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MERCAPTODEXTRAN, A METAL-CHELATING AND DISULPHIDE-REDUCING POLYTHIOL OF HIGH MOLECULAR WEIGHT

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Abstract—A high molecular weight polythiol, mercaptodextran has been synthesized by thiolating various dextrans with *N*- acetyl homocysteine thiolactone. The thiol groups are unusually stable towards autoxidation. They are highly reactive and readily reduce disulphide bonds. Because of these properties mercaptodextran may be used to keep autoxidizable thiols in the reduced form. Mercaptodextran has no radioprotective effect, since it does not penetrate the cellular membrane. Mercaptodextran has a very high affinity for heavy metal ions. Competitive binding experiments show that mercaptodextran has much higher affinity for silver, mercuric, cupric and auric ions than most other thiols (glutathione, penicillamine, *N*-acetyl-DL-penicillamine, cysteamine, mercaptopropionyl glycine, cysteine and diethyl dithiocarbamate) and other chelating agents (EDTA). Only 2,3-dimercaptopropanol (BAL) shows comparable binding properties. The stability constants for the Hg–BAL and Hg–mercaptodextran complexes are both about 10^{20}, and about 100-times higher than for the Hg–penicillamine complex. The high metal-binding ability combined with a low toxicity suggest a possible use of mercaptodextran in acute metal-intoxications.

MOST thiols of low molecular weight possess a high affinity for heavy metal ions forming mercaptides with, e.g. mercuric, lead, cupric and silver ions. Many thiols also demonstrate pronounced reducing properties, e.g. towards disulphides like cystine and homocystine. Certain thiols of low molecular weight, in particular 2,3-dimercaptopropanol (BAL), D-penicillamine and *N*-acetyl-DL-penicillamine have therefore been used in the treatment of lead, mercury and copper intoxications[1] as well as in Wilson's disease (hereditary copper deposition)[2] and in the treatment of cystinuria[3] and homocystineuria. Several thiols, e.g. cysteamine, are also known as radioprotective agents.[4]

Unfortunately, most thiols of low molecular weight are rather toxic compounds, a drawback which limits their use *in vivo*. The high toxicity may, at least partly, be due to interactions of the added thiol with essential SH- and SS-groups inside the cells. Accordingly, a high molecular weight thiol which does not penetrate the cellular membrane, should be less toxic.

The aims of the present studies have been to synthesize a soluble, reactive thiol of molecular weight high enough to avoid penetration into the cells, and low enough to be eliminated slowly through the kidneys. The idea was that such a compound might circulate in the blood-stream without causing harmful effects, and that the thiol groups on the injected macromolecule might offer a local protection of the bone

marrow against ionizing radiation. It was also thought that a polythiol of high molecular weight would be an efficient metal-chelating agent, which possibly might be of use in acute metal-intoxications.

The results of our attempts to synthesize such a polythiol of high molecular weight is mercaptodextran. It can be prepared from various dextrans by substituting the hydroxyl groups of the glucose units with an alipathic chain terminating in a thiol group. The structure of mercaptodextran is: Dextran–O-CH_2.CH_2.NH.CO.CH (NHAc)CH_2.CH_2.SH. The compound is a reactive polythiol of low toxicity, with exceptionally high affinity for heavy metal ions, but with no radioprotective effect.

MATERIALS AND METHODS

Reagents. Dextran T-5, T-10, T-20, T-40, T-70, T-150 and T-500 with average molecular weight of 5000, 10,000, 20,000, 40,000, 70,000, 150,000 and 500,000 respectively, were obtained from Pharmacia AB, Sweden. 2-Aminoethyl hydrogensulphate and *N*-acetyl-homocysteine thiolactone were products of Koch–Light Ltd. The former compound was recrystallized from dilute ethanol before use. Radioactive mercuric chloride ($^{203}HgCl_2$), cobalt chloride ($^{57}CoCl_2$), cadmium chloride ($^{115}CdCl_2$), strontium chloride ($^{85}SrCl_2$), zinc chloride ($^{65}ZnCl_2$) and ^{35}S-cystamine dihydrochloride were obtained from the Radiochemical Center, Amersham, England. Radioactive cupric chloride ($^{64}CuCl_2$), chromic chloride ($^{51}CrCl_2$) and ferric citrate ($^{59}Fe^{+3}$) were obtained from the Institute of Atomic Energy, Kjeller, Norway.

