MOLECULAR PHARMACOLOGY, 20:579–584

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The Binding of *s*-Triazine Metabolites to Rodent Hemoglobins Appears Irrelevant to Other Species

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Received January 19, 1981; Accepted June 29, 1981

SUMMARY

A sulfoxide metabolite of *s*-triazines binds to Cys β-125 of rat hemoglobin rather than to the usually more reactive Cys β-93, which is present in most mammalian hemoglobins. Hemoglobins from species other than rodents and chicken (Cys β-126) do not react with the sulfoxide. An attempt to explain this unexpected finding on a stereochemical basis is presented and the general implications of this phenomenon are discussed.

INTRODUCTION

Herbicides of the *s*-triazine type (Fig. 1) are widely used for pre- and postemergence weed control. The fate of these compounds in the environment has been thoroughly investigated (for review see refs. 1 and 2). In many animals, the *s*-triazines are rapidly metabolized and excreted, and residues in the tissues and blood are generally very low. Following the administration *in vivo* of the radioactively labeled compounds simetryn and dipropetryn (Fig. 1) to rats, a small but persistent amount of the label was found to be bound to the macromolecular content of rat erythrocytes.[3] Similar results were obtained from alkylthio-*s*-triazines (3, 4).

The present work was undertaken in order to investigate the behavior of blood from species other than rat and to elucidate the reaction mechanism causing the binding of *s*-triazines to rat hemoglobin. Other authors (5–8) have also noted binding of compounds chemically different from the class investigated here to blood of rodents, but not to blood of other species. In no case, however, has the binding site been determined.

MATERIALS AND METHODS

All reagents used were of analytical grade and were purchased from E. Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), or Sigma Chemical Company (St. Louis, Mo.). Soluene and Instagel were obtained from Packard Instruments Corporation (Downers Grove, Ill.). Unlabeled and ^{14}C-labeled triazines were synthesized according to the method of Esser *et al.* (2). The corresponding sulfoxides were obtained by a 1 hr incubation, at room temperature, of a 1.05 molar excess of *m*-chloroperbenzoic acid in chloroform, which was added dropwise to the triazine, also dissolved in the same solvent. The excess *m*-chloroperbenzoic acid was extracted with 10% sodium carbonate, followed by water. The chloroform was dried over sodium sulfate and the purity of the sulfoxide derivative was checked by TLC[4] on precoated plates of Silica Gel 60 F-254 (Merck, Darmstadt), developed by one of the following solvents: (a) benzene-chloroform-ethyl acetate-1-propanol, 4:2:4:2; (b) benzene-ethanol, 9:1; or (c) chloroform-acetone, 7:3.

The microsomal oxidation of simetryn was achieved by incubation at 37° of 0.4 μmole of simetryn in the following mixture [according to the method of Dautermann and Muecke (9), which was slightly modified]: 1.00 ml of 20% microsomal suspension corresponding to 200 mg of rat liver, 0.25 ml of 12 mM NADP, 0.25 ml of 20 mM NADPH, 0.25 ml of 128 mM glucose 6-phosphate disodium salt, 0.50 ml of 0.4 mM magnesium chloride, 1.75 ml 0.3 M potassium phosphate buffer (pH 7.4), and 20 μl of 1% bovine serum albumin in 0.1 M Tris buffer (pH 7.4); the mixture was brought to a final volume of 4.0 ml with water. The reaction was stopped after the desired time by adding 4 ml of ethanol. After centrifugation at 15,000 × *g* for 10 min, the supernatant was checked on TLC as described above.

Venous or heart blood from rats, mice, guinea pigs, humans, and chickens was collected with heparin as the anticoagulant. Fresh blood from sheep, cows, and pigs was obtained from the local slaughterhouse.

Rat hemolysate was separated into its hemoglobin components as described by Garrick *et al.* (10). Although in our hands Fractions IV and VI (in the Garrick nomenclature) are not well resolved from the main species, we strictly adhered to their nomenclature by using the main Fraction V as reference. Globin chain separation and

This project was supported in part by Eidgenoessische Technische Hochschule Grant 0.330.077.99/1. It was presented in part as a poster exhibit at the XI Meeting of the Federation of European Biochemical Societies, Copenhagen, 1977.

