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METABOLISM OF DISULFIRAM AND DIETHYLDITHIOCARBAMATE IN RATS WITH DEMONSTRATION OF AN *IN VIVO* ETHANOL-INDUCED INHIBITION OF THE GLUCURONIC ACID CONJUGATION OF THE THIOL*

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Abstract—The metabolism of tetraethylthiuram disulphide (disulfiram) and the corresponding thiol, diethyldithiocarbamate, has been studied in rats, using ^{35}S-labelled compounds. New analytical methods have been worked out. Four low molecular weight metabolites of disulfiram were demonstrated: diethyldithiocarbamate, the S-glucuronide of diethyldithiocarbamate, inorganic sulphate, and carbon disulphide. In addition a small fraction of the radioactive sulphur was found bound to proteins as mixed disulphides. After the intraperitoneal injection of 10 mg disulfiram at most 8% of the titrable –SH groups of plasma proteins and only about 0·1 to 0·2% of the –SH groups of the soluble proteins of liver were found to be blocked. Free disulfiram was never detected in plasma, liver or in urine, indicating that this compound *in vivo* immediately undergoes chemical reactions which ultimately lead to its reduction to the parent thiol diethyldithiocarbamate. The thiol thus formed appeared to be further metabolized at a high rate along several pathways: (1) conjugation with glucuronic acid; (2) oxidation to sulphate; (3) decomposition to carbon disulphide and diethylamine; (4) reoxidation to disulfiram. Evidence was obtained indicating that ethanol lowers the rate by which diethyldithiocarbamate is conjugated with glucuronic acid. The results are discussed in relation to the ethanol sensitizing effect of disulfiram.

IN 1948 disulfiram was introduced by Hald, Jacobsen and Larsen as an adjuvant in the treatment of chronic alcoholism.[1] However, in spite of extensive studies on the disulfiram-ethanol reaction its mechanism is still obscure.

Disulfiram has been proposed to act in two principally different ways. The first possibility is that the drug[2–4] or one of its metabolites (diethyldithiocarbamate,[5, 6] carbon disulphide[7, 8]) interferes with the normal metabolism of ethanol, and thus gives rise to an accumulation of toxic amounts of intermediary products. The generally accepted theory, proposed by Hald *et al.*[2, 3], thus explains the symptoms to be due to an accumulation of acetaldehyde.

The second possible way of action is that ethanol interferes with the normal catabolism of disulfiram, and that ethanol in one way or another makes the drug itself more

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toxic.[9, 10] We considered this mode of action to be the more plausible, the reasons for which have been discussed in a previous paper.[10]

For the elucidation of the latter possibility, detailed knowledge of the *in vivo* metabolism of disulfiram is required. Regarding the over-all absorption and elimination of disulfiram, much information is available in the literature. Thus, the absorption from the intestine both in man and animals is found to be incomplete, 5–20% appears in the feces.[1, 5, 11, 12] Eldjarn showed, using ^{35}S-labelled disulfiram, that the main part of the drug was excreted in the urine.[5, 12] The elimination was slow, and the radioactive sulphur tended to accumulate. More than 50% of the ^{35}S was recovered as sulphate, partly free, partly esterified. Hald *et al.*[1] and Domar *et al.*[11] could not demonstrate any unchanged disulfiram in the urine of humans and animals, while the occurrence of the drug in its reduced, sulphydryl form (diethyldithiocarbamate) was ascertained in rabbit urine[11] and later also in human urine.[13] Of great interest is the recent observation by Kaslander,[14] demonstrating an S-glucuronide of diethyldithiocarbamate in urine of humans receiving disulfiram. Johnston and Prickett[15] showed that disulfiram is decomposed in rat liver homogenate, yielding carbon disulphide. These authors[16] as well as Merlevede and Casier[17] found that large amounts of both disulfiram and diethyldithiocarbamate are eliminated as this volatile substance in the expiratory air of animals and humans.

Whereas the over-all metabolism thus is well known, our knowledge concerning the chemical state of disulfiram in the living organism is scarce, and controversial reports have appeared on the measurements of disulfiram and its metabolites both in animals and humans.[5, 12, 13, 16, 18, 19] However, previous *in vitro* studies permit certain conclusions as to the fate of disulfiram *in vivo*. Johnston[20] demonstrated that disulfiram is reduced by glutathione (GSH). We have recently shown that this disulphide is efficiently converted to the corresponding thiol, diethyldithiocarbamate, by the glutathione-glutathione reductase system of metabolizing erythrocytes.[21] In a preceding paper[22] it was shown that disulfiram reacts with proteins, apparently by a reaction mechanism similar to that demonstrated by Eldjarn and Pihl in the case of some cystamine derivatives,[23] giving rise to mixed disulphides and free diethyldithiocarbamate. These studies suggest that disulfiram *in vivo* will immediately be reduced to its parent thiol. Supposedly, therefore, free disulfiram will hardly be found after therapeutic doses of this disulphide, while some of it may be recovered as mixed disulphides with proteins.

On the background outlined, a detailed reinvestigation of the *in vivo* metabolism of disulfiram was performed, and the effect of ethanol on the normal breakdown was studied. ^{35}S-labelled compounds and new analytical methods were used.

EXPERIMENTAL

Materials

Highly cross-linked dextran, "Sephadex G-25", particle size 100–270 mesh (block polymerized), was purchased from AB Pharmacia, Uppsala, Sweden. ^{35}S-labelled sodium diethyldithiocarbamate and disulfiram were obtained as previously described.[22] ^{35}S-labelled sulphate was purchased from Institutt for Atomenergi, Kjeller, Norway. The calcium salt of 1-diethylthiocarbamylthioglucuronic acid was kindly supplied by Dr. Pluijgers, Organisch Chemisch Instituut, T.N.O., The Netherlands. All other compounds used were commercial products of high purity.

