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THE DISTRIBUTION AND METABOLISM OF THE RADIATION PROTECTIVE AGENT
MAY 78 G KOLLMANN, B SHAPIRO, S LEON DADA17-67-C-7165

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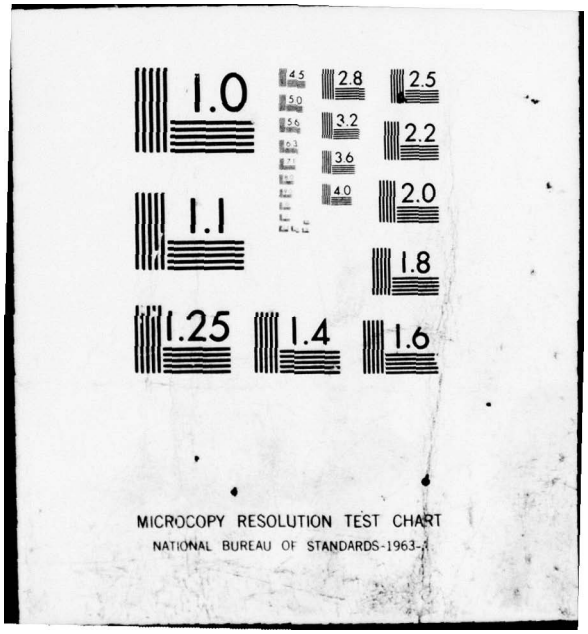
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(6) THE DISTRIBUTION AND METABOLISM OF THE RADIATION PROTECTIVE AGENT AMINOPROPYLAMINOETHYL-PHOSPHOROTHIOATE (WR-2721) IN MICE,

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Aminopropylaminoethyl-phosphorothioate (WR-2721) is the least toxic and most effectuve of the phosphorothioate class of radiation protective agents. In order to investigate the mechanism of action of this agent, distribution and metabolism studies were carried out using 35S-labelled WR-2721. In mice given WR-2721 intraperitoneally, tissue analysis showed only the unchanged compound and the protective agent bound to proteins by mixed disulfide bond. Concentration of the latter form correlated best with the time course of protection.

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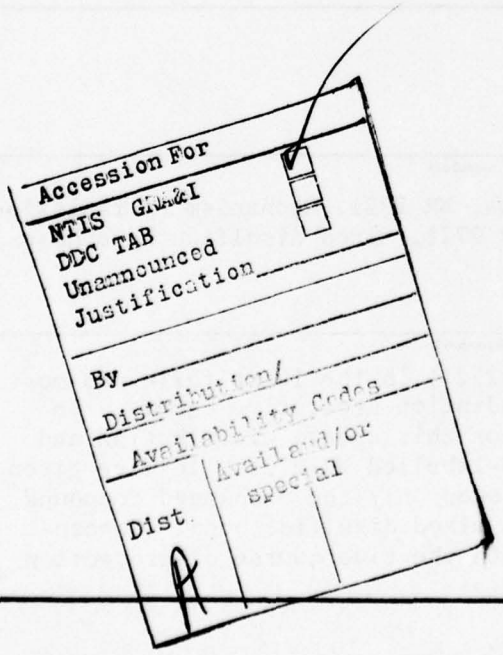
Block 20. Abstract: (continued)

Dephosphorylation to yield the thiol form of WR-2721, necessary for mixed disulfide formation on tissue proteins, was best demonstrated in the kidneys, both in vivo and in vitro, but also occurs in other tissues. Preliminary studies with the disulfide and thiol forms of WR-2721, which are less protective and more toxic than the phosphorothioate, suggest that the presence of the phosphate is necessary to mask the sulfhydryl group during transport to the target site. Formation of a mixed disulfide with tissue protein sulfhydryl groups at the target site appears to yield the final necessary condition for radiation protection.



