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PROTECTION OF CELLS IN TISSUE CULTURE BY MEANS OF CYSTEAMINE AND CYSTAMINE AGAINST THE ACTION OF NITROGEN MUSTARD AND X-RAYS

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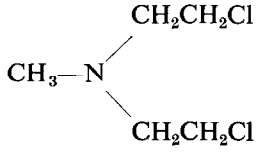
Abstract—The purpose of the present study was to decide whether cysteamine and cystamine protect cells in tissue culture against the action of nitrogen mustard and X-rays. Earle's L-strain of mouse fibroblasts was used in the experiments. Both cysteamine and cystamine afford good protection against nitrogen mustard (HN2), whereas under the same experimental conditions no definite protection of the cultures is obtained against X-irradiation with doses of 1160, 870, 580, 290 and 145 r. The results suggest that the mechanisms of the protective action of cysteamine against X-rays and against nitrogen mustards are different. It is emphasised that radio-protection of isolated cells may be obtained under other experimental conditions and with other cell types. The protection obtained against nitrogen mustard supports the view that destruction of SH groups in the cell plays a central part in the biological effect of HN2.

FOR a study of the mechanism of the protective action of cysteamine (beta-mercapto-ethylamine, $NH_2CH_2CH_2SH$) and cystamine ($NH_2CH_2CH_2S—SCH_2CH_2NH_2$) against nitrogen mustard and X-rays in animal experiments it is of great importance to decide if the protection occurs at a cellular level. The ability of cysteamine to protect isolated cells against X-irradiation has been studied by several investigators[1], but it cannot be excluded with certainty that the protection obtained may be due to anoxia produced by auto-oxidation of the cysteamine in the nutrient medium.[1, 2] Previous studies on the radio-protective effect of cystamine on tissue cultures have given negative results.[3, 4, 5]

Protection by cysteamine against the effect of nitrogen mustard has been demonstrated for plant cells *in vitro*,[6, 7] but the experimental technique employed did not exclude the possibility that it was due to a simple inactivation of the nitrogen mustard by linkage to cysteamine in the nutrient medium, i.e. outside the cells. Cystamine did not afford any protection in these experiments.

The purpose of the present investigation was therefore:

1. To study if cysteamine protects animal cells in tissue culture against the action of the nitrogen mustard HN2, i.e.



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under conditions where a reaction between the two substances in the nutrient medium could be excluded.

2. To study if cysteamine protects animal cells in tissue culture against the action of X-rays under experimental conditions in which anoxia of the cells caused by auto-oxidation of the cysteamine added could be excluded.

MATERIALS AND METHODS

The cells used were Earle's L-strain of mouse fibroblasts, which were grown in a slightly modified Eagle medium[8] to which 10% horse serum was added. The composition of the medium (EHS) was as follows:

Amino acids	g/l.	*Amides*	g/l.
L-arginine	0·021	L-glutamine	0·3
L-cystine	0·012		
L-histidine	0·008	*Vitamins*	mg/l.
L-isoleucine	0·026	Biotin	1
L-leucine	0·026	Choline	1
L-lysine, HCl	0·026	Folic acid	1
D,L-methionine	0·016	Nicotinamide	1
D,L-phenylalanine	0·032	Ca-pantothenate	2
D,L-threonine	0·048	Pyridoxal	1
D,L-tryptophan	0·008	Thiamin	1
L-tyrosine	0·018	Riboflavin	0·1
D,L-valine	0·048	Meso-inosit	1

Salts	g/l.	*Other components*	
NaCl	6·8	Glucose	1 g/l.
KCl	0·4	$NaHCO_3$	1·68 g/l.
$NaH_2PO_4.H_2O$	0·14	Penicillin G	10^5 I.U./l.
$CaCl_2.2H_2O$	0·265	Streptomycin sulphate	100 mg/l.
$MgCl_2.6H_2O$	0·175	Phenol Red	0·01 g/l

Serum factors
Horse serum 10%

The medium without antibiotics was sterilized by filtration through a Jena G5 glass filter. Just before use, the mixture of antibiotics was added, and the medium was bubbled with 5% CO_2 in atmospheric air, which reduced its pH level to about 7·2.

