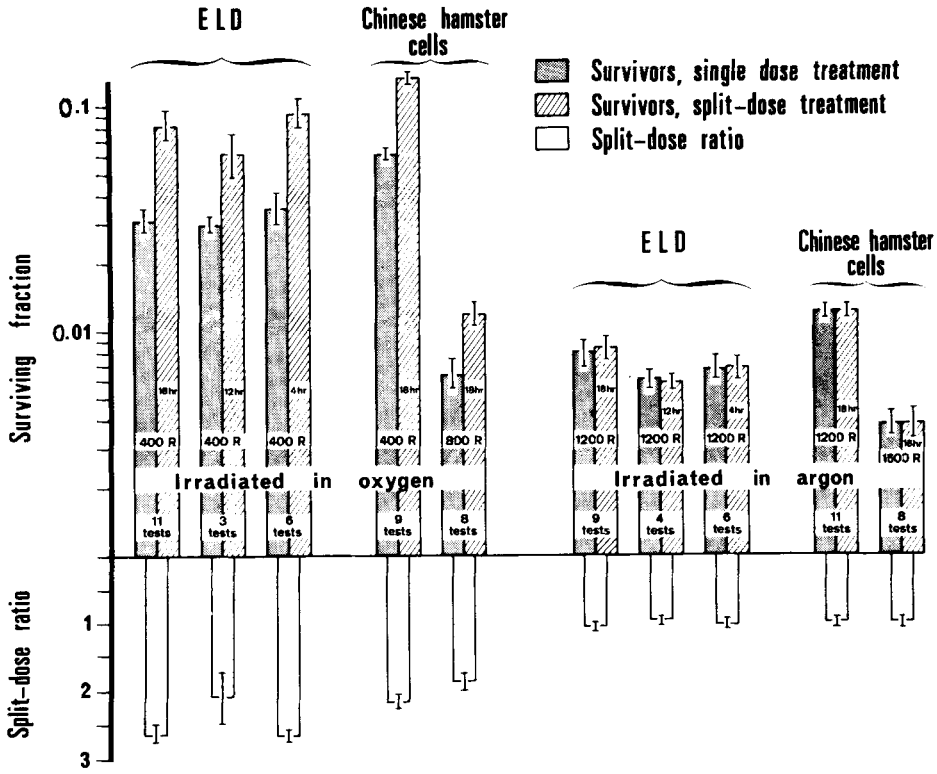

Fig. 4. Survival of ELD and Chinese hamster cells after exposure in argon or oxygen to either a single roentgen dose or to same dose delivered in two equal fractions (upper scale). The time interval between the dose fractions was 4, 12 and 18 hours as indicated in the respective columns, during which period the cells were incubated in air and carbon dioxide. The split-dose ratio calculated from the difference between the survival after single and split doses are indicated (lower scale). Each column shows the mean with S.E. The mean plating efficiency varied between 47 to 70 and 91 to 96 per cent in the test with ELD and Chinese hamster cells, respectively.

A series of five repeat tests was performed to examine the possibility that the difference between the split-dose ratios in argon and oxygen might have been due to some particular effect of the argon treatment before irradiation. Chinese hamster cells were kept in argon for 15 minutes and, within one minute thereafter, irradiated in oxygen with either 700 R or 2×350 R at time intervals of 18 hours. As a particular control, cultures that were kept in oxygen for 5 minutes and subsequently irradiated with the same doses under identical conditions, were employed. The difference in the pretreatment with the two different gases does not affect either the survival or the split-dose ratio, as indicated by the results in Table 6.

