Biochemical Pharmacology, Vol. 39, No. 11, pp. 1807–1812, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

BINDING OF AMINOALKYLPHOSPHOROTHIOATE RADIOPROTECTIVE DRUGS TO RODENT TISSUE PROTEINS

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(*Received* 6 *February* 1989; *accepted* 1 *November* 1989)

Abstract—The formation of protein mixed disulfides which influences the pharmacodynamics of the phosphorothioate radioprotective drugs WR2721 [*S*-2-(3-aminopropyl)aminoethylphosphorothioic acid, Ethiofos] and WR3689 [*S*-2-(3-methylaminopropylamino)ethylphosphorothioic acid] and their metabolites was investigated. WR3689-derived thiols and disulfides bound to rat serum protein to about 45 and 40% of the total drug in the incubation when present at a 400 μM concentration. Metabolites of WR2721 were nearly indistinguishable from the corresponding metabolites of WR3689 in their mixed disulfide binding propensity. Mixed disulfide formation was saturable; binding sites on bovine albumin or rat serum protein amounted to 0.15 and 2.4 μmol/mg protein respectively. The sum of all WR3689 metabolites (when measured by NMR spectroscopy) was reduced to the same degree as drug binding, suggesting that a portion of the bound drug was not NMR observable. Approximately 2–4 nmol WR3689-thiol/mg protein was bound to homogenates of mouse tissues (liver, kidney, lung, brain, and serum) when incubated *in vitro*, whereas after *in vivo* injection drug binding appeared to be limited more by drug distribution than by the capacity for mixed disulfide formation.

Aminoalkylphosphorothioic acids (RSP†) are a class of compounds whose members provide cellular protection against radiation and chemotherapeutic drug toxicity [1]. Represented by the prototype drugs WR2721 [*S*-2-(aminopropylamino)ethylphosphorothioic acid, Ethiofos] and WR3689 [*S*-2-(3-methylaminopropylamino)ethylphosphorothioic acid], these compounds are thought to be prodrugs which are protective after removal of the phosphate functional group [2]. Therapeutic evaluation of these drugs in humans has been compromised by widely varying inter-individual pharmacokinetics and occasionally unanticipated toxicity [3]. Recent work describing the metabolism of these drugs in experimental animals [4] and humans [5] is starting to improve our understanding of their pharmacodynamics.

One aspect of RSP metabolism is the ability of these drugs or their metabolites to bind to serum protein. Tabachnik *et al.* [6] found that cystic fibrosis patients treated with daily doses of WR2721 showed an extended plasma half-life of drug when assayed as total drug (parent + metabolites) in plasma. They ascribed this behavior to long-lived binding of WR1065 (*N*-2-mercaptoethyl-1,3-diaminopropane, the thiol metabolite of WR2721) to plasma protein, presumably by mixed disulfide formation. Fleckenstein *et al.* [7] found that a portion of WR2721 administered to Rhesus monkeys was bound to plasma and could be released by treatments known to reduce disulfide bonds. However, what is unclear from these reports is the extent of protein binding, which form(s) of the drug is capable of binding, and the influence of drug binding on pharmacokinetics *in vivo*.

The cellular thiol/disulfide status appears to be an important determinant of tissue resistance to a variety of damaging treatments [8, 9]. We are investigating the feasibility of using carbon-13 nuclear magnetic resonance (NMR) spectroscopy to non-invasively assess the redox status of tissues. NMR measurement of the ratio of ^{13}C[WR3689-thiol/[^{13}C]WR3689-disulfide is a promising chemical probe of this redox balance [10]. However, binding of a large fraction of the ^{13}C-labeled compound to slowly tumbling soluble protein or to slowly moving membrane constituents may alter significantly the spin–spin relaxation time (T_2) of the drug molecule. Such alterations in T_2 may decrease the sensitivity of NMR detection. Therefore, an accurate measurement of the extent of protein binding is necessary for quantitative redox measurement by this technique.

We report here that both thiol and disulfide metabolites of RSP are capable of binding to rat plasma proteins *in vitro*. Information on the kinetics and concentration dependence is also presented as well as a brief estimate of the magnitude of *in vivo* binding after RSP administration to experimental animals.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley derived rats (200–350 g) were obtained from Tyler Laboratories

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† Abbreviations: RSP, aminoalkylphosphorothioic acids; WR2721, *S*-2-(3-aminopropylamino)ethylphosphorothioic acid; WR3689, *S*-2-(3-methylaminopropylamino)-ethylphosphorothioic acid; redox, reduction–oxidation; $LD_{50/30}$, median lethal dose 30 days after drug injection; and DTT, 1,4-dithiothreitol.

