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INTERACTIONS OF DISULFIRAM AND DIETHYLDITHIOCARBAMATE WITH SERUM PROTEINS STUDIED BY MEANS OF A GEL-FILTRATION TECHNIQUE

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Abstract—Tetraethylthiuram disulphide (disulfiram) and the corresponding thiol diethyldithiocarbamate in concentrations from 10^{-6} to 10^{-4} M have been quantitatively separated by means of gel filtration on a Sephadex G-25 column, using ^{35}S-labelled compounds. Both the thiol and the disulphide possess distribution coefficients (K_d) above unity, showing that they are adsorbed to the gel material. The K_d of the disulphide (2·65) was considerably higher than that of the thiol (1·26). Quantitative recovery of the strong metal-complexing diethyldithiocarbamate was obtained only when the buffer used were freed of complexing metal ions by pretreatment with diphenylthiocarbazone.

The interactions of diethyldithiocarbamate and disulfiram with serum proteins were studied by the gel filtration technique. The thiol was shown only to become loosely and reversibly adsorbed to the proteins. The disulphide reacted with the albumin fraction of the serum proteins by mixed disulphide formation with their—SH groups in an amount which corresponded to the known amount of titrable -SH groups of albumin.

DITHIOCARBAMATES and thiuram disulphides find a variety of applications as fungicides, bactericides and insecticides both in agriculture and clinical medicine.[1] Furthermore, diethyldithiocarbamate, because of its strong metal chelating properties, has been used in the treatment of nickel poisoning,[2] and of the copper accumulation of the hepatolenticular degeneration.[3] Various dithiocarbamates such as diethyldithiocarbamate, also demonstrate protective properties as to the deleterious action of X-rays.[4, 5]

Tetraethylthiuram disulphide (disulfiram) has been used in the treatment of alcoholism for the past 15 years, particularly in Scandinavian and other European countries. This therapeutic use is based on the observation by Hald *et al.*[6, 7] that disulfiram renders human sensitive to ethyl alchohol. Despite extensive studies, the mechanism underlying the ethanol-sensitizing effect of disulfiram is still a matter of discussion.[8] One reason for this may be that our knowledge regarding its *in vivo* metabolism is scarce. To obtain more detailed information, however, new methods for the separate determination of disulfiram and its metabolites seem to be required.

Dithiocarbamates and thiuram disulphides when present in protein-free solutions, are conveniently measured on the basis of their decomposition by hot mineral acids to the amine and carbon disulphide. The amount of either of these products may be estimated by various methods.[1] In studies of the metabolism of disulfiram, however, these methods proved unsuitable, first because they do not allow a determination of

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the disulphide and the parent thiol separately, second because the presence of proteins in the samples makes the measurements inaccurate.

Domar *et al.*[9] described a spectrophotometric method for the simultaneous determination of diethyldithiocarbamate and disulfiram by their reaction with cupric and cuprous ions, respectively. When the samples contained proteins, we found this method as well as a modification of it recently described by Åkerström and Lindahl,[10] to be rather inaccurate. Thus, the presence of proteins led to a varying and incomplete extraction of the compounds to be measured. A partial removal of the protein interference by pretreatment of the samples with pancreatin or Tween 20 was reported by Linderholm and Berg.[11] By this modification they claimed to determine quantitatively disulfiram in blood and plasma, and diethyldithiocarbamate in plasma. However, in the hands of the author, these results could not be reproduced. Similarly, Divatia *et al.*[12] were unable to extract disulfiram quantitatively from whole blood and plasma, but upon the addition of cupric ions they managed to extract and estimate disulfiram as a copper complex. No separate determination of the thiol and the disulphide was obtained by their method.