Table 6

Effect of pretreatment with argon or oxygen on the survival of Chinese hamster cells exposed to oxic irradiation with a single or split doses

Treatment before irradiation	Plating efficiency ±S. E. (%)	Exposure dose in oxygen (R)	Surviving fraction (%)		Split-dose ratio	
			Mean ±S. E. (^{e}log units)	Arithmetic value of mean	Mean ±S. E. (^{e}log units)	Arithmetic value of mean
15 min in argon	85±5	700	−0.149±0.115	0.86	0.830±0.151	2.29
		2×350	0.681±0.127	1.98		
5 min in oxygen	84±5	700	−0.173±0.126	0.84	0.781±0.100	2.18
		2×350	0.608±0.130	1.84		

Survival regression after irradiation with split-doses. The survival regression after split-dose treatments was studied by double exposures of the hamster cells to increasing total roentgen doses. The first exposure was always to a dose of 800 R, the second one to a dose varying from 200 to 800 R, all exposures being made in argon. In each of the five repeat tests, the cells were in two different stages of growth when irradiated for the first time. In one case, the first dose was given 3 hours after plating of the cells, followed by the second dose after an interval of 18 hours. In the second case, the cells were irradiated with the first dose 21 hours after plating, and with the next dose after a further interval of 9 hours. These time intervals were chosen from two previous observations. On the one hand, cells that have been irradiated 3 hours after plating, will not divide for 18 hours. On the other hand, if the first irradiation is made 21 hours after plating, the cells will have completed one division, and the population will have doubled and consist of sister-pairs. In the latter instance, the survivors will not have completed a second division within a further period of 9 hours.

The survival of the irradiated cells in relation to unirradiated controls is illustrated in Fig. 5-A. The numerical value of the survival parameters calculated by least square analysis are presented in Table 7. The slope constants are almost identical whether the exposure to radiation was made before or after the first division of the cells. In contrast, the extrapolation number of the regression line at a zero dose is close to unity when the cells were treated before division, and has a value between 2 and 3 when the cells were treated after division.

Considering that the probability of a cell surviving irradiation with 800 R, used as the first dose, is 0.06 (cf. Figs 2 and 5-A), it can be calculated that the probability of both cells in a sister-pair surviving is $0.06^2 = 0.0036$, and that of