Synthesis of mercaptodextran. Mercaptodextran was synthesized along the same route as previously described for thiolated Sephadex.[5] Dextran with the desired molecular weight (13 g) was dissolved in a mixture of sodium hydroxide (12 g) and 2-aminoethyl hydrogensulphate (4 g) in water (30 ml). The syrup was spread out on a glass plate (20 × 20 cm) and placed in an oven at 115° overnight. The dry, crude aminodextran was powdered and dissolved in cold water (100 ml). The viscous solution was slowly added to 1 l. of 96% ethanol in a Waring blendor. The mixer was operated at full speed for 2 min. The white, crystalline precipitate was filtered off, washed well with ethanol and sucked dry. The aminodextran (13 g) was redissolved in water (400 ml) and neutralized to pH 7–8 with concentrated acetic acid. *N*-acetyl homocysteine thiolactone (2·5 g) was added and the mixture placed in an automatic titrator (Radiometer titrator 11, Radiometer, Copenhagen). The burette contained 1 N sodium hydroxide, and the end-point was set at pH 7·8. A solution of silver nitrate (2·6 g of $AgNO_3$ in 50 ml of water) was added drop by drop under stirring. The automatic titrator maintained the pH at 7·8 ± 0·1 by addition of sodium hydroxide. The pale yellow reaction mixture was allowed to stand for 2 hr at room temperature and pH 7·8. The solution was then saturated with thiourea and acidified with nitric acid to pH 1–2. The clear and colorless solution was gel filtered through a large column (150 × 4 cm) of Sephadex G-25 (coarse grade) equilibrated with distilled water. Mercaptodextran appeared in the excluded fraction. The silver–thiourea complex was adsorbed to the Sephadex particles, and subsequent regeneration of the column therefore required extensive washing with thiourea–nitric acid solution. To the gel filtered fraction was added 9 vol. of ethanol to precipitate the mercaptodextran. The precipitate initially appeared as a colloidal suspension which was readily broken by the addition of a few drops of concentrated hydrochloric acid. The white, crystalline mercaptodextran was centrifuged down, washed with ethanol and dried in vacuum.

Interaction of mercaptodextran with metal ions: gel filtration and potentiometric titration methods. The interaction of a metal ion with mercaptodextran and other complexing agents (EDTA, penicillamine, cysteamine, etc.) were studied in two different ways. The first method determined the competitive binding of the metal ion to mercaptodextran in the presence of a low molecular weight complexing agent. To a mixture of equivalent amounts of mercaptodextran and the other complexing agent to be tested, were added about 1 mg of the radioactive metal ion (e.g. $^{203}Hg^{2+}$). After 5 min at room temperature and pH 7·4, the metal–mercaptodextran complex was separated from the chelate of low molecular weight by gel filtration on a column of Sephadex G-25 (coarse grade, 50 × 2 cm column). The radioactivity associated with the two different metal complexes was subsequently determined in a liquid scintillation spectrometer (Packard Tri Carb, Model 3310).

The second method for studying the interaction of metal ions with the chelating agents involved potentiometric pH–titration experiments. Upon formation of the metal chelates hydrogen ions are liberated. By means of automatic titration equipment (Radiometer titrator TTT 2, fitted with a Titragraph recorder, Radiometer, Copenhagen) these hydrogen ions were accurately titrated. Standard 0·1 N sodium hydroxide solutions were used. Calibrations using dilute nitric acid of known concentration and ionic strength were carried out. The activity coefficients were found to be constant over the entire pH range used in the present study, and was equal to 0·8. In order to establish values of the stability constants, theoretical titration curves were calculated by computer (CDC-3300) using a program designed and written by Klausen and Rudd.[6] On the basis of the suggested complexes and a corresponding set of chosen equilibrium constants, a theoretical calibration curve was calculated. This curve was then compared with the experimental curve by calculating a root-mean-square sum of errors. The equilibrium constants were then varied to minimize this sum of errors.

