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INTRACELLULAR DISTRIBUTION AND BINDING OF RADIATION PROTECTIVE MERCAPTOALKYLGUANIDINES

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BY

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INTRACELLULAR DISTRIBUTION AND BINDING OF RADIATION

PROTECTIVE MERCAPTOALKYLGUANIDINES

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INTRACELLULAR DISTRIBUTION AND BINDING OF RADIATION PROTECTIVE MERCAPTOALKYLGUANIDINES

CHAPTER I

HISTORICAL

Patt and co-workers (1), in 1949, reported that cysteine hydrochloride, when administered to rats a short time prior to x-irradiation, significantly increased the rate of survival of the treated animals over that of the untreated controls. This clearly indicated, for the first time, that it was possible to protect an animal against the lethal effects of a single dose of radiation by administering a relatively simple chemical compound at such a time that it was present in the animal's body during the irradiation. Since this phenomenon was demonstrated, a large number of compounds, diversified in their chemical and biological nature, have been tested for their effectiveness as radiation protective agents [see periodic reviews on this subject by Selle (2)]. Many of the compounds tested have exhibited some degree of protective capacity, but relatively few, including β-mercaptoethylamine (3), S,2-aminoethylisothiuronium bromide hydrobromide (4) and other related aminoalkylisothiuronium salts (5), and a group of dithiocarbamates (6), have been found to provide 100% survival to animals receiving the radiation L. $D._{100}$ dose. This latter group of protective compounds, particularly the aminoalkylisothiur-

onium salts, is the subject of this dissertation.

Bacq and associates (3) discovered the radiation protective activity of β-mercaptoethylamine while screening for protective activity a large number of amines and thiol-containing compounds structurally related to cysteine. Since it seemed imperative to define clearly the elements of structure necessary for protective activity in this compound, Doherty and co-workers synthesized and tested a number of compounds chemically related to β-mercaptoethylamine (7). As a result of this study relating chemical structure to protective activity, a group of active aminoalkylisothiuronium salts, of which S,2-aminoethylisothiuronium bromide hydrobromide and S,3-aminopropylisothiuronium bromide hydrobromide are the parent compounds, were discovered (4, 5). These compounds are more active than β-mercaptoethylamine on a micromolar basis, some being many times more active. They also have a lower therapeutic ratio (ratio of effective dose to lethal dose), a particularly desirable feature since the effective dose of β-mercaptoethylamine is near the dose that produces some deaths in the experimental animals from drug toxicity. From a practical viewpoint, the thiuronium salts have the additional important advantage of being considerably more stable than β-mercaptoethylamine.

The radiation protective ability of these thiuronium compounds has been established by extensive testing with mice (5, 8), in which both the oral and intraperitoneal routes of administration proved equally satisfactory. After the effectiveness of these compounds had been clearly demonstrated, and after the minimal effective dose had been established in the mouse testing program, it seemed desirable to demonstrate their efficacy in higher animals. These experiments, using Macaca mulatta

monkeys as the experimental animal, were performed by Overman and associates (9), who found that S,2-aminoethylisothiuronium bromide hydrobromide, the only one of these compounds so tested to date, was equally as effective in monkeys as had already been observed in mice.

CHAPTER II

INTRODUCTION

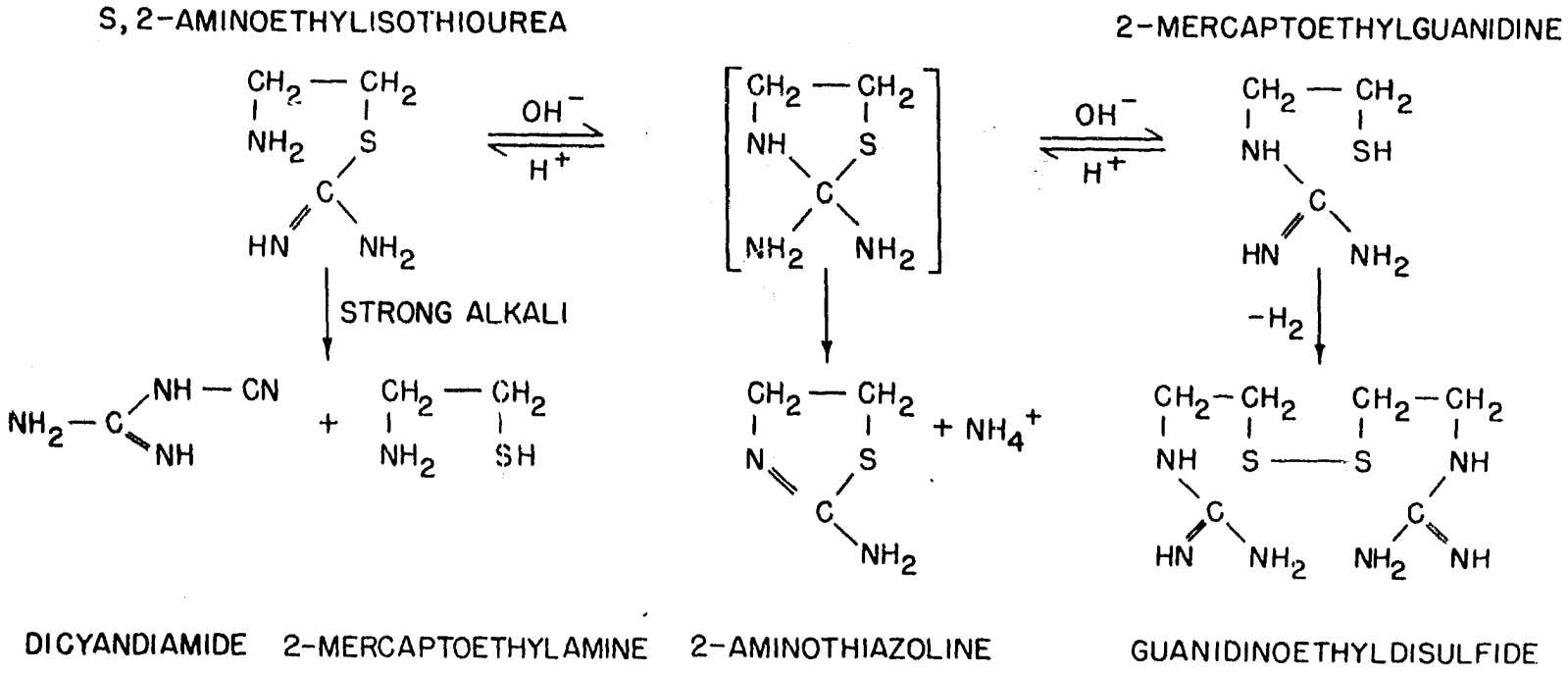
Many investigations, particularly with *in vitro* systems, have been undertaken to determine the mechanism by which radiation produces its deleterious effects on biological material. Both the direct and indirect effects, as they are usually classified, have been discussed in some detail by Bacq and Alexander (10). The direct effects are those produced by the action of the radiation directly on the biological material. The indirect effects are those that result secondarily from the radiation, as for example the radiation-induced formation of free radicals in the medium, radicals which in turn interact with the biological material. Alexander and co-workers (11) concluded that the HO_2 radical is a major factor in the lethality realized from irradiation. Their conclusions were based on a comparison of the protective activity of a number of different substances in a polymer system, whose depolymerization is catalyzed by the HO_2 radical, to the protective activity of these substances in mice. By postulating that protective agents are effective because they successfully combat these radicals, these investigators presumably implicate the indirect effect of radiation and free radicals in general as being significant contributors to the damaging effects of radiation. If it is, therefore, assumed, on the basis of these and other

