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EFFECT OF RADIOPROTECTIVE AMINOTHIOLS ON THE INDUCTION AND REPAIR OF SINGLE-STRAND BREAKS IN THE DNA OF IRRADIATED MAMMALIAN CELLS

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Using structural and functional parameters to characterize DNA injury, it has been shown that sulphydryl compounds protect DNA against injury by ionizing radiation. Protection in shifts of T_m values (JELLUM 1966), changes in specific viscosity (KOLLMANN et coll. 1967) and template activity (LEON et coll. 1971) have been demonstrated. With advances in analytical techniques, it has become possible to investigate radiation injury expressed by breaks of the phosphodiester chains of DNA. The breaks can be quantitatively estimated by the alkaline sucrose gradient technique of MCGRATH & WILLIAMS (1966). Protection by cysteamine against the formation of single-strand breaks in the DNA of mammalian cells after irradiation in oxygen has been demonstrated previously (LOHMAN 1968, ALEXANDER et coll. 1970, LOHMAN et coll. 1970, SAWADA & OKADA 1970 and ANTOKU 1976).

The effect of cysteamine on the formation of single-strand breaks in mammalian cells after irradiation under both oxic and anoxic conditions is now reported. It is expected that such an investigation may be informative in regard to the protecting mechanism of thiols, and also provide data in regard to the possible reaction of neoplasms which consist of a large number of hypoxic cells. In view of the known toxic effect of cysteamine on cellular survival (VOS et coll. 1962, 1970, TAKAGI et coll. 1974) particular attention was paid to estimate the possible toxic effect of cysteamine

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to DNA. MPG (2-mercapto-propionyl-glycine) which has been shown to exhibit a greatly reduced toxicity in comparison to cysteamine complemented the investigation. Complementary experiments were performed to elucidate the effect of cysteamine on the rejoining process following radiation exposure.

Material and Methods

Cell cultures. A Chinese hamster cell line, V-79, maintained under standard tissue culture conditions, was used as the cell material. The culture medium consisted of Eagle's medium in Earle's saline supplemented with 15 per cent fetal calf serum and antibiotics. The mean doubling time of the cells was about 12 to 14 hours under these conditions. For each experiment monodispersed cells were prepared from 6 to 8 day old cultures by treatment with 0.5 per cent trysin solution at 37°C for 10 min.

Labelling. The cells were plated in Pyrex Petri dishes with a medium containing ^{3}H-TdR (specific activity 23.7 Ci/mmole) at a concentration of 1 μCi/ml. The dishes were incubated in a humidified atmosphere of air and 5 per cent CO_2 for 18 hours. The total activity was 0.5 to 1.0 cpm/cell on the average.

Treatment with thiols and quinacrine. Two different aminothiols were used: cysteamine (Sigma Chem. Co.) which has a well documented protective effect in most systems and MPG (2-mercapto-propionyl-glycine, Thiola, Santen Pharmaceutical Co., Ltd, Japan) which has been shown to exhibit a protective effect similar to that of cysteamine but has a greatly reduced toxicity (SUGAHARA et coll. 1970).

Medium containing one of the aminothiols at desired concentration was added to the cells, either immediately or 15 min before transfer of the Petri dishes into the irradiation box. Incubation was performed at 37°C. With the time allowed for flushing of the boxes with gas, the actual incubation periods were 10 or 25 min.

In order to inhibit the repair of breaks which may occur during the irradiation period, in some experiments the cells were treated with quinacrine (5 μg/ml), a substance known to be inhibitory in this regard (FUKS & SMITH 1971).