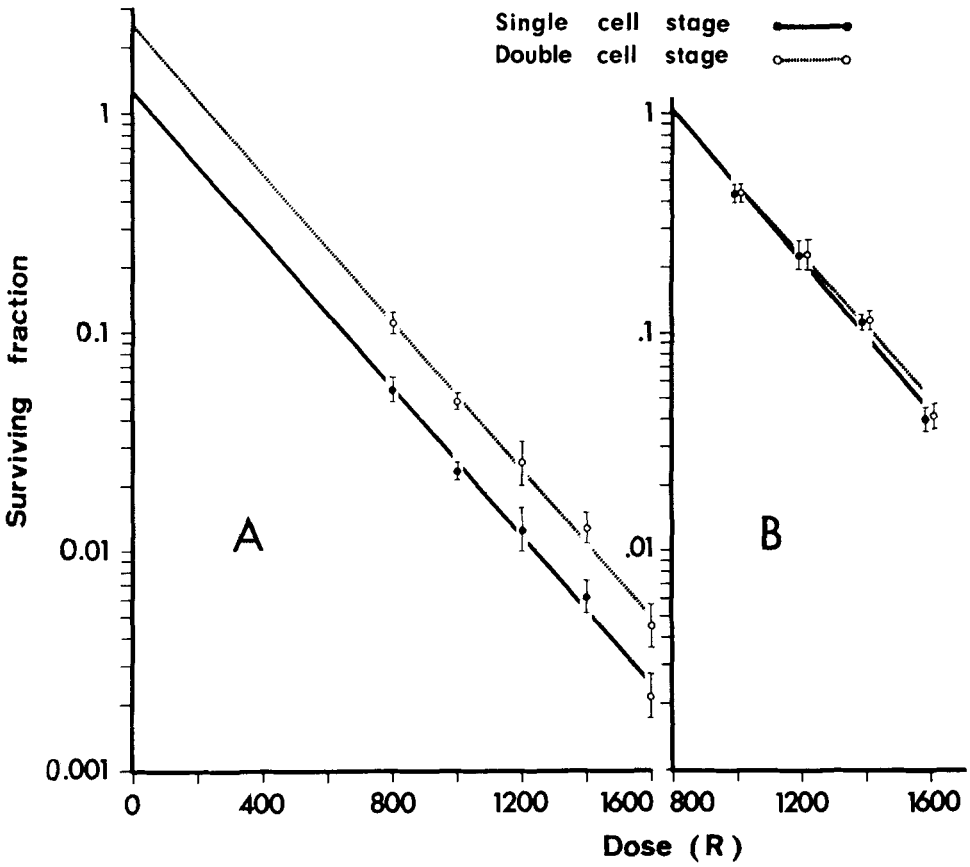

Fig. 5. Survival of Chinese hamster cells after irradiation with increasing doses of roentgen rays in two exposures in argon. The first exposure followed the plating of the cells after a period of 3 hours (single cell stage) or 21 hours (double cell stage) and was, in all cases, to 800 R. The second exposure followed the first exposure after a period of 18 hours (single cell stage) or 9 hours (double cell stage) to a dose varying from 200 to 800 R. Between exposures the cells were incubated in air and carbon dioxide. Means with 95 per cent confidence limits are shown from 5 repeated tests with a mean plating efficiency of 93 ± 5 per cent. Left part of figure (A) illustrates survival relative to the unirradiated cells; right part (B) the survival relative to the survivors of 800 R treatment.

a single cell in a sister-pair is $2 \times (1 - 0.06) \times 0.06 = 0.1152$. This suggests that the overwhelming majority $\left(\frac{0.1152}{0.1152 + 0.0036} = 97\ \%\right)$ of the survivors in the population exposed to an initial radiation with 800 R after cell division will consist of single cells and not of sister-pairs, i.e. the cell multiplicity will be 1 when the second radiation dose is applied.

Table 7

Numerical value of the survival parameters in the tests illustrated in Fig. 5

Time interval between plating and 1st irradiation	Time interval between 1st and 2nd irradiation	Survival of irradiated cells relative to unirradiated controls				Survival of irradiated cells relative to survivors of 800 R			
		Slope constant of survival regression Mean ± S. E. (^{e}log units)	D_0 (R)	Extrapolation number* Mean ± S.E. (^{e}log units)	Arit. value of mean	Slope constant of survival regression Mean ± S. E. (^{e}log units)	D_0 (R)	Extrapolation number** Mean ± S. E. (^{e}log units)	Arit. value of mean
3 hours	18 hours	0.390 ± 0.018	256	0.232 ± 0.096	1.26	0.393 ± 0.027	254	0.031 ± 0.129	1.03
21 hours	9 hours	0.387 ± 0.018	259	0.906 ± 0.076	2.47	0.389 ± 0.025	257	0.023 ± 0.120	1.02

*Calculated from the intersection of the statistical regression line with the ordinate at zero dose.
**Calculated from the intersection of the statistical regression line with the ordinate at 800 R.

In Fig. 5-B the survival of the irradiated cells relative to the survivors of a treatment with 800 R is illustrated. The numerical value of the survival parameters, calculated by least square analysis is presented in Table 7. The slope constants are, again, similar if the data obtained in tests with cells before and after division are considered. However, in this situation the extrapolation numbers are again similar and close to unity in both cases.

Discussion

Slope of survival regression. The results indicate that under anoxic conditions significant differences in radiosensitivity exist between cell strains selected from the same original tumor cell population. It may be concluded that in agreement with the wide intercellular variation of different characteristics (Harris 1964), neoplastic cell populations may also vary with regard to the radiosensitivity of their cellular components. It may be calculated from the relationship between the slope constants that the sensitivity of clone-g and ELT is 24 and 19 per cent less, respectively, than the sensitivity of the original ELD. The decreased sensitivity of clone-g may be attributed to the protective effect of the increased cellular concentration of low molecular sulphydryls, which has been estimated to be 24 per cent higher in this clone than in ELD (Caspersson & Révész 1963, Révész