Table 1. Aminoalkylphosphorothioates and metabolites

Code number	Drug structure
WR2721	$NH_2CH_2CH_2CH_2NHCH_2CH_2S—PO_3H_2$
WR1065 (WR2721-thiol)	$NH_2CH_2CH_2CH_2NHCH_2CH_2S—H$
WR33278 (WR2721-disulfide)	$NH_2CH_2CH_2CH_2NHCH_2CH_2S—SCH_2CH_2NHCH_2CH_2CH_2NH_2$
WR3689	$CH_3NH_2CH_2CH_2CH_2NHCH_2CH_2S—PO_3H_2$
WR255591 (WR3689-thiol)	$CH_3NH_2CH_2CH_2CH_2NHCH_2CH_2S—H$
WR183159 (WR3689-disulfide)	$CH_3NH_2CH_2CH_2CH_2NHCH_2CH_2S—SCH_2CH_2NHCH_2CH_2CH_2NHCH_3$

(Bellevue, WA) and were housed in quarters having a 12-hr light cycle (6:00 a.m. to 6:00 p.m.) with free access to food (Wayne Rodent Blox No. 8604 from Animal Specialities, Hubbard, OR) and tap water. All animal experimentation was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Drugs. The structures of drug molecules used in this investigation are shown in Table 1. Drugs were provided by the Developmental Therapeutics Program, Division of Cancer Treatment of the National Cancer Institute. Drug purity was tested on each lot received by determining the melting point and by thin-layer chromatography (reverse phase; 0.5 M NaCl/methanol, 1:1); if the lot was not acceptable, the material was recrystallized from 50% methanol in water. Preparation of [^{3}H]WR3689 (high and low sp. act. 20 μCi/mg, 4.9 mCi/mmol; and 1.4 μCi/mg, 344 μCi/mmol, respectively) has been described [11]. [^{35}S]WR2721 (0.7 μCi/mg, 150 μCi/mmol) was synthesized in our laboratory as described [12]. Thiol and disulfide metabolites of these drugs were prepared as needed by acid hydrolysis of the appropriate RSP to the thiol and subsequent oxidation of the thiol to disulfide by bubbling a basic solution of the thiol with high purity oxygen. The radiolabeled thiol (sp. act. 12 μCi/mg, 1.8 μCi/mmol) was kept in the reduced form in the presence of polyacrylamide-immobilized dithiothreitol (Calbiochem, La Jolla, CA). All phosphorothioate drugs were stored under argon in a dessicator at room temperature.

Serum collection. Under deep ether anesthesia, rat blood was withdrawn from the descending aorta. Serum was separated from the clotted blood by centrifuging for 4 min at 13,000 *g*, collected, and recentrifuged to remove any red blood cell contamination. In certain experiments, this serum was dialyzed overnight against 0.1 M potassium phosphate buffer, pH 7.4. Protein concentration was measured by the method of Lowry *et al.* [13] or alternatively by the method of Smith *et al.* [14] with bovine serum albumin as the standard. For comparisons of the drug binding to serum with that of its binding to the tissues, the serum was diluted (1:1) to approximate the protein concentration of 50% tissue homogenates.

Tissue collection and homogenate preparation. Mice anesthetized for serum collection were killed by cervical dislocation. Liver, whole brain, left kidney and lung were quickly removed, put on ice, trimmed into 0.5-g pieces, and weighed accurately. Collected organs were placed in a 2-mL microfuge tube and covered with buffer (1 mL total volume, 0.1 M potassium phosphate, pH 7.4). A polytron homogenizer was used to disrupt the tissue and the homogenate was diluted to 50% with buffer. For lung, due to its low weight, a 25% homogenate was prepared. When the binding of drug to various tissues was compared, homogenate prepared from the organs of the eight mice was pooled for each experiment.