Irradiation procedure. Before irradiation, the medium was drained off so that not more than 1 ml was left in the dishes, covering the cells with a fluid layer of less than 0.4 mm. With the lids removed, the dishes were placed in an air-tight plastic chamber which rested in an ice-bath keeping the temperature of the cultures at less than 10°C. Oxygen or argon, with less than 2 ppm oxygen impurity, both supplemented with 1.25 per cent CO_2, was flushed through the irradiation chamber for 10 min before and during exposure at a rate permitting 3 to 4 gas exchanges per min. When argon gas was used, the time allowed for gas exchange was found to be sufficient for complete removal of oxygen from the medium surrounding the cells (LITTBRAND 1971). Further

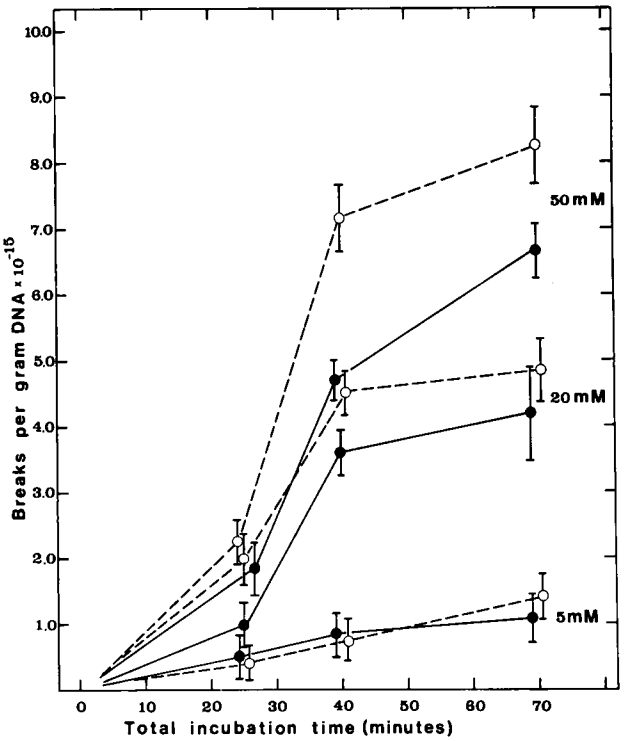

Fig. 1. The number of single-strand breaks in DNA after incubation of Chinese hamster cells in 5, 20 or 50 mM cysteamine for a period varying between 15 and 60 min in air and, subsequently, for a constant period of 10 min in oxygen or in anoxia. Mean ±SE is indicated as calculated from 6 to 10 replicate experiments. ●—● Incubation in air + oxygen. ○---○ Incubation in air + anoxia.

details of the chamber and the practice used to control the atmospheric conditions have been described previously (LITTBRAND & RÉVÉSZ 1969, LITTBRAND).

Radiation was generated with a Siemens roentgen unit at 190 kV and 15 mA; filtration 2.5 mm Al (HVL 0.95 mm Cu); dose rate 3.44 Gy/min at FFD 40 cm, and was measured by a Philips integrating dosemeter. The correction factor for back-scatter of radiation, calculated from survival measurements of cells when irradiated on glass versus plastic, was 1.35±0.05 (LITTBRAND).

Procedure after irradiation. Immediately after irradiation, the cells were rinsed four times in ice-cold saline containing 0.02 M EDTA in order to remove as much residual thiol as possible, and then dissociated with 0.5 per cent trypsin solution for 5 min.

When the effect of aminothiols on the rejoining process of radiation-induced single-strand breaks of the DNA was determined, the cells were irradiated in the absence of aminothiol. Immediately after irradiation, the medium of the cells was drained off and replaced by fresh, warm medium containing the particular aminothiol, and incubated at 37°C for 30 or 60 min.

Gradient centrifugation and determination of breaks. The number of single-strand breaks was determined by the alkaline sucrose-gradient technique, originally in-

Table 1

The number of single-strand breaks in DNA after incubation of Chinese hamster cells in MPG in 3 different concentrations for 15 to 60 min in air and then for 10 min in oxygen. Means calculated from 3 to 5 replicate experiments

Concentration of MPG	Number of breaks/gram DNA $\times 10^{-15}$ Total incubation time		
	25 min	40 min	70 min
10 mM	0.12	0.19	0.37
20 mM	0.30	0.54	0.55
50 mM	0.52	0.75	0.80

troduced by McGRATH & WILLIAMS. A suspension of 2.5×10^4 cells in 0.1 ml was lysed in 0.1 ml of 0.5 M NaOH on top of a 5 to 20 per cent alkaline sucrose gradient at room temperature for 30 min. Centrifugation was performed in a Spinco Ultracentrifuge at 20 000 rpm and 20°C for 90 min. The content of the gradients was collected in 20 fractions and the activity of each fraction was measured in a Packard Tri-Carb Scintillation Spectrometer, model 3320. The average molecular weight of the DNA and the number of single-strand breaks induced by radiation could be calculated from the sedimentation profiles of DNA, obtained by plotting the activity of each fraction expressed as percentage of total activity versus fraction number. A detailed description of the centrifugation technique and the calculation method has been published previously (MODIG et coll. 1974).