Methods

Animal procedure. Male rats of Wistar strain, weighing 240–300 g, were used. The animals were maintained on a nutritionally adequate stock diet until the beginning of the experiment. ^{35}S-labelled diethyldithiocarbamate and disulfiram were administered intraperitoneally in volumes of 2 ml. A fine suspension of the water insoluble disulfiram was obtained by the aid of ultrasonic vibration after the addition of one drop of Tween 20. Ethanol in 3·1 % $NaHCO_3$ (10 %, w/v) was injected subcutaneously in doses of 2 g per kg body weight. Rats not receiving ethanol were given 3·1 % $NaHCO_3$ in equal volumes (20 ml/kg b.w.). Bicarbonate was administered in order to keep the urine pH on the alkaline side, since expected metabolites (e.g. diethyldithiocarbamate) are highly unstable at low pH values.

During the experiment the rat was kept in a sealed metabolism cage, permitting quantitative collection of urine and of the CS_2 expired. The CS_2 was trapped in 2 M KOH in methanol as methylxanthate.[24] The rats were killed by decapitation after a light blow on the head, and the blood from the neck vessels was collected in a tube containing 50 μl heparin. The liver and the blood plasma, the latter being separated from the corpuscles by centrifugation, were immediately frozen and kept at $-20°$ until the examination.

Before the analyses, plasma was diluted with metal-free 10^{-2}M EDTA buffer, adjusted to pH 8·5 by NaOH, in the ratio 1:6.[22] The liver, while kept at 0°, was homogenized in a Potter-Elvehjem homogenizer in the same metal-free EDTA buffer, and the homogenate was subsequently sonified for 2 × ½ min at 20,000 cycles per second and 4 amp., using a Branson S-75 sonifier. The homogenate was made up with the EDTA buffer to a final concentration of 28·5 g liver (wet weight) per 100 ml, and subjected to centrifugation in a Spinco Model L preparative ultracentrifuge for 1 hr at about 150,000 × *g*. The almost clear supernatant, which contained 70–90 % of the total radioactivity in the liver, was then examined for the various ^{35}S-metabolites as described below.

Separation and determination of ^{35}S-metabolites. Four ml of the solutions to be examined (diluted plasma, supernatant of the liver homogenate, and diluted urine) were placed on a Sephadex G-25 column (1·5 × 20 cm) and subjected to gel filtration, the effluent being collected in volumes of 3 or 5 ml. As previously described,[22] a separation of proteins, diethyldithiocarbamate and disulfiram was obtained by this procedure. In addition, a rough estimate of the amount of ^{35}S present as metal complexes of diethyldithiocarbamate could simultaneously be obtained by determining the recovery of the radioactivity introduced, since such chelates are known to adhere firmly to the Sephadex material.[22]

On the small column used, other ^{35}S-metabolites with distribution coefficients close to unity were eluted together with diethyldithiocarbamate.[22] This fraction of the eluate therefore, was further analyzed in order to determine the relative percentages of the various ^{35}S-metabolites present.

The determination of diethyldithiocarbamate was carried out in a closed Quickfit Semi-micro Junior distillation apparatus. Two ml of the eluate, to which μmoles carrier diethyldithiocarbamate (250 μl of a freshly prepared 4 × 10^{-2}M solution) had been added, was acidified by the addition of 2 ml 2·5 × 10^{-2}M HCl from the tap funnel. The CS_2 formed during the acid-provoked decomposition of the thiol was trapped as methylxanthate for 5 min at room temperature.[22] During this period air

was bubbled through the system by applying suction at the trapping side. The counting samples were prepared by transferring 1 ml of the methanolic KOH into the counting vials followed by 10 ml of the scintillation mixture (see below). The mean recovery obtained by this method in 8 experiments with solutions of known amounts of ^{35}S-labelled diethyldithiocarbamate in concentrations ranging from 10^{-6}M to 2×10^{-4}M, was 92·5% (range, 87·1–96·6%). The recovery was independent of the concentrations used.

The ^{35}S-glucuronide of diethyldithiocarbamate was assayed by boiling the eluate with phosphoric acid (final conc. 7·3 M) for 3½ h, the $C^{35}S_2$ formed on the acid-induced decomposition of the conjugated thiol being trapped in 2 M KOH in methanol. The reactions were carried out in a closed distillation apparatus fitted with a reflux condensor. Counting samples were prepared as described above. Corrections for amounts of free diethyldithiocarbamate, which also is measured by this method, were made. To test the method, diluted urine from rats injected with ^{35}S-labelled diethyldithiocarbamate containing known amounts of the S-glucuronide (see below) was employed. In 9 experiments with S-glucuronide in concentrations ranging from 10^{-6}M to 6×10^{-4}M, the mean recovery was 83·3% (range, 78·9–89·2%). The recovery was independent of the concentrations used. The reasons for the incomplete recoveries have not been explored.

Sulphate was determined by measuring the radioactivity before and after precipitation of the sulphate as barium sulphate. The precipitation was carried out by adding 50 μl 10^{-2}M carrier sulphate (Na_2SO_4) and subsequently 50 μl $1{\cdot}3 \times 10^{-2}$M $BaCl_2$ to 2 ml of the eluate. Diethyldithiocarbamate was found partially to co-precipitate. The thiol, therefore, when present in significant amounts, had to be removed beforehand by acidification and aeration (see above).

Radioactivity measurements. Sample preparations and the assay for radioactivity as well as the determination of quenching coefficients were performed as described in detail in a preceding paper.[22] Electrophoretic and chromatographic paper strips were assayed for radioactivity in pieces of 1 cm in vials containing 5 ml of the scintillation mixture.

RESULTS

Metabolites of disulfiram and diethyldithiocarbamate excreted in urine

Large amounts of ^{35}S-labelled metabolites appeared in the urine of rats receiving radioactive disulfiram intraperitoneally. Upon gel filtration of the urine only one peak was obtained, containing metabolites with distribution coefficients close to unity. Thus, no significant amounts of disulfiram could be demonstrated.

The ^{35}S-labelled metabolites present were identified by subjecting the urine to paper chromatography and high-voltage electrophoresis. Evidence was thus obtained that mainly two metabolites of disulfiram were present. The one metabolite, which remained on the origin in the chromatographic system (Fig. 1), and moved towards the anode in the electrophoretic field, was found to be sulphate. This was made likely by demonstrating identical behavior of the authentic ^{35}S-labelled sulphate (see Fig. 3,A). The identity was confirmed by the disappearance of this radioactive peak when the sulphate of the urine had been removed beforehand by $BaCl_2$ precipitation in the presence of sulphate carrier.