The stock cultures were grown in Roux flasks each containing 60–80 ml medium; stock cultures from 4 to 6 days old, seeded with 3 million cells per flask, were used. The cells were brought into suspension by trypsinization with a 0·05% trypsin solution for 15–30 min (trypsin 1:250 "Difco" 0·5 g/l., penicillin G 100,000 I.U./l., streptomycin sulphate 0·1 g/l., NaCl 8·0g/l., KCl 0·4 g/l., glucose 1·0 g/l., Phenol Red 0·02 g/l., $NaHCO_3$ 0·35 g/l.). The cell suspension was diluted in EHS, and a number of Carrel flasks were seeded with 2 ml per flask. Unless otherwise stated, the Carrel flasks were used for the experiments after incubation at 37° for about 20 hr. During the experiments, the medium was changed every second or third day, depending on the number of cells per flask.

After treatment of the cultures (which is described below), the cell growth was followed by daily cell counts in three Carrel flasks from each experimental group. It is stated in the literature that the L-cell can be detached from the glass wall simply by shaking the culture flasks. This was quite impossible in the present experiments, and it appeared that as early as 1 hr after the seeding of the flasks the cells adhere so densely to the glass wall that they could not be brought into suspension, even by vigorous shaking of the flasks. The cells were usually brought into suspension by the addition at room temperature of 0·5 ml of 1·25% trypsin solution (12·5 mg trypsin "Difco" 1:250 per ml dissolved in Hank's saline) to each flask without removal of the medium.

Duplicate counts of the suspension from each flask were performed on an area of 5 mm² in a Bürker-Türk haemocytometer. The figures listed in the accompanying tables represent the average number of cells per 5 mm² obtained by duplicate counts on a total of three flasks. The number of cells per ml is obtained by multiplication of the figures by 2000 and the number of cells per flask by multiplication by 5000.

The cysteamine used was supplied by "Fluka", Switzerland, in 10 g ampoules; on receipt, the cysteamine was transferred to ampoules in amounts of 100 mg under nitrogen. Cystamine was supplied as cystamine dihydrochloride by Nutritional Biochemicals Corporation, Cleveland, Ohio.

The nitrogen mustard HN2 was supplied by A/S Ferrosan under the name of "Erasol"®, dissolved in absolute alcohol in ampoules of 10 mg.

The irradiation of the cultures was performed at the Radium Centre for Jutland. Batches of 11 Carrel flasks were irradiated at a time. The flasks were placed on a circular board, so that the distance from the centre of the beam was the same for all flasks (about 10 cm). A Philips 250 kV unit (250 kV, 15 mA; filter 0·5 mm Cu; distance 60 cm) was used. The doses were measured in air by means of a Philips universal dosimeter, with the ionization chamber placed in the centre of the beam. The doses listed in the tables are those calculated at the centres of the flasks. The dose rate was approximately 70 r/min.

RESULTS

A. Toxicity of cysteamine and cystamine

In preliminary experiments it was necessary to study the toxic effect of cysteamine and cystamine on cultures of L-cells. As it was to be expected that the effect of cysteamine depends on the size of the inoculum, the toxicity was studied in cultures with different initial cell counts. Cysteamine or cystamine was dissolved in EHS immediately before the addition to cultures 20 hr old. At subsequent changes of medium, cysteamine or cystamine was added in the same concentration as at the start of the experiment. The results of a series of experiments are shown in Table 1, in which, as in all subsequent tables, the figures indicate the cell counts per 5 mm² in the haemocytometer, each figure being the average of duplicate counts on three flasks (see also Materials and Methods).

In the first three experiments, the toxicity of cysteamine was studied at different initial cell counts in the flasks. It is seen that at an initial cell count of 47 (experiment I) a cysteamine concentration of 2×10^{-3} M killed all the cells (i.e. the cell count fell to 0). At an initial cell count of 17 (experiment II), all the cells were killed at a concentration of $1{\cdot}0 \times 10^{-3}$ M, and when the initial cell count was reduced to 5 (experiment III), all cells were killed at a concentration of $0{\cdot}5 \times 10^{-3}$ M. A repetition of the experiments showed the same toxicity and the same relationship between the initial cell count and the toxicity,

In experiment IV, the toxicity of cystamine was studied in cultures with an initial cell count of 21. It is seen that a concentration of $0{\cdot}5 \times 10^{-3}$ M (converted to the concentration of the cystamine base, since the hydrochloride was used) killed all the cells. Thus, in molar amounts, the toxicity of cystamine was about twice as high as that of cysteamine (cf. experiments II and IV). However, as one molecule of cystamine by reduction forms two molecules of cysteamine, the toxicity of the two substances, calculated on the basis of their content of sulphur, is nearly identical.