In the present paper we report on a quantitative separation of disulfiram, diethyldithiocarbamate and proteins by means of Sephadex G-25 gel filtration, and the use of this procedure in studies on the interactions of this thiol and disulphide with proteins. In a forthcoming paper the application of the separation procedure in studies of the catabolism of diethyldithiocarbamate and disulfiram in rats will be presented.[13]

EXPERIMENTAL

Materials

Sephadex G-25, particle size 100–270 mesh (block polymerized), was purchased from AB Pharmacia, Uppsala, Sweden. ^{35}S-labelled sodium diethyldithiocarbamate was obtained from The Radiochemical Centre, Amersham, Bucks., England. This product was further purified by recrystallization from ethyl acetate. ^{35}S-labelled disulfiram (tetraethylthiuram disulphide) was prepared by oxidizing radioactive diethyldithiocarbamate with alcoholic iodine as previously described.[8] Scintillators and 1 M hyamine hydroxide in methanol were obtained from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. Other chemicals used were commercial products of high purity.

10^{-2} M EDTA buffer, adjusted to pH 8·5 by NaOH, was used throughout as solvent and as elution buffer. To remove traces of complexing metal ions, the buffer was extracted twice with approximately 0·1 % diphenylthiocarbazone in chloroform, and then three times with pure chloroform. The chloroform left after the final washing was distilled off at low pressure.

Disulfiram and diethyldithiocarbamate in concentrations ranging from about 10^{-6} to 10^{-4} M were used in the experiments. In contrast to the thiol, the disulphide is only slightly soluble in water. Buffer solutions of this compound in concentrations below the saturation range (about 10^{-4} M) were prepared by adding 50 μl of an alcoholic solution of disulfiram to 10 ml of the buffer. This small amount of ethanol did not alter the gel-filtration properties.

Gel-filtration procedure

Highly cross-linked dextran, Sephadex G-25, was used for separation of proteins, diethyldithiocarbamate and disulfiram. Carefully packed columns (1·5 × 17-cm) with

a bed volume of about 30 ml were usually employed. The gel material was thoroughly washed with the metal-free elution buffer prior to use. Four ml of a sample containing the solutes to be separated was introduced into the column, and effluent fractions of 3 or 5 ml were collected by the aid of an automatic fraction collector. The filtration was carried out at room temperature, the elution rate being approximately 1·2 ml per min.

The amounts of disulfiram and diethyldithiocarbamate in the protein-free fractions may be estimated by any of the methods available for their determination in pure solutions (see Introduction). Throughout the present study, ^{35}S-labelled compounds have been used, and the amounts measured by radioisotope technique. An advantage of this technique, beside its high sensitivity, is that it permits a direct determination of the amount of sulphur which becomes attached to the proteins.

Sample preparations and counting procedure

The radioactivity was assayed in a Tri Carb Liquid Scintillation spectrometer (Packard Instrument Co. Inc.). The scintillation solvent used consisted of: 0·5 g 2,5-diphenyloxazole (PPO); 0·05 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene (POPOP); 80·0 g naphtalene (scintillation grade); 385 ml xylene; 385 ml dioxane; and 250 ml absolute ethanol.[14] As a rule the sample preparations contained 0·5 mg water in 10 ml scintillation mixture. Under these conditions the absolute counting efficiency was approximately 55%. Protein-containing counting samples were prepared by initially dissolving the proteins in 1 M methanolic hyamine hydroxide, followed by the addition of the scintillation mixture. Corrections for a reduced counting efficiency when proteins were present, were made by the internal standard technique.

Identification of diethyldithiocarbamate and disulfiram

Diethyldithiocarbamate (concentrations below 10^{-4} M) in the chromatographic eluate was identified by the following criteria: (1) Upon acidification with dilute HCl to pH 3–4 at room temperature, the radioactivity could be removed by bubbling air through the sample. The thiol thus is highly acid-labile, being decomposed to diethylamine and the volatile carbon disulphide. With the low concentrations of diethyldithiocarbamate regularly used, complete removal was obtained by this procedure only after the addition of diethyldithiocarbamate carrier. (2) The radioactivity released by acidification and aeration could be quantitatively trapped in 2 N KOH in methanol, a solution in which carbon disulphide reacts by forming methylxanthate.[1] (3) Addition of cupric ions made the radioactive diethyldithiocarbamate extractable with chloroform due to the formation of the apolar copper complex.