The radioactivity of the other metabolite could be nearly quantitatively recovered as CS_2 upon acid hydrolysis and decomposition (see Experimental). Fig. 1 shows that this metabolite appeared at R_f 0·23 in a chromatographic system of propanol-water (85:15, v/v), and at R_f 0·65 in a system of butanol-acetic acid-water (4:1:1, v/v/v). These R_f values are the same as those given for the S-glucuronide of diethyldithiocarbamate by Kaslander.[14] The identity of this compound as the S-glucuronide was confirmed by using the authentic S-glucuronide (kindly supplied by Dr. Pluijgers) as marker both in the chromatographic (Fig. 1) and in the electrophoretic system. In the latter system, the metabolite as well as the authentic compound demonstrated electrophoretic neutrality at about pH 2 or below, whereas at higher pH it moved towards the anode.

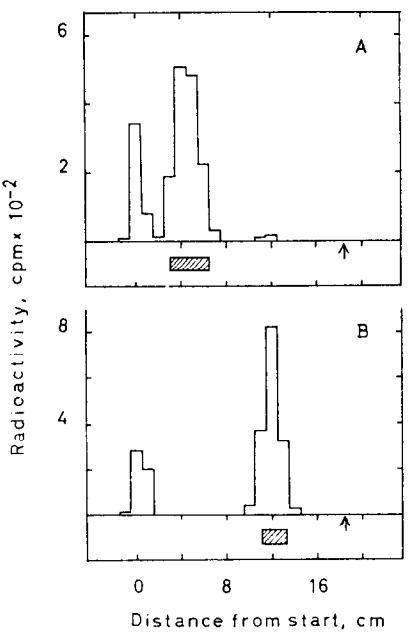


FIG. 1. Distribution of radioactivity on paper chromatograms of urine from a rat given 10 mg ^{35}S-labelled disulfiram intraperitoneally. The urine was collected for a period of 3 hr after the dose. The descending chromatograms (Whatman No. 4 filter paper) were developed with the following solvents:

A. Propanol-water (85:15, v/v).

B. n-Butanol-acetic acid-water (4:1:1, v/v/v).

Carrier 1-diethylthiocarbamylthioglucuronic acid was spotted with ammoniacal silver nitrate (hatched spots). The arrows indicate the fronts.

One may therefore conclude that the main metabolites of disulfiram excreted in the urine were the S-glucuronide of diethyldithiocarbamate and inorganic sulphate. Also when diethyldithiocarbamate was given, the same two compounds were the main metabolites found. It is remarkable that regardless of whether the drug was administered in its oxidized or its reduced form, only traces of free thiol could be demonstrated.

Table 1 shows the amounts of radioactive metabolites recovered in urine from rats receiving disulfiram (10 mg) or diethyldithiocarbamate (25 mg). Within the first hr

mainly the S-glucuronide of diethyldithiocarbamate was excreted. With time increasing amounts of free ^{35}S-labelled sulphate was also found. As to be expected, the rate of ^{35}S excretion was considerably higher when the readily soluble thiol was given, than when the disulphide was given. In the former case the S-glucuronide accounted for a greater fraction of the total radioactivity eliminated (within 4 hr, 76% and 57%, respectively).

TABLE 1. ^{35}S-METABOLITES IN URINE OF RATS AFTER THE ADMINISTRATION OF LABELLED DISULFIRAM (ASSA) OR DIETHYLDITHIOCARBAMATE (ASH)

Expt. no.	Comp. given	Dose (μmoles S)	Time after dose (hr)	^{35}S-metabolites in urine			
				Total (μmoles S)	ASH (%)	S-glucur. (%)	Sulphate (%)
1	ASSA	138	1	10·11	0·00	91·5	8·5
2	,,	,,	2	15·90	0·26	77·5	22·2
3	,,	,,	4	26·45	0·10	57·4	42·5
8	ASH	222	1	46·15	0·03	96·1	3·9
10	,,	,,	2	78·90	0·06	80·6	19·3
10	,,	,,	4	95·31	0·06	76·3	23·6

Metabolites of disulfiram and diethyldithiocarbamate in the expiratory air

After the administration of the ^{35}S-labelled compounds, significant amounts of radioactivity, apparently as CS_2, were found in the expiratory air (Fig. 2). The rate of

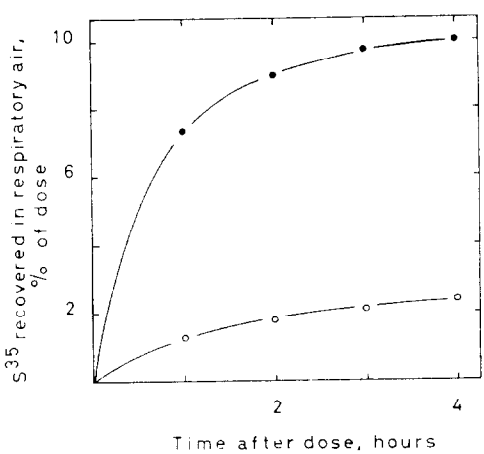


FIG. 2. Carbon disulphide expired by rats after the intraperitoneal administration of ^{35}S-labelled disulfiram and diethyldithiocarbamate. The compounds were injected at zero time. ○ — ○, disulfiram in doses of 10 mg (138 μmoles S). ● — ●, diethyldithiocarbamate in doses of 25 mg (222 μmoles S).

elimination was more rapid when the drug was given in its reduced than in its oxidized form (Fig. 2). Thus, within 4 hr after the intraperitoneal injection of 25 mg diethyldithiocarbamate (222 μmoles S), about 10% of the sulphur was recovered in the expiratory air, whereas after 10 mg disulfiram (138 μmoles S) only about 2% was recovered.

Non protein-bound ^{35}S-metabolites of disulfiram and diethyldithiocarbamate in plasma and liver

As in urine, the S-glucuronide of diethyldithiocarbamate and inorganic sulphate were identified as the main low molecular weight metabolites of disulfiram both in the plasma and in the liver (Fig. 3). No evidence for the occurrence of free disulfiram was obtained. Thus, only 0–2% of the radioactivity was eluted from the Sephadex column in fractions corresponding to the elution volume to be expected for disulfiram.