Cysteamine at a concentration higher than that stated in Table 1 could be used in the radiation protection experiments, if it was allowed to exert its action for only a limited period. The result of one experiment is shown in Table 2. In all four groups, the cysteamine concentration was 3×10^{-3} M, but the cysteamine-containing medium was replaced by a cysteamine-free one after 15, 30, 60 and 90 min at

TABLE 1. CELL COUNTS IN CULTURES OF L-CELLS TREATED WITH CYSTEAMINE OR CYSTAMINE AT VARIOUS CONCENTRATIONS*

Experiment no.	Days	Concentration of cysteamine or cystamine					Controls
		2×10^{-3}M	10^{-3}M	$\frac{1}{2} \times 10^{-3}$M	$\frac{1}{4} \times 10^{-3}$M	$\frac{1}{8} \times 10^{-3}$M	
I	0			47		—	47
Cysteamine	1	14	63	86	85	—	95
	2	5	62	134	187	—	199
	3	1	91	254	362	—	363
	4	2	107	—	—	—	—
	5	—	176	—	—	—	—
	6	0	244	—	—	—	—
II	0			17			17
Cysteamine	1	—	16	28	34	29	31
	2	—	7	32	63	74	83
	3	—	1	46	122	164	173
	4	—	0	82	247	292	290
	5	—	1	116	409	538	519
	6	—	0	153	559	—	—
III	0			5			5
Cysteamine	1	—	4	8	9	9	9
	2	—	1	6	12	18	30
	3	—	1	6	18	39	55
	4	—	0	4	23	76	111
	5	—	0	1	52	152	187
	6	—	—	0	80	283	378
	7	—	—	0	123	444	—
IV	0			21			21
Cystamine	1	0	0	0	24	35	42
	2	0	0	0	24	64	89
	3	0	0	0	13	88	165
	4	—	—	—	—	—	—
	5	—	—	0	14	471	560

* The substances were added to the cultures on day 0, and at the change of medium the substances were added in the same concentration as at the start of the experiment. Experiments I–III show the effect of cysteamine on cultures with various cell counts. Experiment IV shows the effect of cystamine at an initial cell count of 21. The figures listed in this and the following tables represent the average number of cells obtained by duplicate counts on three flasks from each group (see also Materials and Methods).

TABLE 2. EFFECT OF CYSTEAMINE ON THE CELL COUNT*

Days	Duration of treatment in min				Controls
	15	30	60	90	
0			26		26
1	57	54	49	38	58
2	145	143	103	77	151
3	276	274	260	162	280

* Cysteamine was added to the cultures in a concentration of 3×10^{-3}M on day 0, when the cysteamine-containing medium was replaced by cysteamine-free medium at 15, 30, 60 and 90 min after the addition.

37 °C. It is seen that no demonstrable inhibition of growth was obtained when the change was made after 15 or 30 min. A slight inhibition of growth was observed when the change was made after 60 min, while change after 90 min resulted in a considerable inhibition. Since in the subsequent protection experiments the time of cysteamine exposure was chosen as 60 min, it seemed possible to use cysteamine concentrations of up to 3×10^{-3} M.

B. Protection with cysteamine and cystamine against the effect of HN2

In order to study this problem it was necessary to use experimental conditions which excluded the possibility of inactivation of HN2 by linkage to cysteamine or cystamine in the medium. Each Carrel flask was seeded with about 120,000 cells, i.e. a cell count of 24, and about 20 hr later the flasks were treated as follows:

Group I Cysteamine or cystamine alone
Group II Cysteamine or cystamine before HN2
Group III HN2 alone
Group IV No addition to the medium (controls).

Apart from the addition of these substances, all four groups were treated in exactly the same manner.

After empyting the flasks, 2 ml of fresh medium was added, containing cysteamine or cystamine in groups I and II, but not in groups III and IV. The flasks were then incubated at 37° for 1 hr, following which they were emptied again and placed in a rack with the mouths downwards for a few minutes to allow the remaining nutrient medium to drip off. Each flask was then rinsed with 1 ml of Hank's saline and again placed in the rack as before. Finally, 2 ml nutrient medium was added; it consisted of 10% Hank's saline and 90% EHS; in groups I and III with addition of HN2 to the desired concentration and in groups I and IV without any addition. The HN2 solution was prepared immediately before its addition to the cultures by dilution with Hank's saline to 10 times the final concentration. The final dilution (ten times) was done by mixture of 1 part of HN2 solution and 9 parts of EHS. The cultures were then incubated at 37 °C, and daily cell counts were performed on three flasks from each group.

