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ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY

REPORT NO. 102
12 November 1952

STUDIES ON THE PROTECTIVE ACTION OF SULFHYDRYL COMPOUNDS AGAINST X-IRRADIATION*

*Subtask under Effects of Irradiation, AMRL Project No. 6-59-08-013, Subtask, Early Effects of Ionizing Radiation.

MEDICAL RESEARCH AND DEVELOPMENT BOARD
OFFICE OF THE SURGEON GENERAL
DEPARTMENT OF THE ARMY

Washington National Record Center
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STUDIES ON THE PROTECTIVE ACTION OF SULFHYDRYL COMPOUNDS AGAINST X-IRRADIATION*

by

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ABSTRACT

STUDIES ON THE PROTECTIVE ACTION OF SULFHYDRYL COMPOUNDS AGAINST X-IRRADIATION

The Influence of Cysteine on the Radiation Induced Inhibition of Nucleic Acid Synthesis in the Intestinal Mucosa of the Albino Rat.

OBJECT

To study the effect of x-rays on nucleic acid new formation in the intestinal mucosa of cysteine treated and control animals.

RESULTS AND CONCLUSIONS

Cysteine, in a concentration of 850 mg/kg body weight administered intravenously 10 minutes prior to total body irradiation with 880 r/air, counteracts the inhibiting effect of x-rays on nucleic acid synthesis in the intestinal mucosa of the albino rat. The protective action of cysteine for this tissue probably involves such important processes as the nucleic acid cycle. If the protection afforded by cysteine is not merely based on a "shielding" process, the beneficial effect on nucleic acid synthesis must be an indirect one.

RECOMMENDATIONS

Simultaneous investigations on nucleic acid metabolism and on mitotic activity should be done; different time intervals after the irradiation and different chemicals should be studied; besides intestinal mucosa other tissues should be included in the investigations.

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STUDIES ON THE PROTECTIVE ACTION OF SULFHYDRYL COMPOUNDS AGAINST X-IRRADIATION

The Influence of Cysteine on the Radiation Induced Inhibition of Nucleic Acid Synthesis in the Intestinal Mucosa of the Albino Rat.

I. INTRODUCTION

Cysteine and glutathione protect against the damaging effects of x-rays (1). Different theories concerning the mechanism of this protection have been suggested (2). Final conclusions, however, have not been reached. While several investigators maintain that certain biological cycles and systems are protected from x-ray damage by cysteine (3), others report negative results (4). An indirect indication of the protective mechanism is given in publications, which report a faster recovery of certain organ functions in x-irradiated, glutathione treated mice (5).

Earlier studies on the recovery processes in x-irradiated rat intestine, that were done in this laboratory (6), were continued and the effect of cysteine on the metabolic processes in the intestinal mucosa was investigated. The inhibition of nucleic acid synthesis by x-rays (7) was used as a basis for comparative measurements.

II. EXPERIMENTAL

A. Methods and Procedures

This investigation was made with approximately 300 male Sprague-Dawley rats weighing about 300 grams each and included 79 experiments, more than 50 of which were of an exploratory nature in developing the technique. Each experiment, considered as one independent group, consisted of four animals: rat A, cysteine injected and irradiated; rat B, cysteine injected; rat C, saline injected and irradiated and rat D, saline injected control. Before the experiment the animals were kept under identical conditions and given Purina Chow and water ad lib. During the experimental procedure, all samples were treated alike, i.e. all manipulations necessary for studying the effects of cysteine and x-rays on nucleic acid metabolism were done simultaneously with the four animals and the tissue on the same time scale. The handling of the animals during the experiment, as already emphasized by Holmes (8) and by Edwards and Sommers (9), is of extreme importance. Struggling, while being handled for injections and irradiation, decisively influences the nucleic acid figures.