Radioprotection experiments. The animals used were 70-day old male mice of the CBA-strain. Their weight was 26·1 ± 0·5 g. Ten min prior to radiation, groups of mice were given intravenous injections of mercaptodextran (mol wt 40,000 and 70,000 which do not penetrate the cellular membrane, but are excreted slowly in the kidneys). For comparison mice were also injected with cysteamine (50, 100 or 150 mg/kg body wt) and with mixtures of cysteamine and mercaptodextran. The mercaptodextrans were administered in the form of 10 per cent solutions in physiological saline (pH = 7·4) and the amount injected varied between 1500 and 4500 mg mercaptodextran per kilogram body weight (~0·2–0·5 mmole SH). The animals were irradiated with 900 röntgen, in a Müller MG 300 X-ray machine operated at 260 kV and 12 mA and fitted with two filters (0·5 mm Cu and 0·5 mm Al). The dose rate was 76 röntgen/min. The observation period following irradiation was 20 days. All radiation experiments were carried out at the Research Institute of National Defence, Stockholm, Sweden.

RESULTS

General properties of mercaptodextrans. Mercaptodextrans synthesized from dextrans with different molecular weight (from 5000 to 500,000) are white, crystalline powders readily soluble in water, and with the same physical properties as the parent dextrans. The viscosity increases with increasing molecular weight. The degree of substitution is approximately 0·1 mmole SH/g of mercaptodextran with only small

variation from batch to batch (measured by amperometric silver titration). The thiol groups immediately form a deep red colour with alkaline sodium nitroprusside. Oxidation of the SH-groups with iodine or with sodium tetrathionate yields viscous solutions due to disulphide aggregation. Subsequent reduction of the disulphide bonds with potassium borohydride or with excess of a thiol (e.g. cysteamine) restores the normal viscosity. Mercaptodextran also readily reacts with disulphides, e.g. cystine, cystamine and oxidized glutathione, partly reducing them to their corresponding thiols and partly forming mixed disulphides. Thus, experiments with ^{35}S-cystamine and mercaptodextran (mol wt 20,000) showed that 20 per cent of the SH-groups of the dextran molecule formed mixed disulphide. The remaining 80 per cent produced an equivalent amount of cysteamine concomitant with the formation of intra- or intermolecular disulphide bonds on the dextran molecules. The thiol–disulphide exchange between mercaptodextran and low molecular weight disulphides is rapid. Thus, the interaction with, e.g. cystine (diethyl ester) is complete in 2 min at room temperature at pH 7·5 (Fig. 1).

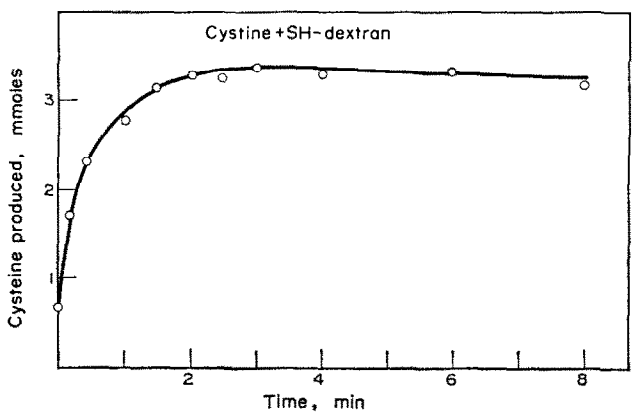

FIG. 1. Rate of thiol–disulphide exchange between mercaptodextran and cystine (diethyl ester). The incubation mixture contained mercaptodextran of mol. wt 20,000 (5·5 mM SH) cystine diethyl ester (2·5 mM), and sodium phosphate buffer (0·1 M, pH 7·4) in a final volume of 1·5 ml. Aliquots (100 μl) were withdrawn at intervals and immediately mixed with 96% ethanol containing 0·03 M HCl (1000 μl). After centrifugation, the thiol content of the supernatant fluid was determined using amperometric silver titration.