The results of two experiments are shown in Table 3. The protective action of cysteamine was studied at a concentration of 3×10^{-3} M. It is seen that both experiments revealed protection (cf. groups II (protected) and III (unprotected)).

In the first experiment, the cysteamine concentration used showed only a slight toxic effect or none at all (cf. groups I (cysteamine-treated) and IV (untreated)) while the toxic effect observed in the second experiment was relatively great; but nevertheless protection was also obtained in the latter experiment.

To obtain a rough measure in the degree of protection, groups of protected and unprotected cultures were treated with varying concentrations of HN2, and for each concentration cell counts were performed on three protected and three unprotected cultures 7 and 14 days after treatment.

The results appear from Table 4. The protection by cysteamine in concentrations of $3{\cdot}0 \times 10^{-3}$ and $1{\cdot}5 \times 10^{-3}$ M and by cystamine in a concentration of $0{\cdot}75 \times 10^{-3}$ M was studied. A comparison of protected and unprotected cultures shows that in all three experiments the HN2 concentration required to give the same effect had to be four times as high in the protected as in the unprotected cultures.

C. Protection with cysteamine and cystamine against the effect of X-irradiation

The technique employed was that described above, with the exception that the rinsing after initial incubation of the cultures at 37 °C for 1 hr (after the addition of cysteamine or cystamine to the medium) was done with 1 ml of EHS instead of with 1 ml of Hank's saline. After the rinsing, no fluid was added until after the irradiation.

TABLE 3. CELL COUNTS IN CULTURES TREATED WITH CYSTEAMINE AND/OR NH2*

		HN2 $\frac{1}{56} \times 10^{-4}$M				HN2 $\frac{1}{112} \times 10^{-4}$M		
	I	II	III	IV	I	II	III	IV
0	26				36			
1	34	21	27	28	16	16	26	50
2	78	26	21	74	16	16	20	127
3	145	40	18	151	35	19	15	193
4	305	72	30	324	124	18	12	362
5	—	139	34	—	138	29	11	—
6	650	179	45	671	265	31	13	676
7	765	201	53	783	301	40	10	739
8	—	342	61	—	—	34	8	—
9	—	373	93	—	—	66	12	—
10	—	478	169	—	—	120	11	—
11	—	497	230	—	—	215	12	—

* Cysteamine, 3×10^{-3}M. The treatment was performed on day 0 (i.e. about 20 hr after the seeding) as follows:
I Cysteamine alone
II Cysteamine before HN2
III HN2 alone
IV Controls

TABLE 4. CELL COUNTS IN CULTURES 7 AND 14 DAYS AFTER TREATMENT WITH CYSTEAMINE OR CYSTAMINE AND/OR HN2*

Concentration of HN2	Cysteamine 3×10^{-3}M				Cysteamine $1{\cdot}5 \times 10^{-3}$M				Cystamine $0{\cdot}75 \times 10^{-3}$			
	7 days		14 days		7 days		14 days		7 days		14 days	
	+	−	+	−	+	−	+	−	+	−	+	−
$\frac{1}{2} \times 10^{-4}$M	0	0	0	0	0	0	0	0	3	0	1	0
$\frac{1}{4} \times 10^{-4}$M	3	0	34	0	4	0	2	0	11	1	10	1
$\frac{1}{8} \times 10^{-4}$M	24	0	321	0	14	1	119	0	22	3	242	2
$\frac{1}{16} \times 10^{-4}$M	126	3	609	20	107	4	494	2	143	9	657	11
$\frac{1}{32} \times 10^{-4}$M	177	10	853	162	324	10	—	27	324	18	—	199

* + signifies treatment with cysteamine or cystamine before HN2 in various concentrations.
− signifies HN2 without previous treatment with cysteamine or cystamine.

It is emphasised that, just like the HN2 treatment considered in section B, X-irradiation was performed at room temperature. As no X-ray unit is available in the Institute of General Pathology, the cultures had to be transported to another institute (see above), where the irradiation was performed. About $2\frac{1}{2}$ hr elapsed from the time the cultures were taken out of the incubator until they were placed there again after the irradiation and addition of 2 ml EHS.

All the experimental groups were treated in exactly the same manner, apart from the addition of cysteamine or cystamine and the irradiation.