SUMMARY

The distribution and metabolism of the radiation protective agent amino-propylaminoethyl-phosphorothioate (WR 2721) were studied in homogenates of mouse tissues to which WR 2721 was added *in vitro* and in homogenates of tissues from mice injected intraperitoneally with WR 2721 20 minutes or 60 minutes prior to sacrifice. The kidneys, both *in vitro* and *in vivo*, are the most active organs in the dephosphorylation of WR 2721. The tissues show only WR 2721 itself and protein bound agent (by mixed disulfide formation). Radiation protection which decreases at 60 minutes, as compared with 20 minutes, correlates best with the concentration of tissue protein-bound agent, which decreases at 60 minutes. Preliminary studies with the disulfide and thiol forms of WR 2721, which are less protective and more toxic than WR 2721, suggest that the presence of the phosphate is necessary to mask the sulfhydryl group during transport to the target site. However, at the target site, formation of a mixed disulfide with tissue protein sulfhydryl groups is necessary for protection.

INTRODUCTION

Distribution and metabolism studies of a number of radiation protective agents of the sulfhydryl-disulfide and phosphorothioate classes in mice *in vivo* have all revealed the formation of a mixed disulfide between the protective agent and tissue protein sulfhydryl groups (1-4). Moreover, this "protein-bound" protective agent has been the metabolic form which appeared to correlate best with protection. Indeed, mixed disulfide protein-bound agent may be the major and perhaps the only protective form of these agents in mice *in vivo*.

Evidence that mixed disulfide protein-bound protective agent is the protective form is still circumstantial, and it would be important to further test the hypothesis. For this reason, the distribution and metabolism of aminopropylaminoethyl-phosphorothioate (WR 2721) was studied. This agent appears to be the least toxic and most effective of the phosphorothioate class. The compound was labeled with ^{35}S and analyses were carried out on homogenates of mouse tissues to which WR 2721 was added *in vitro* and on homogenates of tissues from mice injected intraperiteoneally with WR 2721 20 minutes or 60 minutes prior to sacrifice. A small radiation mortality study was run concurrently with the distribution and metabolism studies in order to be sure that the analyzed mice were really being studied under conditions of protection. Limited studies were also carried out with the thiol and disulfide forms of WR 2721.

MATERIALS AND METHODS

Preparation of ^{35}S-WR 2721 and its thiol and disulfide forms

Thiophosphoryl-chloride-^{35}S was used for the preparation of trisodium phosphorothioate-^{35}S and this reagent (specific activity 15 mCi/mM) was then

reacted with N-(2-bromoethyl)-1,3-propanediamine-dihydrobromide to prepare WR 2721, following the general procedure established by Åkerfeldt (5).

The thiol form of the protective agent was prepared by hydrolyzing the phosphorothioate in 2.5 N hydrochloric acid with nitrogen continuously bubbled through the solution to prevent oxidation of the thiol to the disulfide. The presence of the thiol was spot tested with sodium nitroprusside and 2,2'-dithiobis-(5-nitropyridine). The disulfide was prepared by overnight bubbling of oxygen through a 2×10^{-3} M solution of the thiol. Oxidation was assumed to be complete when there was a negative test with sodium nitroprusside and with 2,2'-dithiobis-(5-nitropyridine).

Mortality Studies

The mice were 8-10 week old C57 BL/6J males weighing 20-25 g. The animals were injected intraperitoneally with a volume of non-labeled WR 2721 solution in phosphate buffer pH 7.5 (less than 0.5 ml) to give a dose of 400 mg/kg body weight and placed in 200 ml beakers topped with wire screening. Control mice received equal volumes of buffer intraperitoneally. At 20 or 60 minutes after injection, the animals were irradiated by means of a "Gammator 50" (Radiation Machinery Corporation) 400 Ci ^{137}Cs source utilizing a rotating platform. The exposure rate was 117 R/min. Radiation times were from 3 to 16 minutes. After irradiation, the animals were housed in 8"x12"x6" plastic cages with wire cage tops. The cages contained natural pine chips, and the mice received Purina laboratory chow and tap water, ad lib. The condition of the mice was checked daily and all dead mice were autopsied. Two small groups of mice were given the sulfhydryl and disulfide forms of WR 2721, 200 mg/kg intraperitoneally and irradiated 20 minutes later.