arguments, that the major effect of x-irradiation, in the dose range of most importance with the in vivo experiments below (approximately 1100 roentgens or less), is that of free radical production in the aqueous medium and, further, that the protective agent acts to combat these radicals, two questions remain to be answered in explaining the mechanism of action of protective compounds such as the aminoalkylisothiuronium salts. The first question is a consideration of how these compounds are capable chemically of combating the free radicals formed. Before discussing this, some aspects of the chemistry of these thiuronium salts must be considered. It was observed quite early that solutions of S,2-aminoethylisothiuronium bromide hydrobromide (AET) apparently underwent an intra-transguanylation reaction at pH 7-8. AET, which has neither a mercaptan nor a guanido group, gave, at pH 7-8, an immediate, positive nitroprusside test for the mercaptan group and, also, a positive Sakaguchi test for the guanido group (5). This strong indication that an intra-transguanylation had occurred at this pH has since been rigorously confirmed by Khym and coworkers (12). Using ion-exchange chromatography, the products formed when various thiuronium salts were allowed to stand at each of several different pH's were separated and identified. The results of this study are summarized with AET in Figure 1, which illustrates both the intra-transguanylation reaction and, also, other type products that may result in certain pH ranges. These investigators have found that, at neutral pH, almost all of the compounds tested undergo this transformation to the mercaptoalkylguanidine, a factor considered important in their ability to protect the animal(5).

It is of interest to note that both the oral and the intraperi-

Figure 1

Formation of Mercaptoethylguanidine and Other Products from Aminoethylisothiourea



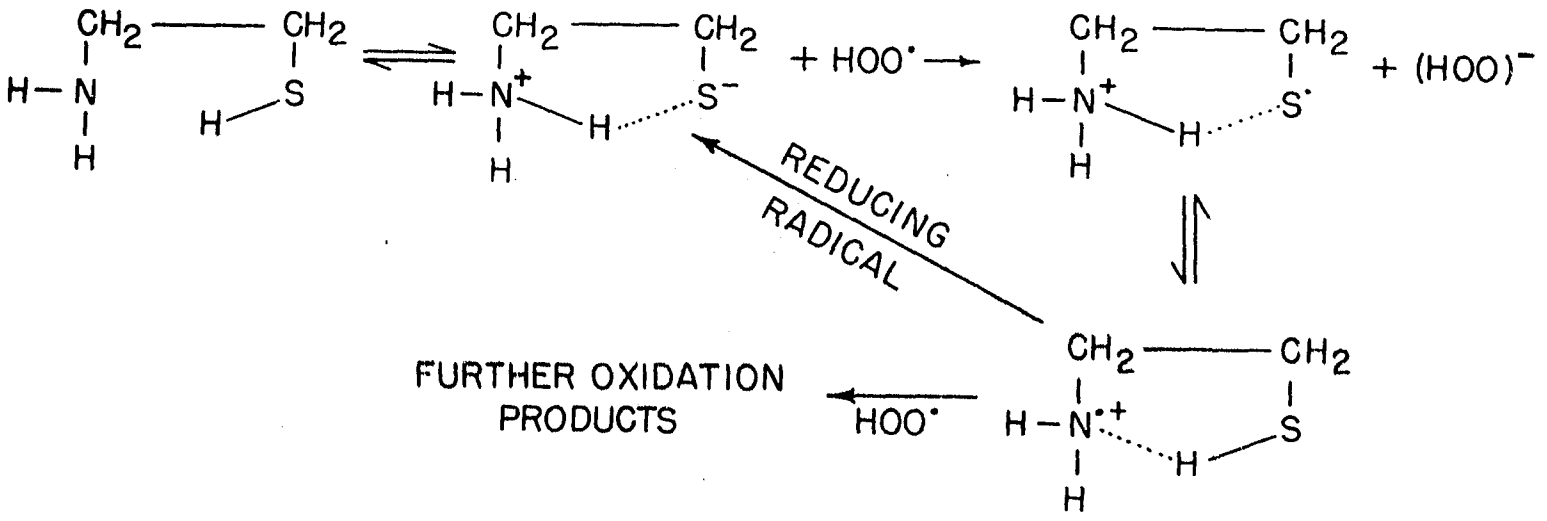
toneal routes of administration are satisfactory, though approximately twice the amount of compound is necessary, in most cases, when given by the oral route.

Doherty and Shapira (13), in considering how the mercaptoalkylguanidines are chemically capable of combating free radicals, have postulated that their chemical structure is such that they can donate electrons to quench the highly reactive free radicals, such as OH and HO_2, formed in the aqueous medium. Though this loss of an electron, of course, converts them into free radicals, they are much more stable, through resonance, than the free radicals produced initially in the medium by the radiation, so that the tendency for a detrimental radical reaction with the biological material is greatly reduced. This mechanism for chemical protection has previously been postulated for β-mercaptoethylamine, as shown in Figure 2, by these investigators (7), who reasoned that the more negative sulfur atom of mercaptoethylguanidine should be more active as an electron donor, paralleling its greater protective activity, than β-mercaptoethylamine. In an extension of this postulate, they have also proposed that replacement of the sulfur by other atoms having a high electron density should, on theoretical grounds, also give compounds with high protective activity, providing their toxicity or other biological complication is not limiting.

The second question in explaining the mechanism of action is a consideration of how this group of mercaptoalkylguanidines exert their postulated free radical quenching action in the intact biological system, that is, in vivo. This is the subject to which the series of experiments reported and discussed here are directed.

Figure 2

Postulated Interaction of Mercaptoethylamine with Free Radicals

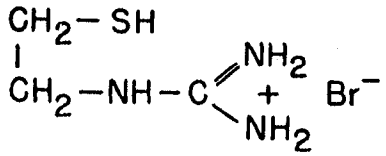


It has been shown by Congdon and associates (14) that mercaptoethylguanidine exerts a definite biological effect, particularly on hematopoietic tissue. They found that protected animals receiving 900 roentgens resemble, in their assays, control animals receiving only 450 roentgens. A radiation dose reduction factor of two is, therefore, obtained in these protected animals. It was the objective of the experiments reported here to investigate the biochemical basis for these biological effects.

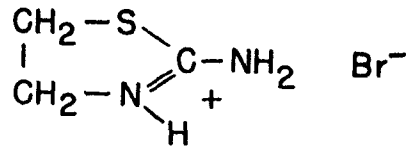
The cellular distribution and binding of three closely related mercaptoalkylguanidines were investigated. These compounds, mercaptoethylguanidine hydrobromide (MEG), D-mercaptobutyl-2-guanidine hydrobromide (D-MBG-2), and L-mercaptobutyl-2-guanidine hydrobromide (L-MBG-2), Figure 3, had most desirable properties for this investigation. MEG is quite active as a protective agent, possessing the particular advantage of being effective against increased doses of radiation. By giving larger doses of this agent, the radiation L. $D._{50}$ can be elevated to more than twice its normal value (8). On a micromolar basis, D-MBG-2 and L-MBG-2 are two of the most highly effective protective agents known, when evaluated against a lethal dose of radiation (900 roentgens). Also, of considerable importance for these experiments, they have equal toxicity though differing in protective activity by a factor of more than two (15). Thus, this pair of optical isomers, whose only chemical difference is their configuration about an asymmetric carbon, possess a marked difference in protective activity. In addition to MEG, D-MBG-2, and L-MBG-2, a fourth compound, 2-aminothiazoline hydrobromide (2-AT), Figure 3, was included in several experiments, since it is only partially effective at