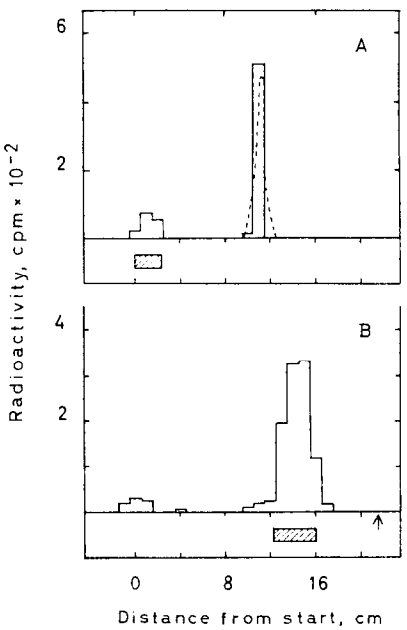


FIG. 3. A. Electrophoretic pattern of the low molecular weight ^{35}S-metabolites of plasma from a rat which 1 hr earlier had received 10 mg labelled disulfiram. The metabolites were separated from the proteins by gel filtration, and the eluate was freeze-dried to a small volume of sufficiently high radioactivity. After the addition of calcium diethylthiocarbamylthioglucuronate carrier, 10 μl of this solution was subjected to high-voltage electrophoresis (45 V/cm) for 40 min at − 3°, using a buffer pH 3.5, consisting of acetic acid-pyridine-water (10:1:89, v/v/v). Electrophoresis of ^{35}S-labelled sulphate was carried out parallelly, and the distribution of this radioactivity was assayed in an end-window GM strip counter (the broken curve).

B. Chromatographic pattern of the low molecular weight ^{35}S-metabolites of supernatant of the liver homogenate prepared from a rat which 1 hr earlier had received 40 mg labelled disulfiram. The isolation procedure was carried out as described in Fig. 3,A. The descending paper chromatogram was developed with n-butanol-acetic acid-water (4:1:1, v/v/v).

The carrier 1-diethylthiocarbamylthioglucuronic acid was spotted with ammoniacal silver nitrate (hatched spots). The arrow indicates the front.

Moreover, the small amount of radioactivity sometimes present in these fractions was most likely due to tailing of metabolites with lower distribution coefficients, since no significant amount of it could be identified as disulfiram by the previously described methods.[22]

Table 2 and 3 demonstrate that already one hour after the administration of 10 mg disulfiram, all the non protein-bound radioactivity present both in plasma and in liver occurred as the sulphate and the S-conjugate. Significant amounts of free thiol were found only when higher doses of disulfiram were given (Table 5 and 6). Apparently disulfiram is subjected to an immediate *in vivo* reduction to the parent thiol, and the diethyldithiocarbamate formed undergoes a rapid catabolism.

TABLE 2. NON PROTEIN-BOUND ^{35}S-METABOLITES IN PLASMA FROM RATS GIVEN LABELLED DISULFIRAM (ASSA, 10 MG) OR DIETHYLDITHIOCARBAMATE (ASH, 25 MG)*

Expt. no.	Comp. given	Time after dose (hr)	Total	ASH	S-glucur.	Sulphate	%† identified
1	ASSA	1	169	0	37	132	100
2	,,	2	93	0	15	68	89
3	,,	4	64	0	7	54	95
4–6‡	ASH	1/4	1561	567	772	142	95
7	,,	1/2	672	77	377	124	86
8	,,	1	305	2	106	128	77
9	,,	2	148	0	13	127	95
10	,,	4	65	0	10	52	95

* All amounts are expressed as m-μmoles sulphur per ml plasma.
† Per cent of total non protein-bound ^{35}S-metabolites.
‡ Mean of three experiments.

TABLE 3. NON PROTEIN-BOUND ^{35}S-METABOLITES IN SUPERNATANT OF LIVER HOMOGENATE FROM RATS GIVEN LABELLED DISULFIRAM (ASSA, 10 MG) OR DIETHYLDITHIOCARBAMATE (ASH, 25 MG)*

Expt. no.	Comp. given	Time after dose (hr)	Total	ASH	S-glucur.	Sulphate	%† identified
1	ASSA	1	309	4	242	18	86
2	,,	2	95	0	56	25	85
3	,,	4	67	0	42	18	90
4–6‡	ASH	1/4	3211	15	2559	49	82
7	,,	1/2	2075	4	1815	88	92
8	,,	1	312	2	207	56	85
9	,,	2	130	1	67	39	82
10	,,	4	56	0	21	21	75

* All amounts are expressed as m-μmoles sulphur per g liver (wet weight).
† Per cent of total non protein-bound ^{35}S-metabolites.
‡ Mean of three experiments.

The high rate by which diethyldithiocarbamate is metabolized, was more clearly demonstrated when administering this compound as such. This thiol, owing to its great solubility in polar solvents possesses three experimental advantages as compared to the corresponding disulphide: (1) It allows a more exact reproduction of the dose from test to test; (2) it is rapidly and probably quantitatively absorbed from the peritoneum with little peritoneal reaction; (3) it gives rise to an initial higher *in vivo* concentration of ^{35}S with the same dose of sulphur. Already 15 min after the injection of 25 mg labelled diethyldithiocarbamate, only about 0·5% of the low molecular weight ^{35}S-metabolites present in liver was recovered as free thiol (Table 3).

Nearly all of the thiol had been converted to its S-glucuronide, and a small portion had been oxidized to sulphate. In plasma the percentage of free thiol was higher, about 35%, but within the next 45 min the amount fell to below 1% (Table 2). These results are in accordance with the view that the glucuronic acid conjugation takes place in the liver.

No significant amounts of metal complexes of diethyldithiocarbamate, which could have been expected metabolites,[22] were detected in plasma, liver or in urine with the available methods.