In Vitro Studies

Tissues and plasma were removed from the mice immediately after sacrifice. The tissues were homogenized in 1 to 2 ml of ice-cold distilled water. ^{35}S-WR 2721 was added to give a concentration of 0.4 mg protective agent per ml homogenate. The homogenates were either kept at 0°C or at 37°C for fifteen minutes and then chromatographed.

In Vivo Studies

The mice were 8-10 week old C57 BL/6J males weighing 20-25 g. The animals were injected intraperitoneally with ^{35}S-WR 2721 in a volume required to give a dose of 400 mg/kg body weight. Several mice were given the thiol form of WR 2721. All mice were then placed in a 200 ml beaker topped with wire screening. At 20 minutes or 60 minutes after injection, urine was expressed from the bladder by local pressure, and blood was removed from the retro-orbital venous sinus. The mice were then sacrificed by cervical dislocation in a refrigerated room. The abdominal cavity was opened and liver, spleen, intestines and kidneys removed and placed in tared containers. The urinary bladder was excised and included in the analysis of the excreta to be sure all urine was collected.

The organs were weighed, minced and homogenized in a tapered ground glass tissue grinder with 1 to 20 ml of ice-cold distilled water. Forty microliters of each homogenate were removed for paper chromatography. The heparinized blood was centrifuged and aliquots of plasma chromatographed. The bladder, feces and urine were combined and homogenized with the distilled water used to rinse the beaker in which the mouse was held before sacrifice. Aliquots of this were also chromatographed.

Measurement of Total ^{35}S Activity in the Tissues

The homogenates were diluted as needed to give a conveniently measurable amount of radioactivity. Three milliliter aliquots of the homogenate were mixed with 7 ml of Insta-gel (Packard). The mixture was heated to 40°-42°C in a water bath for a few minutes, shaken vigorously until the gel became translucent and cooled. The samples were counted in a Packard Tri-Carb Scintillation Counter for a sufficient period of time to accumulate at least 1000 counts. All results were corrected for quenching. The quenching correction curves were obtained by dissolving the pure radioactive protective agent in water containing increasing amounts of each tissue homogenate (recorded in terms of mg dry weight). The pure compound in Insta-gel without homogenate served as the 100% standard. Aliquots of all homogenates were dried in an oven at 100°C for 4 hours, then placed in a desiccator overnight prior to dry weight measurements.

Chromatography

Two descending chromatographic systems were employed with Whatman No. 1 filter paper strips. System A solvent consisted of ethanol:isopropanol: 1N HCl (60:60:40) and the strips were run at 5°C for 40 hours. System B solvent consisted of ammonia (29%) : ethanol:water (10:60:30), and the strips were run as for system A. All paper strips were dried and scanned for radioactive areas with an automatic strip scanner and a GM flow counter. The radioactive areas on the paper strips were recorded on chart paper as peaks. Planimetry of the areas under the peaks provided a measurement of the relative amount of S-35 in each peak. These areas were recorded as percentages of the total radioactivity on the strip.

Calculations

From the total counts injected into each animal and the total counts found in the tissues, plasma and excreta, the percent of administered protective agent sulfur (S-WR-2721) in each was determined. Percent in the carcass was obtained by subtracting percents in tissues, plasma and excreta from 100% (injected). Multiplying the percents in the tissues, plasma and excreta by the known amount of protective agent sulfur injected gave the amount of S-WR-2721 in each. These values were divided by the respective tissue dry weights to determine the concentration of S-WR-2721 in each tissue. By multiplying the percent of total activity in each metabolic product, separated on chromatography of each tissue, by the concentration of S-WR-2721 in that tissue, the concentration of each metabolic product in micrograms S-WR-2721 per mg dry weight of tissue was obtained. Averages of the data for the mice in each group were determined.