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The cysteine (2ml) was administered into the tail vein of the animal ten minutes prior to irradiation in a concentration of 850 mg/kg body weight in distilled water, adjusted with 2N NaOH to pH 7-8. The animals were irradiated in pairs (rat A and rat C) in a well ventilated lucite cage with x-rays from a Kelley-Koett deep therapy unit with 200 kv, 6ma, 1 mm Al inherent filtration, 1 mm Al plus 0.5 mm Cu added, 30 cm target distance, 40 r/min/air, a total of 880 r.

Immediately after irradiation the animals received intraperitoneally 20 to 50 microcuries of P^{32}. Two hours later they were sacrificed and the entire small intestine was removed for study. Recently made radioautographs by Holt and Warren (10) showing the uptake of injected P^{32} by the rat intestine justify the use of the entire small intestine in preference to the intestinal mucosa alone. Their radioautographs show a strong concentration of P^{32} in the intestinal mucosa, while the muscular layers of the intestine show practically no P^{32}.

The small intestine was rinsed with water and further processed in a timed procedure. One part, about 2 gms, of the intestine was taken for the inorganic tissue phosphorus determination (ITP) and its specific activity, while the remaining part was used for the determination of the desoxypentose nucleic acid phosphorus (DNA) and its specific activity.

The separation of the DNA for measuring and comparing the relative changes in the inhibition of nucleic acid new formation by x-rays could be done by any one of the many published methods. The method recently described by Kelly and Jones (11) for studying the direct and indirect effects of x-rays and beta-rays on nucleic acid new formation in different tissues was chosen. This method, based on that of Levene, Klein and Beck (12) lends itself to the procedure because of its simplicity and its congruency with the methods originally applied by Hevesy and his co-workers in their pioneer investigations. Following the Kelly and Jones procedure the tissue used for DNA extraction was put in a cooled mortar, covered with about 7 grams of Berkshire sand and ground for five minutes after adding 2 ml of 5% saline solution. After this 5 ml of saline solution were added and the grinding continued for five more minutes. At this point the whole homogenate was transferred into a test tube. The residue in the mortar was rinsed into the test tube with 3 ml of saline solution, the test tube thus containing the tissue homogenate in 10 ml of saline solution. After stirring for one minute with a

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glass rod the tube was placed in a boiling water bath for five minutes, 0.25 ml glacial acetic acid added and the solution made basic with a mixture of 0.4 gm sodium hydroxide and 0.1 gm sodium acetate. The basic mixture was kept in the boiling water bath for about one hour until the tissue was almost completely dissolved. One ml of glacial acetic acid and 0.7 ml of 5% dialyzed ferric hydroxide solution were then added. After standing a short time another ml of acetic acid was added and the solution was centrifuged. The supernatant liquid was decanted, treated with an equal volume of methyl alcohol, centrifuged and the resulting supernatant discarded. In order to purify the desoxypentose nucleic acid the residue was dissolved in 5 ml of 1 mol sodium hydroxide. After adding 0.2 ml of a saturated solution of disodium phosphate and an equal volume of methyl alcohol the solution was heated for 15 minutes in a water bath at 65°C and then centrifuged. The supernatant was placed in an ice bath, acidified with 3 mol hydrochloric acid, diluted with an equal volume of methyl alcohol and centrifuged for 10 minutes. These repeated treatments produce a residue high in DNA content. This nucleic acid containing residue was then redissolved in sodium hydroxide and reprecipitated with hydrochloric acid and methyl alcohol and finally dissolved in 5 ml of 0.1 mol sodium hydroxide. Since Klein and Beck found that nucleic acid was chemically pure after 3 precipitations and since in the reported experiments, after several precipitations, the absolute values but not the ratio of the values changed, not more than 3 precipitations were done.

The tissue taken for the determination of the inorganic tissue phosphorus (ITP) and its specific activity was put into a cooled mortar and about 7 gms of Berkshire sand and 2.0 ml of 25% trichloroacetic acid were added. After grinding the homogenate was quantitatively transferred into a test tube with 8 ml of trichloroacetic acid.