The experimental set-up was as follows:

Group I Cysteamine or cystamine alone

Group II Cysteamine or cystamine before irradiation

Group III Irradiation without any pre-treatment

Group IV Controls.

In two preliminary experiments an X-ray dose of 580 r was chosen. No protection was obtained in these experiments. As the experimental conditions were not quite the same as in section B, it was decided to investigate protection both against HN2 and against irradiation under identical experimental conditions.

The experimental set-up in these experiments (Tables 5 and 6) was as follows:

Group I Cysteamine or cystamine alone

Group II Cysteamine or cystamine before irradiation (580 r)

Group III Irradiation (580 r) alone

Group IV Controls

Group V Cysteamine or cystamine before $\frac{1}{12} \times 10^{-4}$ M HN2

Group VI $\frac{1}{12} \times 10^{-4}$ M HN2 alone

TABLE 5. CELL COUNTS IN CULTURES TREATED ON DAY 0

Days	Cystamine $1{\cdot}5 \times 10^{-3}$M; X-ray dose 580 r; HN2 $\frac{1}{12} \times 10^{-4}$M						Cystamine $0{\cdot}75 \times 10^{-3}$M; X-ray dose 580 r; HN2 $\frac{1}{12} \times 10^{-4}$M					
	I*	II	III	IV	V	VI	I*	II	III	IV	V	VI
0	31						24					
1	19	14	29	43	12	23	48	29	27	51	37	26
2	30	9	47	88	14	17	104	42	46	113	31	22
3	61	9	47	204	—	—	256	54	59	269	—	—
4	136	16	50	404	14	5	477	57	55	412	42	7
5	—	—	—	—	—	—	—	—	—	—	—	—
6	419	16	52	852	12	5	—	80	78	—	59	7
7	—	18	69	—	—	—	—	110	99	—	—	—
8	—	12	88	—	39	4	—	150	150	—	93	5
9	—	15	107	—	—	—	—	169	169	—	—	—
10	—	16	193	—	104	3	—	272	257	—	271	5
11	—	29	223	—	—	—	—	321	309	—	—	—
12	—	—	—	—	—	—	—	—	—	—	—	—
13	—	172	501	—	349	4	—	525	480	—	450	4

* I Cystamine alone
II Cystamine before irradiation (580 r)
III Irradiation (580 r) alone
IV Controls
V Cystamine before HN2, $\frac{1}{12} \times 10^{-4}$M
VI HN2, $\frac{1}{12} \times 10^{-4}$M, without pretreatment

The results of two experiments with cystamine are shown in Table 5. In the first experiment, a cystamine concentration of $1{\cdot}5 \times 10^{-3}$ M, which has a relatively great toxic effect, was used. The experiment revealed good protection against HN2 in spite of the toxic effect of the substance, while it did not seem to afford any protection against X-irradiation. In the second experiment, a cystamine concentration of $0{\cdot}75 \times 10^{-3}$ M, which had no toxic effect, was used. Once more, good protection against HN2, but none against X-irradiation, was obtained.

The results of three experiments with cysteamine are shown in Table 6. In all three experiments, a cysteamine concentration of $1{\cdot}5 \times 10^{-3}$ M was used, but to decide if protection against irradiation could be obtained if the cells were treated at different times after seeding (see Discussion), the treatment of the cells were performed at 2, 24 and 48 hr after the seeding.

TABLE 6. CELL COUNTS IN CULTURES TREATED AS IN TABLE 5 WITH MODIFICATIONS*

Cysteamine $1{\cdot}5 \times 10^{-3}$M; X-ray dose 580 r; HN2 $\frac{1}{12} \times 10^{-4}$M

Days	Treatment at 2 hr after seeding						Treatment at 24 hr after seeding						Treatment at 48 hr after seeding					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI	I	II	III	IV	V	VI
0	22						28						21					
1	20	19	15	18	16	11	41	30	32	47	40	38	31	21	23	32	—	—
2	35	25	25	35	15	9	89	47	46	111	27	20	48	29	25	56	—	—
3	67	34	42	56	15	5	177	54	53	203	27	17	93	38	27	107	—	—
4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	185	56	54	157	28	3	390	77	70	440	35	10	193	37	40	232	—	—
6	239	55	58	239	33	5	—	78	64	—	—	—	222	26	39	277	—	—
7	—	63	75	—	49	8	—	80	77	—	38	10	298	28	30	350	—	—
8	—	69	78	—	68	6	—	96	77	—	—	—	—	42	42	—	—	—
9	—	80	101	—	—	—	—	134	121	—	67	8	—	37	36	—	—	—
10	—	110	107	—	131	10	—	131	142	—	—	—	—	45	47	—	—	—
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	—	164	186	—	249	12	—	249	240	—	225	11						
13	—	—	—	—	—	—	—	291	251	—	—	—						