et coll. 1963a). The role of the inherent sulphydryls in influencing intrinsic radiosensitivity is further supported by the finding that clone-a, with the same sensitivity as ELD, has a thiol concentration of the same magnitude (RÉVÉSZ et coll. 1963a). The increased radioresistance of ELT with a sulphydryl concentration of the same magnitude as ELD (CASPERSSON & RÉVÉSZ 1963, RÉVÉSZ et coll. 1963a), can be associated with the duplicated genome of this strain, as discussed in other reports (GLAS & RÉVÉSZ 1963, RÉVÉSZ et coll. 1963b, RÉVÉSZ & LITTBRAND 1964). The possible necessity of inactivating a larger number of sensitive sites has probably accounted for the radioresistance in such a case. However, since the extrapolation number of the ELT survival curve is similar to the extrapolation of ELD whether oxic or anoxic irradiation was used (cf. Fig. 2) some other factors in addition to, or instead of, an increased target number may also play a role. The observations of SILINI & HORNSEY (1962) and BERRY (1963) on an increased extrapolation number of tetraploid tumors in comparison to diploid ones may not be appropriate in this context. The cell lines in their tests were not sublines of each other and the change in the extrapolation number may be associated therefore to some difference between lines other than ploidy.

The difference in radiosensitivity demonstrated between the cell strains irradiated in anoxia was shown to be reduced to statistically insignificant variations when the exposures to roentgen radiation was made in oxygen. This implies that the oxygen enhancement ratio is different for the different cell strains. Statistical analysis of the data indicated that the differences in the oxygen enhancement ratio were significant (Table 3). A reduction of the sensitivity differences in this gas as compared to anaerobic conditions has been previously noted also when the cell strains used in the present tests were investigated in vivo (RÉVÉSZ et coll. 1963b, RÉVÉSZ et coll. 1967). Similar observations were reported by SILINI & HORNSEY (1962) in their in vivo tests. The relationship between the values of oxygen enhancement ratios of diploid and tetraploid cell lines used by these authors (2.7/3.1) was similar to the relationship observed in our case 2.48/2.83.

The oxygen effect can apparently mask variation in cellular sensitivity whether due to a difference in ploidy or to the -SH content. If a proportionately increased sulphydryl concentration accounts for the relative resistance of some cell lines in anoxia, then the disappearance of this resistance in oxygen would be in agreement with the observation of a considerable decrease of the sulphydryl protection in an oxygenated medium (DEWEY 1963, HOWARD-FLANDERS 1960, SHALEK & SMITH 1968). It can also be argued that the correlation between radiosensitivity and ploidy observed when treatment is made in anoxia may imply the principally genetic character of the radiation injury to the cell. The decrease or abolition of such a correlation when treatment is made in oxygen suggests the prevalence of

some non-genetic mechanism operative in the presence of this gas and involving structures and systems other than the genetic apparatus alone. This conclusion is in agreement with the postulate based on observations made in experiments with micro-organisms that DNA is more heavily involved in killing by roentgen-radiation under anoxic as compared to aerobic conditions (ALPER 1963).

Extrapolation numbers. The extrapolation number (n) of the survival curves of ELD and its substrains, as well as the Chinese hamster cells, were similar and had values around 2 when irradiations were administered in the presence of oxygen, and values close to unity when the irradiations were given in the absence of the gas. The relatively low value of n of the oxic survival curves may be attributed to the fact that the cells used in the survival tests were derived from 6- to 8-day old cultures, i.e. were in a stationary phase of growth. HAHN (1968) reported that the survival curve of such cells extrapolate to lower values than cells derived from cultures in the phase of exponential growth.

The extrapolation number to unity found throughout in the tests when cells were irradiated under anaerobic conditions, was found (RÉVÉSZ & LITTBRAND 1969) to be independent of the growth phase of the cultures from which the cells were derived. This is also indicated by the experiments (cf. Table 7) in which the cell population used has completed one division after plating and before irradiation. The cells in such a population may be regarded as having full metabolic activity and being in an early phase of exponential growth. When the survival regression of such cells was establised the extrapolation number was unity (cf. Fig. 5-B), as in the case of cells which were, as a routine, derived from cultures in their plateau phase and which had probably an impaired metabolism.