The specific activities of the inorganic tissue phosphorus and of the purified DNA were determined in the following manner. One aliquot of the final solution was taken for the determination of the phosphorus using the method of Fiske and Subbarow, and another aliquot was used for counting the P^{32} activity with a Geiger-Mueller counter and a scaler. Both measurements were related to the same amount of solution, so that the ratio of "activity counts to phosphorus amount" gave the specific activity directly. Following Hevesy the ratio of the specific activities of DNA to ITP was then taken as a figure for evaluating the influence of cysteine on the x-ray effect.

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B. Results

The results for 23 experiments are presented in Table 1 and Figure 1. Table 1 gives the specific activities of the nucleic acid fraction (DNA) and the specific activities of the inorganic tissue phosphorus (ITP). The percentage inhibition of nucleic acid synthesis, calculated from the ratio of DNA-activity to ITP-activity, is given in column 5 of Table 1. This figure represents a relative measure of the influence of cysteine on the x-ray induced inhibition of nucleic acid new formation in comparison with the controls.

TABLE 1

PERCENTAGE INHIBITION OF NUCLEIC ACID SYNTHESIS AS CALCULATED FROM THE SPECIFIC ACTIVITIES OF DNA AND ITP (INDIVIDUAL EXPERIMENTS PRESENTED).

Animal	DNA Specific Activity	ITP Specific Activity	DNA/ITP x 100	% Inhibition of Nucleic Acid Synthesis
A	560	30520	1.90	0
B	540	28440	1.90	
C	339	31320	1.10	39
D	502	28250	1.80	
A	260	21250	1.20	50
B	582	24770	2.40	
C	329	28200	1.20	59
D	708	24400	2.90	
A	241	19400	1.20	20
B	341	22600	1.50	
C	149	18650	0.80	68
D	487	19730	2.50	
A	500	33850	1.50	44
B	854	31800	2.70	
C	240	21200	1.10	39
D	678	38300	1.80	
A	410	30975	1.30	43
B	655	28300	2.30	
C	430	34800	1.20	43
D	761	36875	2.10	
A	324	31750	1.00	17
B	369	29650	1.20	
C	228	24900	0.92	58
D	595	27525	2.20	
A	250	24300	1.03	64
B	692	23800	2.90	
C	337	36325	0.92	62
D	640	26500	2.41	
A	230	18050	1.27	39
B	344	16450	2.09	
C	224	19050	1.17	47
D	416	18900	2.21	
A	354	29400	1.42	45
B	645	25000	2.58	
C	391	25000	1.56	42
D	638	23800	2.68	
A	208	20200	1.03	39
B	323	22200	1.46	
C	208	20400	1.02	46
D	407	21200	1.90	

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TABLE 1 (Continued)

Animal	DNA Specific Activity	ITP Specific Activity	DNA/ITP x 100	% Inhibition of Nucleic Acid Synthesis
A	716	56400	1.27	35
B	1156	59400	1.95	
C	655	52000	1.26	55
D	1510	55600	2.71	
A	673	57000	1.18	43
B	1175	57800	2.04	
C	532	52900	1.00	54
D	1013	46800	2.16	
A	547	36600	1.50	41
B	1046	41250	2.53	
C	515	37100	1.39	42
D	1246	52000	2.40	
A	765	58600	1.30	40
B	1413	65500	2.16	
C	555	63000	0.88	51
D	1256	69250	1.81	
A	530	44600	1.19	48
B	1080	47400	2.28	
C	438	39800	1.10	41
D	901	48500	1.86	
A	409	36600	1.12	36
B	671	38300	1.75	
C	401	32800	1.22	42
D	745	35400	2.10	
A	516	43200	1.19	60
B	1400	46600	2.99	
C	538	44200	1.21	46
D	943	42200	2.23	
A	698	34400	2.03	7.3
B	695	31750	2.19	
C	495	51000	0.97	69
D	738	23450	3.12	
A	472	35100	1.35	35
B	798	38400	2.07	
C	310	33400	0.93	60
D	867	37000	2.34	
A	691	64600	1.07	24
B	1170	83400	1.40	
C	588	71600	0.82	49
D	1233	76100	1.62	
A	420	41350	1.02	22
B	721	54900	1.31	
C	421	49200	0.85	53
D	907	49850	1.82	
A	583	43850	1.34	38
B	1123	52000	2.16	
C	533	72100	0.74	55
D	1164	70200	1.66	
A	479	37350	1.28	2.3
B	610	46500	1.31	
C	414	48000	0.86	44
D	751	49100	1.53	