For the identification of disulfiram (concentrations below 10^{-4} M) the following criteria were used: (1) Only a few per cent of the radioactivity could be removed on acidification to pH 3–4, in agreement with the higher acid-stability of the disulphide than of the parent thiol. (2) Complete volatilization of the radioactivity was obtained after the addition of diethyldithiocarbamate carrier. This criterion is based on the fact that the radioactive disulphide undergoes instantaneous exchange reactions with the much greater amount of carrier thiol. The radioactivity in non-decomposed disulfiram remaining after acidification therefore is negligible. (3) The radioactive disulfiram was directly extractable with chloroform.

The above tests were performed to ascertain that diethyldithiocarbamate and

disulfiram were eluted from the Sephadex column unchanged, and at different elution volumes. Later we considered it sufficient as a routine to carry out the following identification test in the case of both the thiol and the disulphide: 2 ml of the eluate, to which 20 μmoles diethyldithiocarbamate carrier had been added, was acidified by an equal volume of $2{\cdot}5 \times 10^{-2}$ M HCl (to pH 3–4). After 1 min the CS_2 formed by the decomposition was removed by bubbling water-saturated air through the solution for 3 min at room temperature. When only ^{35}S-labelled diethyldithiocarbamate and disulfiram were present, complete volatilization of the radioactivity was obtained by this procedure.

RESULTS AND DISCUSSION

Separation of diethyldithiocarbamate and disulfiram by gel filtration

Figure 1. A shows that under the conditions described disulfiram appeared in the effluent from the Sephadex G-25 column after a larger volume than the corresponding thiol, diethyldithiocarbamate, and Fig. 1, B shows that these compounds when present in a mixture, were separated by this filtration. Identification tests demonstrated that both compounds were recovered unchanged.

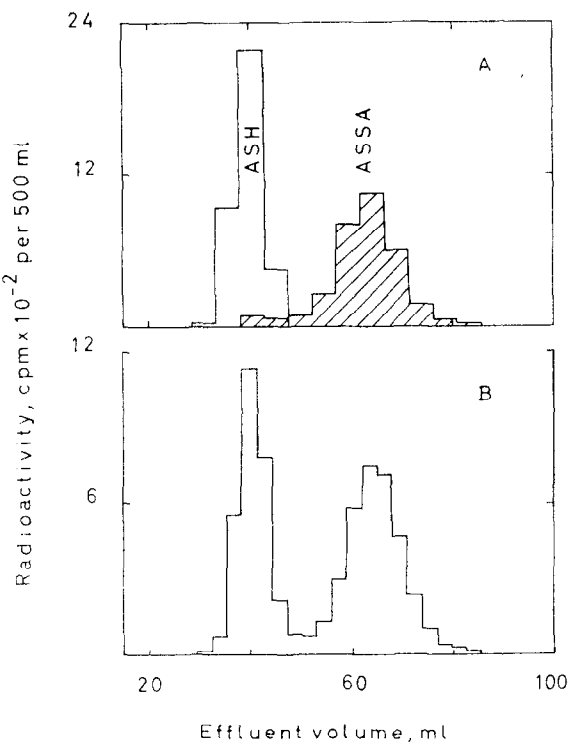

FIG. 1. Separation of diethyldithiocarbamate (ASH) and disulfiram (ASSA) on a Sephadex G-25 column. The ^{35}S-labelled compounds were dissolved in 10^{-2}M metal-free EDTA buffer.

A. Radioactive elution pattern obtained with a solution of ASH ($3{\cdot}50 \times 10^{-5}$ M) or of ASSA ($1{\cdot}55 \times 10^{-5}$ M). Recoveries: ASH (open columns), 98·5%; ASSA (hatched columns), 97·5%.

B. Radioactive elution pattern obtained with a mixture of ASH ($1{\cdot}70 \times 10^{-5}$ M) and ASSA ($1{\cdot}05 \times 10^{-5}$ M) of the same specific activity. Recoveries: ASH 100·1%; ASSA, 92·5%.