Results

In a preliminary series of experiments the effect of cysteamine and MPG on the possible induction of DNA breaks was tested. Cell cultures were incubated with varying concentrations of cysteamine or MPG at a temperature of 37°C under aerobic conditions for 15 to 60 min, and thereafter at a temperature below 10°C for another 10 min, either in the presence of oxygen or in anoxia. This type of treatment was tested in order to resemble the conditions of the experiments in which incubation with these substances was combined with radiation exposure.

The number of single-strand breaks after treatment with cysteamine is presented in Fig. 1. Increasing concentrations of cysteamine in the medium and prolonged incubation time result in an increasing number of breaks. A period of anoxia clearly enhances break formation. The enhancement is directly related to the cysteamine concentration.

Treatment of cells with varying concentrations of MPG also leads to the formation

Table 2

The effect of cysteamine and MPG on the induction of single-strand breaks in the DNA of Chinese hamster cells by exposure to 39 Gy in the presence of oxygen or in anoxia. The number of single-strand breaks and the dose modifying factors (DMF) are shown. Means ± SE indicated, calculated from results in 6 to 12 replicate experiments

Thiol	Concentration	Treatment time before irradiation (min)	Breaks/gram DNA × 10^{-15}					
			Irradiation in oxygen			Irradiation in anoxia		
			Controls	Thiol treated cells	DMF	Controls	Thiol treated cells	DMF
Cysteamine	5 mM	10	5.93 ± 0.27	4.09 ± 0.27	1.45	2.50 ± 0.19	2.02 ± 0.19	1.24
		25	5.15 ± 0.28	3.63 ± 0.20	1.41	2.50 ± 0.20	2.11 ± 0.11	1.18
	20 mM	10	5.77 ± 0.27	3.28 ± 0.16	1.76	2.57 ± 0.12	2.22 ± 0.16	1.15
		25	5.77 ± 0.27	3.74 ± 0.23	1.54	2.57 ± 0.12	3.43 ± 0.16	0.74
MPG	10 mM	10	5.77 ± 0.20	4.45 ± 0.16	1.30	2.61 ± 0.13	2.15 ± 0.19	1.21
		25	6.00 ± 0.21	5.15 ± 0.51	1.16	2.61 ± 0.13	2.93 ± 0.23	0.89
	20 mM	10	6.24 ± 0.31	4.91 ± 0.20	1.27	3.00 ± 0.20	1.90 ± 0.15	1.57
		25	5.80 ± 0.20	5.26 ± 0.17	1.10	3.00 ± 0.20	3.08 ± 0.20	0.97

of breaks, though to a lesser extent than with cysteamine at comparable concentrations and incubation times (Table 1). Thus the number of breaks induced by 50 mM MPG during a total incubation time of 70 min is considerably less than with 5 mM cysteamine.

In regard to these results, and in order to minimize the number of breaks due to cysteamine and MPG, in the radiation experiments, it was decided to use incubations with the thiols for not longer than 25 min and at concentrations not exceeding 20 mM.

In one series of experiments Chinese hamster cells were exposed to a standard dose of 39 Gy in the presence of cysteamine or MPG. The thiols were used at two different concentrations and added to the cells either 10 or 25 min before radiation exposure. Irradiation was made in the presence of oxygen or under anoxic conditions. Cells not treated with the thiols but otherwise handled in the same way served as controls. After irradiation the average molecular weight of the cellular DNA was measured, and the number of single-strand breaks calculated in 6 to 12 replicate experiments.