A. Cysteine treated, irradiated.
B. Cysteine treated, non-irradiated.
C. Non-cysteine treated, irradiated.
D. Non-cysteine treated, non-irradiated.

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Figure 1 shows the percentage values of Table 1 plotted as percentage synthesis rather than inhibition in order to illustrate the relationship of the cysteine treated animals to the non-cysteine treated group.

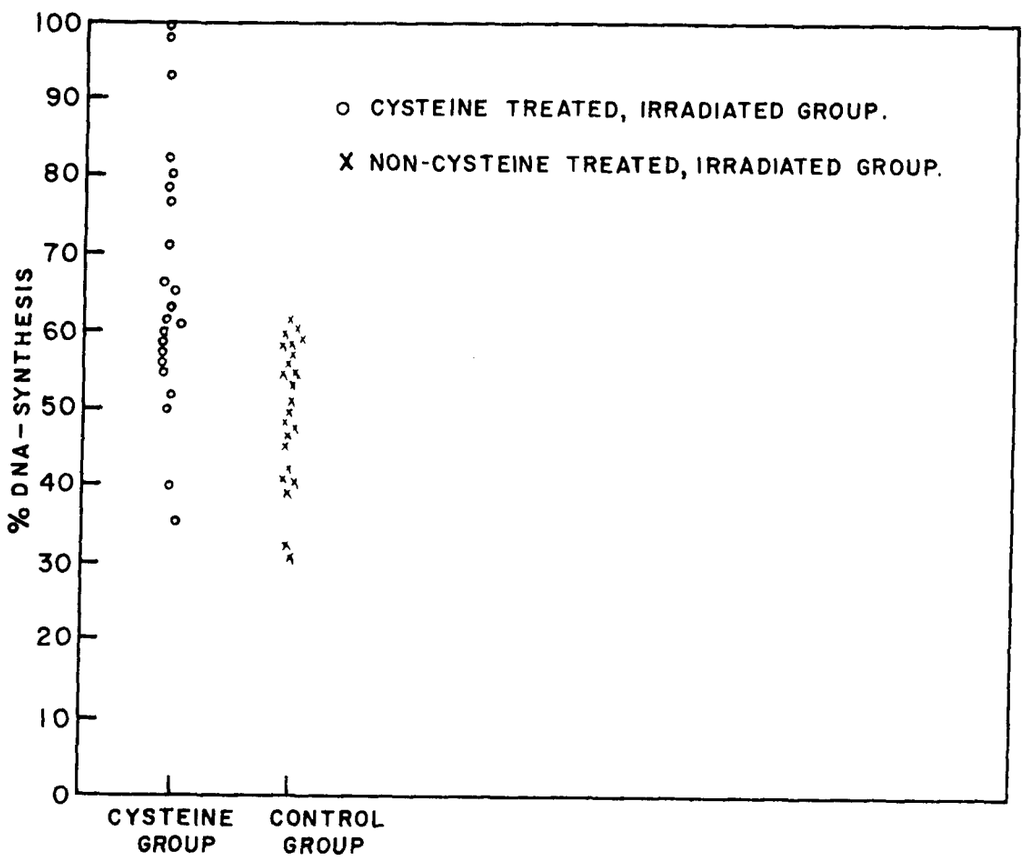

FIG. 1. DNA SYNTHESIS IN X-IRRADIATED, CYSTEINE TREATED AND NON-CYSTEINE TREATED ANIMALS (880r).