The difference between the extrapolation numbers when irradiation was made in argon and oxygen, as well as the difference in the oxygen enhancement ratio of closely related cell lines suggest that oxygen does not act as a simple, dose-modifying factor. It may be inferred that, in addition to a quantitative difference, there is also a qualitative difference in the lethal damage caused by irradiation in the prescence and absence of oxygen. In view of recent findings that indicate the oxygen dependency of the recovery process, it is conceivable that the difference concerns the repair of the radiation damage (LITTBRAND & RÉVÉSZ 1969). As an alternative explanation, the extrapolation to unity and a corresponding nearly exponential dose-effect relationship may denote that a single damaging event is sufficient to abolish the reproductive capacity of anoxic cells. The dose-effect relationship after anoxic treatment with the sparsely-ionizing roentgen rays, is indeed similar to that observed after treatment with densely-ionizing radiations (BARENDSEN 1962, BARENDSEN et coll. 1963, DEERING & RICE

1962). On the other hand, an extrapolation number larger than unity, noted after aerobic treatment, may be regarded as indicating that more than one damaging event, of the same or a different kind (genetic or non-genetic) must be accumulated for any lethal effect.

The crossing of the extrapolated survival regression of the oxic and anoxic cells (Fig. 2) implies that the effect of oxygen in enhancing sensitivity varies with the magnitude of the exposure dose. In the dose range before the crossing, a negative enhancement, i.e. a protecting effect, may prevail. Experimental evidence for such a protection was not obtained in the tests performed in 100 per cent oxygen with either single exposure doses in the range of 50 to 125 R or with multiple doses of 100 R, though this latter treatment may be particularly suitable for discovering small differences in survival. The differences between the survival of oxic and anoxic cells were always insignificant and an oxygen enhancement ratio close to unity was indicated. With increasing doses after the crossing, the oxygen enhancement ratio could be expected to increase from unity towards a maximal value defined by the ratio between the slope constant of the oxic and anoxic survival regressions (RÉVÉSZ & LITTBRAND 1967a). This expectation received experimental proof in tests in which the survival curve of oxic cells was determined after exposure to doses between 200 and 600 R and that of anoxic cells after exposure to doses between 800 and 1 800 R. It is evident that the enhancement of radiosensitivity by oxygen is a function also of the exposure dose and is therefore more complex than expressed by the formula proposed by HOWARD-FLANDERS & ALPER (1957). A more detailed discussion of this problem has been published elsewhere (LITTBRAND & RÉVÉSZ 1969).

Recovery and the oxygen effect. In considering the relationship between the split-dose ratio and the extrapolation number (ELKIND & SUTTON 1960), the split-dose ratio around 2 and 1 found in the oxic and anoxic experiments, respectively, is in agreement with the theoretical expectations. It has been shown that a summation of survival curves with diverse extrapolation numbers (DITTRICH 1960) or slope constants (POWERS & TOLMACH 1963) may result in a common composite curve with a decreased extrapolation number. The close agreement between extrapolation number and split-dose ratio excludes this possibility, i.e. the heterogeneous response of the cell population as an explanation of the findings. The recovery observed when cells were pretreated in argon and subsequently irradiated in oxygen (Table 6) also contradicts the argument that such pretreatment is, in some way, responsible for the impaired recovery when irradiation is made anoxically. The data of the different experiments are thus concordant in indicating that recovery requires oxygen. The results of experiments in which recovery was studied at varying oxygen tensions strongly suggest that

oxygen is required as the energy source for the recovery process (LITTBRAND & RÉVÉSZ 1969).

Since the cells in the experiments immediately after exposure to radiation in anoxia were put under aerobic conditions, it may also be concluded that post-treatment access to oxygen does not restore recovery. Recovery is, indeed, dependent upon the presence of oxygen during or very shortly after the radiation treatment. It has been argued (LITTBRAND & RÉVÉSZ 1969) that the damage induced by radiation may be recoverable only during a limited time period. If oxygen is present during the critical period, the recovery process may begin and continue whilst its energy requirement is satisfied.