* Cysteamine ($1{\cdot}5 \times 10^{-3}$M) was used in place of cystamine: the other conditions are as in Table 5. As shown in this table, the treatment was performed at different times after the seeding of the cultures.

Only in the first two experiments was the protection against HN2 studied at the same time. It is seen that in both experiments good protection against HN2, but none against irradiation, was obtained. Nor was any protection against X-irradiation obtained in the third experiment. However, it should be noticed that cysteamine seemed to exert a slight inhibitory action on the growth of the cultures in the last two experiments listed in the table.

The protective action of cysteamine and cystamine against lower and higher X-ray doses were also studied.

In four experiments the X-ray dose applied was 290 r. In these experiments, cystamine concentrations of $0{\cdot}75 \times 10^{-3}$ and $0{\cdot}38 \times 10^{-3}$ M and cysteamine concentrations of $0{\cdot}5 \times 10^{-3}$ and $0{\cdot}75 \times 10^{-3}$ M were used.

One experiment was suggestive of a slight protective effect by $0{\cdot}38 \times 10^{-3}$ M cystamine, while the remaining three experiments did not reveal any protection.

For three experiments an X-ray dose of 145 r was used. Neither did these experiments reveal any evidence in favour of a protective effect. Protection against HN2 was studied in two of the experiments; both showed distinct protection. Finally, the results of two experiments, in which X-ray doses of 870 and 1160 r were used, are shown in Table 7. Again, no protection against irradiation, but good protection against HN2 was obtained.

In recapitulation, it may be said that it was not possible to obtain significant protection against the effect of X-irradiation under the experimental conditions used, whereas good protection against HN2 could be obtained under exactly the same conditions.

TABLE 7. CELL COUNTS IN CULTURES TREATED AS IN TABLE 5*

Days	Cystamine $0{\cdot}75 \times 10^{-3}$M; X-ray dose 870 r; HN2 $\frac{1}{1{\cdot}2} \times 10^{-4}$M						Cystamine $0{\cdot}75 \times 10^{-3}$M; X-ray dose 1160 r; HN2 $\frac{1}{1{\cdot}2} \times 10^{-4}$M					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
0	31						30					
1	59	32	36	55	—	—	44	28	24	45	—	—
2	121	41	46	127	—	—	111	29	32	121	30	20
3	—	—	—	—	—	—	192	—	—	219	—	—
4	429	39	40	456	35	16	375	30	31	412	32	10
6	—	20	22	—	—	—	—	20	20	—	—	—
8	—	15	13	—	98	11	—	16	15	—	59	8
11	—	14	10	—	—	—	—	11	10	—	130	7
13	—	13	18	—	—	—	—	10	9	—	290	3
18	—	79	103	—	—	—	—	7	7	—	—	—
20	—	121	114	—	—	—	—	10	9	—	—	—
22	—	180	164	—	—	—	—	4	11	—	—	—

* The X-ray doses were 870 r and 1160 r; HN2, $\frac{1}{1{\cdot}2} \times 10^{-4}$M; Cystamine, $0{\cdot}75 \times 10^{-3}$M

DISCUSSION

For a more detailed discussion of previous investigations on the toxic and protective effects of cysteamine the reader is referred to a recently published survey.[1] The toxicity, as shown above, depends on the number of cells present in the cultures. This dependence should be expected, especially if the explanation of the toxic effect is that offered by Eldjarn and Pihl,[11] viz. that cysteamine forms mixed disulphides with SH-bearing proteins in the cell, resulting, *inter alia*, in an inactivation of enzymes whose activity depends on the presence of a free SH group.

On the assumption that a certain number of SH groups per cell are to be blocked in order to obtain a toxic effect, this dependence between the toxicity and the number of cells in the cultures should be expected.

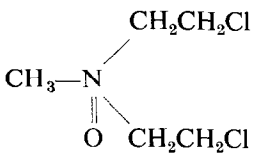
As regards the possibility of protecting the L-cell against the action of HN2 the present experiments showed that both cysteamine and cystamine afford good protection under conditions where inactivation of HN2 by linkage to cysteamine or cystamine in the medium can be excluded.