Although the reactivity of the SH-groups of mercaptodextran is high, they are comparatively stable towards oxidation by air. Under conditions which oxidize cysteamine in 30 min, the SH-groups of mercaptodextran require about 20 hr for complete oxidation to the corresponding disulphide (Fig. 2). Even in the presence of ferric and cupric ions, which strongly catalyse the autoxidation of thiols of low molecular weight,[7,8] the stability of mercaptodextran was good (Fig. 2). The figure also demonstrates that mercaptodextran "protects" the thiol groups of cysteamine, i.e. the autoxidation of the latter is considerably diminished in the presence of mercaptodextran. Similar results have been obtained with other readily autoxidizable thiols (not shown). Mercaptodextran may consequently be used as a soluble, high molecular weight thiol-protecting agent, in a similar way as dithiothreitol[9] and thiolated Sephadex[10] have been used.

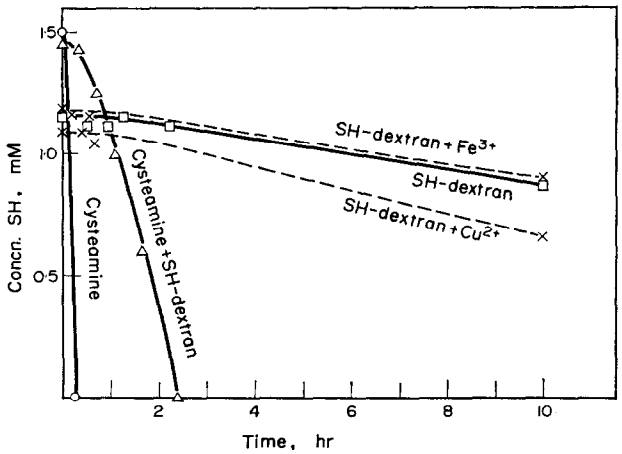

FIG. 2. Stability of mercaptodextran and its effect on the autoxidation of cysteamine. The incubation mixture used for testing the rate of autoxidation of mercaptodextran in the presence and absence of heavy metal ions contained: Mercaptodextran of mol. wt 40,000 (1·2 mM SH) and Na–phosphate (0·1 M, pH 7·4) in a final volume of 3 ml. Some flasks also contained $FeCl_3$ (3×10^{-5} M) or $CuCl_2$ (3×10^{-5} M). Aliquots (100 μl) were withdrawn at intervals and the SH-content was determined by amperometric silver titration. The incubation mixture used to study the stabilizing effect of mercaptodextran on the autoxidation of cysteamine contained: cysteamine (1·5 mM), Na–phosphate (0·1 M, pH 7·4) and mercaptodextran (2 mM SH) in a final volume of 3 ml. Aliquots (100 μl) were withdrawn at intervals, and the SH-content was determined after precipitation of the mercaptodextran as described in Fig. 1.

Failure of mercaptodextran to protect against ionizing radiation. The protection experiments clearly demonstrated that neither mercaptodextran with mol wt 40,000 or 70,000 had any radioprotective effect in all concentrations tested (Table 1). All mercaptodextran-treated animals died after 8–15 days following radiation, like the control animals. The animals which received 50 mg/kg body wt of cysteamine, showed

TABLE 1. RADIOPROTECTIVE EFFECT OF MERCAPTODEXTRAN AND CYSTEAMINE

Protective substance	Amount injected (mg/kg body wt)	Radiation dose (r)	No. animals	Survival* (%)
Mercaptodextran (mol. wt 40,000)	1500	900	5	0
	3000	900	5	0
	4500	900	5	0
Mercaptodextran (mol. wt 70,000)	1500	900	5	0
	3000	900	5	0
	4500	900	5	0
Cysteamine	50	900	5	40
	100	900	5	100
	150	900	5	100
Cysteamine + mercaptodextran (mol. wt 40,000)	50 + 3000	900	5	40
	100 + 3000	900	5	100
	150 + 3000	900	5	100

