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DETOXICATION OF SODIUM ^{35}S-SULPHIDE IN THE RAT

C. G. CURTIS, T. C. BARTHOLOMEW, F. A. ROSE and K. S. DODGSON
Department of Biochemistry, University College, Cardiff CF1 1XL, Wales

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Abstract—When sodium ^{35}S-sulphide is administered to rats either intraperitoneally, orally or intravenously it is oxidized primarily to inorganic ^{35}S-sulphate which is eventually excreted in the urine. Intravenously administered sulphide has a transient existence in blood and the lack of an accumulative effect of sulphide poisoning is due to the rapid rate of oxidation. The blood is not the principle site of sulphide oxidation *in vivo*. Sulphide detoxication occurs in both plasma and red cells *in vitro* by binding to proteins.

EARLY studies[1] showed that when sodium ^{35}S-sulphide was administered to rats the bulk of the radioactivity was recovered in urine as oxidized forms of inorganic sulphur and as ester sulphate. The tissues responsible for sulphide oxidation have not been established with certainty and there have been conflicting views on this point. Thus, it has been reported[2] that inhaled hydrogen sulphide exists only momentarily in the blood and its destruction occurs to a considerable extent in the plasma. In contrast, other work[3] pinpoints the red cells as the major site of sulphide destruction. The oxidation of S^{2-} ions to SO_4^{2-} involves the transfer of eight electrons and these steps are by no means well-defined at present. *In vitro* studies have demonstrated the oxidation of sulphide to thiosulphate by metals and metallo–protein complexes including ferritin,[4,5] and the catalytic oxidation of sulphides by haem and haemoglobin has also been investigated.[6–8] Heat-labile and heat-stable systems capable of oxidizing sulphide to thiosulphate have been reported in rat liver and kidney[9–11] and a protein-bound sulphite has been suggested as an intermediate, although this has been disputed by Sorbo[12] who implicated polysulphides in the transformation. The present report is concerned with the distribution and fate of administered sodium ^{35}S-sulphide and forms part of a wider investigation of the metabolic activity of reduced sulphur compounds *in vivo*.

MATERIALS AND METHODS

Radioisotopes. Sodium ^{35}S-sulphate and sodium ^{35}S-sulphide were obtained from the Radiochemical Centre, Amersham, Bucks, England.

Experimental animals. These included young, (4–6 weeks, 30–50 g body wt.) and adult M.R.C. hooded rats (200–300 g body wt.). In some experiments animals received intraperitoneal and oral injections of materials while under general ether anaesthesia and were subsequently placed in metabolism cages designed to permit separate collection of urine and faeces. In experiments in which urine and bile were collected via ureter and bile duct cannulae, animals were first lightly anaesthetized with ether, the

jugular vein was cannulated and phenobarbitone (Nembutal, veterinary grade) was injected via the cannula.[13]

Paper chromatography. Samples were subjected to descending chromatography on Whatman No. 1 chromatographic paper.

Paper electrophoresis. Samples (5–10 μl) were subjected to horizontal electrophoresis on Whatman No. 1 paper in 0·1 M-sodium acetate–acetic acid buffer, pH 4·5, with a potential gradient of 8 v/cm.

Thin-layer chromatography. Samples (5 μl) were applied to silica-gel plates and chromatograms were developed in a saturation chamber of the type described by Davies.[14]

Cellulose acetate membrane electrophoresis. Samples (2 μl) were applied to cellulose acetate membranes and subjected to horizontal electrophoresis for 2 hr in a barbitone buffer, 50–70 mM, pH 8·6.[15]

Detection of radioactive areas on chromatograms and electrophoretograms. These were located with a Packard Model 7200 radiochromatogram scanner and the relative amount of radioactivity associated with each area was estimated from the record of the scanner.[16] Alternatively, radioactive areas were located by radioautography on X-ray film.

Whole-body radioautography. This was carried out as described by Powell, Curtis and Dodgson.[17]

Measurement of radioactivity. This was carried out in a Beckman, Model LS 100 scintillation spectrometer. Samples (50 μl) of the ^{35}S-labelled materials were added to the scintillation cocktail (10 ml) which consisted of 2,5-diphenyloxazole (5 g) dissolved in Triton X-100 (500 ml, scintillation grade) and toluene (1 l.). Sufficient counts were recorded to ensure that the error was less than 3 per cent. The total ^{35}S in blood and plasma was measured as follows. Samples (0·25–0·50 ml) were dissolved in N NaOH (3 ml), diluted to 20 ml with water and counted. Standards were prepared by adding known amounts of ^{35}S to blood or plasma (0·5 ml) in N NaOH (3 ml) and diluting to 20 ml with water. For the measurement of trichloroacetate—insoluble ^{35}S, samples (0·25–0·50 ml), of blood and plasma were added to trichloroacetate (3 ml of a 10% solution). The precipitates were resuspended three times in trichloroacetate with intermediate centrifugation. The final precipitates were dissolved in N NaOH (3 ml) and diluted to 20 ml prior to counting.