In vitro *binding experiments.* Drug solutions were freshly prepared in 0.1 M potassium phosphate buffer, pH 7.4, at a concentration calculated to give the final dilution designated in the figure legends. For the determination of the concentration dependence of [^{3}H]WR3689 binding, the bovine albumin (Cohn Fraction V, Sigma Chemical Co., St. Louis, MO) or serum was mixed with drug solution at a ratio of 90% serum or 90% albumin (5 g/100 mL) to 10% drug solution in individual polyethylene tubes and incubated in a water bath maintained at 37°. In certain experiments, incubation time was varied from 0 min to 24 hr, with drug concentration held at 400 μM, while in other experiments the drug concentration was varied from 67 μM to 135 μM, with incubation time held constant at 1 hr. For reference, uniform distribution without excretion of WR3689 after *in vivo* injection of a radioprotective dose (450 mg/kg) results in a drug concentration of 1.8 mM; a similar calculation for the $LD_{50/30}$ for WR3689 (909 mg/kg) yields 3.7 mM.

In vivo *binding experiments.* Male Sprague–Dawley derived rats (175–200 g) were dosed with [^{3}H]WR3689, and the extent of binding in blood and plasma was determined at various times after injection. High specific activity [^{3}H]WR3689 (20 μCi/mg) was supplemented with sufficient unlabeled drug to bring the specific activity of the injectate to 0.133 μCi/mg. Total injected drug was 400 mg/kg. On average 3μCi of radiolabeled drug was injected i.p. into each animal. At specified times after injection (15, 30, 45, and 60 min) the animals were anesthetized with ether and blood was drawn from the descending aorta with a heparinized syringe. The red cells were removed and the plasma was processed as described below for mixed disulfides. Tissues (liver, kidney, lung, and brain) were removed, weighed, and immediately homogenized, and protein mixed disulfides were determined as described below.

Mixed disulfide determination. The method of Livesey and Reed [15] was modified slightly for these measurements. From each incubation or tissue sample an aliquot was removed for scintillation counting to determine the total radioactivity in the sample. In preliminary experiments, a portion of the homogenate was passed through a centrifugal ultrafiltration membrane (NP-66, Amicon Co., Danvers, MA) to determine the total amount bound by any mechanism, including electrostatic or other non-covalent interactions. In another aliquot, protein was separated from low molecular weight components of the sample by precipitation; a 0.5-mL portion of the incubation mixture was mixed with an equal volume of acetonitrile, sonicated and centrifuged at 13,000 *g* for 3 min. The supernatant fraction was counted for radioactivity which represented total unbound drug in the incubation. The pellet was washed twice by suspending in absolute ethanol, sonicating, and centrifuging. The protein was suspended in 0.5 mL of 50 mM morpholinopropane sulfonic acid, pH 8.0, containing 25 mM 1,4-dithiothreitol (DTT). This mixture was incubated at 37° for 60 min. At the end of this reduction step, the protein was separated by re-precipitating with 1 vol. of acetonitrile and centrifuging. An aliquot of the supernatant fraction was removed for counting, and the pellet was washed twice in ethanol. The supernatant fraction represents mixed disulfides reduced by the DTT, while the activity adhering to the pellet represents other covalent binding to protein.

NMR measurements. NMR spectra of phosphorothioates and their metabolic products were acquired with a Varian VXR-300 spectrometer with a dual tuned (^1H,^{13}C) 5 mm probe. For standard ^{13}C acquisition, a pulse time of 8.7 μsec (65° pulse), 5.4 sec recyle delay, and a sweep width of 218.77 ppm (16501.7 Hz) were used. Heteronuclear-broad-band coupling was accomplished via a Waltz-16 sequence centered at 4 ppm in the proton spectrum. For standard ^1H acquisition, a pulse time of 20 μsec, recycle delay of 3 sec, and a sweep width of 13.3 ppm (4000 Hz) were used.

Statistical analysis. Data are expressed as the mean of at least three determinations for each data point. Standard deviation is shown in the figures when appropriate.

RESULTS

Magnitude of binding in vitro. The abilities of WR2721 and WR3689 and the thiol and disulfide metabolites of each to bind to tissue were investigated using rat serum as a model tissue. Initially, the binding assay measured drug electrostatically bound, bound as mixed disulfides, and the amount covalently bound in a form which could not be released by dithiothreitol. The amount of electrostatically bound material was variable but negligible (<1% of total drug); non-specific covalent binding was consistently low (<10% of total drug). The majority of drug binding was represented as mixed disulfides with serum protein thiols or disulfides. Figures 1 and 2 show the percent of drug present as mixed disulfides after incubation of rat serum with 0.4 mM WR2721, WR3689, or their