* Observation period = 20 days. All animals (male mice of the CBA-strain) which did not survive, died 8–15 days following the radiation.

about a 40 per cent survival rate, and cysteamine used in higher doses (100 and 150 mg/kg body wt) gave 100 per cent protection. Mixtures of mercaptodextran and cysteamine had the same protective effect as cysteamine alone. The deaths in the mercaptodextran-treated animals were not due to toxic effects of the compound, since dextran-treated, but non-irradiated animals have survived for months, even after daily injections of more than 2 g of mercaptodextran per kilogram body weight for several weeks.

Affinity of mercaptodextran for metal ions. Preliminary qualitative experiments were carried out using the nitroprusside colour reaction and amperometric silver titration to determine free thiol groups before and after addition of various metal ions. These studies showed that Ag^{+}, Hg^{2+}, Cu^{2+} and Au^{3+} ions were firmly chelated to mercaptodextran. These complexes were so stable that the bound ions could not be removed by treatment with large excess of cysteine, cysteamine, glutathione or EDTA. The Pb^{2+}, Sr^{2+} and Ba^{2+} complexes with mercaptodextran were less stable than those above.

In another series of experiments mercaptides of low molecular weight were made by adding mercuric chloride to glutathione, cysteine, cysteamine, penicillamine and EDTA. Upon addition of excess mercaptodextran to these complexes followed by gel filtration, it was found that the bound mercuric ions were immediately picked up by the SH-groups of the dextran, liberating the free low molecular weight thiol.

The competitive binding of mercuric ions ($^{203}Hg^{2+}$) to mercaptodextran in the presence of equivalent amounts of added penicillamine, glutathione, *N*-acetyl-DL,

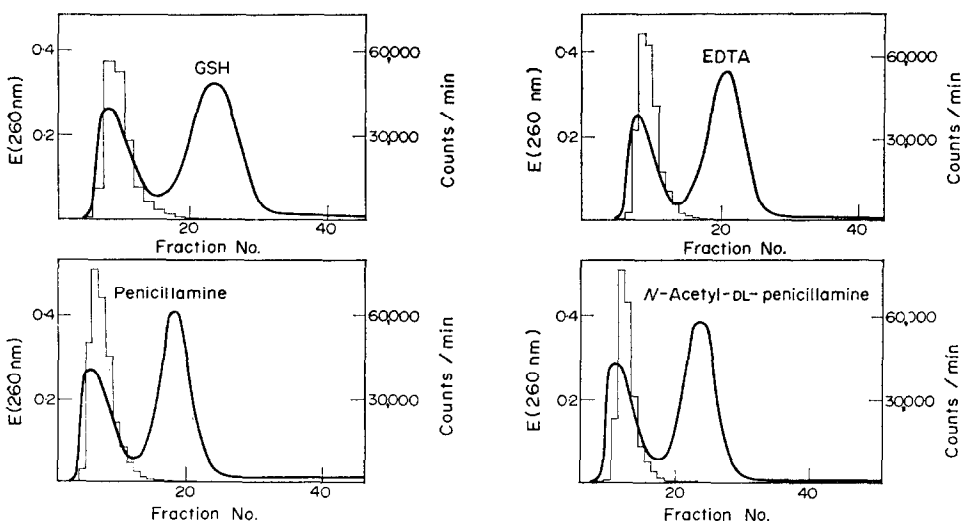

FIG. 3. Competitive binding of mercuric ions to mercaptodextran in presence of chelating agents. The incubation mixture contained: Mercaptodextran of mol. wt 20,000 (5 mM SH), glutathione, penicillamine, *N*-acetyl-DL-penicillamine or EDTA (5 mM) and Na–phosphate buffer (0·1 M, pH 7·4) in a final volume of 2 ml. After addition of $^{203}HgCl_2$ (1 mg in 100 μl of water) and standing for 5 min at room temperature, the mixtures were gel filtered on columns (50 × 2 cm) of Sephadex G-25, coarse grade. The u.v.-absorption of the effluent was continuously monitored using an LKB-Uvicord (LKB, Stockholm, Sweden). Fractions of 4 ml were collected, and the ratioactivity in each fraction was determined in a Packard liquid scintillation counter (Model 3310). Continuous curve = absorption at 260 nm, bar graphs = counts/min. The mercaptodextran and/or mercaptodextran–metal complex appeared in the excluded fractions (first peak).