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[3] J. A. Rose, unpublished results (1972–1974).

[4] The abbreviations used are: TLC, thin-layer chromatography; *p*-CMB, *p*-chloromercuribenzoic acid.

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assignment were carried out according to the method of Garrick *et al.* (11).

Blood incubations *in vitro* were carried out with a final concentration of 10 ppm of the herbicides and their derivatives; if needed, up to 20 μl of ethanol were used to dissolve the triazines for a 5-ml blood incubation. The test tubes, placed in a 37° bath, were open toward the air, and gentle agitation was applied. After centrifugation (1,000 × *g* for 10 min) the packed red cells were washed with 0.15 M Tris-HCl in 0.9% NaCl (pH 9.1) and lysed with three times their volume of 0.005 M Tris-HCl (pH 8.5). Ghosts were centrifuged at 30,000 × *g* for 20 min and the lysate dialyzed against the pH 8.5 buffer to equilibrium. Radioactivity was counted in Packard Tri-Carb liquid scintillation counter (Model 3385). Quenching was corrected either with the AES channel method or by using an internal standard.

The percentage values of the radioactivity in plasma and hemolysates, as seen in Figs. 2, 4, and 5, were calculated on the assumption that the radioactivity found in the whole incubation mixture was 100%. The radioactivity in colored solutions was measured following the procedure below.

The desired amount of lysate was mixed with 2 ml of soluene-2-propanol, 1:1. After 1 hr of incubation, 0.5 ml of hydrogen peroxide was added in order to bleach the samples. When the oxygen production was finished, 15 ml of Instagel were added and the radioactivity was counted. Quenching was corrected as above.

Incubation of glutathione with simetryn sulfoxide was performed in 50 mM Tris buffer (pH 9.1) at 37°, using a 1.3 M excess of the sulfoxide, dissolved in ethanol, over glutathione. Immediately after mixing, the sulfoxide formed a suspension which was allowed to stand overnight. Monitoring of the reaction was achieved by TLC. At the end of the incubation period the unreacted sulfoxide was extracted with chloroform and the water phase was lyophilized. Hydrolysis of the resulting material was performed with 6 N HCl for 24 hr at 110°. After drying under vacuum, the mixture was analyzed on a Liquimat III amino acid analyzer (Kontron AG, Zurich, Switzerland), using a standard single-column program for hydrolysates.

Rapid kinetic experiments were performed on a Durrum-Gibson stopped-flow apparatus (Model D-110, Durrum Instruments Corporation, Palo Alto, Calif.) equipped with a 2-cm observation tube. Equal volumes of hemoglobin and *p*-CMB, both of which were dissolved in 0.1 M bis-Tris buffer (pH 7.4), were mixed at 20°, and the absorption changes at 255 nm were monitored with the aid of an 8-bit resolution transient recorder Model TM-109, R. Maurer, Luzerne, Switzerland), connected via an IEEE bus interface, to an MINC-II computer operated under MINC-VI.0 system software (Digital Equipment Corporation, Maynard, Mass.). The experimental data were then analyzed in terms of a three-exponential model described by the equation:

$$Y = 1 - \{a[1 - \exp(-k_1 t)] + (1 - a - b)$$
$$[1 - \exp(-k_2 t)] + b\,[1 - \exp(-k_3 t)]\}$$

where a and b represent the fractions of the fastest and the slowest phases, respectively, and k_1, k_2, and k_3 represent the pseudo-first order rate constants for the three simultaneous processes.

The program used for the approximation was based on the method of steepest descent (12), and the iterative procedure in all cases gave the same solutions, regardless of the value given as the initial guess.

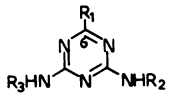
RESULTS

Distribution of ^{14}C-labeled simetryn between plasma and corpuscular elements of whole rat blood. Subsequent to administration *in vivo* of the labeled simetryn to rats, either p.o. or i.v., the initial peak of radioactivity in the plasma was followed by a decline, with a half-time of 2 hr. During the first 3 hr a steady increase of radioactivity in the corpuscular compartment was observed and, after 24 hr, about 95% of the remaining radioactivity was found within the erythrocytes. The simetryn concentration in blood 24 hr after administration was 2 ppm.