The separation of the low molecular weight disulfiram and diethyldithiocarbamate cannot be explained by the molecular sieve properties of the gel matrix used. However, it is known that substances may interact with the Sephadex material, thus showing a behavior differing from that which might be expected from their molecular size only.[15] The distribution coefficients (K_d) for these solutes are greater than unity. The K_d-value for diethyldithiocarbamate was found to be 1·26 and for disulfiram 2·65, as determined from the formula:

$$K_d = \frac{V_e - V_0}{a.W_r}$$

where V_e is the elution volume, V_0 the void volume, a the weight in g of the dry gel, and W_r the water regain.[16] Thus, not only disulfiram but also diethyldithiocarbamate showed evidence of adsorption to the gel material, although the former to a much larger extent. On the small column used in the experiments of Fig. 1, diethyldithiocarbamate will not be separated from low molecular weight compounds with unrestricted diffusibility (K_d-values about 0·7–0·8[15]). However, this may be obtained by increasing the bed volume of the column to about 60 ml (Fig. 2).

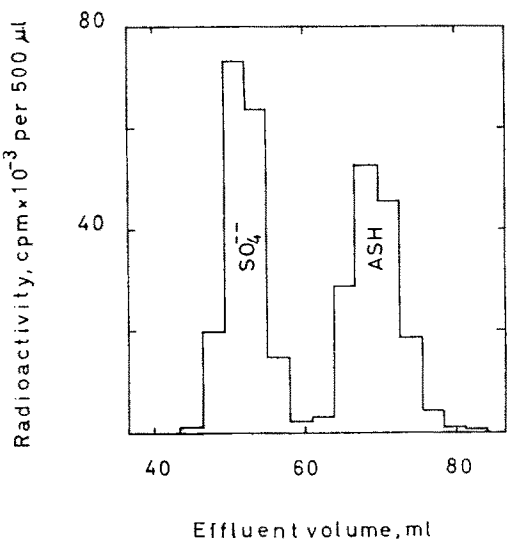

FIG. 2. Separation of diethyldithiocarbamate (ASH) and sulphate on a Sephadex G-25 column (1·5 × 35-cm). The ^{35}S-labelled ASH and sulphate were dissolved in 10^{-2} M metal-free EDTA buffer. Recoveries: SO_4^{--}, 106%; ASH, 92%.

It was noticed in preliminary tests that when employing diethyldithiocarbamate in concentrations as small as 10^{-4}M or below, the relative recovery of the radioactivity from the column decreased with decreasing amounts of the thiol introduced. This phenomenon, however, was absent when a metal-free buffer was used. Obviously the traces of complexing metal ions originally present in the buffer interfered with the flow of small amounts of diethyldithiocarbamate, probably by forming chelates with the thiol. In accordance with this interpretation, chelates of diethyldithiocarbamate with copper, iron, manganese, and zinc were found to adhere firmly to the top of the

Sephadex column. When using the metal-free buffer, the mean recovery in 9 experiments with diethyldithiocarbamate and 8 experiments with disulfiram, in the concentration range from 5×10^{-7}M to 4×10^{-5} M, was 94·2% (range 86–100%) and 95·5% (range 90–99%), respectively. The recovery percentage was independent of the concentrations used.

Interaction of diethyldithiocarbamate with serum proteins

As pointed out above, proteins seriously interfere in earlier methods for the assay of diethyldithiocarbamate and disulfiram indicating that an interaction between these compounds and proteins takes place. We have therefore studied this interaction by means of the gel filtration technique. Diethyldithiocarbamate or disulfiram was dissolved in human serum diluted with the metal-free EDTA buffer. After 15 min of incubation at room temperature, an aliquot of the solution was subjected to gel filtration.

Fig. 3,A shows that when ^{35}S-labelled diethyldithiocarbamate had been added to