RESULTS

Mortality Studies

Figure 1 shows the survival curves of mice irradiated at 20 minutes or 60 minutes after an intraperitoneal injection of 400 mg/kg WR 2721. $LD_{50/30}$ for control animals was 800 R, for 20 minute animals 1400 R and for 60 minute animals 1060, giving a DRF of 1.75 for 20 minute animals and a DRF of 1.32 for 60 minute animals. Figures 2 and 3 show the survival patterns for mice receiving 200 mg/kg of the disulfide and thiol forms of WR 2721, respectively. Mice receiving the disulfide showed no protection, whereas those receiving the thiol showed some protection.

In Vitro Homogenate Study

Chromatograms of tissue homogenates showed only the original WR 2721 in most tissues. In the kidney homogenates at 0°C and 37°C and in the small intestines homogenates at 37°C, a second peak on the starting line (protein-bound dephosphorylated protective agent) was also seen. Table 1 shows the

in vitro metabolism of WR 2721 compared with aminopentyl-aminoethyl-phosphorothioate (WR 2823). The kidneys are the major organs of dephosphorylation for both WR 2721 and WR 2823. Electrophoretograms of plasma to which the thiol or disulfide forms of WR 2721 were added showed all activity bound to the plasma proteins. WR 2721 does not alter its structure or bind to plasma protein in vitro. The ratio of binding to albumin compared to binding to globulin was 0.57 for the thiol and 1.15 for the disulfide.

In Vivo Distribution

Table II shows the distribution in four tissues, plasma and excreta of ^{35}S-WR 2721 in per cent ^{35}S injected and in microgram S^{2721} per mg dry weight at 20 or 60 minutes after 400 mg/kg WR 2721 injected intraperitoneally. Excretion at 60 minutes is about twice that observed at 20 minutes and all tissue concentrations are significantly lowered at 60 minutes.

Metabolism

Chromatograms of tissue homogenates of mice given WR 2721 showed only two compounds: WR 2721 and protein-bound agent. In the excreta, a compound at R_f 0.15 was found, identified as the disulfide. The protein-bound agent appeared to be a mixed disulfide with protein sulfhydryl groups since, after treatment with cysteine, it ran as the thiol.

Chromatography of tissue homogenates from mice given thiol or disulfide form of WR 2721 showed protein-bound agent as with WR 2721 but also had a disulfide peak and a third peak at R_f 0.30 of unknown identity, possibly a mixed disulfide.

Table III shows the metabolism of WR 2721 at 20 or 60 minutes in per cent of tissue S^{2721} in each chemical form. In all tissues, there is a considerable per cent of protective agent protein-bound at 20 minutes, decreasing in the

liver, kidneys and small intestines at 60 minutes. The per cent original WR 2721 increases in these tissues at 60 minutes. Table IV shows the metabolism of WR 2721 at 20 or 60 minutes in micrograms S^{2721} per mg dry weight. Concentration of WR 2721 does not change significantly in liver, kidneys and small intestines but concentration of protein-bound agent had dropped markedly in these tissues. Spleen shows decrease in both forms whereas plasma shows a large decrease in WR 2721 and no change in protein-bound agent.

DISCUSSION

The decreased protection at 60 minutes as compared with 20 minutes after injection of WR 2721 appears to correlate best with decreased concentration of protein-bound protective agent in the tissues. The plasma protein-bound agent was unchanged but plasma protein is probably the carrier of dephosphorylated agent to and from the tissues. The plasma WR 2721 has dropped markedly at 60 minutes as might be expected from distribution to the tissues, metabolism and excretion.

The kidneys show the greatest metabolism of WR 2721 *in vivo* as well as *in vitro*. Initially it was thought that all dephosphorylation *in vivo* took place in the kidneys. However, in a nephrectomized mouse, dephosphorylation did take place. Therefore, although dephosphorylation was not detected in tissue homogenates (except kidneys), the tissues *in vivo* can remove the phosphate group.