The number of breaks per gram DNA in the presence and absence of the thiols is indicated in Table 2 which also shows the dose modifying factors (DMF). The latter were expressed by the ratio between the number of breaks determined in the absence and presence of the particular thiol. Since the relationship between radiation dose and yield of breaks is linear (MODIG et coll.) this ratio will actually be numerically identical to the ratio calculated for the two radiation doses which correspond to the number of breaks measured in the absence and presence of the thiols.

Table 3

The effect of 5 and 20 mM cysteamine on the induction of single-strand breaks in the DNA of quinacrine-treated and untreated cells, irradiated in the presence of oxygen or in anoxia. The cells were treated with quinacrine for 40 min and with cysteamine for 10 min before irradiation. Means calculated from 2 to 3 replicate experiments

Quinacrine	Cysteamine concentration	Breaks/gram DNA $\times 10^{-15}$					
		Irradiation in oxygen 39 Gy			Irradiation in anoxia 91 Gy		
		Controls	Thiol treated cells	DMF	Controls	Thiol treated cells	DMF
5 μg/ml	5 mM	9.88	6.83	1.44	7.81	5.83	1.34
	20 mM	9.19	5.40	1.70	7.63	4.89	1.56
Absent	5 mM	6.06	4.50	1.35	6.68	4.88	1.37
	20 mM	5.93	3.19	1.86	6.46	3.82	1.69

In the presence of oxygen both cysteamine and MPG reduce the initial yield of single-strand breaks (Table 2). When anoxic conditions prevail, the reduction is smaller in relation to that under oxic conditions, and in some instances even sensitization is observed. At comparable concentrations cysteamine exhibits, in general, a larger protective effect than MPG. It is also noted that better protection is obtained when the particular substance is added to the cells 10 min rather than 25 min before radiation exposure.

In order to establish the effect of the thiols in the absence of repair processes which may proceed during the irradiation period, in another experimental series the cells were pretreated with quinacrine. This compound is known to greatly inhibit repair of single-strand breaks (FUKS & SMITH, VOICULETZ et coll. 1974) when added to bacteria or mammalian cells. A quinacrine concentration of 5 μg/ml for 40 min before radiation exposure was used. The exposure doses were chosen to give similar effects in regard to the initial yield of breaks in oxygen (39 Gy) and anoxia (91 Gy). Cysteamine was added to the cells in 5 or 20 mM concentration 10 min before irradiation.

The results presented in Table 3 show that quinacrine-treatment increases the initial yield of breaks both in thiol-treated and untreated cells by an average factor of about 1.6 in oxygen and 1.2 in anoxia. As also estimated from the DMF values, quinacrine-treatment influences only slightly the protective effect of cysteamine concerning the induction of breaks. In contrast to the results presented in Table 2, the data in Table 3 show that the DMF expressing the protective effect of cysteamine in anoxia is only slightly smaller than that in oxygen. However, it should be noted,