penicillamine or EDTA is shown in Fig. 3. It is seen that when a mixture of mercaptodextran and any of the latter chelating agents compete for the mercuric ions, the metal ions are exclusively bound to mercaptodextran. Figure 4 (top) on the other hand, shows that when 2,3-dimercaptopropanol (BAL) compete with mercaptodextran, the mercuric ions are bound to both compounds. Moreover, from the data in Fig. 4 it can be calculated that the amount of mercuric ions bound to mercaptodextran very closely equals the amount complexed with BAL. Thus, mercaptodextran and BAL have approximately equal affinity for mercuric ions. It should be noted that Figs. 3 and 4 show results from experiments using mercaptodextran of mol wt 20,000. The same results have been obtained with mercaptodextrans of mol wt 10,000, 40,000, 70,000, 150,000 and 500,000 (not shown).

Experiments similar to those described in Figs. 3 and 4 were carried out using cupric ions ($^{64}CuCl_2$). It was found that the cupric ions, like the mercuric ions, were complexed much stronger to mercaptodextran than to EDTA, penicillamine, 2-mercaptopropionyl glycine ("Thiola"), sodium diethyl dithiocarbamate (Fig. 5) or any of the following other low molecular weight chelating agents (not shown): *N*-acetyl-DL-penicillamine, glutathione, cysteine, cysteamine and dithiothreitol. 2,3-Dimercaptopropanol (BAL) on the other hand, had approximately the same ability to bind cupric ions as mercaptodextran (Fig. 4, bottom).

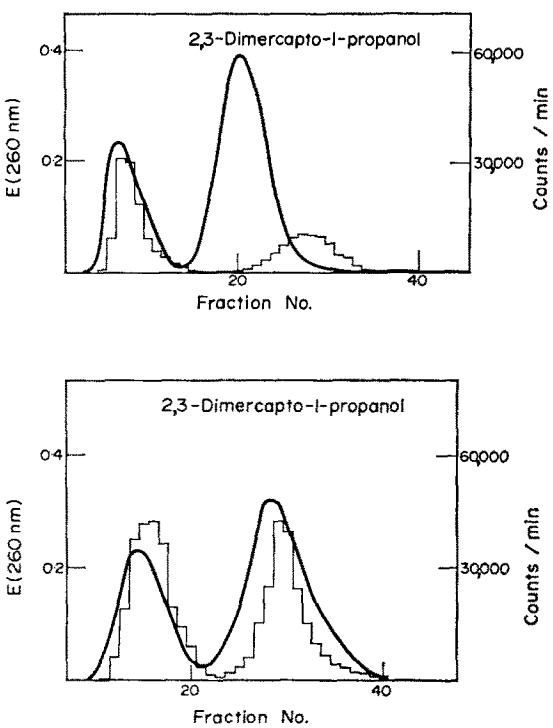

FIG. 4. Competitive binding of mercuric ions (top) and cupric ions (bottom) to mercaptodextran in the presence of 2,3-dimercaptopropanol (BAL). The experimental conditions were the same as in Fig. 3. The BAL–Hg^{2+} complex had distribution coefficient greater than 1 on the Sephadex column, and was therefore eluted after the "salt fraction" including free (excess) of BAL.