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III. DISCUSSION

Attempts to determine the manner in which sulfhydryl compounds afford protection against x-ray damage have frequently been made. Different physiological systems have been investigated.

The erythropoietic function of the bone marrow under glutathione and under cysteine treatment has been studied with negative results by Hennessy, Folsom and Glover (4). However, the studies of Patt, Smith and Jackson (3) on peripheral blood indicate positive results, while negative results in blood studies are reported by Lorenz (4). The studies of Smith, Patt and Tyree (3) indicate that cysteine owes its capacity to alter radio-sensitivity to factors other than its reducing power, while Cronkite, Brecher and Chapman (5) suggest that humoral factors may be competitively protected from the destructive effects of "activated water" by sulfhydryl compounds. No protective action of cysteine on lymph node cultures is reported by Trowall (13), indicating that the protection against cellular destruction by x-rays is not necessarily involved in the beneficial action of this sulfhydryl. Straube, Patt, Smith and Tyree (3) find partial protection of tumors from growth inhibiting action of x-rays by cysteine and Hall reports similar findings in tumor cell cultures (3). Skipper and Mitchell (14), checking the uptake of C^{14} in DNA-purines under x-irradiation, report no interference of glutathione with x-ray induced inhibition of nucleic acid synthesis in mice tissue, while Forssberg (15) finds beneficial effects of cysteine on C^{14} incorporation in desoxypentose nucleic acid irradiation experiments. Sallmann (16) reports protective action of cysteine against microscopical irradiation damage in the lens of rabbit eyes, when exposed to 1500 r, while the histo-chemically determined turnover of DNA was not noticeably influenced by the sulfhydryl compound. Limperos (3) claims a definite protective action of reducing agents on DNA metabolism.

The reported results with intestinal mucosa require special discussion and careful interpretation. Two factors must be considered: the reliability of the observed changes and the possible causes for the effect. The reliability can be proved by significance calculations and by the fact that the magnitude of the observed inhibition is in full agreement with the figures given by Hevesy and others for the inhibiting effect of x-rays on DNA synthesis in the intestinal mucosa of the rat (7). Distribution studies of the afforded protection also may confirm the results. In each survival-protection experiment several animals will succumb early to the irradiation insult, a relatively large number of animals will show a medium or medium-high protection and only a

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few animals will be highly protected and survive. The result of a cysteine-survival irradiation experiment done in conjunction with the DNA studies is presented in Figure 2. It illustrates the response of the particular animal strain used to the chosen experimental conditions: 880r/air, 850mg cysteine/kg body weight, pH 7 to 8, injection i. v. 10 minutes prior to irradiation.