Oxygen may play a dual role in determining the reaction of cells to radiation: on the one hand, it may sensitize the cells in enhancing the radiation damage and, on the other hand, it may protect them in permitting recovery. Apparently, in the dose range around the crossing of the extrapolated survival curves, the protecting effect can wholly compensate for the injury due to the sensitizing effect. With larger doses, the protecting effect has a decreasing significance in relation to the sensitizing effect. The relative importance of the two antagonistic effects has been shown to be dependent also upon the oxygen concentration (RÉVÉSZ & LITTBRAND 1967b, LITTBRAND & RÉVÉSZ 1969). As an example a concentration of 0.5 μM oxygen in the medium permits the development of almost maximal recovery while it sensitizes only slightly (LITTBRAND & RÉVÉSZ 1969). This indicates that an oxygen enhancement ratio close to some known maximal value cannot be simply taken as evidence for the prevalence of total anoxic conditions in a certain experimental system.

Present observations in relation to earlier studies. The results presented in this paper are in full agreement with preliminary observations (LITTBRAND & RÉVÉSZ 1964, ROBINSON & RÉVÉSZ 1961). They also confirm the data obtained in experiments in which the effects on radiosensitivity of cysteamine, triacetoneamine-N-oxyl or oxygen in varying concentrations were studied on radiosensitivity, cells irradiated similarly in anoxia or 100 per cent oxygen being used as controls (LITTBRAND & RÉVÉSZ 1969, to be published, RÉVÉSZ & LITTBRAND 1970). The findings reported by PHILLIPS (1968) are also in agreement with our observation on an oxygen dependent extrapolation number. This author irradiated bone marrow cells in vitro in the presence of oxygen or nitrogen. In subsequent in vivo tests with the spleen colony technique, the aerobic survival curves extrapolated to 1.5 while the anaerobic curves extrapolated close to unity. In an in vitro system with a long gassing equilibrium time, HALL et coll. (1966) found a reduction of the extrapolation number to unity when the radiation survival of HeLa cells was studied after storage in severe nitrogen hypoxia for

36 hours. A 6-hour storage resulted in no reduction of the extrapolation number. In in vitro experiments with L-cells, HUMPHREY et coll. (1963) noted a reduction of the extrapolation number from 12 to 3 when cells were irradiated under hypoxic conditions in nitrogen. Under conditions similar to those of the present material a 20-second flushing with nitrogen before irradiation reduced the extrapolation number of the survival curve of Chinese hamster cells from 3.8 to 2.4 (ELKIND et coll. 1968). In contrast, when these cells were irradiated in a different in vitro system, the extrapolation number in nitrogen was similar to the aerobic one. Experiments with HeLa cells in vitro gave similar results in that the extrapolation number failed to change whether oxygen or nitrogen was the gas phase (NIAS et coll. 1967).

In a general agreement with the present results, no repair of the radiation damage was noted when ascaris eggs were treated with fractionated radiation doses under hypoxic conditions (LIECHTI 1929). A decreased split-dose ratio was calculated also by HALL & CAVANAGH (1967) in experiments with *Vicia faba* when irradiation was applied in nitrogen instead of aerobic conditions. In contrast, in split-dose experiments with Chinese hamster cells exposed in nitrogen, recovery was demonstrated by ELKIND et coll. (1965). When cellular survival curves were determined in experiments in vivo, extrapolation numbers dependent (BELLI et coll. 1967, PHILLIPS 1968, VAN PUTTEN & KALLMAN 1968) or independent (HEWITT & WILSON 1961, REINHOLD 1966) of the presence of oxygen during the irradiation were reported.