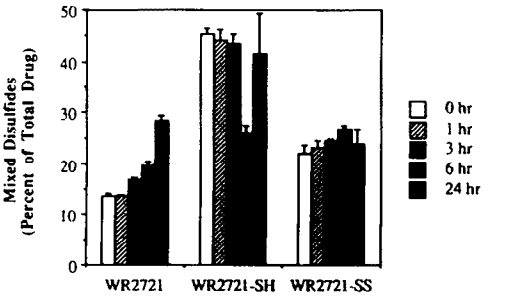

Fig. 1. Formation of protein mixed disulfides between rat serum protein and WR2721 and metabolites. Each drug was incubated individually at 400 μM with dialyzed rat serum protein for the indicated time. Drug binding to protein was then determined on each individual incubation. Bars indicate means ± SD (N = 3).

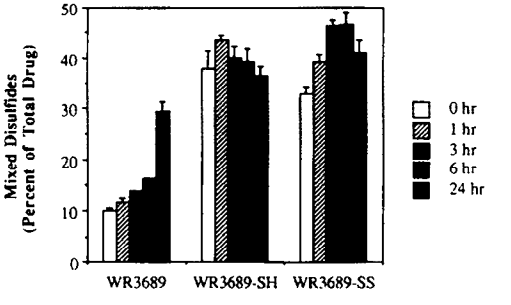

Fig. 2. Formation of protein mixed disulfides between rat serum protein and WR3689 and metabolites. See legend of Fig. 1 for experimental details.

metabolites respectively. Thiols formed mixed disulfides with serum protein to a greater extent than did the phosphorothioate forms of these drugs; binding was rapid and relatively constant over time. The disulfide of WR2721 was less efficient at forming mixed disulfides with protein than the disulfide of WR3689. Binding of phosphorothioates increased over time, suggesting that phosphate hydrolysis [2] was required prior to binding to protein.

Dependence on drug concentration. The extent of drug binding as a function of drug concentration was investigated to determine (a) whether binding to a model tissue was saturable, and (b) the number of binding sites per unit of tissue protein. Assuming thermodynamic and kinetic equilibrium [16], the fraction bound (β) may be related to the drug concentration (X) by:

$$\beta = \frac{1}{1 + \frac{K}{nP} + \frac{X}{nP}}$$

where K is the dissociation constant of the drug–protein complex, P is the protein concentration, and n is the number of binding sites. In the case of covalent complex formation (as with mixed disulfide formation), K approaches 0 and this relationship

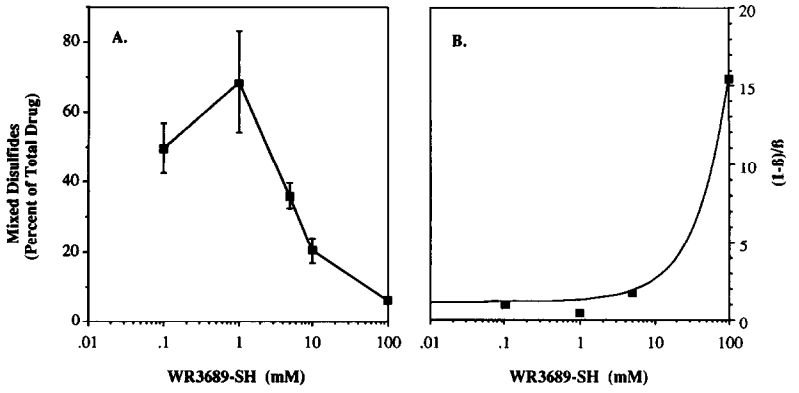

Fig. 3. (A) Saturation of drug binding to bovine albumin. The protein (50 mg/mL) was mixed at a 9:1 ratio with solutions containing various amounts of WR3689-SH. At the end of 1 hr, mixed disulfides were determined. Points indicate the mean of three measurements with bars indicating SD. (B) Binding site analysis. Data from panel A was plotted against the function $(1 - \beta)/\beta = X/nP$ as defined in the text. The solid line indicates the unweighted linear least square fit to the curve, plotted on a logarithmic scale to show all the points.