TABLE 4. ^{35}S BOUND TO THE SOLUBLE PROTEINS OF LIVER AND TO THE PROTEINS OF PLASMA FROM RATS GIVEN LABELLED DISULFIRAM (ASSA, 10 MG) OR DIETHYLDITHIOCARBAMATE (ASH, 25 MG)*

Expt. no.	Comp. given	Time after dose (hr)	In supernt. of liver homogenate	In plasma	^{35}S released from plasma prot.-^{35}S by GSH (%)†	
					Total	Identified as ASH
1	ASSA	1	18	36	92	86
2	,,	2	17	29	92	87
3	,,	4	10	11	94	77
4–6‡	ASH	1/4	53	45	83	74
7	,,	1/2	42	32	81	63
8	,,	1	35	30	56	—
9	,,	2	34	22	45	32
10	,,	4	18	15	75	—

* The amounts are expressed as m-μmoles sulphur per g liver (wet weight) or per ml plasma.
† Per cent of total radioactive sulphur bound to the proteins.
‡ Mean of three experiments.

TABLE 5. EFFECT OF ETHANOL ON THE AMOUNTS OF ^{35}S-METABOLITES IN PLASMA FROM RATS GIVEN LABELLED DISULFIRAM (ASSA) OR DIETHYLDITHIOCARBAMATE (ASH)*

Expt. no.	Comp. given	Ethanol (g/kg)	Total ^{35}S	Protein-bound ^{35}S	Non protein-bound ^{35}S-metabolites			
					ASH	S-glucur.	Sulphate	%† identified
14	ASSA	0	966	77	13	375	427	92
15	,,	0	722	62	15	261	297	87
16	,,	2	912	66	102	282	383	91
17	,,	2	781	84	44	232	334	88
4	ASH	0	1697	53	474	904	153	93
5	,,	0	1624	41	729	697	135	99
6	,,	0	1497	42	498	714	138	93
11	,,	2	1700	59	889	675	146	104
12	,,	2	1685	44	1056	496	80	100
13	,,	2	1439	42	881	385	101	98

* ASH (25 mg) was injected simultaneously with the ethanol, and the rats were killed after 15 min. ASSA (40 mg) was injected twice with an internal of 5 hr. Ethanol was given 20 min after the last dose, and the rats were killed 30 min after the ethanol injection. All amounts are expressed as m-μmoles sulphur per ml plasma.
† Per cent of total non protein-bound ^{35}S-metabolites.

It will be seen from Table 1–3 that the over-all metabolism of disulfiram is much slower than that of diethyldithiocarbamate. The main reason for this is most likely a protracted and perhaps incomplete absorption of the insoluble disulphide from the peritoneum. In fact it was observed that disulfiram tended to aggregate in the abdominal cavity.

TABLE 6. EFFECT OF ETHANOL ON THE AMOUNTS OF ^{35}S-METABOLITES IN SUPERNATANT OF LIVER HOMOGENATE FROM RATS GIVEN LABELLED DISULFIRAM (ASSA) OR DIETHYLDITHIOCARBAMATE (ASH)*

Expt. no.	Comp. given	Ethanol (g/kg)	Total ^{35}S	Protein-bound ^{35}S	Non protein-bound ^{35}S-metabolites			
					ASH	S-glucur.	Sulphate	%† identified
14	ASSA	0	2106	36	16	1462	137	78
15	,,	0	1549	50	21	1070	123	82
16	,,	2	1638	66	25	953	246	78
17	,,	2	1516	66	63	993	154	84
4	ASH	0	3500	46	11	2840	28	83
5	,,	0	3210	45	21	2451	105	82
6	,,	0	3080	67	14	2386	14	80
11	,,	2	2488	47	77	1979	10	85
12	,,	2	3592	66	70	2683	137	82
13	,,	2	3408	65	68	2941	10	90

* The experimental conditions were as described in Table 5. All amounts are expressed as m-μmoles sulphur per g liver (wet weight).

† Per cent of total non protein-bound ^{35}S-metabolites.

Protein-bound metabolites of disulfiram and diethyldithiocarbamate in plasma and liver

It appears from Table 4 that substantial amounts of radioactivity, when administered intraperitoneally as disulfiram, were bound to the plasma proteins and to the soluble proteins of liver (proteins in supernatant of the homogenate used). Even when the drug was injected in its reduced, sulphydryl form, a significant fraction of the radioactive sulphur became attached to the proteins. Regardless of whether disulfiram or diethyldithiocarbamate was given, the amount of radioactivity linked to the proteins decreased gradually with time, but at a rate slower than the fall in the total radioactivity. Thus during the first hours, the protein-^{35}S constituted an increasing percentage of the total radioactivity present.

Disulfiram has been shown to form mixed disulphides with protein —SH groups *in vitro*.[22] In order to establish whether the protein-bound radioactivity *in vivo* likewise arose from a fixation of the diethyldithiocarbamate residue, the protein-containing eluate from the Sephadex column was treated with 5×10^{-5}M GSH.[22] Table 4 shows that the ^{35}S bound to plasma proteins of rats receiving disulfiram as well as of rats receiving diethyldithiocarbamate was liberated to a large extent after the addition of GSH, the greater fraction of which was identified as diethyldithiocarbamate. Also a GSH-provoked splitting of the liver protein-^{35}S was found, but in this case the release of ^{35}S was less complete.

Evidence was thus obtained that both disulfiram and diethyldithiocarbamate give rise to mixed disulphide formation *in vivo*. Previous *in vitro* studies have shown that

diethyldithiocarbamate in contrast to disulfiram only becomes loosely adsorbed to proteins. The demonstration of mixed disulphides even after the injection of the thiol, therefore, suggests that some *in vivo* oxidation to the corresponding disulphide takes place. In this connexion it may be mentioned that naturally occurring substances as cytochrome c and methaemoglobin have been shown to oxidize diethyldithiocarbamate *in vitro*.[10, 25, 26]

Effect of ethanol on the metabolism of disulfiram and diethyldithiocarbamate

Ethanol in subcutaneous doses of 2 g/kg body weight appeared to have no influence on the total amount of radioactive sulphur recovered from plasma (Table 5) or liver (Table 6), either when administered as disulfiram or as diethyldithiocarbamate. Moreover, no significant difference in the amount of protein-bound ^{35}S was detected. However, regarding the relative amounts of the various low molecular weight metabolites, a substantial increase in the free diethyldithiocarbamate and a corresponding decrease in the S-glucuronide appeared upon ethanol administration. This change in the catabolism was most clearly shown when using the sulphydryl form of the drug. Thus, Table 6 shows that 15 min after the rats had received both diethyldithiocarbamate (25 mg) and ethanol (2 g/kg b.w.), the liver had a free thiol level that was on the average 5 times that of the control. The mean plasma level of these rats (Table 5) was 1·7 times that of the control level. Similarly, also in rats receiving disulfiram (40 mg × 2) higher values of free diethyldithiocarbamate in plasma and liver were found upon ethanol administration (Table 5 and 6). In this case the individual variation from test to test was greater. Table 5 and 6 furthermore show that concomitant with the increase of free thiol, a decrease in the S-glucuronide was usually found when ethanol in addition to the drugs was given. No constant and significant change in the sulphate levels was revealed.

