Binding of Drugs to Human Serum Albumin XVII: Irreversible Binding of Mercaptopurine to Human Serum Proteins

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Abstract □ Studies on the binding of mercaptopurine to human serum, human serum albumin, or orosomucoid gave varying results, with binding ranging from 20 to 90%. Binding has been shown to be affected by oxidizing agents, which increase binding at low concentrations, and by reducing agents, which decrease binding. Repeated equilibrium dialyses and gel filtration experiments indicated that mercaptopurine is partly irreversibly bound to serum proteins. This binding is oxidation dependent and probably involves the sulfhydryl group of the drug.

Keyphrases □ Mercaptopurine—irreversible binding to human serum proteins □ Binding—irreversible, of mercaptopurine to human serum proteins □ Antineoplastics—mercaptopurine, irreversible binding to human serum proteins

Loo *et al.* (1) reported that mercaptopurine is bound 19% in human plasma. However, preliminary studies in this laboratory using equilibrium dialysis to measure the binding of mercaptopurine to serum proteins *in vitro* gave varying results. Moreover, Scatchard analyses of data obtained from experiments with serum, purifed human serum albumin, and orosomucoid gave very low n values, which were not compatible with reversible binding to a specific binding site. The variance obtained with different serum samples was even greater than that seen when identical samples were compared. However, aged serum samples generally gave higher binding (up to ~90%).

Further investigation indicated that binding was influenced by the presence of reducing or oxidizing agents, *e.g.*, mercaptoethanol and hydrogen peroxide. The present paper shows that the varying binding of mercaptopurine to serum proteins *in vitro* can be explained by irreversible binding phenomena involving the reactive mercapto group of the drug.

EXPERIMENTAL

Drugs—6-Mercaptopurine was used in the unlabeled[1] or ^{14}C-labeled[2] form. Radiochemical purity (>96%) was checked by TLC.

Serum—Two types of serum samples were used: (*a*) pooled normal serum obtained from healthy persons and stored frozen for a long time, and (*b*) freshly prepared normal serum from three healthy persons and used within several days. The blood was collected in untreated glass tubes without any additions.

Equilibrium Dialysis—Serum protein binding was determined by equilibrium dialysis at 20° against an isotonic phosphate buffer (2), pH 7.4, using standard membranes[3]. Duplicate determinations were performed, and the amount of serum and buffer was 500 μl on each side of the dialysis cell. The drug was dissolved in the buffer. The equilibration time was 17–19 hr. Control experiments indicated that equilibrium was reached within 15 hr. After equilibration, radioactivity was determined in duplicate with 200 μl of sample aliquots from both sides of the dialysis cells in 5 ml of Instagel.

Gel Filtration—Gel filtration was performed with Sephadex G-25[4] in a 25 × 1.5-cm column and a 0.005 M phosphate buffer (pH 7.4) with 0.1 M potassium chloride.

RESULTS AND DISCUSSION

Equilibrium Dialysis—Quantitative studies on the binding of [^{14}C]mercaptopurine to serum proteins by equilibrium dialysis gave varying results. The variation was particularly large between fresh and aged serum samples, and addition of reducing or oxidating agents significantly affected binding. Table I summarizes some of the results obtained. Each group of results was obtained with identical serum samples at the same time but with different treatments. Thus, the binding to freshly drawn serum was ~20–30%, while serum stored frozen for ~3 years bound ~90% of the drug. If the freshly drawn serum was exposed to air, binding significantly increased with time. In contrast, addition of reducing substances (cysteine, dithiothreitol, or mercaptoethanol) profoundly impaired the binding of both aged and fresh serums.

The difference in the binding capacity of fresh and aged serums was probably due to serum oxidation since addition of small amounts of hydrogen peroxide increased binding (Table I). However, when hydrogen peroxide was increased to millimolar concentrations, the detected binding decreased. Later experiments with gel filtrations indicated that this effect was mainly due to the oxidation of mercaptopurine to the dimeric form, which has a lower affinity for albumin.

These results suggest that the binding of mercaptopurine to the serum proteins depends on the oxidation–reduction properties of the system. Thus, the sulfhydryl group of the mercaptopurine may be involved in the binding. Such binding involving a covalently bound S-group should not be easily reversible.