In contrast to the situation *in vivo*, *in vitro* the labeled compound remained largely in the extracellular compartment and the fraction of radioactivity in the erythrocytes was low. It did not increase above the approximately 15% reached within 1 hr even after 24 hr of incubation (Fig. 2).

Metabolic modification of simetryn by incubation with rat liver microsomes. Incubation of simetryn with the microsomal fraction of rat liver cell homogenate generated several labeled metabolites (Table 1), accounting for about one-third of all radioactivity present. TLC

s-triazine frame:



NAME	R_1	R_2	R_3
SIMETRYN	$-S-CH_3$	$-C_2H_5$	$-C_2H_5$
SIMETRYN-SULFOXID	$-\overset{O}{\overset{\|}{S}}-CH_3$	$-C_2H_5$	$-C_2H_5$
DESAETHYL-SIMETRYN	$-S-CH_3$	$-C_2H_5$	$-H$
AMETRYN	$-S-CH_3$	$-CH(CH_3)_2$	$-C_2H_5$
AMETRYN-SULFOXID	$-\overset{O}{\overset{\|}{S}}-CH_3$	$-CH(CH_3)_2$	$-C_2H_5$
DIMETHAMETRYN	$-S-CH_3$	$-CH(CH_3)-CH(CH_3)_2$	$-C_2H_5$
DIPROPETRYN	$-S-C_2H_5$	$-CH(CH_3)_2$	$-CH(CH_3)_2$
SIMAZIN	$-Cl$	$-C_2H_5$	$-C_2H_5$
ATRAZIN	$-Cl$	$-CH(CH_3)_2$	$-C_2H_5$

FIG. 1. *Structures and common names of triazines*

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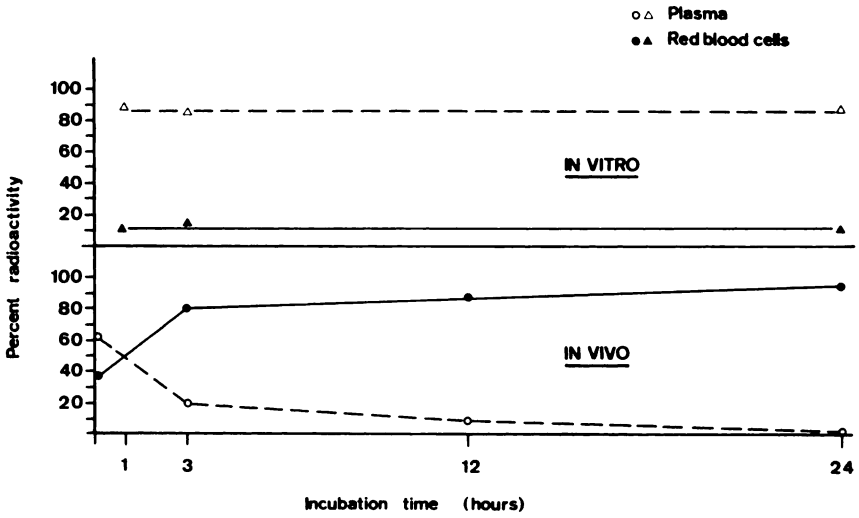

FIG. 2. *Distribution of ^{14}C-labeled simetryn between plasma and red blood cells of rat*

The distribution of radioactivity after incubation of simetryn with rat blood *in vitro* (*top panel*) and after i.v. injection of the herbicide (*bottom panel*). The percentage was calculated assuming that the radioactivity in the whole blood was 100%. The values plotted were obtained from hemolysates and plasmas without dialysis.

demonstrated that the main resulting component was the sulfoxide derivative (Fig. 1). The same derivative, but with better yield (>90%), was obtained upon oxidation of simetryn with *m*-chloroperbenzoic acid.

Incubation of rat blood in vitro with the ^{14}C-labeled simetryn sulfoxide. In contrast to the incubation of blood with unmodified simetryn (Fig. 2) a massive incorporation into erythrocytes *in vitro* could be observed with the sulfoxide derivative. Exhaustive dialysis removed only a minor part of the radioactivity (Table 2). Separation of the hemolysate into its component hemoglobins resulted in a chromatogram identical with that obtained from a hemolysate after administration *in vivo* of the unmodified simetryn (Fig. 3); i.e., all six rat hemoglobins bound the sulfoxide derivative.