On the basis of the investigations of Eldjarn and Pihl,[11] it is reasonable to assume that cysteamine is firmly bound as mixed disulphides to SH-bearing proteins in the cells. That cysteamine is bound in the cells also appeared in my experiments from the fact that cysteamine could not be removed from the cells if they were kept in a cysteamine-free medium for $1\frac{1}{2}$ hr at room temperature. No diffusion of cysteamine from the cells into the cysteamine-free medium seems to occur.

As stated in a previous survey,[1] a possible explanation of the protective effect is therefore that SH groups on the proteins of the cell are protected against the action of HN2 through the formation of mixed disulphides with cysteamine, since HN2 will have a considerably lower affinity for S—S bonds than for the ionised free SH groups.

Accordingly, the mechanism of action should then consist in a change in the point of attack of HN2 in the cell: SH groups are protected, while the strongly reactive HN2 attacks groups which are of less significance for the continued existence of the cell.

The possibility of protecting cells *in vitro* against the effect of nitrogen mustard by means of cysteamine and cystamine has also been studied by Deysson and Truhaut.[6, 7] They studied the protective action of the two substances against the effect of HN3 on *Allium cepa* roots and against the effect of nitromine



on *Pisum sativum*. In both cases, they found good protection by cysteamine, but no protection by cystamine. However, in their experiments the nitrogen mustard was mixed *in vitro* with cysteamine or cystamine in isotonic saline (Knop solution), and these mixtures were added to the cultures. The protection obtained by cysteamine must be supposed to be due to simple inactivation of the nitrogen mustard in the medium, while the lack of protection by cystamine may be explained by the fact that this substance only to a slight extent inactivates nitrogen mustard.* Simultaneous addition of cystamine and nitrogen mustard to the cells excludes the possibility that cystamine may react with the cellular elements before the nitrogen mustard has exerted its action. Accordingly, protection by the mechanism of action outlined above cannot either be obtained by the experimental technique employed by Deysson and Truhaut.

If the mechanism of the protective action of cysteamine is that proposed above, the results of the present experiments also lend support to the view that the destruction of SH groups in the cells plays a central role in the biological effects of nitrogen mustard.

As regards the possibility of protecting L-cell cultures against X-rays, the present studies show that no definite protection could be obtained with cysteamine or cystamine, while these substances afforded good protection against HN2 under exactly the same experimental conditions. Accordingly, the experiments suggest that the mechanism of the radio-protective action by cysteamine and cystamine differs from that of their protective action against HN2. According to the theory advanced by Eldjarn and Pihl,[11, 12] the mechanism should in both cases be that the substances form mixed disulphides with protein-bound SH groups and thus protect these proteins against the effect of both X-rays and HN2.

A review of the literature[1] seems to show that previous investigations on the possibility of protecting tissue cultures against the action of X-rays by means of cystamine have all given negative results,[3, 4, 5] but protection has been obtained with isolated thymocytes.[13] Cysteamine has afforded protection of various cell types, but it cannot be definitely excluded that the protection obtained in these cases was due to anoxia in the nutrient medium produced by auto-oxidation of the cysteamine added.[2]

Anoxia of the cells caused by auto-oxidation of the cysteamine added is unlikely under the present experimental conditions, since the cells were rinsed with freshly

* When cysteamine is mixed with HN2 in Hank's saline, an appreciable inactivation of HN2 occurs within 2–3 min, whereas cystamine inactivates HN2 at a much slower rate (unpublished experiments).

aerated medium after the treatment with cysteamine and since the irradiation was then performed without any medium covering the cells.

Nevertheless further experiments are necessary before definite conclusions are drawn as to the possibility of protecting the L-cell with cysteamine against irradiation. So far it is only possible to conclude that no protection can be obtained under the present experimental conditions,* and it must be emphasized that the L-cell used cannot be regarded as a normal animal cell, since a rough count of its chromosomes showed that it had a modal chromosome number of 62, while the normal diploid chromosome number in the mouse is 40.

* As the maximum radio-protective effect of cysteamine in experiments with mice is obtained if the compound is injected shortly (from 0 to 30 min) before the irradiation, a couple of experiments were done, in which the cultures were irradiated from 20 to 30 min after the addition of cystamine or cystamine. No protection was obtained.

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