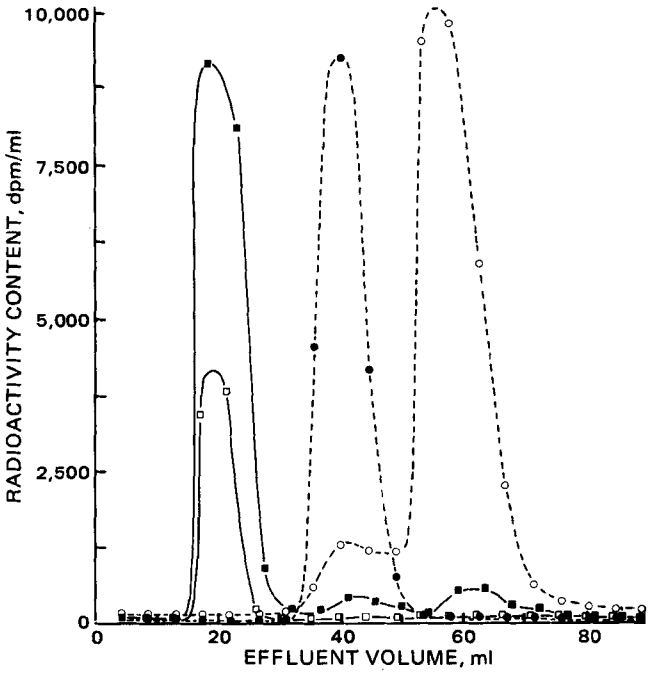

Figure 1—*Aged human serum with [^{14}C]mercaptopurine chromatographed in 0.1* M *potassium chloride with 0.005* M *phosphate, pH 7.4 (■). The protein peak fraction was rechromatographed under the same conditions (□). As controls, untreated [^{14}C]mercaptopurine was run alone (○) and after oxidation with hydrogen peroxide in excess (●) on the same column.*

[1] Wellcome Foundation, England.
[2] CEA (Commissariat á l'Energie Atomique), Gif-Sur-Yvette, France.
[3] Technicon.
[4] Pharmacia Fine Chemicals.

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Table I—Binding [a] of 6-Mercaptopurine to Human Serum Proteins at Various Conditions

Sample	Mercaptopurine Concentration, μM	Binding, Percent	Binding, Relative
Fresh serum	2.5	30.8	100
Fresh serum + mercaptoethanol (4.8 mM)	2.5	6.5	21
Fresh serum aged for 4 hr in air	2.5	54.2	176
Aged serum	2.5	86.3	100
Aged serum + mercaptoethanol (4.8 mM)	2.5	26.0	30
Fresh serum	0.7	64.2	100
Fresh serum + hydrogen peroxide (0.25 mM)	0.7	95.1	148
Fresh serum + hydrogen peroxide (2.5 mM)	0.7	86.3	134
Aged serum	0.7	92.0	

[a] Binding was studied by equilibrium dialysis at 20° with an isotonic phosphate buffer (pH 7.4).

To test whether the binding is reversible or not, repeated equilibrium dialysis was performed. The buffer side of the dialysis cell was replaced with fresh buffer after equilibrium was attained. The cell was then equilibrated again. As shown in Table II, the free fraction on the buffer side reduced to about half of the original free fraction after the second equilibration. This result indicates an irreversible binding of mercaptopurine and an equal distribution of the initially unbound drug on the protein side between the two sides after buffer replacement. Nothing of the bound fraction was dissociated from the protein and redistributed to both sides of the dialysis cell. A repeated equilibrium dialysis was also performed with salicylic acid as a control. In this experiment, the original free fraction on the buffer side was recovered after the second equilibration, which indicates reversible binding as expected.

Gel Filtration—Another experiment was performed with gel filtration to test whether binding is reversible. A sample solution of [^{14}C]mercaptopurine in aged serum was run on a Sephadex G-25 column. The peak fraction containing the proteins and bound drug, *i.e.*, the first eluted peak, was run a second time. The chromatograms obtained are shown in Fig. 1. The second run gave only one peak, indicating that no free drug was present. These results support the hypothesis that binding is irreversible.

The same experiment was performed under identical conditions with salicylic acid, which is known to exhibit reversible binding to serum albumin. As expected, the second run showed that salicylic acid dissociated from the protein fraction.

When [^{14}C]mercaptopurine was run alone on the column, the chromatogram showed two peaks. Their relative size was directly correlated to the presence of any oxidizing agent, *e.g.*, hydrogen peroxide, which completely transferred the mercaptopurine into the dimeric form when added in excess. As shown in Fig. 1, the dimer was eluted between the protein and monomer peaks.

Table II—Repeated Equilibrium Dialysis of [^{14}C]-Mercaptopurine (14,000 dpm/cell) and Human Serum

	Radioactivity, dpm: Protein Side	Radioactivity, dpm: Buffer Side	Fraction Bound, %
First equilibration	ND[a]	1500	90.0
	ND	1317	
Second equilibration after replacement of buffer	11,727	817	93.5
	12,097	720	

[a] Not detected.

These results are compatible with the hypothesis that the sulfhydryl group of mercaptopurine is involved in an oxidation-dependent reaction with free sulfhydryl groups in proteins and/or in an oxidation-independent S–S interchange with the protein molecules. The mercaptopurine dimer does not bind or is only poorly bound to serum proteins.

Recently, similar results were found in a study on the binding of mercaptopurine to rat hepatic microsomal proteins (3). After oxidation of the labeled microsomal protein with performic acid, 75% of the radioactivity was released, suggesting that the drug was bound *via* a disulfide bond.

Binding studies *in vitro* with mercaptopurine and similar compounds should be performed with great care, excluding air and oxidizing agents. Biological samples with mercaptopurine, *e.g.* blood, plasma, or serum, should also be handled under well-controlled conditions, and results obtained under poorly defined conditions should be judged skeptically.

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