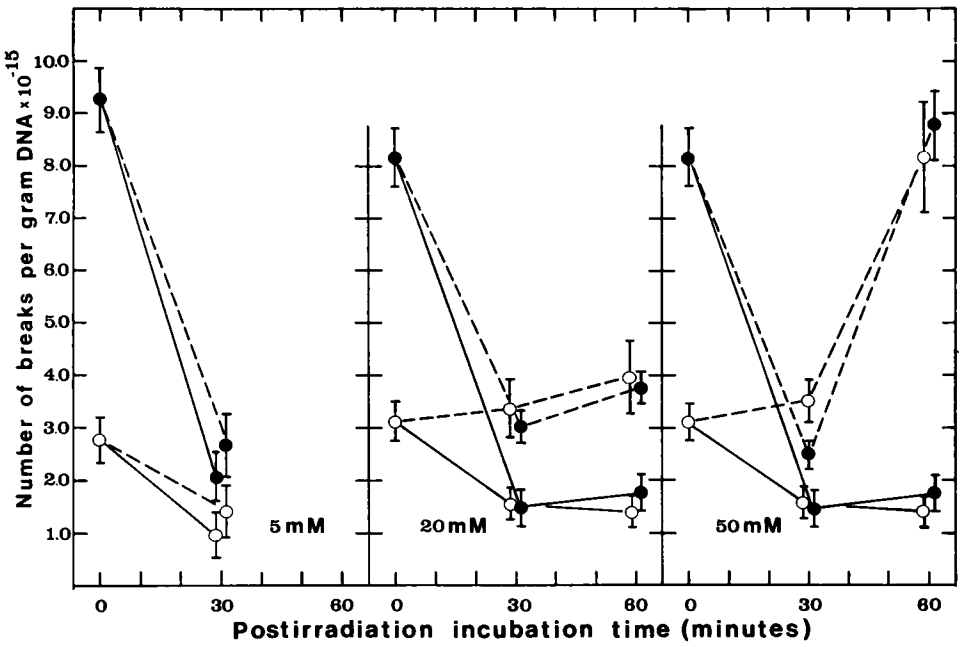

Fig. 2. The effect of cysteamine on the post-irradiation rejoining of single-strand breaks in the DNA of Chinese hamster cells. The cells were exposed to a dose of 39 Gy in the presence of oxygen or in anoxia, and immediately thereafter, cysteamine was added at different concentrations. The number of DNA breaks after irradiation and following incubation was then determined after 30 or 60 min incubation in air. Mean ± SE is shown, calculated from 6 to 16 replicate experiments. ● Oxic irradiation. ○ Anoxic irradiation. --- Cysteamine present. — Cysteamine absent.

that the anoxic exposure doses differ (39 Gy and 91 Gy in the experiments of Table 2 and 3, respectively).

The effect of cysteamine on post-irradiation repair of the radiation induced breaks was investigated in another series of experiments in which the cells were exposed to 39 Gy in oxygen or in anoxia. Immediately after exposure cysteamine at 5, 20 or 50 mM concentration was added to the cells, which were incubated in air at 37°C.

Fig. 2 presents the number of breaks immediately after irradiation and following 30 or 60 min incubation. Cysteamine-untreated cells show a rapid decrease of the number of breaks in the oxically irradiated cases, and a less fast decrease in the anoxically irradiated cases during a 30 min incubation. No significant further decrease occurred during a subsequent incubation for 30 min. This finding is similar to that described in previous experiments (MODIG et coll.), and can be attributed to a rapid rejoining of the breaks during the oxic, and less fast rejoining during the anoxic incubation period. Cysteamine appears to inhibit this rejoining. The inhibition is greater with increasing concentration of the thiol and increasing incubation time. At the largest concentration of cysteamine (50 mM) and longest incubation time (60 min) this thiol not only inhibits rejoining but clearly enhances production of breaks.

Discussion

It has been shown that thiols protect DNA of mammalian cells from the induction of single-strand breaks by oxic irradiation. LOHMAN (1968) and LOHMAN et coll. (1970) found that 32 mM cysteamine protects the DNA of T-cells with a maximum DMF of about 4, and similarly, 30 mM cysteamine protects the DNA of L5178Y lymphoma cells with a DMF of about 1.5 (ALEXANDER et coll. 1970). GINSBERG et coll. (1969) showed cysteamine protection of DNA of several E. coli strains in stationary phase cultures. SAWADA & OKADA (1970) calculated a DMF of about 6, by treating L5178Y lymphoma cells with 50 mM cysteamine. On the other hand, ORMEROD & STEVENS (1971) failed to find any protection with cysteamine against the formation of breaks in the DNA of the same type of cells. Recently ANTOKU (1976) reported on the protective effect of cysteamine under anoxic conditions. He found no protection of DNA breaks by cysteamine in L5178Y lymphoma cells at radiation doses below 200 Gy; on the other hand, at a dose of 450 Gy a protective effect was noted with a DMF of 1.6.

The results obtained in the present investigation indicate that both cysteamine and MPG are effective in protecting the DNA of mammalian cells against the formation of single-strand breaks by irradiation with doses of 39 and 91 Gy, given in the presence of oxygen or in anoxia. On a molar basis cysteamine is more efficient than MPG. This difference is probably not due to a decreased incorporation of MPG by the cells, since previous experiments (RÉVÉSZ et coll. 1974) demonstrated that both compounds raise the intracellular non-protein sulphydryl content to about the same extent at equimolar concentrations, and suggested a similar incorporation rate for the two substances. A possible explanation of the difference between the effect of cysteamine and MPG, may be the absence of a basic aminogroup in the MPG-molecule and, as a consequence, a possible failure to bind to DNA. It has been shown that the presence of an aminogroup in thiols is of importance for their protective effect (DOHERTY et coll. 1957).