The experiments with thiol and disulfide support the hypothesis that phosphorothioates are less toxic because they are transported with the sulfur marked. The thiol and disulfide were extremely toxic and had to be given in such low dose that little or no protection could be achieved. Their interaction

with plasma proteins in vitro was of interest and may indicate a slightly different transport in vivo. The thiol bound preferentially to gamma-globulin, which has no sulfhydryl but has disulfide groups, whereas the disulfide bound preferentially to albumin which has free sulfhydryl groups. As one might expect, the disulfide as well as mixed disulfide with tissue proteins appeared in all the tissues. Another product, possibly a mixed disulfide with a small molecule, was also seen.

Protection by mixed disulfide formation with tissue protein sulfhydryls has been postulated previously. That such a bond is protective has been shown in enzymes (6,7) and erythrocytes (8). The mechanism is not known but may simply be the lesser affinity of disulfides, as compared with sulfhydryls, for radiation-induced radicals. The results presented here further support the hypothesis that protection by the phosphorothioate protective agents is mediated by the formation of a mixed disulfide with tissue protein sulfhydryl groups.

Legends to the Figures

Fig. 1. Survival curves of mice irradiated at 20 minutes or 60 minutes after an intraperitoneal injection of 400 mg/kg WR 2721. Each point represents 10 mice.

Fig. 2 Survival curves of mice irradiated 20 minutes after intraperitoneal injection of 200 mg/kg WR 2721-S-S (the disulfide form). Each curve represents 10 mice.

Fig. 3 Survival curves of mice irradiated 20 minutes after intraperitoneal injection of 200 mg/kg WR 2721-SH (the thiol form). Each curve represents 10 mice.

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Fig. 1. Survival curves of mice irradiated at 20 minutes or 60 minutes after an intraperitoneal injection of 400 mg/kg WR-2721. Each point represents 10 mice.