A calculation of the stoichiometry of binding, based on the specific radioactivity of the sulfoxide and on the hemoglobin concentration, surprisingly indicated a ratio of one molecule of sulfoxide per hemoglobin tetramer.

The assignment of the modified protein to the parent hemoglobin fraction was carried out by individually modifying the separated hemoglobins with the sulfoxide. These modified hemoglobins were subsequently chromatographed with carrier hemolysate which showed that the introduction of the sulfoxide residue had altered the net charge of the protein to be more acidic. Accordingly, the modified hemoglobins eluted prior to the corresponding nonmodified hemoglobins (Fig. 3).

Species differences. The incubation *in vitro* of whole blood of a wide variety of species with the sulfoxide derivative of simetryn demonstrated that, of all mammals tested, only the rodents (rats and guinea pigs) bound significant amounts of the label to their hemolysates. Considerable incorporation was also observed in chickens (Fig. 4).

Interaction of other s-triazines and their sulfoxide derivatives with hemoglobins of different species. Incubations *in vitro* of rat blood with other ^{14}C-labeled *s*-triazines and the respective sulfoxide resulted in findings analogous with those obtained with simetryn. The herbicides themselves did not react, but the sulfoxide compounds were rapidly bound to the red blood cell contents of rat tissue. Again, exhaustive dialysis could not remove the radioactive compounds from the hemolysate.

In Fig. 5 the results from the incubation of various *s*-triazines with the blood of a variety of animals are summarized. Only red blood cells from the small rodents (rats, mice, and guinea pigs) bound the sulfoxide derivatives.

Modification of cysteine with simetryn sulfoxide. When the tripeptide glutathione (γ-L-glutamyl L-cysteinylglycine) was allowed to react under the same conditions used for the blood samples, with ^{14}C-labeled simetryn sulfoxide, a product was formed which on acid hydrolysis yielded, in addition to the three amino acids glycine, glutamic acid, and cysteine, a labeled compound assumed to be the modified cysteine. Dialysis of rat

TABLE 1

TLC identification of the compound formed by incubation of ^{14}C-labeled simetryn with rat liver microsomes

Compound	Incubation (20 min)
Simetryn	69.5%
Simetryn sulfoxide	21.5%
N-desethyl simetryn	6.0%
Unidentified polar Derivatives	3.0%

TABLE 2

Percentage distribution of radioactivity in rat blood after incubation in vitro of simetryn and simetryn sulfoxide

Blood Component	Simetryn[a]		Simetryn sulfoxide, 10 ppm[a]
	18 ppm	3 ppm	
	%	%	%
Plasma	90	87	15
Hemolysate	10	13	85
Hemolysate after dialysis	<1	<1	50

[a] Final concentration.

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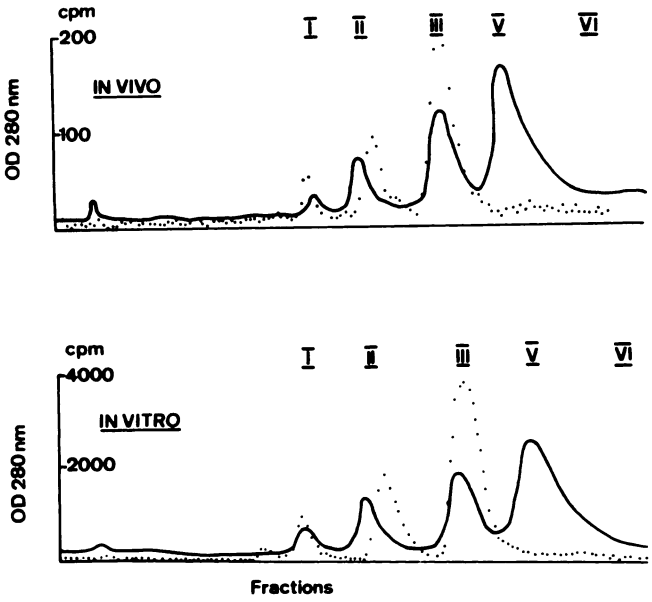

FIG. 3. *Separation of modified hemoglobins*

The chromatographic separation of rat hemolysates (10) after administration *in vivo* of simetryn (*top panel*) and after incubation *in vitro* of the sulfoxide derivative (*bottom panel*).——, Optical density; ····, radioactivity. The corresponding radioactive peaks are slightly shifted to the *left*. *Roman numerals* indicate various rat hemoglobins. Hemoglobin IV was not resolved.

hemolysates, subsequent to incubation with the sulfoxide, yielded after hydrolysis the same modified compound as verified by amino acid analysis. Blocking of the —SH groups of hemoglobin with iodoacetamide prior to incubation with the sulfoxide completely inhibited its binding reaction.