Figure 3

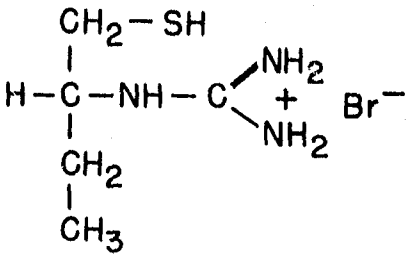
Structural Formulas of MEG, 2-AT, D-MBG-2, and L-MBG-2



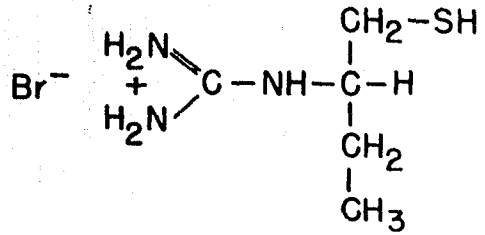
MERCAPTOETHYLGUANIDINE·HBr
(MEG)



2-AMINOTHIAZOLINE·HBr
(2-AT)



D-MERCAPTOBUTYL-2-GUANIDINE·HBr
(D-MBG-2)



L-MERCAPTOBUTYL-2-GUANIDINE·HBr
(L-MBG-2)

the maximum tolerated dose (5).

Both the tissue distribution and the intracellular distribution of these compounds were studied by radioisotopic tracer techniques. It was thus found that each of the compounds was bound intracellularly. The nature of this binding was then investigated by dialysis against suitable solvents. Finally, the effect of radiation on the intracellular distribution and on the type of binding was determined using D-MBG-2.

CHAPTER III

EXPERIMENTAL

Radioactive Compounds

C^{14}- or S^{35}-labeled AET, D,2-aminobutylisothiuronium bromide hydrobromide, and L,2-aminobutylisothiuronium bromide hydrobromide were prepared using either C^{14}- or S^{35}-labeled thiourea. The three S^{35}-labeled compounds were initially prepared at equal specific activity (1.2 mc/mM), as were also the three C^{14}-labeled compounds (1.0 mc/mM). The actual specific activity of the compounds at the time of a particular experiment is shown below the respective table. 2-AT was prepared from AET(S^{35}), so that both had the same specific activity [the details of preparation of these radioisotopic compounds will be published soon (16)].

Preparation of the Mercaptoalkylguanidine Salt from the Corresponding Aminoalkylisothiuronium Salt

Approximately 20 minutes before injection, the aminoalkylisothiuronium salt was neutralized with one equivalent of sodium hydroxide. As may be seen in Figure 1, this transguanylation reaction separates to opposite ends of the molecule the sulfur and carbon atoms introduced as thiourea during the synthesis. Any large-scale metabolic cleavage of the initial compound should result in a difference in the relative distribution of activity obtained with each of the isotopically labeled compounds,

since the radioactive tracer in the S^{35}-labeled mercaptoalkylguanidine salt and the radioactive tracer in the C^{14}-labeled mercaptoalkylguanidine salt are at opposite ends of the molecule.

Tissue Distribution Study

The S^{35}-labeled compound was injected intraperitoneally into a 101xC_3H mouse, 20-25 gms., and, after 45 minutes, the animal was stunned, decapitated, and bled. The desired tissue samples were removed immediately. The entire liver, spleen, and brain, as well as a portion of the skeletal muscle, were removed and washed in isotonic saline. The kidney and heart were removed and washed with saline after being cut open so that much of their contents would be eliminated. The sample of bone marrow was obtained by perfusing the femurs with distilled water. The blood was collected in distilled water as the animal was being bled. The sample of intestinal mucosa was prepared by lightly scraping the walls of the upper several centimeters of the small intestine, after it had been perfused with approximately 5 ml. of isotonic saline and cut open. These samples were then individually homogenized in distilled water, and aliquots of each plated on small, concave glass planchets (approximately 1 in. diameter). Activity was determined on each of these aliquots, which had comparable dry weights, and the specific activity of each calculated (data expressed in terms of counts per second per mg. dry weight).

Intracellular Distribution Study

Rats, Sprague-Dawley males, 230-280 gms., and mice, either C_3H or 101xC_3H, 20-25 gms., were used in the course of the experiments reported

(the actual number and type of animals used in a particular experiment are given in the respective table). The labeled compound was injected intraperitoneally into the test animal, then 45 minutes later, the animal was stunned, decapitated, and bled. All subsequent operations were performed in either a 4° C. cold room or in refrigerated centrifuges. The liver or spleen, depending on the particular experiment, was immediately removed, washed once in cold, isotonic saline, and then twice in cold, 0.25 M sucrose. The organ was homogenized in 9 volumes of the isotonic sucrose solution, and cellular fractions separated following essentially the procedure of Hogeboom and co-workers (17). The erythrocytes, whole cells, and debris were first removed (10 minutes at 100 x g; all g values are for maximum diameter). This precipitate was suspended in 4 ml. of the sucrose solution and re-homogenized. The new precipitate, which was sedimented by a second centrifugation (10 minutes at 110 x g), was discarded. The nuclei were next sedimented (10 minutes at 1200 x g), then the mitochondria (10 minutes at 12000 x g), and, finally, the microsomes (60 minutes at 82000 x g). After the first sedimentation of each particulate fraction, the precipitate was suspended in 4 ml. of sucrose solution, and then re-sedimented at a slightly higher speed. The resulting precipitate was suspended in 3 ml. of sucrose solution and used in the subsequent experiments. Activity[a] and dry weight (dry weight was compar-

[a]The activity was measured using the Model 192 Ultrascaler and Model D47 Gas Flow Counter, with 'Micromil' window, sold by the Nuclear Instrument and Chemical Corporation, Chicago, Illinois. With this instrument, the background is approximately 17 counts per minute. This background was less than 10% of most of the samples counted, and was neglected in the calculations. In those instances where it was greater than 10%, however, this background was subtracted from the measured activity before calculating specific activities.

able for all fractions) were determined on 0.20-ml. aliquots of each of the fractions, which had been plated on small, concave glass planchets (approximately 1 in. diameter). Total nitrogen (12) was also determined on a suitable aliquot from each fraction, and the specific activity calculated (data expressed in terms of counts per minute per μg. of nitrogen).

Dialysis Procedures

Both total and equilibrium dialysis techniques were utilized. In the total dialysis experiments, after dialysis against a solvent was complete, a suitable aliquot of the non-dialyzable material was plated and the activity determined. This activity was compared to that of a similar amount of the material from the undialyzed sample, and the approximate "percentage of original activity" calculated. In the equilibrium dialysis experiments, the procedure of Doherty and Vaslow (18) was followed, with the exception that the conventional apparatus was replaced by a slightly modified one, which had certain desirable characteristics for these experiments. One milliliter of the material to be dialyzed was placed in a small dialysis tube and allowed to come to equilibrium with 9 ml. of dialyzing solvent contained in a larger outer jacket. In this modified apparatus it was possible to accentuate very nicely any non-dialyzable activity in the tube, so that the procedure was useful for clearly establishing whether the removal of activity present in the dialysis tube could be effected by the solvent. After equilibrium had been established in the dialysis, equal aliquots from the contents of the dialysis tube and from the surrounding liquid were plated, and the activity of each determined and compared.