Several reports have appeared on a cytotoxic effect of cysteamine. VOS et coll. (1962, 1970) showed a toxic effect on cellular survival at cysteamine concentrations in the range 0.1 to 2.0 mM. The toxicity disappeared at higher concentrations. The toxic effect was inhibited by anoxia, low pH and KCN, and was suggested to be due to the formation of thiolate ions (RS^-) or toxic oxidation products. The decreased toxicity of cysteamine at concentrations above 2.0 mM was explained by a gradual inhibition of the enzymes responsible for thiol oxidation with increasing sulphydryl concentrations. A similar concentration-dependent toxicity was observed also by TAKAGI et coll. (1974), who found that cysteamine kills HeLa cells more effectively at concentrations between 0.5 to and 5.0 mM than at 30 mM. They explained the toxic action of cysteamine by the generation of a peroxide which is decomposed by cysteamine at larger concentrations. SAWADA & OKADA (1970) found that cysteamine induced single-strand breaks, and it also inhibited the repair of the breaks to a larger

Table 4

The dose modifying factors (DMF) for cysteamine and MPG before and after correction for the toxic effect of the substances

Thiol	Concentration	Incubation time (min)	DMF in oxygen		DMF in anoxia	
			Uncorrected	Corrected	Uncorrected	Corrected
Cysteamine	5 mM	10	1.45	1.52	1.24	1.36
		25	1.41	1.62	1.18	1.45
	20 mM	10	1.76	1.98	1.15	1.68
		25	1.54	2.11	0.74	1.80
MPG	10 mM	10	1.30	1.31	—	—
		25	1.16	1.19	—	—
	20 mM	10	1.27	1.30	—	—
		25	1.10	1.17	—	—

extent at 0.5 mM than at 5 mM. In the experiments of SAWADA & OKADA the toxic effect could partly be inhibited by KCN, similarly as in the investigation by VOS et coll. and which concerned the effect of cysteamine on cellular survival.

A toxic effect of cysteamine and MPG is clearly demonstrated in the present experiments by the finding that both compounds induce an increasing degradation of DNA in unirradiated cells with increasing concentration and incubation time. In oxygen the toxic effect was about 10 times higher with cysteamine than with MPG after 60 min of incubation. Cysteamine also had a greater toxicity when the cells were treated with it under anoxic conditions for comparable periods. This difference in the thiol effect between oxic and anoxic conditions may be explained by the fast oxidation of cysteamine in the presence of oxygen which rapidly decreases the concentration and, consequently, toxicity.

The toxic effect of cysteamine on DNA breaks observed may not be related to the toxic effect of this substance on cellular survival observed by VOS et coll. and TAKAGI. The toxicity to DNA gradually increases with increasing concentration of the substance and incubation time, while the toxicity to the survival is apparent only in the concentration range 0.1 to 5.0 mM. As indicated, toxicity to survival may be attributed to thiolate ions (RS^-) or peroxides. The mechanism of the toxic effect to DNA is not known. It may be due to the generation of strand-breakage in the living cell directly, or the thiols may render the DNA-molecules more fragile to the formation of breaks during lysis and gradient centrifugation.

In view of the finding that cysteamine in itself induces DNA degradation, the failure of ORMEROD & STEVENS to observe any protection by 30 mM cysteamine may be due to a compensation of the protective effect by toxicity. A similar explanation may be given to the failure of ANTOKU (1976) to detect DNA protection by cysteamine

at radiation doses below 200 Gy in anoxia, while he calculated a DMF of 3 to 4 in experiments performed with doses in the range 100 to 300 Gy in oxygen. His anoxic experiments were performed with an incubation time of 40 min which may allow for the development of greater toxic effects than during the 10 min incubation he used in the oxic experiments.