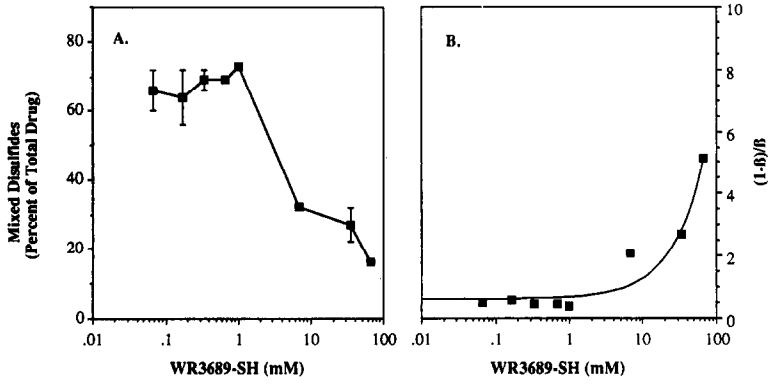

Fig. 4. Saturation of drug binding to rat serum protein (A) and binding site analysis (B). Data are plotted as in Fig. 3.

simplifies to:

$$\frac{1}{\beta} = 1 + \frac{X}{nP}.$$

This may be rearranged to yield

$$\frac{(1-\beta)}{\beta} = \frac{X}{nP}.$$

Therefore, plotting the ratio of free/bound drug against the total drug concentration yields a straight line whose slope may be used to calculate the number of binding sites.

Figure 3A shows the dependence of binding of WR3689-thiol to bovine albumin on total drug concentration. If drug binding were saturable, the fraction bound would decrease as the drug concentration increases. This relationship was observed with WR3689-thiol when incubated with albumin. The mathematical relationship is plotted in Fig. 3B, with a linear least squares fit to the experimentally determined points (the plotted line is curved because of the logarithmic abscissa). The experimental points fit this mathematical relationship well, with an r^2 of 0.984 over three orders of magnitude in drug concentration. The slope of this line is 0.144, inferring 0.139 μmol bound/mg albumin. Using this figure and the average molecular weight of bovine albumin (69,000 daltons), the average number of binding sites per albumin was calculated as 9.92. A similar experiment was performed with rat serum protein, resulting in the data shown in Fig. 4, A and B. In this experiment, serum binding amounted to 2.4 μmol bound/mg serum protein.

Loss of NMR signal by protein binding. We directly tested the loss of NMR signal by measuring the intensity of signal for [^{13}C]WR3689 enriched in the carbon position alpha and beta to sulfur when incubated in the presence or absence of albumin. Table 2 shows that the magnitude of signal loss paralleled the extent of protein binding observed in Figs. 3A and 4A at similar concentrations of WR3689-thiol. However, at the biologically relevant concentrations of 2–3 mM, signal loss was low and constant, which should allow correction of observed signal intensity for protein binding.

Table 2. [^{13}C]WR3689-SH protein binding by NMR

Sample	^{13}C (alpha) (arbitrary integration units)	^{13}C (beta)
WR3689-SH = 10 mM		
RSH + buffer	181.8	89.3
RSH + albumin	182.1	90.0
WR3689-SH = 3 mM		
RSH + buffer	148.4	108.8
RSH + albumin	119.3	97.5

NMR measurements were done on a Varian VXR-300, using a standard ^{13}C pulse sequence, locked on D_2O and broad band Waltz-16 decoupling with a recycle time of 5.5 sec.

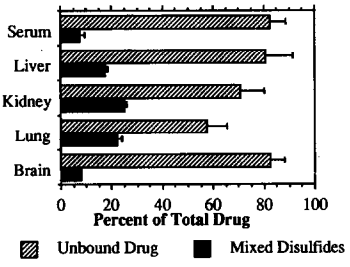

Fig. 5. Binding of WR3689-thiol to mouse tissues *in vitro*. Homogenates of mouse tissues were incubated with WR3689-thiol (2 mM) in a 9:1 (v/v) ratio and incubated for 1 hr; then protein mixed disulfides were determined. Results are plotted as means ± SD (N = 3).

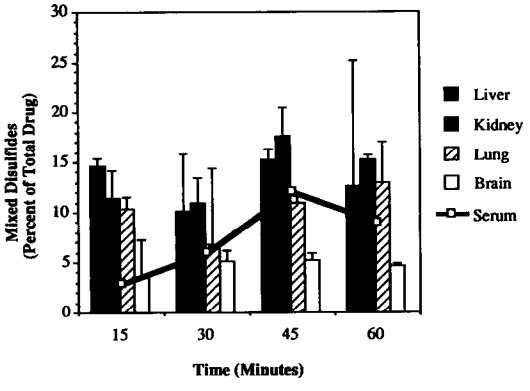

Fig. 6. Binding of WR3689 to mouse tissues *in vivo*. C3H mice were intraperitoneally injected with ^{3}H-labeled WR3689 and killed at the times indicated; protein mixed disulfides were determined. The percentage of drug in each tissue which was in protein mixed disulfide form is shown. Bars indicate the mean ± SD for three mice per time point.