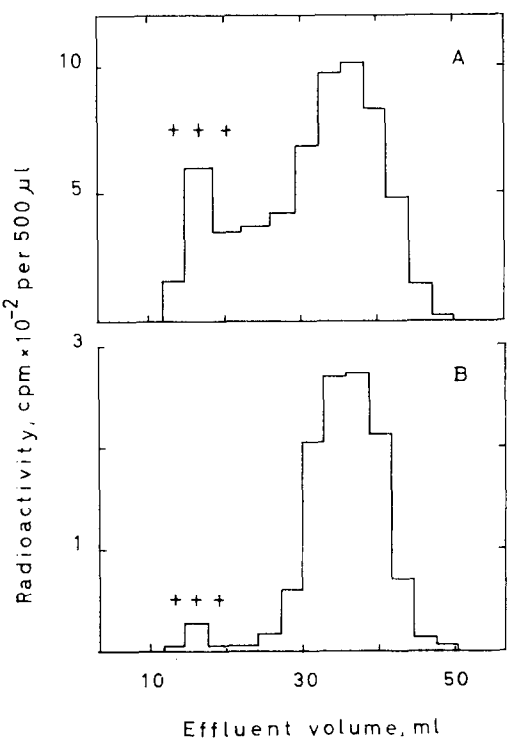

FIG. 3. Separation of diethyldithiocarbamate (ASH) and serum proteins on a Sephadex G-25 column. The ^{35}S-labelled ASH was dissolved in human serum diluted with 10^{-2} M metal-free EDTA buffer.

A. Radioactive elution pattern obtained with a solution of $4{\cdot}05 \times 10^{-5}$ M ASH in serum diluted 3 times (protein conc.: 2·0 g/100 ml). Radioactivity recovered: 99·5%, of which 14·5% in the fractions containing proteins.

B. Radioactive elution pattern obtained with a solution of $7{\cdot}6 \times 10^{-6}$ M ASH in serum diluted 6 times (protein conc.: 1·0 g/100 ml). Radioactivity recovered: 92·0%, of which 3·4% in the fractions containing proteins.

+ + + indicates the protein-containing fractions.

serum diluted with buffer in the ratio 1:3, a broad radioactive peak was obtained upon gel filtration. The separation of the radioactivity from the proteins was incomplete, as much as 14·5% being recovered in the fractions containing proteins. This incomplete separation, despite widely different distribution coefficients, suggested that the thiol becomes loosely attached to the serum proteins, and that a gradual release of the radioactivity takes place during the passage through the column. This interpretation was supported by the finding that a renewed filtration of the protein-containing fraction led to a further release of diethyldithiocarbamate, resulting in no more than 4·5% of the original radioactivity being eluted together with the proteins. It should be noted, however, that a nearly complete separation of the radioactive thiol from the proteins was obtained by only one run through the column provided that the serum dilution was increased to the ratio 1:6 (Fig. 3,B).

The reversible interaction between diethyldithiocarbamate and proteins was conveniently studied by the Sephadex filtration procedure described by Hummel and Dreyer.[17] The inset of Fig. 4 demonstrates the radioactive profile obtained when 2 ml serum diluted in the ratio 1:2 in the presence of the thiol had been subjected to gel filtration. The peak gives directly the amount of radioactivity bound to the proteins. This amount corresponds to that of the free thiol removed, the latter appearing as the following "trough". Figure 4 demonstrates that as the concentration of diethyldithiocarbamate was increased in the range from 10^{-5}M to 4×10^{-4}M, increasing amounts of the thiol became attached to the serum proteins.

Linderholm and Berg[11] have previously pointed out on the basis of extraction experiments that diethyldithiocarbamate appears to be adsorbed to proteins. Our data

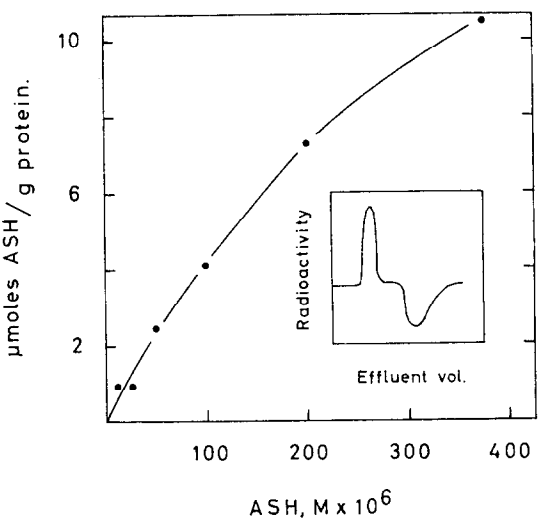

FIG. 4. Amounts of radioactive sulphur adsorbed to the serum proteins with increasing concentrations of ^{35}S-labelled diethyldithiocarbamate (ASH). ASH in varying amounts was added to serum diluted with an equal volume of 10^{-2} M metal free EDTA buffer (protein conc.: 3·6 g/100 ml). Two ml of this solution was then subjected to gel filtration on a column (1·5 × 20–cm) which beforehand had been equilibrated with an elution buffer (10^{-2} M EDTA) containing ASH in the same concentrations as that of the serum dilutions introduced. The inset shows the typical elution profile obtained (plot from experiment with 5×10^{-5} M ASH).