Irradiation Experiment

Six 101xC$_3$H mice, 24-25 gms., were separated into three groups of two each. Each of the six was injected intraperitoneally with 0.55 mg. of D-MBG-2(S^{35})[b]. Two of the groups were subjected to irradiation[c] (approximately 940 roentgens, 175 roentgens per minute); the remaining group served as the non-irradiated controls. One of the former groups was irradiated 20 minutes after injection, the normal procedure with these protective compounds; the other was first irradiated, then injected 5 minutes after completion of the irradiation. Forty-five minutes after injection, regardless of the time of irradiation, each animal was sacrificed, the liver removed, and the cellular fractions isolated as described under "Intracellular Distribution Study".

Preparation of Liver Homogenate

Homogenates were prepared from both mouse and rat livers. The desired compound was administered intraperitoneally to the animal and, after 45 minutes, the liver was removed and homogenized in cold, isotonic sucrose. Following the second sedimentation of the material composed of erythrocytes, whole cells, and debris at 100 x g, as previously described, the final precipitate was discarded and the resulting supernatants combined for use in the experiments.

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[b]The dose listed for each compound is calculated for the salt form, in this case D-MBG-2·HBr. This dose is determined by calculation from the amount of the original isothiuronium salt actually weighed.

[c]The mice, caged in a revolving lucite container, were irradiated using a Philips X-Ray machine, at 250 kvp, 15 ma., with 1 mm. Al filter inherent, and 1 mm. Al added, filter, target to object distance of 60 cm.

CHAPTER IV

RESULTS

Tissue Distribution Study

The distributions of MEG, D-MBG-2, and L-MBG-2(S^{35}) in several tissues are shown in Table 1. The data in this table demonstrate their general distribution throughout the animal body and indicate that all three compounds are more highly concentrated in several of the tissues, including the liver, bone marrow, and spleen.

Intracellular Distribution Study

The intracellular distribution was determined for each compound in both liver and spleen. Most of the results were obtained using mouse liver, but several experiments were included using rat liver and spleen to eliminate the possibility that the observed results in the case of the mouse liver were unique for this tissue. Table 2 shows the intracellular distribution of MEG(S^{35}) in mouse liver at three dose levels, 0.55 mg., 2.85 mg., and 5.70 mg. The data in this table indicate that a selective distribution was obtained on administering MEG. The nuclei have the highest specific activity of the particulate fractions, while the microsomal fraction has the lowest. This selective distribution was maintained with increased dose, though the actual amount bound increased quite markedly. In Table 3, the intracellular distributions of D-MBG-2 and

Table 1

TISSUE DISTRIBUTION OF MEG, D-MBG-2 AND L-MBG-2(S^{35})

COMPOUND ⟶	MEG	D-MBG-2	L-MBG-2
TISSUE	DISTRIBUTION* (counts/second/mg. dry weight)		
Liver	0.73	1.0	1.0
Bone Marrow	2.6	3.8	4.0
Spleen	1.7	1.7	1.6
Intestinal Mucosa	1.2	1.1	0.88
Kidney	1.0	2.4	2.2
Blood	0.25	0.25	0.17
Brain	0.36	0.48	0.44
Skeletal Muscle†	0.23	0.34	0.34
Heart	1.0	1.4	1.3

Specific activity, 0.3 mc/mM; 0.55 mg. of each compound was injected.

*These results were obtained using one animal for each compound.

†Taken from hind legs.

Table 2

INTRACELLULAR DISTRIBUTION OF MEG(S^{35}) IN MOUSE LIVER

DOSE OF COMPOUND (mg.) ⟶	0.55	2.85	5.70
CELL FRACTION	DISTRIBUTION* (counts/minute/μg. of N)		
Nuclei	1.2	8.9	34.8
Mitochondria	1.2	7.9	28.1
Microsomes	0.41	2.9	12.5
Soluble	4.5	15.7	37.7

Specific activity, 1.2 mc/mM

*These results were obtained using one animal at each dose level.

Table 3

INTRACELLULAR DISTRIBUTION OF D-MBG-2 AND L-MBG-2(S^{35}) IN MOUSE LIVER

DOSE OF ISOMER (mg.) ⟶	(D)0.55	(L)0.55	(D)1.10	(L)1.10
CELL FRACTION	DISTRIBUTION* (counts/minute/μg. of N)			
Nuclei	0.76	0.98	1.5	1.6
Mitochondria	1.34	1.12	2.3	1.8
Microsomes	0.93	0.39	1.9	0.92
Soluble	4.0	4.0	7.5	10.6

Specific activity, 1.2 mc/mM

*The results given for the 0.55-mg. dose represent the average of results obtained using two animals for each isomer; those given for the 1.10-mg. dose represent results obtained using one animal for each isomer.

L-MBG-2(S^{35}) in mouse liver are shown for 0.55 mg. and 1.10 mg. doses. When the distribution results obtained at the lower dose level for both compounds are compared, a significant difference is found in the amount bound to the microsomal fraction, where the specific activities are found to differ by a factor of slightly more than two, comparable to their relative protective activities. It is further observed from column four of this table that the higher dose of the L-isomer was sufficient to bring the amount bound to this fraction up to the level previously obtained with the D-isomer at the lower dose.

Table 4 shows the intracellular distribution obtained in mouse liver after injection of each of the three C^{14}-labeled compounds. On comparing these results with those obtained with the S^{35}-labeled compounds (Tables 2 and 3), it appears that essentially the same relative distribution pattern was obtained for the respective compounds. It seems unlikely, then, that any metabolic cleavage of the initial compound occurred, since these two radioactive tracers are on opposite ends of the molecule.

That the observations on the intracellular distribution found with these compounds in mouse liver are not unique for this tissue is illustrated by similar results obtained in rat liver and spleen. Table 5 shows the selective distribution obtained with MEG(S^{35}) in these two tissues and Table 6, the intracellular distribution obtained with the D- and L-isomers(S^{35}) in rat spleen.

Nature of Binding to Cellular Constituents

The nature of the binding by the cellular constituents of the radioactivity (referred to from here on as "activity") resulting after

Table 4

INTRACELLULAR DISTRIBUTION OF MEG, D-MBG-2, AND L-MBG-2(C^{14}) IN MOUSE LIVER

COMPOUND AND DOSE (mg.) ⟶	MEG(2.85)	D-MBG-2(0.55)	L-MBG-2(0.55)
CELL FRACTION	DISTRIBUTION* (counts/minute/μg. of N)		
Nuclei	1.6	0.16	0.15
Mitochondria	1.5	0.31	0.30
Microsomes	0.85	0.18	0.11
Soluble	4.9	1.04	0.94

Specific activity, 0.5 mc./mM

*These results were obtained using one animal for each compound.

Table 5

INTRACELLULAR DISTRIBUTION OF MEG(S^{35}) IN RAT LIVER AND SPLEEN

ORGAN ⟶	Liver	Spleen
CELL FRACTION	DISTRIBUTION* (counts/minute/μg. of N)	
Nuclei	16.3	6.0
Mitochondria	18.8	7.6
Microsomes	6.7	8.1
Soluble	16.8	5.7

Specific activity, 0.4 mc./mM; MEG was administered at a dose of 142.5 mg. per kg.

*The liver distribution results were obtained using a portion of the liver from one animal; the spleen distribution results were obtained by combining the spleens from two animals.