Globin chain separation and location of the bound radioactivity. Separation of the isolated and modified rat hemoglobin Fraction V into the globin chains resulted in a chromatogram similar to that obtained by Garrick *et al.* (11). Radioactivity from the ^{14}C-labeled sulfoxide derivative was found on the β-chain exclusively (Fig. 6).

Kinetics of p-CMB binding to the sulfhydryl groups of purified rat oxyhemoglobins. The binding of *p*-CMB to rat oxyhemoglobins, purified from rat hemolysate, is complex. The process could be resolved into three phases, the first characterized by a second-order rate constant of $3–5 \times 10^5$ M^{-1} sec,$^{-1}$, as compared with 5.8×10^5 M^{-1} sec^{-1} reported for the Cys β-93 of human oxyhemoglobin (13); the second and third were characterized by rate constants of $6–8 \times 10^3$ M^{-1} sec^{-1} and 70–150 M^{-1} sec^{-1}, respectively. Numerous experiments, carried out with several different concentrations of both the protein and the *p*-CMB, gave the same results within experimental error. Preincubation of rat hemoglobin with the sulfoxide derivative led to the modification of the —SH group with an intermediary reaction rate, but left the rapidly reacting one unaltered.

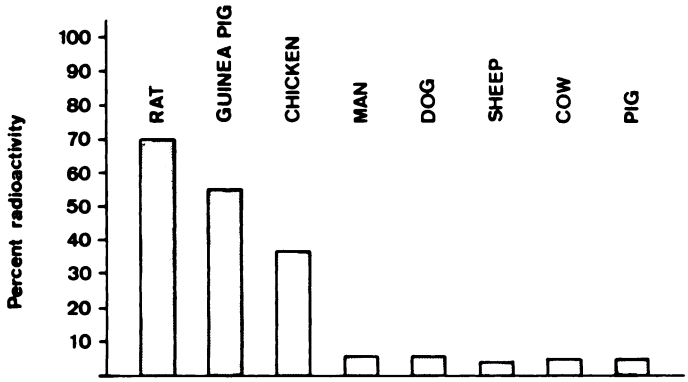

FIG. 4. *Binding of ^{14}C-labeled sulfoxide to hemolysates of various species*

Covalent binding of the ^{14}C-labeled simetryn sulfoxide to macromolecular red cell contents of different species was measured after exhaustive dialysis of the hemolysates. The values are expressed in percentages of the total radioactivity found in the hemolysate.

DISCUSSION

The above results show that *s*-triazines per se do not appreciably bind to the content of erythrocytes. The *s*-triazines, carrying an alkylthio group on the C-6 atom, are partially metabolized to the corresponding sulfoxide. These compounds, in turn, bind covalently to certain hemoglobins, but not to all. The evidence for this lies in the observation that the formerly dialyzable sulfoxide can no longer be separated from the hemolysate by this procedure. Moreover, on DEAE-cellulose chromatography, the radioactivity coelutes with the protein, slightly ahead of each of the clearly separated hemoglobins of rat blood. From the specific radioactivity it can be calculated that one sulfoxide reacts with one tetrameric hemoglobin molecule. Upon separation of the globin chains, >95% of the radioactivity is shown to be associated with the β-chains.

Incubation of the ^{14}C-labeled sulfoxide derivative of simetryn with glutathione leads upon hydrolysis to the formation of a radioactively labeled compound which gives, besides glycine, glutamic acid and small amounts of cysteine, a modified labeled amino acid. The same radioactively labeled compound is obtained by hydrolysis of β-globin chains of rat hemoglobin which had been previously incubated with the ^{14}C-labeled sulfoxide. It therefore appears likely that the binding site for the sulfoxide is a cysteine residue. This is in keeping with the observation that preblocking of —SH groups with iodoacetamide prevents binding of sulfoxide completely. On this basis we propose the reaction scheme illustrated in Fig. 7 (14).