A common interpretation of the contradictory results is difficult at present. Apart from differences in the cellular material and experimental conditions, the statistical analysis of the significance of the data is often incomplete and uncertainty often concerns also the degree of hypoxia. The interpretation of split-dose experiments in vivo is, in addition, still complicated by the possible repopulation and re-oxygenation of the irradiated tissue during the time interval between the treatments. While recovery of hypoxic cells in vivo was reported by many investigators (BERRY et coll. 1967, FOWLER et coll. 1965, HORNSEY 1967) recent carefully conducted experiments also indicated that the recovery of such cells may be substantially decreased (ELKIND et coll. 1968, BELLI et coll. 1967) or absent (PHILLIPS & HANKS 1968). In a recent paper, SUIT & URANO (1969) reported that acutely hypoxic mammary carcinoma cells repaired damage rapidly and extensively. In contrast, chronically hypoxic cells were less able to repair radiation injury.

The danger of hypoxic cells. Considering the varying oxygen gradients prevailing in human tumors (THOMLINSON & GRAY 1955), and the finding (LITTBRAND & RÉVÉSZ 1968) that cells can survive for a prolonged period of time even when

less than 0.004 μM O_2/l is available in the medium, the present results and conclusions may carry some radiotherapeutic implications. While well oxygenated portions of a tumor and normal tissues with unimpaired blood supply will suffer a larger initial radiation damage than the anoxic tumor cells, the oxic cells can probably recover but the damage to anoxic cells may not be repaired. Fractionated irradiation may therefore result in a greater additivity of the injury in the anoxic cells than in oxygenated cells. Fractionation of the radiation dose can be expected to reduce the anoxic protection ratio which, with approximately small doses, may have a value even less than 1. The potential danger of surviving therapeutic radiation would seem to be greatest with the hypoxic rather than anoxic cells since the former will not only suffer less injury but also have the capacity of repairing a part of the damage (LITTBRAND & RÉVÉSZ 1969). Transfer of cells from the initially anoxic to the hypoxic compartment as a result of partial re-oxygenation may constitute a particular hazard with regard to the success of therapy.

Conclusions

Cultures of Chinese hamster cells, an Ehrlich ascites tumor and three of its substrains were exposed to roentgen radiation in oxygen or argon with an oxygen content less than 2 ppm. Significant differences in radiosensitivity between the ascites tumor and the substrains were demonstrated when irradiation was administered in argon. In oxygen the radiosensitivities were similar implying differences in the oxygen enhancement ratio between different cell strains. The survival curves after anoxic irradiation with either single or split exposure doses were exponential with extrapolation numbers to a zero dose close to unity. In oxygen the survival curves were sigmoid and the extrapolation numbers around 2. This difference between the extrapolation numbers implies a crossing of the survival curves corresponding to a dose range where theoretically no oxygen enhancement of sensitivity can be expected to occur. Experiments performed with single exposures to 125 R or less, and with three exposures to 100 R, proved the correctness of this expectation and indicated that survival is independent of the presence or absence of oxygen in this dose range.

The split dose ratios calculated from the survival after single exposure and two exposures to the same dose separated by an interval of 4, 12 or 18 hours, agreed well with the extrapolation numbers and were around 2 and close to 1 when irradiations were given in the presence and absence of oxygen, respectively.

Differences in the cellular concentration of non-protein bound sulphydryl groups and the ploidy grade were considered to explain the differences in the anoxic radiosensitivity between the Ehrlich ascites tumor cells and its substrains. The role of oxygen in the expression of radiation damage was considered as a

dual one acting as an essential modifying agent to both the process of sublethal recovery and lethal damage.

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SUMMARY

The survival parameters of different cell lines were studied after exposure to roentgen radiation. Oxygen affected both production and repair of radiation damage. The extent of these processes varied in the different cell lines.

ZUSAMMENFASSUNG

Die Überlebensparameter verschiedener Säugetier-Zell-Linien wurden nach Röntgenbestrahlung untersucht. Sauerstoff beeinflusste sowohl die Bildung als auch die Reparation des Strahlenschadens. Das Ausmass dieser Prozesse variierte in verschiedenen Zell-Linien.

RÉSUMÉ

L'auteur a étudié les paramètres de survie de différentes lignées cellulaires de mammifères après exposition aux rayons de roentgen. L'oxygène a une influence aussi bien sur l'apparition que sur la réparation des radio-lésions. L'importance de ces processus est variable dans les différentes lignées cellulaires.

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