Table 6

INTRACELLULAR DISTRIBUTION OF D-MBG-2 AND L-MBG-2(S^{35}) IN RAT SPLEEN

COMPOUND ⟶	D-MBG-2	L-MBG-2
CELL FRACTION	DISTRIBUTION* (counts/minute/μg. of N)	
Nuclei	0.98	0.90
Mitochondria	1.04	0.92
Microsomes	1.28	0.87
Soluble	1.34	1.20

Specific activity, 0.4 mc/mM; each isomer was administered at a dose of 27.5 mg./kg.

*The spleens from two animals given the isomer were combined before fractionation.

administering one of these compounds, was investigated both in the individual cellular fractions and in whole homogenates. The results from a series of equilibrium dialyses carried out on each of the liver cellular fractions suggested several generalizations. First, the activity present in each of the particulate fractions was not completely removable by either water, 0.5% sodium cyanide, 1% MEG (non-radioactive), or 0.5% lithium thioglycollate. The activity present in the soluble cytoplasmic fractions was much more subject to dialysis against water than was that in the particulate fractions. Finally, the activity in each of the fractions, including the soluble cytoplasmic fraction, from animals treated with MEG was more resistant to removal by water dialysis than was that in the corresponding fractions from either the D- or L-MBG-2

treated animals.

Since it was evident that the total activity present in the cell was held by more than one type of binding, the maximum activity that could be removed by dialysis against each of several solvents was then determined, as shown in Table 7, using homogenates prepared from the liver of mice treated with the particular compound. A significant portion of the total activity was removed by water dialysis, with an additional quantity being removed by 2 M guanidine hydrochloride. With the guanidine, between 60 and 85%, depending on the compound, of the original activity was dialyzable. Of the remaining more tightly bound activity, 5 to 30% (based on original activity present) could be removed by dialysis against water after treatment with either an oxidizing agent, for example performic acid (19), or a reducing agent, for example lithium thioglycollate or sodium borohydride. Between 5 and 10% of the original activity remained non-dialyzable against any of these agents.

In order that the material to which this non-dialyzable activity was bound might be characterized to some degree, the Schneider procedure (20) was used to separate further each of the cellular fractions. It was found that, in each, the acid-soluble fraction contained the most activity, the protein fraction nearly as much, while the nucleic acid fraction had by far the least. A similar finding was made when the activity was determined on Schneider fractions of the liver homogenate from a mouse given 2.85 mg. of MEG(S^{35}). Each of the three Schneider fractions from this homogenate was then dialyzed against water in an equilibrium dialysis. The activity originally present in the acid-soluble fraction was almost completely dialyzable, while that in both the nucleic acid

Table 7

DIALYSIS OF LIVER HOMOGENATES FROM MICE RECEIVING MEG, D-MBG-2, OR L-MBG-2(S^{35})

SOLVENT →	H_2O	Guanidine[a]	LTG[a]	LTG+Guanidine[a]	$NaBH_4$[b]	PFA[b]
COMPOUND ADMINISTERED	MAXIMUM NONDIALYZABLE (Percentage of Original Activity)					
MEG	50	40	15	14	12	11
D-MBG-2	35	25	15	10	7	6
L-MBG-2	25	15	14	8	7	6

Each compound was administered at a dose of 0.55 mg.

[a]Guanidine was 2 M guanidine hydrochloride; LTG was 1% lithium thioglycollate; LTG+Guanidine was a mixture containing 2 M guanidine hydrochloride and 1% lithium thioglycollate. An aliquot of the homogenate was allowed to dialyze to completion against the respective solvent, then this solvent was removed by dialysis against water.

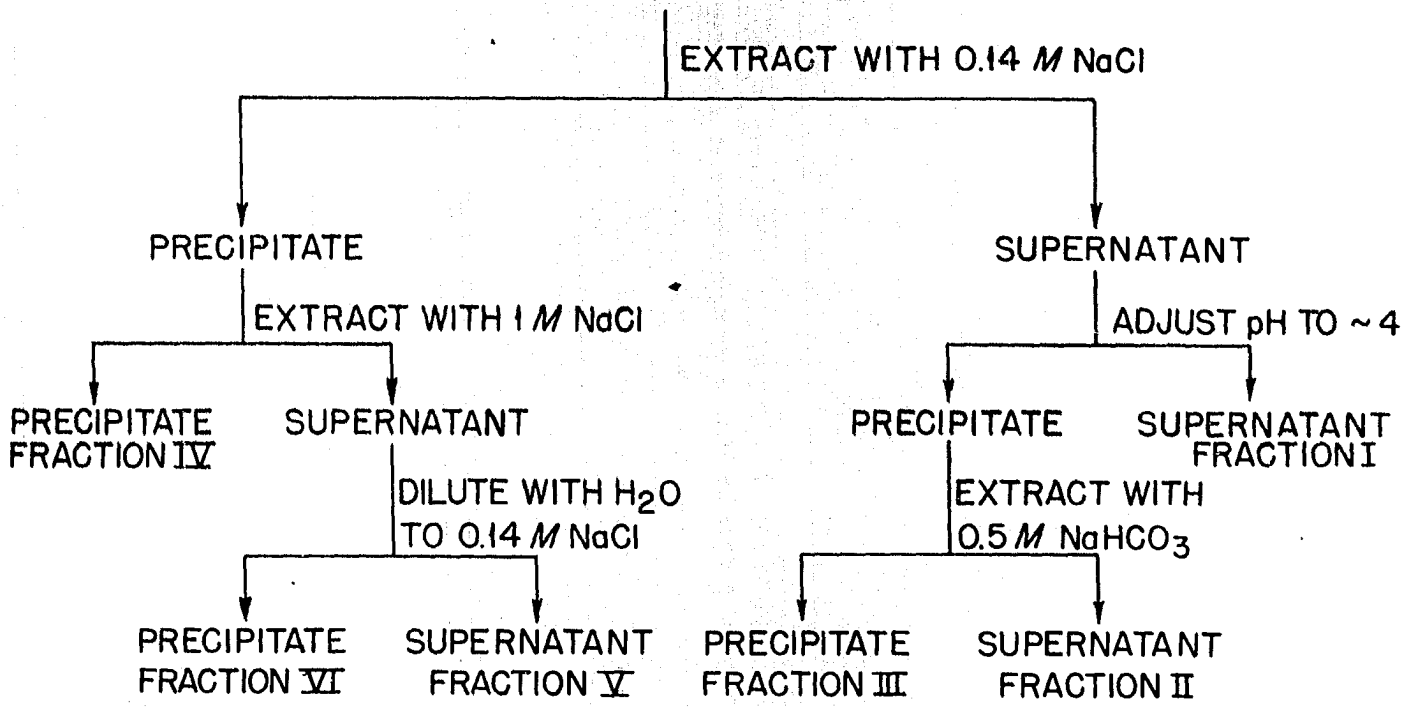
[b]$NaBH_4$ was 5% sodium borohydride solution; PFA (performic acid) was prepared by mixing 5 ml. of formic acid with 0.6 ml. of 30% hydrogen peroxide immediately before use. The trichloroacetic acid-insoluble material of the homogenate, prepared by adding 2.5 ml. of cold, 10% trichloroacetic acid to 1.0 ml. of the homogenate and separating the resulting precipitate by centrifuging, was treated with either the $NaBH_4$ or PFA. The resulting material was then dialyzed against water. The activity present in the trichloroacetic acid-soluble fraction from this separation was found to be completely dialyzable against water.

and protein fractions was only slightly affected. Subsequent dialysis against 1% lithium thioglycollate removed the activity from the nucleic acid fraction, but removed only a portion of the remaining activity from the protein fraction. Approximately the same amount of activity was removed from this latter fraction by a 2 M guanidine hydrochloride-1% lithium thioglycollate mixture. An additional amount could be removed with either 5% sodium borohydride or performic acid treatment, followed by water dialysis. A small amount of activity still remained bound against all these agents. From these results, it was evident that the non-dialyzable activity was bound to the material contained in the protein fraction, by a type of chemical bond not easily, if at all, affected by either these oxidizing agents, reducing agents, or denaturants.