TABLE 7. EFFECT OF ETHANOL (2 G/KG S.C.) ON THE AMOUNTS OF CARBON DISULPHIDE EXPIRED BY RATS GIVEN ^{35}-LABELLED DIETHYLDITHIOCARBAMATE (25 MG)

Time after dose (min)	Carbon disulphide (μmoles S)	
	− ethanol	+ ethanol
15	7·34	21·75
15	6·97	12·51
15	10·05	12·85
30	9·54	16·19
30	19·51	20·19
60	14·87	20·55

These data indicate an interference with the conjugation of diethyldithiocarbamate by ethanol. Additional support for such an interference is seen in Table 7, which shows that rats given both diethyldithiocarbamate and ethanol expired more CS_2 than rats receiving only diethyldithiocarbamate. The CS_2 most likely is derived from the free thiol, and not from the considerably more stable conjugate. A delayed conjugation, therefore, would be expected to give rise to increased CS_2 elimination via the lungs.

On the basis of these results the conclusion seems warranted that ethanol inhibits the *in vivo* glucuronic acid conjugation of diethyldithiocarbamate. Relatively high doses of the sulphur compounds were required to detect the inhibitory effect, and it was revealed only during a short time after the dose, *i.e.* only under conditions when the conjugating system had been stressed by high substrate concentrations.

DISCUSSION

Metabolism of disulfiram and diethyldithiocarbamate in rats

In the present report the catabolism of disulfiram and diethyldithiocarbamate after their intraperitoneal administration to rats has been studied. Four low molecular weight metabolites were demonstrated: diethyldithiocarbamate, the S-glucuronide of diethyldithiocarbamate, sulphate, and carbon disulphide. In addition, a definite fraction of the sulphur was bound to proteins, for the greater part as mixed disulphides on their —SH groups.

In spite of the high sensitivity of the methods used, free disulfiram was never detected in plasma, liver or in urine. This is in agreement with previous *in vitro* studies showing that disulfiram has a high reactivity towards protein —SH groups and naturally occurring thiols such as GSH and coenzyme A.[20–22, 27] With the doses employed, therefore, the disulfiram absorbed will at once undergo spontaneous chemical reactions which ultimately lead to a complete reduction of the disulphide. The rapid appearance of diethyldithiocarbamate and of its S-glucuronide supports this conclusion. Moreover, the fact that the same metabolites were recovered regardless of whether the disulphide or the thiol had been given, indicates that diethyldithiocarbamate is an obligatory intermediate in the catabolism of disulfiram. The present results, therefore, confirm the assumption made by several authors on the basis of both *in vivo*[5, 11–13, 16] and *in vitro*[10, 21] studies that disulfiram will be reduced in the living organism. Previous evidence for the occurrence of free disulfiram is probably obtained by the use of unspecific methods.[5, 12, 19]

The reduction of disulfiram to diethyldithiocarbamate is most likely accomplished via spontaneous exchange reactions with GSH and protein —SH groups.[21, 22] The GSSG which represents the final oxidation product in these reactions, will be enzymatically reduced by the glutathione reductase of the cells. In addition, the disulphide-reducing system of animal mitochondria reported by Eldjarn and Bremer[28] may participate in the *in vivo* reduction of disulfiram. Other normally occurring reducing agents such as cysteine and homocysteine probably play a quantitatively minor role, although these may be of importance in extracellular fluids such as plasma.

It appears from the above that efficient mechanisms in protecting protein —SH groups against oxidation exist intracellularly. The proteins of the extracellular fluids, on the other hand, are not protected to the same extent. Consistent with this, we found as much as about 8% of the plasma protein —SH groups[22] blocked during the disulfiram treatment. More remarkable is the finding that also a significant amount of the radioactive sulphur was attached to the soluble proteins of liver. This amount, however, corresponded only to a very small portion of the total protein —SH groups present. Rogulski[29] has recently shown, using amperometric technique, that rat liver homogenates contain protein —SH groups in an amount of 15·5 μmoles/g tissue, 70% of which was found in the proteins of the cytoplasma, i.e. in the particle-free supernatant. According to this, the supernatant analyzed by us should contain about 12,000 mμmoles protein

—SH groups per g liver tissue. However, at most 18 m-μmoles radioactive sulphur per g tissue was found attached to these proteins after the administration of 10 mg disulfiram (Table 4). Assuming that this radioactive sulphur was bound entirely to proteins by mixed disulphide linkages, not more than about 0·1 to 0·2% of the titrable —SH groups present was blocked.

Despite the high plasma levels of free diethyldithiocarbamate observed during the first $\frac{1}{2}$ hr after its administration (Table 2), only traces of the free thiol were recovered from the urine (urinary pH always kept above 7). One reason for this may be a blocked glomerulus filtration of diethyldithiocarbamate, which at least in part may be due to an adsorption of the thiol to the proteins of blood.[22] A contributing factor would be if some glucuronic acid conjugation of the thiol takes place also in the kidney. Thus, in rats this organ has been shown to participate in the conjugation of other compounds.[30] Earlier studies have revealed free diethyldithiocarbamate in urine both of rabbits and humans after disulfiram treatment,[11, 13] a disparity from our data which may be ascribed to species differences.

Diethyldithiocarbamate, either formed *in vivo* by reduction of disulfiram or injected as such, appears to be metabolized at a high rate along several pathways: (1) oxidation to sulphate, (2) decomposition to carbon disulphide, (3) conjugation with glucuronic acid. In addition, evidence was obtained that a small amount is oxidized to disulfiram.