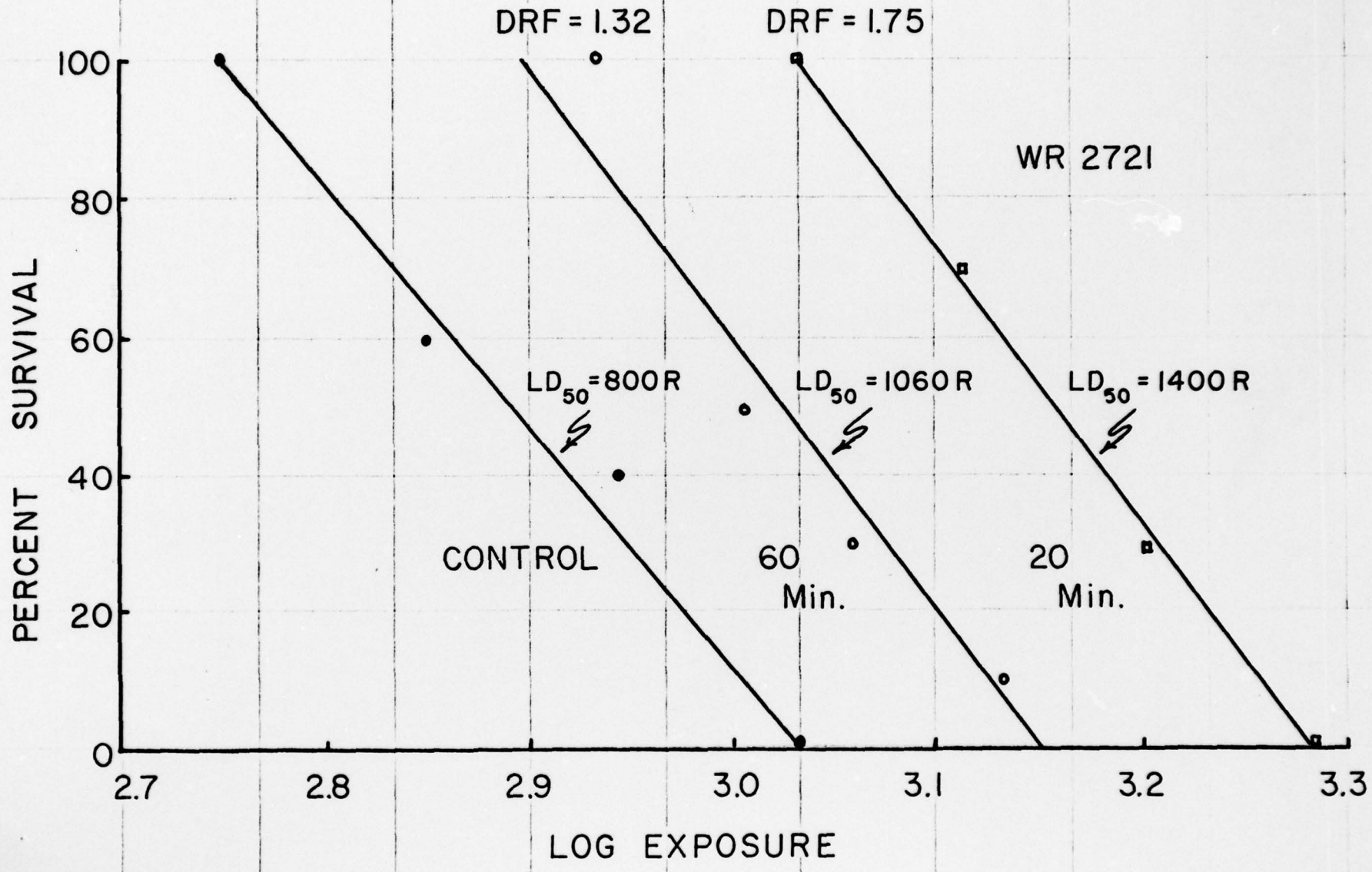

Fig. 2. Survival curves of mice irradiated 20 minutes after intraperitoneal injection of 200 mg/kg WR 2721-S-S (the disulfide form). Each curve represents 10 mice.