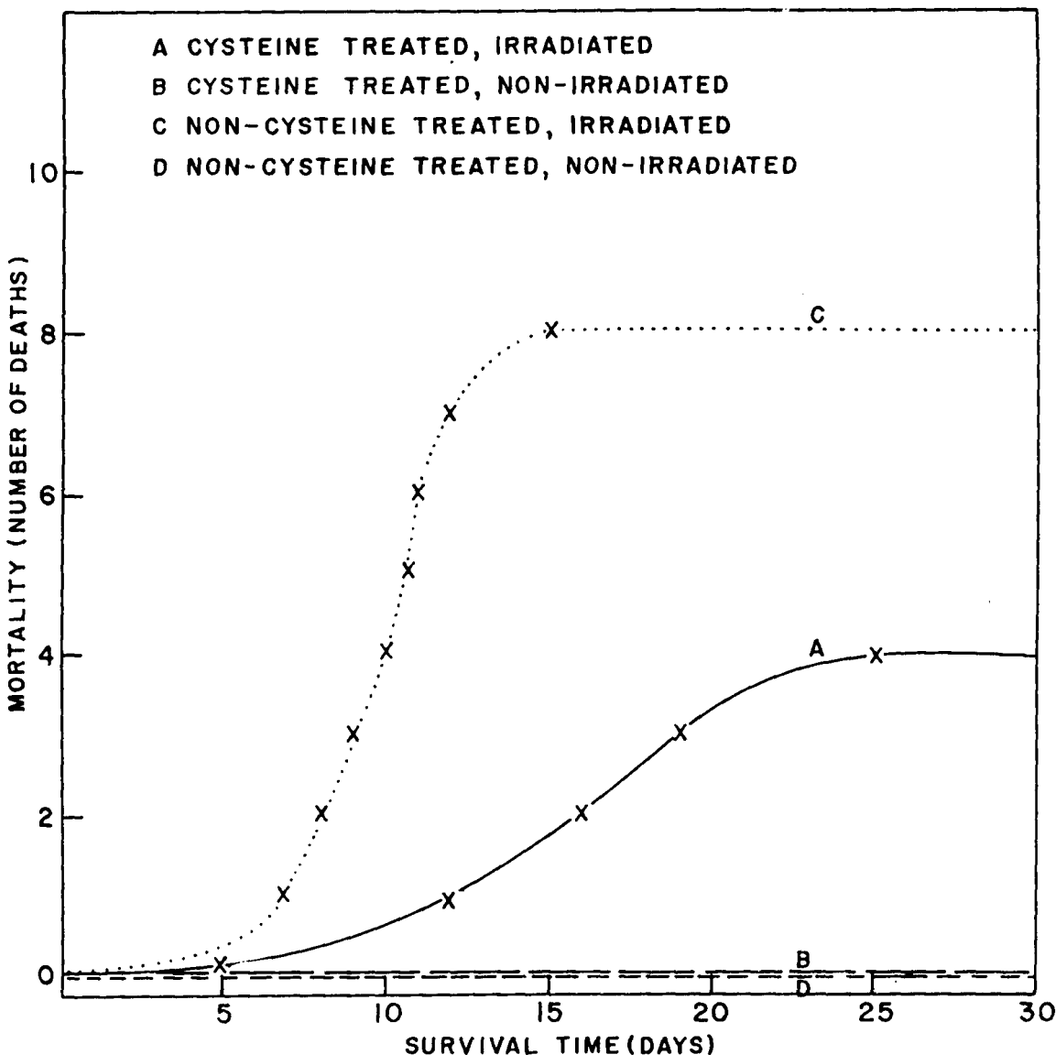

FIG. 2. SURVIVAL CURVES OF IRRADIATED, CYSTEINE TREATED AND NON-CYSTEINE TREATED ANIMALS. (880r).

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The frequency curve A of Figure 3 for the afforded protection is obtained by integrating the area between curves C and A of Figure 2 for each 3 day interval.