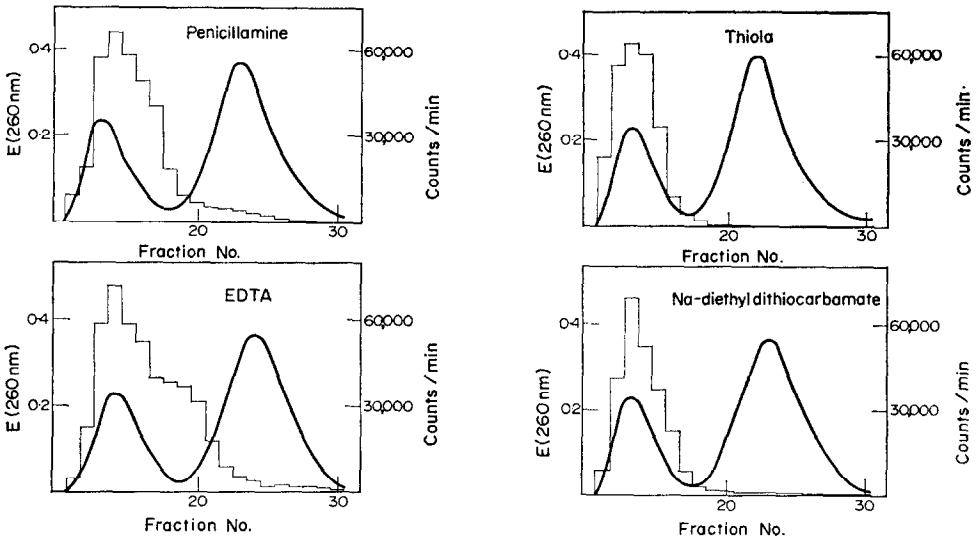

FIG. 5. Competitive binding of cupric ions to mercaptodextran in the presence of penicillamine 2-mercaptopropionylglycine ("Thiola"), EDTA or Na–diethyl dithiocarbamate. The experimental conditions were the same as in Fig. 3. except that $^{64}CuCl_2$ (1 mg) was added instead of mercuric ions.

Figure 6 shows some typical experiments where the binding of Zn^{2+}, Co^{2+}, Cd^{2+} and Sr^{2+} ions to mercaptodextran in the presence of other chelating agents have been studied. Co^{2+} ions had approximately the same affinity for BAL as for mercaptodextran, whereas Cd^{2+} showed greater affinity for BAL (Fig. 6). Zinc ions were exclusively bound to mercaptodextran in the presence of penicillamine, whereas the affinity of strontium ions was greater for EDTA than for mercaptodextran. Also ferric

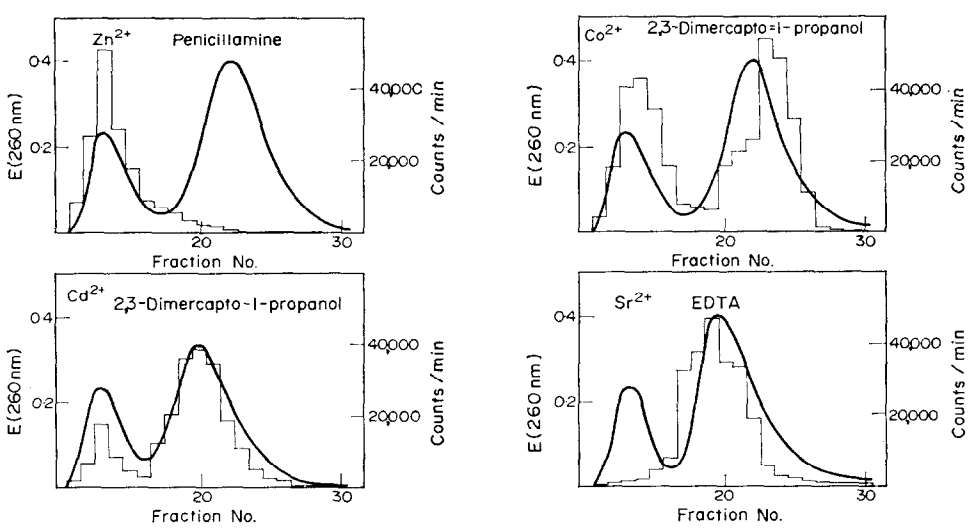

FIG. 6. Competitive binding of zinc, cobalt, cadmium and strontium ions to mercaptodextran in the presence of penicillamine, 2,3-dimercaptopropanol (BAL) or EDTA. The experimental conditions were similar to those described in Fig. 3.

and chromic ions had greater tendency to form complexes with EDTA than with mercaptodextran.