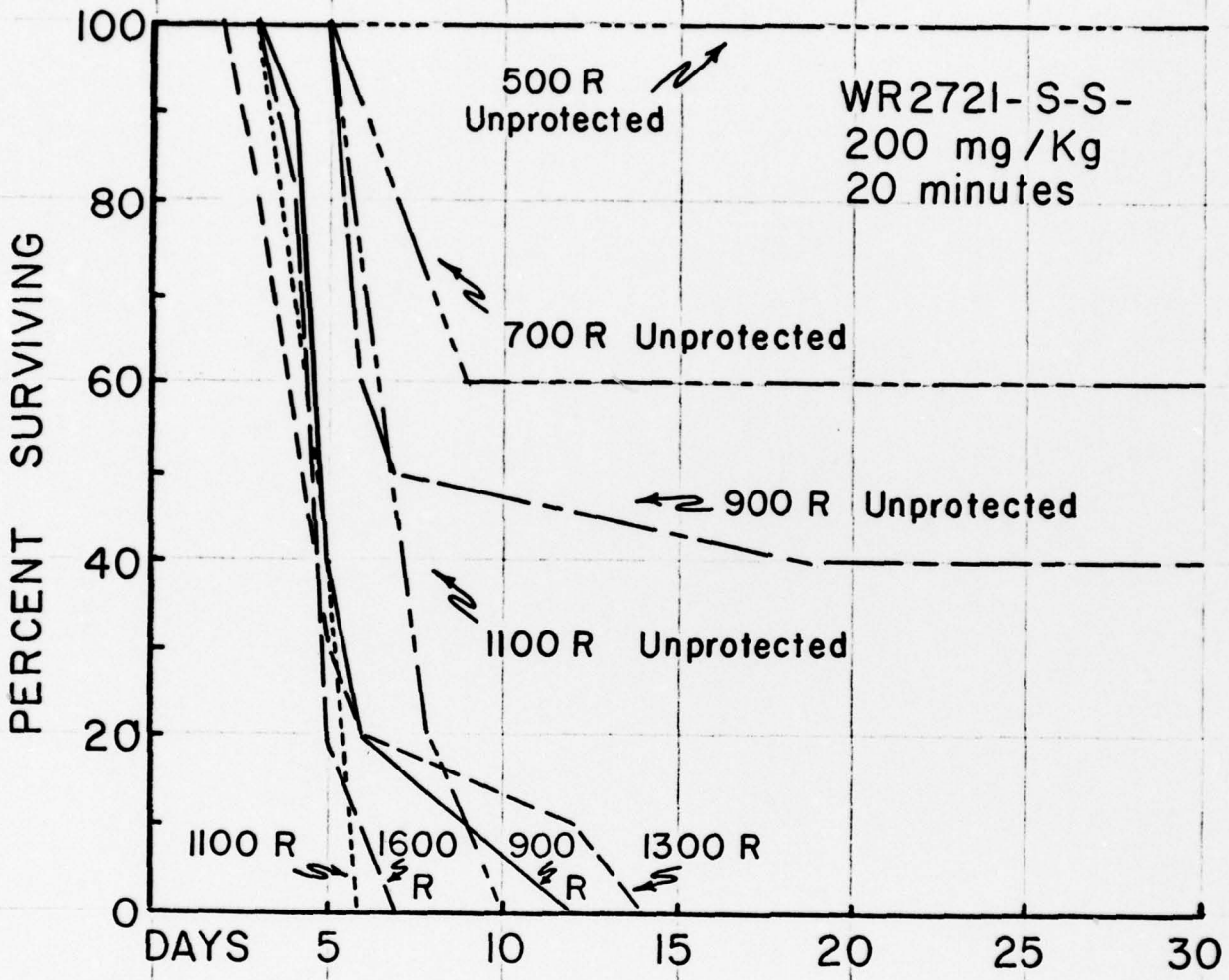

Fig. 3. Survival curves of mice irradiated 20 minutes after intraperitoneal injection of 200 mg/kg WR 2721-SH (the thiol form). Each curve represents 10 mice.