confirm their assumption. Moreover, repeated runs through the Sephadex column of the protein-containing fraction, brought the amount of radioactive sulphur which was eluted with the macromolecules down to a negligible amount. Consequently, no evidence was obtained suggesting a spontaneously irreversible interaction between diethyldithiocarbamate and serum proteins. Such interaction might have been a possibility knowing that several other thiols under certain conditions form mixed disulphides via exchange reactions with disulphide bounds of proteins.[18-20]

Interaction of disulfiram with serum proteins

Gel filtration of serum dilutions after ^{35}S-labelled disulfiram had been added revealed elution patterns quite different from those obtained in similar experiments with diethyldithiocarbamate. With a solution of $3{\cdot}4 \times 10^{-5}$M disulfiram in serum diluted 20 times with the EDTA buffer, three distinct radioactive peaks were obtained (Fig. 5, A). The first peak was localized to the protein-containing fractions. The radioactive sulphur of the second and the third peak were *in toto* identified as diethyldithiocarbamate and disulfiram, respectively. When decreasing the serum dilution, i.e. increasing the serum concentration of the solvent and keeping the amounts of disulfiram constant, the disulfiram peak decreased and the two other peaks increased correspondingly.

The radioactive sulphur eluted from the column together with the proteins was in this case firmly anchored to the macromolecules. Thus, a renewed filtration of this fraction led to only insignificant liberation of the radioactivity. On the other hand, when the protein-containing fraction were preincubated with $2{\cdot}5 \times 10^{-3}$M GSH for $\frac{1}{2}$ hr at room temperature, a nearly quantitative release of the radioactivity occurred. The ^{35}S liberated was identified as diethyldithiocarbamate (Fig. 5,B). This observation indicates that the radioactivity was attached to the proteins by a mixed disulphide linkage to their -SH groups. The thiol-disulphide exchange reaction which apparently has taken place corresponds to the reactions previously observed between several cystamine derivatives and a number of proteins:[21, 22]

$$\text{Protein—SH} + \text{ASSA} \rightarrow \text{Protein—S—SA} + \text{ASH} \qquad (1)$$

TABLE 1. INTERACTION OF DISULFIRAM (ASSA) WITH SERUM PROTEINS*

Protein conc. (g/100 ml)	ASSA added (μM)	^{35}S recovered (%)†			ASSA interacted (m-μmoles/ml serum)
		Prot. bound	ASH‡	ASSA	
1·12	45	47·3	43·4	4·4	279
0·56	48	37·1	37·6	25·3	434
0·33	34	35·7	38·9	26·3	443

* ASSA was dissolved in human serum diluted with the metal-free EDTA buffer. The various ^{35}S-solutes formed were separated on a Sephadex G-25 column.

† per cent of total ^{35}S.

‡ diethyldithiocarbamate.

Table 1 shows that upon the addition of disulfiram to serum under various conditions, the same amount of radioactivity was recovered in the form of diethyldithio-

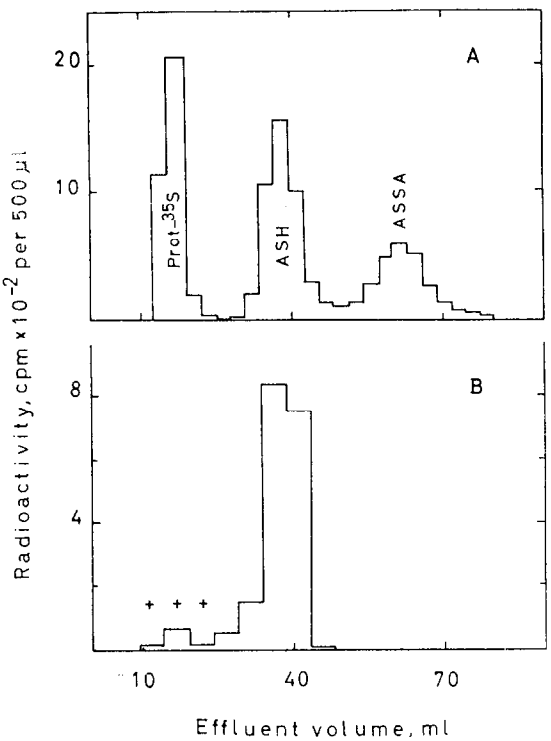

FIG. 5.