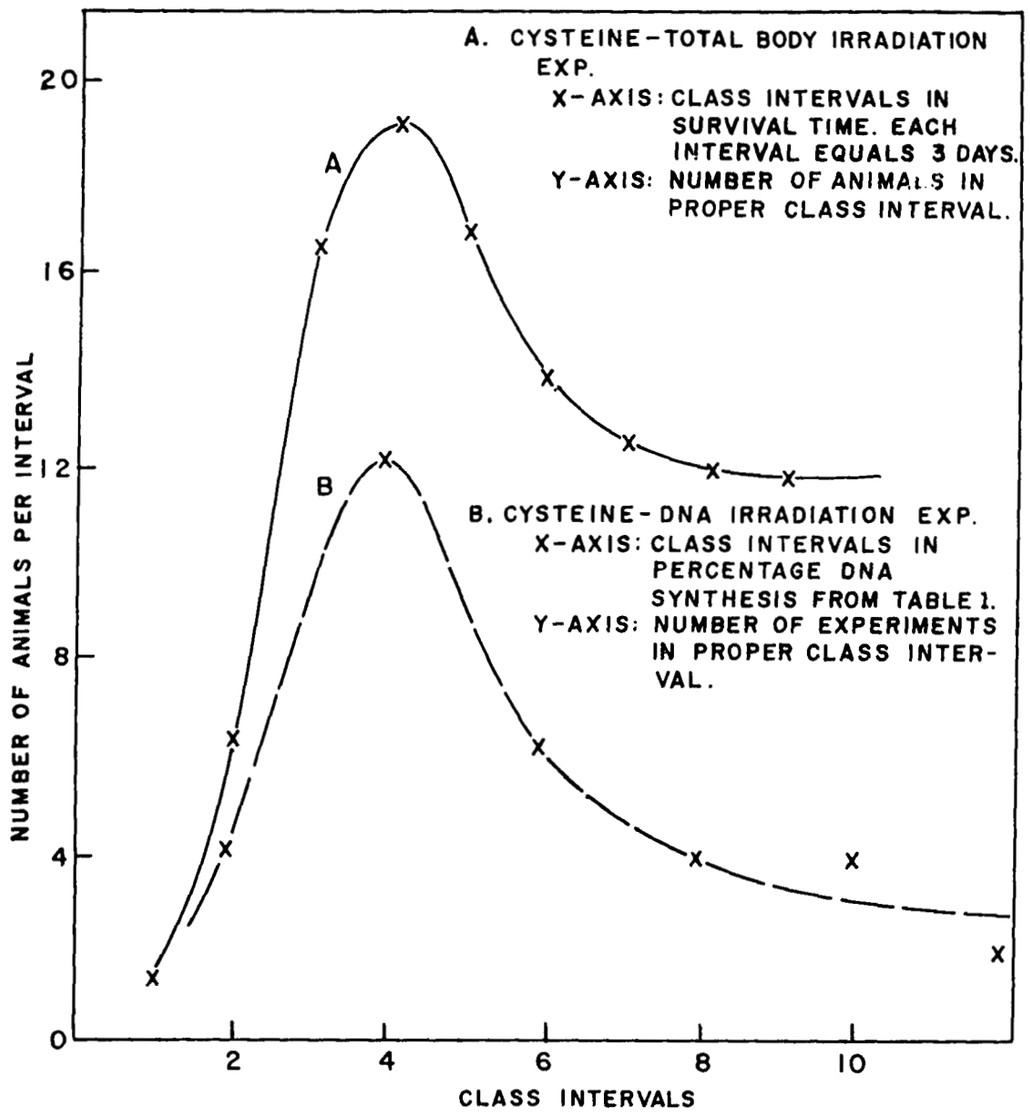

FIG. 3 FREQUENCY CURVE OF AFFORDED PROTECTION.

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A frequency curve (curve B of Figure 3) with a similar trend is obtained when the number of DNA experiments of certain percentage range (10%) inhibition protection, taken from Table 1, is plotted against the proper class intervals of the percentage inhibition. The right skewed frequency curve B indicates that the reported DNA studies follow the well established survival distribution curve for total body x-irradiation protection, lending credence to the reliability of the reported findings.

As to the cause of the observed beneficial influence of cysteine on the radiation induced nucleic acid synthesis inhibition, present discussions in the literature offer the following possible explanations.