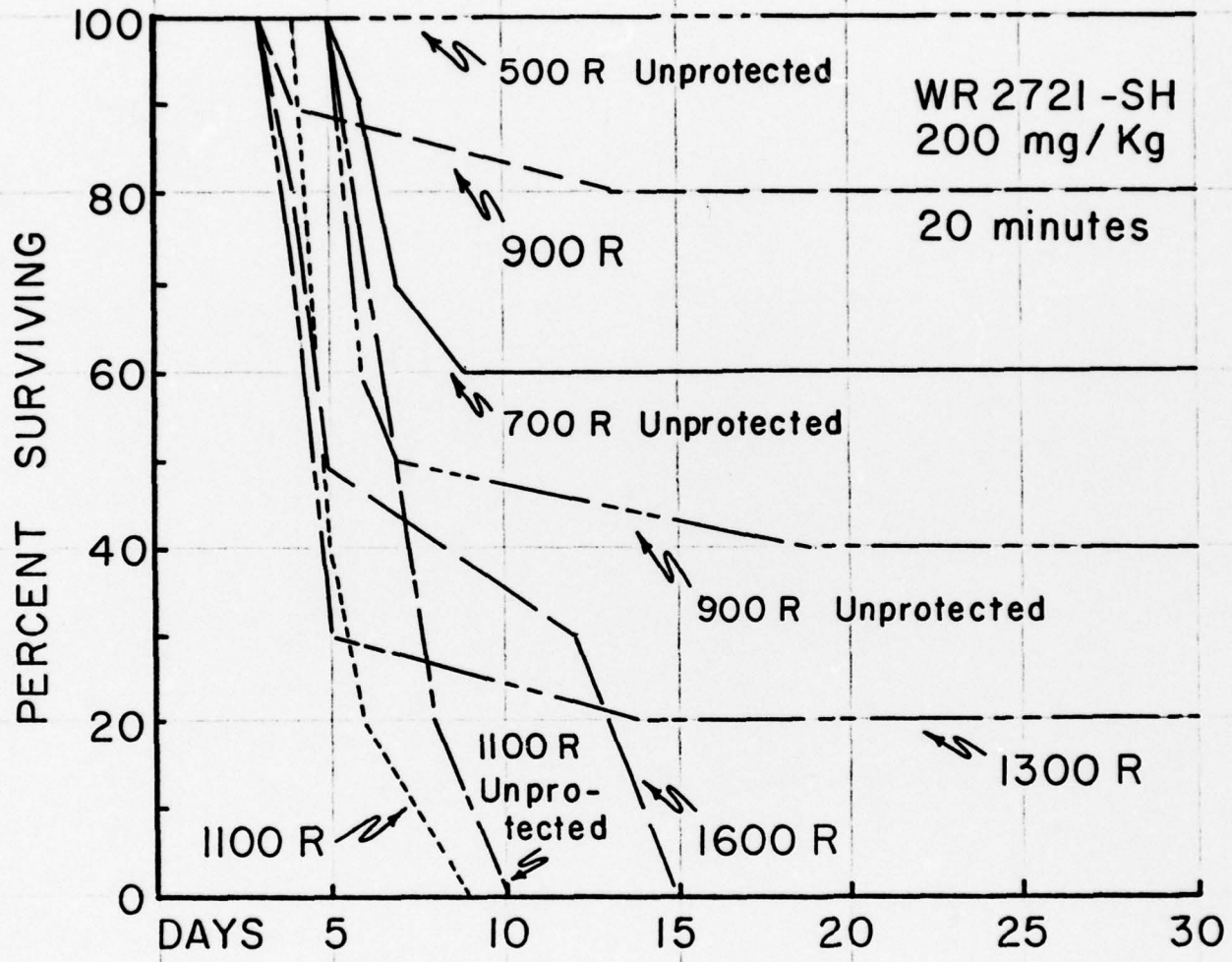

TABLE I

In Vitro Metabolism of
WR 2823 and WR 2721*

	^{35}S-2823		^{35}S-2721	
	0°	37°	0°	37°
Spleen	96	85	100	100
Liver	96	75	100	100
Kidneys	92	45	72	26
Sm. Int.	93	83	100	77
Plasma	100	100	100	100

* Values are % S^{agent} remaining after 15 min. incubation

TABLE II

Distribution of WR 2721 in Mice
after 400 mg/kg I.P.

	% ^{35}S inj.		µg S^{2721}	mg dry wt.
	20'	60'	20'	60'
Spleen	0.27	0.21	.173	.136
Liver	5.23	4.42	.219	.184
Kidneys	1.63	1.02	.318	.185
Sm. Int.	3.36	2.87	.206	.171
Plasma	1.77	1.04	23.7*	13.8*
Excreta	13.8	27.2	____	____

*Plasma values given in µg S^{2721} per ml plasma

TABLE III

Metabolism of WR 2721 in Mice after
400 mg/kg I.P.

Percent of Tissue S^{2721} in each chemical form

	Protein bound		2721	
	20'	60'	20'	60'
Spleen	44	44	56	56
Liver	36	23	64	77
Kidneys	52	22	48	78
Sm. Int.	48	27	52	73
Plasma	51	86	49	14
Excreta	--	--	57*	74*

* Remainder was in disulfide form

TABLE IV

Metabolism of WR 2721 in Mice after
400 mg/kg I.P.

	Micrograms S^{2721} per mg dry wt.			
	Protein bound		2721	
	20'	60'	20'	60'
Spleen	.076	.061	.097	.076
Liver	.079	.041	.140	.143
Kidneys	.165	.040	.153	.145
Sm. Int.	.099	.046	.107	.120
Plasma*	12.1	11.8	11.6	1.8

* Plasma values given in µg S^{2721} per ml plasma

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