A. Separation of disulfiram (ASSA), diethyldithiocarbamate (ASH) and protein—^{35}S on a Sephadex G-25 column. ASSA (final conc.: $3{\cdot}4 \times 10^{-5}$ M) was dissolved in serum diluted 20 times with the EDTA buffer. Protein concentration was 0·33 g/100 ml. Radioactivity recovered: 94·0% of which 35·7% in the protein-containing fractions, 38·0% in the ASH-containing fractions, and 26·3% in the ASSA-containing fractions.

B. Liberation of A^{35}SH from protein—^{35}S by GSH. The protein-containing fractions obtained in the experiment of Fig. 5, A were combined and the solution incubated with $2{\cdot}5 \times 10^{-3}$ M GSH for ½ hr at room temperature. The solution was then subjected to renewed filtration on a Sephadex G-25 column. Radioactivity recovered: 99·5% of which 5·8% in the protein-containing fractions. The ^{35}S separated from the proteins was identified as ASH.

+ indicates the protein-containing fractions.

carbamate as that which was found linked to the proteins. This stoicheiometrical agreement is in accordance with equation (1), and adds further support to the conclusion that disulfiram forms mixed disulphides with protein-SH groups. Recently Owens and Rubinstein[23] have shown that the disulfiram analogue, tetramethylthiuram disulphide, gives exchange reactions with the —SH group of coenzyme A, but that no mixed disulphide can be isolated. Neither did various other thiols of low molecular weight give rise to detectable amounts of mixed disulphides with tetramethylthiuram disulphide, the final product of the reactions being the corresponding disulphide of the parent thiol and dimethyldithiocarbamate.

Figure 6 shows that the serum-protein bound sulphur was attached exclusively to the albumin fraction. This is in agreement with the fact that nearly all reactive —SH groups of serum proteins are localized to this fraction. The content of free —SH groups of human albumin, as determined by various methods, is 0·68 mole per mole

albumin.[24] Knowing that the serum concentration of albumin is about 4·2 g/100 ml and the molecular weight of this protein is about 66,000,[24] the amount of free —SH groups can be calculated to be approximately 440 μmoles per ml serum. Table 1 shows that this corresponds remarkably well with the amount of disulfiram which was found

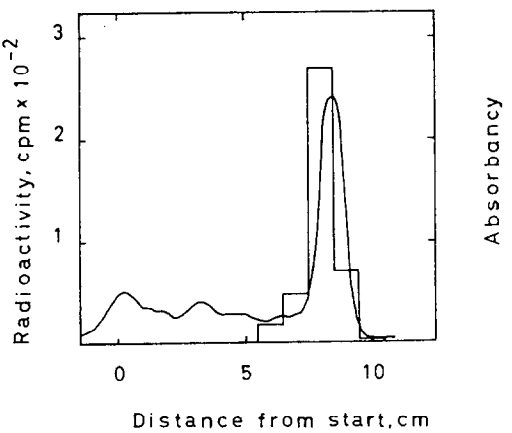

FIG. 6. The electrophoretic pattern of the protein—^{35}S formed by the addition of labelled disulfiram to diluted serum. The protein—^{35}S isolated by gel filtration was freeze-dried to a small volume (suitable protein concentration) and subjected to low-voltage electrophoresis. The distribution of radioactivity of one of two parallel strips was assayed in pieces of 1 cm in vials containing 5 ml of scintillation mixture. The other strip was stained with amido-schwartz and scanned in a Spinco Analytrol scanner (the curve).