A different approach was then taken in studying the distribution and binding of the activity within the liver cells. Of particular interest was the further characterization of this material to which was bound the activity so resistant to removal by treatments thus far used. The combined homogenate of the liver from two rats given MEG(S^{35}), 200 mg./kg., was separated by the procedure shown schematically in Figure 4, which is based on the method of Mirsky and Pollister (21) for separating RNA and DNA from the other cell constituents. Using this procedure, in which advantage is taken of the different solubility characteristics of the cell constituents, six fractions, including ones relatively rich in RNA (Fraction II) and in DNA (Fraction VI), can be obtained. All six fractions contained a significant amount of activity, though a majority of the resulting total activity was found in Fraction I. Each of these six fractions was dialyzed against water, which removed two-thirds of the

Figure 4

SEPARATION SCHEME FOR LIVER HOMOGENATE



activity originally present in Fraction I, but only a small amount of the activity initially contained in the other fractions. Approximately half the activity originally present in the homogenate was thus water-dialyzable, most of which was in Fraction I. Subjecting each of the water-dialyzed fractions then to an equilibrium dialysis against three different solvents, 2 M guanidine hydrochloride, 1% lithium thioglycollate, and a mixture of 2 M guanidine hydrochloride-1% lithium thioglycollate, resulted in complete equilibrium of activity in three of the fractions with at least one of the solvents. Only the guanidine-thioglycollate mixture brought the activity in Fractions I and V to equilibrium. Guanidine alone brought the activity in Fraction II to equilibrium, and also had a pronounced effect on the activity in Fractions III, IV, and, particularly, VI. It was found that lithium thioglycollate alone had a much less pronounced effect than did the guanidine alone on these latter three fractions, while the mixture of the two had an effect very similar to that of guanidine alone. None of these solvents, however, was able to bring the activity in Fractions III, IV, or VI to equilibrium.

All fractions were found to contain significant amounts of protein (22). Fraction I had twice the protein present in either Fractions III, IV, V, or VI; Fraction II had considerably less. Fractions III and IV had the highest specific activity (counts per minute per mg. of protein). Fractions I, V, and VI each had about two-thirds, while Fraction II had only about one-half, this specific activity.

Each of these six fractions was separated by the Schneider procedure and the relative activity, shown in Table 8, of each of the resulting Schneider fractions was determined. The dialysis experiments

Table 8

RELATIVE DISTRIBUTION OF ACTIVITY
AFTER SCHNEIDER PROCEDURE

SCHNEIDER FRACTION ⟶	Acid-Soluble	Nucleic Acid	Protein
FRACTION	RELATIVE ACTIVITY*		
I	+++	-	+
II	-	++	-
III	±	±	+
IV	±	±	+
V	-	-	+
VI	±	-	++

*- represents nil amount; ± represents a trace amount; +, ++, and +++ represent proportionally larger amounts of activity.

reported above revealed that the Schneider protein fractions of Fractions III, IV, and VI contained the non-dialyzable activity. With this fact in mind, the data in Table 8 indicates that this non-dialyzable activity of the homogenate is almost equally distributed among protein-type material of quite different character, since the basis for this separation was the different solubility characteristics of the cell constituents.

In Vitro Binding Experiment

Liver homogenate from an untreated mouse was incubated with MEG(S^{35}) in pH 7 buffer at 40° C. for 16 hours. Dialysis of the homogenate against water resulted in the removal of less than half of the original activity. Dialysis against 1% lithium thioglycollate removed the majority, though not all, of the original activity. Thus, binding of at

least a superficially similar character was obtained both *in vitro* and *in vivo*.

Effect of Radiation on the Intracellular Distribution and Binding of D-MBG-2

Table 9 summarizes the results of an experiment to determine whether radiation has any immediately observable effects on the intracellular distribution pattern of a protective compound. When D-MBG-2(S35) was injected before irradiating the mice, the intracellular distribution was very similar to that obtained in the controls, who received D-MBG-2 but no radiation, though, by comparison, a reduced amount of bound activity was found in each of the four fractions from the irradiated mice. When, on the other hand, D-MBG-2 was injected five minutes after a similar dose of radiation, quite a different distribution was obtained. On comparing corresponding cellular fractions from this latter group of animals to those of the control group, the amount of activity bound in the mitochondrial fraction from the pre-irradiated animals was found to be increased considerably; that bound in the microsomal fraction may, also, have been slightly increased. A rapid alteration of the ability of these components to bind this compound after irradiation, as reflected in the specific activity of the respective fractions, was clearly indicated.

The binding of the activity to the cellular fractions from both these groups of irradiated mice was compared, by equilibrium dialysis, to that of the fractions from the non-irradiated controls. Corresponding fractions of all three groups behaved similarly in both water and 5% lithium thioglycollate dialysis, with the exception that the increased amount of activity bound to the mitochondrial fraction obtained from

Table 9

EFFECT OF RADIATION ON INTRACELLULAR DISTRIBUTION OF D-MBG-2(S^{35}) IN MOUSE LIVER

CELL FRACTION	DISTRIBUTION* (counts/minute/μg. of N)		
	Compound Injected Only	Compound Injected; Then Irradiated	Irradiated; Then Compound Injected
Nuclei	0.33	0.29	0.32
Mitochondria	0.65	0.40	1.13
Microsomes	0.48	0.34	0.53
Soluble	1.30	0.90	0.90

Specific activity, 0.4 mc/mM

*These values represent the average of results obtained with two mice each. The particulate fractions were re-suspended in water, rather than 0.25 M sucrose.

those mice which were irradiated before receiving the compound was found to be more tightly bound toward both solvents than was that of either of the other mitochondrial fractions. A significant portion of this increased amount of activity bound to this fraction proved to be rather tightly bound.

Comparison of Intracellular Distributions of MEG and 2-AT

The data in Table 10 illustrate the intracellular distributions of MEG and 2-AT(S^{35}) in mouse liver, when given in equimolar amount and at equal specific activity. Since it has been previously mentioned that 2-AT has never given 100% survival to irradiated mice, this table actually compares the intracellular distribution of a highly protective compound

Table 10

INTRACELLULAR DISTRIBUTION OF MEG AND 2-AT(S^{35}) IN MOUSE LIVER

COMPOUND ⟶	MEG	2-AT
CELL FRACTION	DISTRIBUTION* (counts/minute/μg. of N)	
Nuclei	8.1	3.2
Mitochondria	6.7	2.5
Microsomes	3.6	2.1
Soluble	19.4	16.6

Specific activity, 0.7 mc./mM; dose of MEG was 3.14 mg.(15.7 μM), dose of 2-AT was 1.6 mg.(15.7 μM).

*These values represent the average of results obtained with two mice each.

with an essentially non-protective one. The lack of selectivity in the distribution of 2-AT, when compared to that obtained with MEG, as well as the relatively small amount of activity bound to the particulate fractions, are clearly evident from these results.