Drug binding to tissues. Homogenates of mouse tissues were used to assess the relative binding of WR3689-thiol to protein from different organs *in vitro*. Figure 5 shows that at a total drug concentration of 2 mM, mouse kidney bound more drug per milligram of protein than liver, lung, brain, or serum. When a series of mice was injected with WR3689 (as the phosphorothioate) and tissues were extracted to determine the amount of mixed disulfide formation in each sample (Fig. 6), the biodistribution of the total drug radioactivity was similar to that reported earlier [11]. In terms of bound drug, tissues with greater drug clearance rates such as kidney and liver were found to have more mixed disulfide than tissues known to take up the drug poorly, such as brain [10]. Note in particular the data presented in Fig. 6 on the time course of serum binding of drug. The extent of drug binding in serum varied with time, first increasing and then slightly decreasing. One might explain this relationship by noting that at early times after injection, serum contained more of the drug in all forms, with a small, fixed proportion bound to protein. As the soluble forms of the drug were distributed to the tissues, the bound proportion occupied more of the total. In general, the extent of *in vivo* binding was approximately equal to the extent of binding after *in vitro* incubation and, within the limits imposed by the drug biodistribution, bound material occupied a fixed percentage of the total drug in a tissue.

DISCUSSION

The disposition of many drugs is influenced by the binding of drug or metabolites to serum proteins. However, for relatively few drugs is the formation of protein mixed disulfides an important aspect of drug pharmacodynamics (cf. captopril pharmacokinetics [17]). In this paper, we report that the disposition of WR2721 and WR3689 was influenced markedly by the binding of drug metabolites to protein, both in serum and in other tissues.

As the data of Fig. 1 and Table 2 suggest, an appreciable amount of WR3689-thiol is bound to serum protein and to tissue homogenates. This binding may be a limiting factor in our effort to measure a thiol/disulfide ratio in tissues of a live mouse by ^{13}C-NMR spectroscopy [9]. However, time–course studies (Fig. 1) show that these forms of the drug generate mixed disulfides relatively quickly and to a constant extent. Therefore, although some of the drug may be present in tissues in an NMR-invisible form, this fraction of the drug should be similar between drug thiol and disulfide and should remain approximately constant over the time course of NMR measurement and, therefore, should not skew our measurements of thiol redox. The identity of the binding site may also be important, inasmuch as the extent of binding at 3 mM drug (~40%) was greater

than the extent of signal loss observed by NMR (20%) at the same drug concentration.

The data presented in Figs. 3 and 4 may be used to caution against the over-interpretation of *in vitro* binding data to the *in vivo* situation. For example, total radioactivity (representing drug in all forms) in liver *in vivo* showed that 1.3 mM WR3689 equivalents were present in the tissue 30 min after injection. This amount of WR3689 or its metabolites is less than that used in many of the *in vitro* experiments and therefore may show greater binding than would be inferred from experiments performed *in vitro* at higher drug concentrations. The identification of the chemical species (phosphorothioate, thiol, and disulfide) present *in vivo* would aid in identifying the individual form(s) of the drug which binds to protein.

The importance of these observations on interpreting the mechanism of action of these drugs involves tempting speculation. Radioprotective drugs are thought to operate by any of several mechanisms [18], some of which are influenced by tissue binding. The scavenging action of thiol metabolites is likely to be reduced if a portion of the drug in tissue is bound in mixed disulfide linkage with protein, particularly if these mixed disulfides are formed at sites distant from the nucleic acid in the cell. However, changes in the activity of enzymes involved in DNA repair or cell cycle progression may well include mixed disulfide formation as an intermediate step. The relatively old theory of protein protection by mixed disulfide formation is supported by our finding of large amounts of disulfide formation *in vivo*. Correlative work such as that of Eldjarn and Pihl [19] would be necessary to determine if mixed disulfide formation was a requisite part of protection by phosphorothioate drugs.

Acknowledgements—The technical assistance of David Honari, Norma Nelson, Lay Chin, Kevin Sadeghi and Dan von Seggern is appreciated. This work was supported in part by Research Grants CA36485, CA42045, and EY07028 from the National Institutes of Health, DHHS.

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