Stability constants. Potentiometric pH–titrations were carried out to determine the stability constants of the Hg^{2+}-mercaptodextran complex. Also the Hg^{2+}–BAL and Hg^{2+}–penicillamine complexes were titrated for comparison. From the inflexion points on the titration curves it was inferred that Hg^{2+} and mercaptodextran preferentially formed a 1:1 (molar ratio) complex, but also 1:2 and 2:1 complexes were present. The computer program calculated the following values for the stability constants of the 1:1 complexes:

$$\frac{[\text{Hg–S–dextran}]}{[Hg^{2+}]\,[\text{SH–dextran}]} = 10^{20\pm1}$$

$$\frac{[\text{Hg–BAL}]}{[Hg^{2+}]\,[\text{BAL}]} = 10^{20\pm0\cdot3}$$

$$\frac{[\text{Hg–penicillamine}]}{[Hg^{2+}]\,[\text{penicillamine}]} = 10^{18\pm0\cdot3}$$

(cf. Kuchinskas and Rosen[11] who found a value of 17·5 for the same complex)

Thus, the stability constants of the mercaptodextran–mercury and the BAL–mercury complexes had the same value (approximately 10^{20}), and about 100-times higher than the Hg–penicillamine complex. With regard to stability constants and metal chelates in general, we refer to an excellent monograph by Catsch.[12]

DISCUSSION

The present results show that thiolation of dextrans readily yields soluble polythiols. Depending upon the molecular weight of the dextran used as starting material, mercaptodextrans with average molecular weight varying from 5000 to 500,000 may be prepared. Gel filtrations studies have shown that only a small degree of hydrolysis of the dextran molecules takes place during the synthesis of mercaptodextran. The thiolated compounds have retained most of the properties of the parent dextrans, and show in addition some characteristic features due to the introduced thiol groups. Firstly, the oxidation potential of the SH-groups is high as judged from their ability to interact with disulphides, i.e. the mercaptodextrans are efficient electron donating agents which readily reduce disulphides of low molecular weight. This property, combined with a low tendency to autoxidize in air, make the compounds suitable as thiol-stabilizing agents.

The second and probably most important feature is the high affinity of mercaptodextran for metal ions, particularly Ag^{+}, Hg^{2+} and Cu^{2+}. Of all chelating agents tested (including several thiols of low molecular weight and EDTA) only 2,3-dimercaptopropanol (BAL)[13] has comparable affinity for these ions. Thus, the affinity of mercuric ions for mercaptodextran and BAL, is about 100-times higher than for penicillamine. Moreover, it is evident from animal experiments[14] that mercaptodextran, like the parent dextrans (which may be used as plasma substitutes), is of exceptionally low toxicity. In this context it should be mentioned that less toxic derivatives of BAL, i.e. BAL–glycosides, are known,[15] and also 2,3-dimercaptopropane sulponic acid is a compound with low toxicity.[16,17] The high metal-binding

ability of mercaptodextran together with its low toxicity, suggest that this polythiol may be of use in *in vitro* and *in vivo* metal-detoxification problems. Studies on the application of mercaptodextran in the treatment of acute metal poisoning of animals will be published elsewhere.[14]

The idea of using a high molecular weight, reactive thiol as a radio-scavenger circulating in the blood stream only, gave negative results. Thus, mercaptodextrans with mol wt 40,000 and 70,000 which circulate in the blood for a couple of hours, gave no protection against lethal doses of X-rays. We have also tried mercaptodextrans with lower as well as higher molecular weights, in the presence and absence of small amounts of cysteamine which can be shuffled in and out of the cells, but with negative results. Thus, it is quite clear as pointed out by several authors,[4,18] that in order for the thiol-containing compound to exert protective effect against ionizing radiation, it must be able to penetrate into the cells.

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