The number of DNA breaks which were estimated in the irradiated cells treated with the compounds (cf. Table 2) were corrected in an attempt to calculate the net protective effect, without toxic interference. The correction was made by substracting the number of breaks attributable to toxicity according to the data presented in Fig. 1 and Table 1, from the number of breaks actually measured in the irradiated cells incubated with the compounds at comparable concentrations. Table 4 indicates the DMF values before and after such correction for toxicity. Best protection by cysteamine occurs after 25 min of incubation if the corrected values are considered in contrast to the uncorrected values which indicate 10 min of incubation to be more protective. This difference suggests that the decreased protective effect of cysteamine after prolonged incubation of the cells is actually due to the antagonistic toxic effect of the substance. The DMF-value which indicates a paradoxical sensitizing action of cysteamine (cf. 25 min treatment with 20 mM cysteamine in anoxia) is changed by the correction for a more reasonable value indicating some protection, and may be regarded, therefore, as an artefact due to toxicity. Since the toxicity of MPG is low, the DMF values for this substance are only slightly altered by correction.

Cysteamine was shown to inhibit the rejoining of breaks when added to the cells after irradiation. The inhibition was again dependent upon the concentration of drug and incubation time. Similar observations were made by SAWADA & OKADA, who found that cysteamine inhibited both normal DNA synthesis and the rejoining of breaks. ANTOKU also found that 50 mM cysteamine caused a slower rejoining of breaks both after oxic and anoxic irradiations. It is conceivable that the reduced repair capacity of the cells in the presence of cysteamine is also due to the enhancement of DNA degradation by this substance. This explanation is supported by the observation that at a low thiol concentration, i.e. when toxicity is small, the inhibitory effect of cysteamine is also decreased (cf. Fig. 2).

The mechanism of thiol protection of DNA is not known. Several investigators favour the idea of a transfer of radiation induced transients from the biologic macromolecular target to the sulphur of the protector in exchange for a hydrogen atom. Thus, in a model system, LOMAN et coll. (1970) have shown that cysteine effectively protects thymine against the attack of $OH^{\cdot}$ radicals. DEJONG & BLOK (1972) suggested that cysteamine reacts with phage-DNA radicals, thereby modifying the radiation injury in such a way that the yield of single-strand breaks is reduced. MILVY (1971) demonstrated by means of ESR that spin migration from DNA to the sulphur of cysteamine is favoured over radical recombination at low radiation density.

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SUMMARY

The effect of cysteamine and 2-mercapto-propionyl-glycine on the induction and repair of single-strand breaks in the DNA of Chinese hamster cells by irradiation in vitro in the presence of oxygen or in anoxia was investigated. The substances protected against the formation of breaks by irradiation both under oxic and anoxic conditions. The substances also exhibited a toxic effect which resulted in degradation of DNA. Cysteamine inhibited the rejoining of breaks after radiation exposure.

ZUSAMMENFASSUNG

Der Effekt von Cysteamin und 2-Mercapto-propionyl-glycin auf die Induktion und Wiederverbindung von Einzelstrang-Brüchen der DNS in chinesischen Hamsterzellen nach Bestrahlung in vitro in Gegenwart von Sauerstoff oder in Anoxie wurde untersucht. Die Verbindungen zeigten einen Schutzeffekt gegen die Bildung von Strang-Brüchen nach Bestrahlung unter oxischen sowie anoxischen Bedingungen. Die Verbindungen zeigten auch einen toxischen Effekt, der zur Degradierung der DNS führte. Eine Hemmung der Wiederverbindung von Brüchen nach Cysteamin-Behandlung wurde nachgewiesen.

RÉSUMÉ

Les auteurs ont étudié l'effet de la cystéamine et de la 2-mercapto-propionyl-glycine sur l'induction et la réparation des brisures d'un seul brin de l'ADN de cellules de hamster chinois par irradiation in vitro en présence d'oxygène ou en anoxie. Ces substances ont protégé contre la formation de brisures par irradiation aussi bien en anoxie qu'en présence d'oxygène. Ces substances ont aussi montré un effet toxique qui a déterminé une dégradation de l'ADN. La cystéamine a inhibé la réparation des brisures après exposition aux radiations.

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