It has previously been shown that disulfiram, like many other sulphur containing substances,[31] is excreted in urine as sulphate.[5, 12] In contrast to Eldjarn, we have not demonstrated any esterified sulphate although it is well known that a small fraction normally is eliminated in this form. Since the naturally occurring free sulphate is an intermediate in the formation of esterified sulphate,[32, 33] the latter fraction cannot be of any importance in the therapeutic action of disulfiram. Therefore, we did not make any further effort in demonstrating an esterified fraction. The present data do not permit any conclusions as to the route by which diethyldithiocarbamate is converted to sulphate. At least a small portion may be derived from the carbon disulphate formed by decomposition of the thiol, since about 30% of the carbon disulphide administered to mice and guinea pigs appears in the urine as inorganic sulphate.[34]

Merlevede and Casier[17] found that about 45% of a single oral dose of 500 mg disulfiram to humans was expired as carbon disulphide within 80 hr, while as much as 80% of a single dose of 500 mg diethyldithiocarbamate appeared in this form within 7 hr. These values are much higher than those obtained in rats. Although species differences may explain some of the discrepancy, the main reason for the high values obtained in humans is most likely related to the route by which the drugs were given. Thus, with normal pH of the gastric juice, oral administration must lead to a complete decomposition of diethyldithiocarbamate as well as to some decomposition of the more acid-stable disulfiram. In support of this interpretation is the finding that diethyldithiocarbamate gave rise to maximum concentration of carbon disulphide in the expiratory air already within 10 to 30 minutes after its administration.[17] The fact that these authors in contrast to others,[35] did not find any ethanol-sensitizing effect of diethyldithiocarbamate may thus be explained by the gastric decomposition of the drug.

Recently Kaslander reported the occurrence of the S-glucuronide of diethyldithiocarbamate in urine of humans receiving disulfiram.[14] Some 0·75% of the dose was recovered in this form, but it should be stressed that the author considered this value to

be a minimum value because of the many and laborious steps involved in the isolation procedure. In rats we found this metabolite to account for the greater portion of the radioactive sulphur eliminated both when given as disulfiram and as diethyldithiocarbamate. The glucuronic acid conjugation of diethyldithiocarbamate proceeded at a high rate as judged from the rapid appearance of large amounts of the S-glucuronide not only in the liver in which these processes generally occur, but also in the plasma.

Kamil *et al.*[36] observed that disulfiram fed to rabbits led to an increased urinary excretion of total glucuronic acid. A similar increase was found upon ethanol administration, and the ethyl glucuronide (about 1% of dose) was isolated from the urine. When ethanol and disulfiram were given simultaneously, the increase was 2 to 3 times greater than when these substances were given separately. This finding was explained by a slowing up of the ethanol oxidation by disulfiram, making more of the ethanol available for conjugation. However, on the basis of present knowledge it may as well be a summation effect, assuming that diethyldithiocarbamate in rabbits, like in rats, is conjugated.

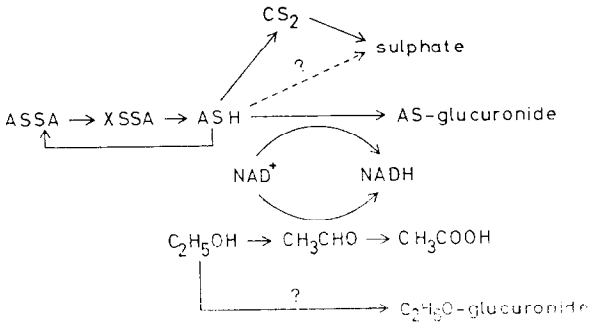
Our results deviate in many ways from those obtained by others in studies on the *in vivo* metabolism of disulfiram. Thus, we have found that disulfiram in rats undergoes an instantaneous reduction to the corresponding thiol and that no free disulfiram occurs. Furthermore, conjugation with glucuronic acid plays a major role in the metabolism of diethyldithiocarbamate. In contrast to what has previously been assumed, only a negligible fraction of the intracellular protein —SH groups appears to be blocked as mixed disulphides upon the disulfiram treatment. Therefore, with regard to the therapeutic action of disulfiram it seems of great interest to re-investigate the normal breakdown of this drug also in man.

Effect of ethanol on the metabolism of diethyldithiocarbamate

Evidence was obtained that ethanol lowers the rate by which diethyldithiocarbamate is conjugated with glucuronic acid. Thus, under the conditions used, an increased level of free diethyldithiocarbamate and a decreased level of the S-glucuronide was found both in liver and in plasma when moderate doses of ethanol had been given. The fact that ethanol also led to an increased expiration of carbon disulphide may well be secondary to the increased level of non-conjugated diethyldithiocarbamate. Similarly, Cooke and Taylor[37] in the case of progesterone have recently reported an ethanol-induced inhibition of the glucuronic acid conjugation in rat liver homogenate. It is difficult to state, however, whether this *in vitro* effect is analogous to the present *in vivo* observation because the concentrations of ethanol used by Cooke and Taylor were much higher, 0·3 to 0·6 M.

Regarding the mechanism by which ethanol inhibits the conjugation of diethyldithiocarbamate, two possibilities may be considered. The one is that ethanol and disulfiram compete for enzymes and substrates involved in the conjugation process since ethanol likewise may be conjugated with glucuronic acid.[36] The other possibility, explaining the inhibition as a competition for NAD^+, is perhaps more plausible. Thus, the NAD^+-dependent oxidation of both ethanol[38, 39] and UDP-glucose[40] is catalyzed by enzymes present in the soluble fraction of the liver cells. Furthermore, the hepatic NAD^+:NADH ratio decreases on both the *in vivo* and the *in vitro* administration of ethanol.[41–44] This alteration has previously been suggested to explain a variety of

metabolic changes caused by ethanol.[45, 46] Both these mechanisms of inhibition may of course be involved simultaneously.



The scheme shows in summary the catabolism of disulfiram and diethyldithiocarbamate in rat along with the steps of the ethanol metabolism which may be relevant to the ethanol-induced inhibition of the glucuronic acid conjugation.