On the other hand, the tested hemoglobins, whether or not they are able to bind sulfoxide, have the "reactive" sulfhydryl in position β-93 in common. The kinetic experiments show that the *p*-CMB combination rate constant for the first phase is very similar to the one reported for the —SH β-93 in human hemoglobin (13). Since in the major β-chain of rat only two cysteines are present, namely β-93 and β-125 (11), it appears likely that the modified cysteine is the one in position β-125. Detailed structural analysis by sequencing the labeled cyanogen-bromide peptide from ^{14}C-labeled sulfoxide-reacted hemoglobin will appear in a separate publication (15).

The observation that, even upon prolonged incubation of rat hemoglobin with large excess of sulfoxide, only about one-half of the β-chains are modified remains without easy explanation since β-125 is an external resi-

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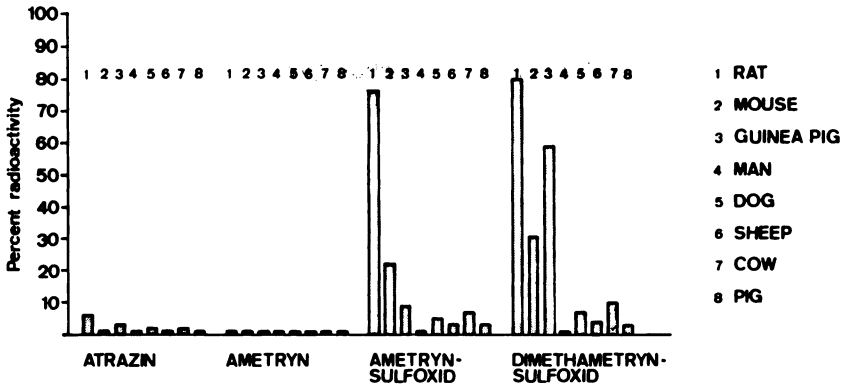

FIG. 5. *Binding of triazines to blood of various species*
Blood of different species was incubated with various triazines and sulfoxides. Radioactivity was measured after dialysis of the hemolysates.

due distant from the area of conformational changes which accompany the binding of oxygen or other ligands to heme (16). At any rate, titration of —SH groups, before and after treatment with the sulfoxide derivative, confirms the stoichiometry of one cysteine residue bound per tetramer. Furthermore, the separation of the individual polypeptide chains by high-performance liquid chromatography gave a ratio of 2:1:1 for α:β-reacted to β-unreacted (15). These findings are in perfect agreement with the titration experiments.

Looking at the model of the hemoglobin molecule offers a possible explanation of the unexpected finding that the Cys β-93, being the —SH group rapidly reactive with *p*-CMB, does not react with the sulfoxide. Between the two nonaromatic nitrogen atoms of the triazine derivatives, on which the residues *R*1 and *R*2 are bound (Figs. 1 and 7), lies a distance of 5 Å. The stereochemical situation around Cys β-93 would allow a molecule of the size of the above-mentioned sulfoxide to bind only in one particular position and would subsequently inhibit a rotation of the two neighboring carboxyl groups of His β-146 and Asp β-94. In contrast to the situation on β-93, β-125 is located on the surface of the hemoglobin tetramer, allowing a free rotation of the bound, frog-shaped (Fig. 7), triazine derivative molecule as well as of the neighboring amino acid residues.

In guinea pig β-globin chains the β-125 residue is also a cysteine (17) and therefore the same considerations can be applied to this protein. The cysteine found in chicken hemoglobin in position β-126 (18) offers slightly less access for the sulfoxide as compared with the rat β-125, but it is still a good candidate for the binding site. The Cys residues β-23 and α-135, in chicken hemoglobin, are not exposed to the solvent and are therefore not available for modification.