to interact with the serum proteins. The finding that a complete blockage of the protein —SH groups is obtained with slight excess of disulfiram indicates that the equilibrium of reaction (1) is displaced far toward the right side. This fact is in accordance with the relatively high redox potential of disulfiram.[25]

On the basis of these results it is warranted to conclude that disulfiram interacts with proteins via mixed disulphide formation on —SH groups. Diethyldithiocarbamate on the other hand does not become chemically bound to proteins, but is only loosely adsorbed to them. When proteins possess prosthetic groups, however, other interactions may also take place. Thus it has previously been shown that the heme group of cytochrome c and hemoglobin may react with both disulfiram and diethyldithiocarbamate.[8, 26, 27]

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REFERENCES

1. G. D. THORN and R. A. LUDWIG, *The Dithiocarbamates and Related Compounds*. Elsevier, Amsterdam—New York (1962).
2. F. W. SUNDERMAN and F. W. SUNDERMAN, Jr., *Amer. J. med. Sci.* **236**, 26 (1958).
3. F. W. SUNDERMAN, Jr., J. C. WHITE, and F. W. SUNDERMAN, *Amer. J. Med.* **34**, 875 (1963).
4. Z. M. BACQ, A. HERVE and P. FISCHER, *Bull. Acad. Roy. Med. Belg.* **18**, 226 (1953).
5. D. W. VAN BEKKUM, *Acta physiol. pharmacol. neerl.* **4**, 508 (1956).
6. J. HALD, and E. JACOBSEN, *Lancet*, **255**, 1001 (1948).
7. J. HALD, E. JACOBSEN and V. LARSEN, *Acta pharm. tox., Kbh.* **4**, 285 (1948).

8. J. H. STRÖMME, *Biochem. Pharmacol.* **12**, 157 (1963).
9. G. DOMAR, A. FREDGA and H. LINDERHOLM, *Acta chem. scand.* **3**, 1441 (1949).
10. S. ÅKERSTRÖM and P. E. B. LINDAHL, *Acta chem. scand.* **16**, 1206 (1962).
11. H. LINDERHOLM and K. BERG, *Scand. J. clin. lab. Invest.* **3**, 96 (1951).
12. K. J. DIVATIA, C. H. HINE and T. N. BURBRIDGE, *J. lab. clin. Med.* **39**, 974 (1952).
13. J. H. STRÖMME, *Biochem. Pharmacol.* **14**, 390 (1965).
14. H. A. VODOPICK and R. M. KNISELEY, *J. lab. clin. Med.* **62**, 109 (1963).
15. B. GELOTTE, *J. Chromatog.* **3**, 330 (1960).
16. P. FLODIN. *Dextran Gels and their Applications in Gel Filtration*, p. 35. Dissertation, Uppsala, Sweden. (1962).
17. J. P. HUMMEL and W. J. DREYER, *Biochim. Biophys. Acta*, **63** ,530 (1962).
18. E. KATCHALSKI, G. S. BENJAMIN and V. GROSS, *J. Amer. chem. Soc.* **79**, 4096 (1957).
19. P. ALEXANDER and L. D. G. HAMILTON, *Arch. Biochem. Biophys.* **88**, 128 (1960).
20. L. LIBENSON and M. JENA, *Arch. Biochem. biophys.* **100**, 441 (1963).
21. L. ELDJARN and A. PIHL, *J. biol. Chem.* **223**, 341 (1956).
22. L. ELDJARN and A. PIHL. *On the Mechanism of Chemical Protection against Ionising Radiation, Progress in Radiobiology*, p. 249. Oliver and Boyd, London (1956).
23. R. G. OWENS and J. H. RUBINSTEIN, *Contr. Boyce Thompson Inst.* **22**, 241 (1964).
24. R. CECIL and J. R. MCPHEE, *Advanc. Protein Chem.* **14**, 255 (1959).
25. E. C. GREGG and W. P. TYLER, *J. Amer. chem. Soc.* **72**, 4561 (1950).
26. D. KEILIN and E. F. HARTREE, *Proc. roy. soc., Lond.* **B 129**, 277 (1940).
27. J. H. STRÖMME, *Biochem. Pharmacol.* **12**, 937 (1963).