On the mechanism of the disulfiram-ethanol reaction

Up to now it has been generally accepted that the disulfiram-ethanol reaction is caused by acetaldehyde accumulated in toxic amounts owing to a disulfiram inhibition of enzymes involved in the metabolism of ethanol. However, we have previously pointed out that the disulphide reducing systems of the cells probably are sufficiently active to prevent such an *in vivo* disulphide inhibition during the disulfiram treatment.[10, 21] We therefore proposed, as a hypothesis for further investigation, that ethanol induces a forced oxidation of diethyldithiocarbamate to disulfiram, and that the unpleasant symptoms should be due to a general "disulphide poisoning".[10] The present data give no support for this hypothesis. Thus, although demonstrating some *in vivo* oxidation of the thiol, we failed to show any significant increase in this oxidation after ethanol administration, which would have appeared as an increase in the protein-bound radioactive sulphur. However, the results agree with the first tentative conclusion that the enzyme —SH groups are effectively protected against oxidation by disulfiram. Thus, at most 0·1 to 0·2% of the —SH groups of the soluble liver proteins was found blocked after injections of disulfiram in doses several times higher than those required to cause ethanol sensitization in man. It seems very unlikely that these blocked —SH groups should particularly belong to enzymes involved in the metabolism of ethanol.

After the administration of ethanol, a decreased rate of glucuronic acid conjugation of diethyldithiocarbamate was the only change observed in the metabolism of disulfiram. We considered a competition for NAD^+ to be a possible explanation of this inhibition. This would indicate that a marked reduction in the NAD^+: NADH ratio occurred when both the drug and ethanol were given. It may be questioned whether such a shift, as a causal factor in many of the metabolic changes observed during the disulfiram-ethanol reaction, has any bearing on the therapeutic action of disulfiram. Thus, a lack of NAD^+ may explain the lowered oxidation of acetaldehyde observed by disulfiram[47–50] since NAD^+ is a necessary cofactor in this oxidation. The reason why the NAD^+-dependent oxidation of ethanol suffers less after disulfiram treatment,[49–51]

may be that the ethanol dehydrogenase[52] has 5 to 6 times lower K_m for NAD^+ than the acetaldehyde dehydrogenase.[53, 54] Ethanol is known to lower the activity of the citric acid cycle *in vitro*, an effect attributed to a decrease in the NAD^+:NADH ratio.[45, 55] If this decrease is accentuated by disulfiram, this explains why the oxidation of glucose to CO_2 is lowered in rats during disulfiram-ethanol treatment as compared to the oxidation when only ethanol was given.[56] Vitale *et al.*[57] considered a competition for NAD^+ to be responsible for a pyruvate-induced reduction in the metabolism of ethanol, results which recently have been reproduced in rat-liver slices.[55] Similarly, the accumulation of pyruvate in blood of animals and humans observed during the disulfiram-ethanol reaction [58, 59] may be due to a relative lack of NAD^+ for its oxidation.[55] Other reports, however, have suggested that the pyruvate on the contrary increases the regeneration of NAD^+ from NADH by way of its conversion to lactate and glycogen.[42, 60] Against this latter view may be pointed out that injections of nicotinamide and thiamine, which both supposedly lead to a facilitated oxidation of pyruvate, offer protection against the toxic reactions induced by pyruvate administration to rabbits.[61, 62] Moreover, nicotinamide, which is shown to increase the *in vivo* level of nicotinamide-adenine dinucleotides,[63, 64] as well as to prevent a marked drop in the rat liver NAD^+:NADH ratio caused by ethanol alone,[43] also protects against the disulfiram-ethanol reaction.[65, 66] In this connexion it is of interest that Büttner *et al.* [67, 68] recently have proposed a reduction in the NAD^+:NADH ratio to be the reason for the ethanol-sensitizing effect of sulfonylureas.[69–71] They found a decrease in this ratio in the liver of rats upon the *in vivo* administration of tolbutamide and ethanol. In contrast to other workers they failed to show any decrease with ethanol alone, and they observed only a small and statistically insignificant decrease when disulfiram and ethanol were given.[68]

Based on the above it seems as if a decreased NAD^+: NADH ratio may have some bearing on various metabolic changes observed in experiments when using high doses of disulfiram. However, it appears less likely that such a shift should represent a primary factor in the pathogenesis of the disulfiram-ethanol reaction in man. In other words, it is difficult to see how therapeutic doses of disulfiram to man will accentuate the ethanol-induced demand for NAD^+ to a degree sufficient to cause a clinical reaction like the disulfiram-ethanol reaction, knowing that ethanol is metabolized at a rate of about 7 g per hour whereas the daily dose of disulfiram is only 200–500 mg.

Conjugation with glucuronic acid is one of the main *in vivo* routes by which exogenous as well as endogenous substances are detoxicated or inactivated.[33] Therefore, an interference with this system, as we have shown when both disulfiram and ethanol were injected, may be expected to cause serious reactions, either via an inhibition of the conjugation of endogenous compounds, or by way of a decreased detoxication of the drug itself. Remarkable in this connexion is the explicit declaration of Child *et al.*[72] that acutely ill rats, provoked by disulfiram-ethanol treatment, resembled most closely rats which were given toxic doses of disulfiram alone. Disulfiram, moreover, is shown to be more toxic in sub-totally hepatectomized rats than in laparotomized non-hepatectomized controls.[73] A possible mechanism for the disulfiram-ethanol reaction may thus be that an ethanol-induced shift in the NAD^+: NADH ratio lowers the capacity for the formation of UDP-glucuronic acid from UDP-glucose, and that, when in addition to ethanol, an exogenous glucuronic acid acceptor (*i.e.* disulfiram) has been administered, an acute insufficiency of this detoxication system develops. It

should be stressed that the inhibition of the glucuronic acid conjugation in rats did not appear pronounced, and that caution therefore should be exercised when judging the relevance of this effect as to the ethanol-sensitizing effect of disulfiram in man. However, if the capacity for conjugation is relatively lower in this species, an insufficiency of this system may more readily develop.

In summary, the data confirm our previous tentative conclusion that disulfiram treatment hardly gives rise to any *in vivo* mixed disulphide inhibition of enzymes.[10, 21] Furthermore, they indicate that the therapeutic action of disulfiram is exerted through its corresponding thiol, diethyldithiocarbamate, or through the further metabolism of this compound. It appears likely that the observed interference of ethanol with the glucuronic acid conjugation of diethyldithiocarbamate is related to its ethanol-sensitizing effect. The final proof for this, however, requires further *in vivo* studies primarily in man.

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