The binding of this activity in the cellular fractions from the MEG treated mice was found to be much tighter, in equilibrium dialysis, than was that from the 2-AT treated mice. Neither dialysis against water nor against 1% lithium thioglycollate was found to be effective in removing completely the activity from the particulate fractions of either group of animals.

Binding of MEG in Blood

Eldjarn (23) has reported that the activity bound to serum

proteins after the administration of S^{35}-labeled cysteamine (mercapto-ethylamine) or cystamine (aminoethyldisulfide) could be removed completely by dialysis against several reducing agents. Since, with the tissue studied here, the activity bound after treatment with either of the three mercaptoalkylguanidine salts used could not be removed completely by any of these agents, or by other agents active toward the disulfide bond, it seemed desirable to determine whether MEG, as representative of these three, was bound in the blood in a similar way to cysteamine or cystamine. The erythrocytes and plasma were obtained by centrifuging the blood from a rat given MEG(S^{35}) by intraperitoneal injection. The activity bound to the erythrocytes was found, by equilibrium dialysis, to be completely removable by water, while that in the plasma required further treatment with 1% lithium thioglycollate to effect complete removal. These results for MEG are, therefore, similar to those previously obtained with mercaptoethylamine. They indicate, however, that the binding of these compounds in the blood, or serum, unfortunately cannot be taken as completely representative of their binding in the tissues.

CHAPTER V

DISCUSSION

The three structurally similar mercaptoalkylguanidine salts used in this investigation are each known to be active in protecting mice against radiation death, based on 100% 30-day survival of mice receiving an otherwise lethal dose of x-irradiation (900 roentgens); but the dose necessary, 2.4 μM (0.55 mg.) of D-MBG-2, 4.9 μM (1.10 mg.) of L-MBG-2, and 14 μM (2.85 mg.) of MEG, is different. Thus, these chemically analogous agents have significantly different activities in the biological system. At least two plausible explanations can be presented for experimental evaluation to account for this difference in biological activity. One explanation postulates that the observed difference in protective activity is due to a corresponding difference in the tissue distribution of the respective compounds, while the other explanation postulates that the observed protective activity difference is due to a corresponding difference in the intracellular distribution of the compounds. Both of these explanations assume that the actual protective activity observed *in vivo* results from the known high biological specificity for structure, since the actual chemical protective capacity, *in vitro* and *in vivo*, should be essentially the same for such closely related compounds. The former explanation, that the observed protective activity difference is

due to the tissue distributions of the respective compounds, predicts that certain sensitive tissues can concentrate the more active compounds preferentially. The results shown in Table 1, however, clearly eliminate this possibility from further consideration as a plausible explanation. The distribution of each compound, when given in equal amounts, in corresponding tissues is quite similar, qualitatively and quantitatively. The data in this table indicate, also, that all three compounds are well distributed throughout the tissues of the animal body. Each is concentrated in both those tissues generally thought to be more vital metabolically and those tissues considered particularly radiation sensitive. The compounds' distribution patterns, though they do not account for the differences mentioned, undoubtedly are a significant factor in their ability to protect the animal against radiation death.

The latter explanation, that the observed protective activity difference is due to a different intracellular distribution for each of the compounds, predicts a selective affinity among the cellular fractions for the compounds, an affinity which is relatively different for the three. This explanation further implies that the intracellular distribution of the compound is in some way involved in the protection it affords. In Tables 2 through 6 are tabulated the results showing the selective intracellular distribution obtained with these compounds. In addition to showing a selective intracellular distribution, these results indicate a direct relationship between intracellular distribution and protective activity. This significant correlation was made in comparing the intracellular distribution obtained with 2.4 μM of D-MBG-2 to that obtained with the same dose of L-MBG-2. Thus, two enantiomorphs, which have a

comparable toxicity and tissue distribution, were found to have a significant difference in their intracellular distribution patterns in both the mouse liver and rat spleen, a difference in direct relationship to their respective protective activities. The actual difference was found in the relative amount of compound bound to the microsomal fraction of the two organs studied, the larger amount bound (approximately one and one-half to two and one-half times) being obtained with the more active D-isomer. Additional support is given this point of the argument by the finding that increasing the dose of the L-MBG-2 to its effective level, 4.9 μM, correspondingly increased to the amount previously obtained with 2.4 μM of the D-isomer the amount of L-isomer bound to the microsomal fraction. It is, therefore, evident that this latter explanation more adequately accounts for the results obtained experimentally.

When the intracellular distribution results obtained with the effective dose of D-MBG-2 are compared quantitatively with those obtained with the effective dose of MEG, it is noted that several times more MEG than D-MBG-2 is bound to each cellular component (an amount reflected in the dose necessary for protection). A logical explanation for this difference is that the MEG, being less specific structurally, is bound less selectively by the cellular components than is the more biologically specific structure D-MBG-2. Thus, assuming certain components or sites within the cell are more radiation sensitive than others, the D-MBG-2 must be of such a character structurally that it is preferentially bound by these sites, and less is, therefore, required for protecting the animal against radiation death. The assumption that these sensitive sites actually exist within the cell is consistent with the capacity of the D- and

L-isomers to protect at a fraction of the dose of MEG, though all three have essentially the same tissue distribution, both qualitatively and quantitatively.

The extent to which the microsomal fraction is implicated in radiation damage by the experimental findings already discussed is not altogether clear, but at least two possibilities are consistent with these results. The most direct possibility is that the microsomal fraction is the cell fraction most affected by radiation; hence, failure to protect it, though adequate protection is provided the other cell components, results in a radiation death similar to that of unprotected animals. An equally good possibility, as far as these results are concerned, is that cell components in addition to the microsomal fraction are normally also severely damaged by radiation. Thus, when one of the cell components, in this case the microsomal fraction, is not protected adequately, the normal radiation death occurs. Only direct experimentation can define clearly any role of the microsomal fraction in radiation death, of course, but the possible importance of this fraction in this regard is suggested by these results.

The investigation of the nature of the binding of these compounds to the cellular components indicates that both covalent and non-covalent bindings are involved. Some of the compound, represented by that which was dialyzable against either water or guanidine, is held relatively loosely, so that it can be easily displaced. The water presumably removed only that portion of the compound which was relatively accessible to this agent and, also, loosely bound, either by secondary valence forces between the cellular material and the compound, or by the

attraction of oppositely-charged groups. The guanidine, which removed some activity not subject to water dialysis, presumably could act as a displacing agent, to remove that portion of the compound which was held by stronger ionic attractions, or as a denaturant, to modify the spatial geometry of the cellular material such that secondary forces would no longer be correctly oriented to bind the compound. From the known properties of these two dialyzing solvents, however, no covalent bonds should be affected, so that the total amount of compound removed by either should represent only that which was held by the non-covalent bonds described.

Of the remainder of the compound, which is evidently bound to the cellular material covalently, all but a small amount was removed by any one of several reducing or oxidizing agents. It seems reasonable to assume that this portion of the compound is bound to the cellular material through a disulfide type of bond, since these agents are extensively used under similar conditions to remove material held together by such a bond. The relatively small amount of each compound which was still bound to the cellular material appears to be held by some bond not subject to the agents used. Since the performic acid oxidation procedure used has been found by previous workers to be sufficiently drastic to attack even very resistant disulfide bonds, it seems doubtful that this represents additional compound bound by such a bond. Guanidine dialysis of a product previously oxidized with performic acid also failed to remove this last amount of compound. As a result of these findings, it seems most likely that this unremoved compound, which is fairly well distributed among the protein-like material of the cell, is bound by a covalent bond through some portion of the molecule other than the mercaptan group. It

is of interest to note that each of the particulate fractions of the cell, as far as could be determined, has some compound held to it by each type of binding.