On the other hand, *p*-CMB, with its rodlike shape and relatively small diameter (2.5 Å), is able to bind to Cys β-93 (13) without severely interfering with its own free rotation and those of neighboring residues. Why the rate of *p*-CMB binding to Cys β-125 is slower than that to Cys β-93 remains an open question at present. However, it is interesting to note that all residues in the vicinity of Cys β-125 are hydrophobic. Furthermore, it is noteworthy that, in the case of rodents, intraerythrocytic glutathione does not prevent the binding of sulfoxide to the special —SH groups of hemoglobin.

Finally, we would like to emphasize that the covalent modification of a protein can depend on very specific stereochemical conditions, and that therefore the observed covalent binding of a drug, or its metabolites, to proteins of one species is sometimes irrelevant to the analogous proteins of many other species. These considerations must be borne in mind when reporting binding of drugs to specific proteins. Detailed structural analysis

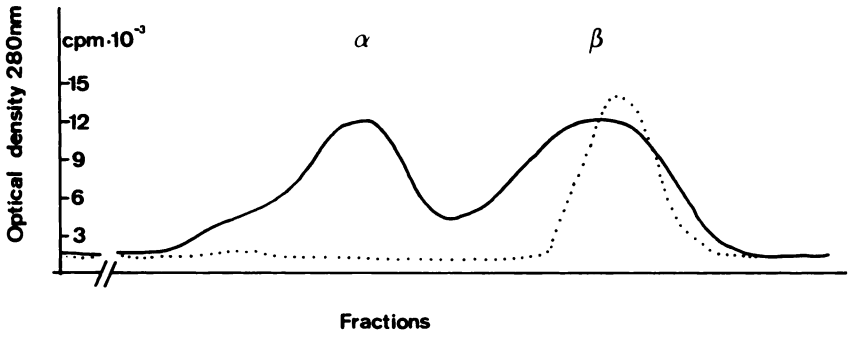

FIG. 6. *Globin chain separation*
Rat hemoglobin Fraction V after incubation with labeled simetryn sulfoxide. The chain separation was achieved according to the method of Garrick *et al.* (11). ——, Optical density at 280 nm; ····, radioactivity.

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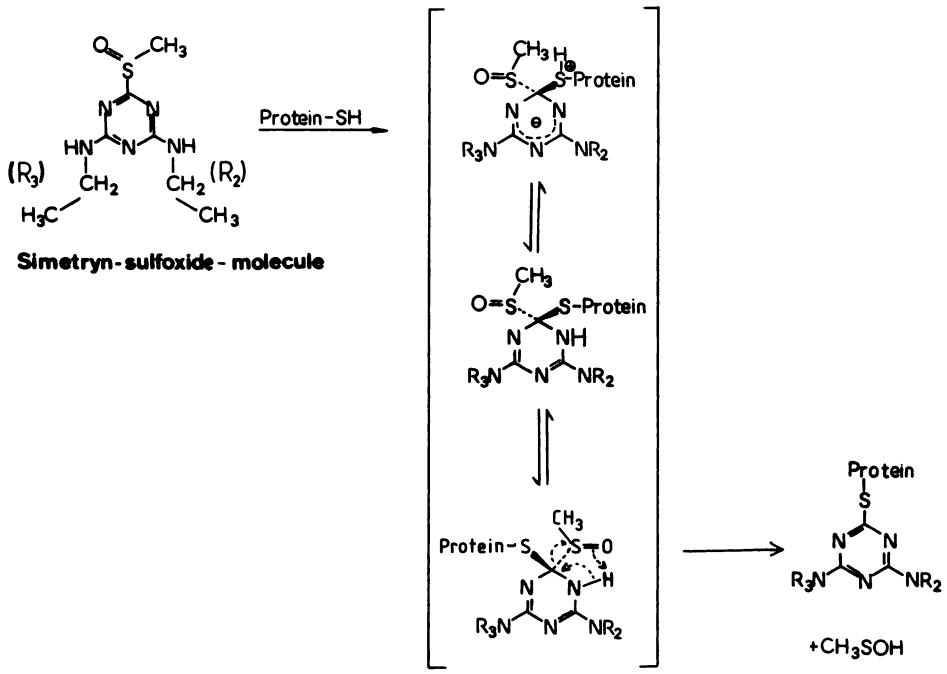

FIG. 7. *Tentative scheme of the reaction of simetryn sulfoxide with the protein sulfhydryl group*

is necessary for interpretation of undesirable consequences of the use of a drug under consideration.

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