Cysteine could influence an x-ray effect on the nucleic acids. This possibility has been discussed by Hevesy (7). From the fact that at least several tens of thousands of r were necessary (7) at that time to produce in vitro changes in DNA solutions, he concluded that the usual therapeutic doses used were not able to cause degradation of nucleic acid in biological systems. Furthermore, he proved this conclusion in experiments with C^{14}. The markedly reduced incorporation of C^{14} into the purines of DNA in x-irradiated rats, as confirmed by Skipper and Mitchell (14), was taken as an indication of a reduced rate of formation of DNA under x-irradiation rather than a direct effect of x-rays on the nucleic acid. Since Hevesy's early work the concept of x-ray dosage necessary to influence DNA in vitro as well as in vivo has changed. Scholes and Weiss (18) found decomposition of DNA with doses of 4000 r in in vitro experiments and Limperos (3) was able to depolymerize DNA in vivo with doses of only 250 r to 1000 r. Scholes and Weiss irradiated 0.05% solutions of DNA and PNA and found formation of ammonia and inorganic phosphate. Limperos extracted DNA from rat thymus 24 hours after irradiation and found marked changes in the viscosity of the nucleic acid fraction. He also showed that reduced oxygen tension protects DNA against the damaging effects of x-rays, indicating an indirect mechanism for the action of x-radiation on DNA in vivo. Nothing is known, however, concerning short time in vivo experiments, so that more studies are necessary to decide whether or not a direct or an indirect effect is responsible for the observed changes. Recent experiments of Feinstein and Butler (19) on the effect of whole body x-irradiation on rat intestine and intestinal nucleoproteins are interpreted as a substantiation of the idea that one effect of whole body irradiation is the degradation of nucleic acid.

A second possibility of cysteine influence concerns cell permeability problems, also discussed by Hevesy. Existing changes in permeability for phosphorus under x-irradiation are, however, according

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to Hevesy, not great enough to explain the magnitude of the observed inhibiting effect. A similar conclusion is drawn by Lacassagne (20), who explains the observed decrease in nucleic acid new formation after x-irradiation by changes in metabolic processes rather than by changes in cell permeability. In this connection the recent studies by Entenman and Weinman (21) on the effect of x-irradiation on the incorporation of inorganic P^{32} into phospholipids should be mentioned. They found in in vitro experiments changes in the uptake of inorganic P^{32} by liver slices, the magnitude of which was the same in samples with or without cysteine treatment.

A third possibility of cysteine influence is that on the P^{32} uptake by pentose nucleic acid. Pentose nucleic acid is believed to be used in DNA synthesis (22), and the accumulation of PNA in the cytoplasm of irradiated cells is thoroughly discussed by Mitchell (22) in connection with possible changes in the P^{32} uptake by PNA and with the blocking of the reduction of ribonucleotides to desoxyribonucleotides by x-rays.

Another possibility of cysteine influence is its incorporation in and its interference with certain enzyme systems. Lacassagne (20) maintains the existence of enzymes which catalyse the synthesis of new pentose nucleoproteins. Mitchell (22) and Langendorff (23) believe enzymes are responsible for the synthesis of DNA from PNA. Langendorff especially suggests a blocking of this enzymatic activity by activated water products. The sulfhydryl compounds may be intimately involved in these enzyme systems.

Still another possibility is the effect on certain processes going on in fast growing and full grown tissues. Hevesy found an inhibition of nucleic acid new formation in growing tissue as well as in full grown tissue. The percentage of inhibition in both these cases was nearly the same. From this fact one might speculate that other factors besides growth per se play a part in the nucleic acid synthesis. The state of secretory activity of organs may be one of these factors.

Which of these possibilities is responsible for the beneficial influence of cysteine cannot be said at present. It may be that a simple shielding effect, as discussed by Brues and Patt (2), is responsible for the observed protection by cysteine.

IV. CONCLUSIONS

Specially arranged experiments show that the inhibiting effect of x-rays on nucleic acid synthesis in intestinal mucosa of the albino rat can be influenced by pretreatment of the animal with cysteine.

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In connection with other investigations (3, 19), it may be concluded that the protective action of cysteine involves such vital processes as the nucleic acid cycle. If cysteine affords it protection by other means than by a purely "shielding" effect, the mechanism of the effect must be an indirect one.

V. RECOMMENDATIONS

Considering the importance of the nucleic acid cycle in cellular metabolism DNA investigations under x-irradiation should be done in connection with mitotic studies. Different time intervals after the irradiation and different chemicals should be investigated for their influence on the process. Other tissue besides intestinal mucosa should be included in the studies.

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