A comparison of the effects of radiation on liver cells from an animal subjected to radiation prior to being treated with protective compound, as compared with that of these cells from animals subjected to radiation subsequent to being treated with protective compound, leads to some interesting observations. The modification of the capacity of the cellular components to bind the protective compound, particularly the mitochondrial fraction from the unprotected animals receiving radiation, suggests that some change in their nature has resulted from the radiation treatment. Since this increased binding capacity was evidenced so soon following irradiation, it seems reasonably certain that this modification is a more immediate effect of radiation. Whether this effect reflects a change sufficient in its later stages to account for the death of the animal, or whether there are more important latent changes, not manifest at this early time, which eventually result in the death of the animal, are possibilities whose final answer remain for further investigation. It is clear, however, that radiation does cause an immediate alteration in the cell, either in certain processes that are ultimately evidenced by binding of the compound, or in the number and character of the sites within the cell available to the compound. These alterations, since they are not observed if the cell has been treated with the protective compound previous to irradiation, are evidently ones against which the protective compound is effective. In this regard, it is interesting to note the results of van Bekkum (24), who observed that the oxidative phosphor-

ylation of spleen mitochondria from rats receiving 1100 roentgens was markedly depressed. He was not able to observe a similar effect, even with higher doses of radiation, in liver mitochondria, but the possibilities here were not exhausted, since he performed his experiments at only one post-irradiation interval, 4 hours.

In arriving at an hypothesis to explain the mechanism of action of the mercaptoalkylguanidines within the cell, a number of factors, including both the chemical and biological aspects of protection, must be taken into due account. Only a few of the many hypotheses that can be advanced to explain various aspects of this problem stand the test of all the known evidence. One hypothesis that appears, on this basis, to be most acceptable proposes that the biological protective action of these compounds may be explained as due to their preferential localization within the cell in such a manner as to counteract the radiation-produced free radicals, thus sparing the critical biological material from much of the damaging effects of these radicals. Taking into account the theory already advanced that these compounds may exert their chemical protection through a radical quenching capacity, it follows that, to be effective, the protective compound must be localized with respect to the biological material it will protect. This localization seems imperative since it is known that the distance traveled by the radiation-produced free radicals before reacting can be expected to be very short, becoming shorter as the reactivity becomes greater. On the basis of this argument, it seems justified to assume that only that amount of protective compound which is on or very near any radiation sensitive sites of the biological material can be significantly effective in destroying the damaging free

radicals. By this explanation, the observed close relationship between the intracellular distribution of these compounds and their protective activity may be readily understood. In addition to being at the proper sites within the cell, it is also necessary that the compound be bound in such a way that it can function as a radical quenching agent. One type of binding possible with these compounds that would meet this criteria is that in which the compound is held by attractive forces only, having no covalent bonds between it and the biological material. Eldjarn (23) has previously argued, in the case of cysteamine and cystamine, that this "free" compound, as he terms it, is too randomly distributed to be effective as a radical quenching agent. It should be pointed out that the distribution data obtained with the compounds studied here indicate that the loosely-held material is not randomly distributed. In fact, at least part of this loosely-held compound may be complexed to certain sites on the biological material, much as is thought to be obtained with an enzyme-substrate complex. Thus, though the compound is loosely held to certain sites, it would still be selectively distributed. Evidence that this type of binding of protective compound to the biological material may indeed provide protection to the biological material has been obtained in several *in vitro* experiments, including those of Doherty (25), who observed protection of chymotrypsin from radiation damage after complexing it with a known chymotrypsin inhibitor before irradiation, of Sutton (26), who observed protection of catalase from radiation damage after complexing it with cyanide, a known inhibitor, before irradiation, and of Okada (27), who obtained protection of the desoxyribonuclease system by complexing it with desoxyribonucleic acid. The results obtained in the dialysis

experiments reported here are consistent with those which would be expected if much of each of these mercaptoalkylguanidine salts existed in such a complex with the biological material, since a major portion of each compound was dialyzable from the cellular constituents by either water or guanidine.

Another type of binding possible with these mercaptans, which has been proposed by Eldjarn (23) as being responsible for the effectiveness of cysteamine and cystamine, is that obtained by disulfide bonding of the compound through thiol groups of the biological material. This proposition is based on his observation that the time when the maximum amount of compound was observed to be bound to the proteins of the blood through disulfide bonds coincided with that period when maximum protection was obtained. It should be noted that this period is also one during which the maximum concentration of an intraperitoneally administered compound would be expected both in the blood and the tissues, so that a clear correlation between disulfide-bound compound and protective activity can not be made independent of this important consideration. With the mercaptoalkylguanidine salts used here, a relatively small percentage of compound bound in this manner was found by the dialysis experiments. For this reason, it seems questionable that this could be the major binding involved in protection by these compounds, particularly in view of the results obtained with the D- and L-isomers, with which both the amount of compound present and the percentage bound in this way are relatively small. Though it is impossible at the present time to evaluate absolutely the relative contribution of each of these two types of binding to the protection obtained, it appears, on the basis of relative amounts of each

present, that the compound bound more loosely should be considered to be much more important in protective capacity. Since, however, there is no good basis at present to rule out the contribution of either type of binding to the ultimate protection afforded by the compound, it seems more satisfactory to conclude that both types may, in fact, contribute to the protection, with the greater contribution to be expected from the loosely bound compound.

CHAPTER VI

SUMMARY

Both the tissue distribution and intracellular distribution of mercaptoethylguanidine hydrobromide (MEG), D-mercaptobutyl-2-guanidine hydrobromide (D-MBG-2), and L-mercaptobutyl-2-guanidine hydrobromide (L-MBG-2) were studied in an attempt to determine how these radiation protective compounds may function *in vivo*. The tissue distribution of all three compounds, which differ by as much as five to one in their protective activity in mice, was similar, both qualitatively and quantitatively. Their intracellular distribution in mouse liver, rat liver, and rat spleen was selective and, further, a relationship between protective activity and intracellular distribution was observed. The implication that the microsomal fraction might be involved in radiation death, as suggested by these results, is discussed.

An investigation of the nature of the binding of each of these compounds to the cellular constituents was made, using dialysis techniques in several solvents, including water, denaturants, and oxidizing or reducing agents previously shown to be effective in breaking the disulfide bond. The results indicated that three general types of binding were involved — one relatively loose, one rather tight, and the other intermediate. An hypothesis, based on the selective intracellular distribution,

the relationship between intracellular distribution and protective activity, and the nature of the intracellular binding reported here, as well as the available animal testing data, has been proposed to explain how these compounds may provide protection in the animal body. This hypothesis states that the protective agent is localized within the cell in such a way that it covers preferentially radiation sensitive sites, being held for the most part in a relatively loose binding, similar to that of an enzyme-substrate complex, so that it may effectively quench radiation-produced free radicals formed in the medium.

The effect of radiation on the subsequent ability of the cellular constituents to bind D-MBG-2 was described and discussed. The ability of the mitochondrial fraction to bind this compound was found to be markedly increased if the animal was irradiated prior to administering it. In comparison, it was observed that the binding of this compound, if administered before irradiation, was quite similar to that in non-irradiated controls.

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