EXPERIMENTAL AND RESULTS

Distribution of radioactivity in urine and faeces of rats following the administration of sodium ^{35}S-sulphide

In preliminary experiments, free-ranging rats received either intraperitoneal, (3 male and 3 female) or oral (3 male and 3 female), injections of sodium ^{35}S-sulphide (5 μmoles in 0·5 ml, 0·154 M phosphate buffer, pH 7·4, per 200 g body wt.). Urines were collected into hydrogen peroxide (3 ml of 100 vol. percentage, adjusted to pH 8·5 with ammonia solution, sp. gr. 0·88) at intervals of 12, 24 and 48 hr after injection and then frozen. Faeces were collected into ammoniacal peroxide (3 ml) for 48 hr, water (10 ml) was added and the mixture centrifuged in order to remove debris. The debris was washed 3 times with water and the pooled supernatant and washings were frozen until required. Subsequently hydrogen peroxide was removed by warming in

a hot water bath and each sample was diluted to 50 ml with water and counted for total ^{35}S. In a separate series of experiments, male and female rats were injected either intraperitoneally or orally with the same quantity of sodium ^{35}S-sulphide as above and the urines were collected into *N*-ethylmaleimide (20 μmoles in 3 ml of water) for 24 hr. Samples (5 μl) of each urine were subjected to paper chromatography in butan-1-ol–acetic acid–water (15:12:25 by vol.) and to paper electrophoresis. After intraperitoneal and oral administration of sodium ^{35}S-sulphide, (Table 1) 84–93 and 52–69 per cent respectively of the injected radioactivity appeared in the urine over the 48 hr experimental period, the major proportion appearing during the first 12 hr. The chromatograms and electrophoretograms obtained from the 24 hr urine samples showed one major radioactive component with a mobility identical to that of inorganic ^{35}S-sulphate. Only trace amounts of other ^{35}S-labelled materials were detected.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN URINE AND FAECES FOLLOWING INTRAPERITONEAL AND ORAL ADMINISTRATION OF SODIUM ^{35}S-SULPHIDE (5 μMOLES PER 200 g BODY WT.) TO RATS

Injection route	Animals	% Injected radioactivity recovered					
		Urine				Faeces	Total
		12 hr	24 hr	48 hr	Total	48 hr	
Intraperitoneal	Male 1	72·2	9·6	5·9	87·7	18·5	106·2
	Male 2	76·0	9·1	8·2	93·3	7·3	100·6
	Male 3	76·1	8·6	6·5	91·2	7·9	99·1
	Female 1	72·1	8·7	6·2	87·0	7·1	94·1
	Female 2	69·5	9·6	7·3	86·4	7·6	94·0
	Female 3	65·4	11·3	7·0	83·7	4·6	88·3
Oral	Male 1	48·7	10·3	10·1	69·1	3·1	72·2
	Male 2	45·6	10·4	6·2	62·2	4·7	66·9
	Male 3	35·5	8·2	8·3	52·0	18·8	70·8
	Female 1	42·5	16·4	7·7	66·6	6·4	73·0
	Female 2	48·0	12·1	6·9	67·0	5·6	72·6
	Female 3	49·3	9·7	4·1	63·1	6·1	69·2

Experiments with cannulated rats

Sodium ^{35}S-sulphide (5 μmoles in 0·5 ml 0·154 M phosphate buffer, pH 7·4, per 200 g body wt.) was administered intravenously to rats (2 male) with bile-duct and ureter cannulae. Urine and bile were collected into *N*-ethylmaleimide (5 μmoles in 100 μl water) at hourly intervals over a 6 hr period. Urine and bile samples were diluted with water to 5 ml and each sample was assayed for total ^{35}S. The urines were also subjected to thin-layer chromatography and paper electrophoresis. The results (Table 2) showed that 45 per cent of the injected radioactivity appeared in the urine within 6 hr, with small amounts (4·7–5·0 per cent) in the bile. The major portion of the radioactivity excreted in the urine in the first 5 hr was identical in mobility with inorganic ^{35}S-sulphate. Only trace amounts of other labelled materials were present. In the 5–6 hr urine sample these unidentified materials represented 15 per cent of the total ^{35}S excreted.

TABLE 2. THE DISTRIBUTION OF RADIOACTIVITY IN BILE AND URINE OF RATS, FOR THE 6 HR PERIOD FOLLOWING INTRAVENOUS ADMINISTRATION OF SODIUM ^{35}S-SULPHIDE (5 μMOLES PER 200 g BODY WT.)

Animal 1 (male)	Urine vol. (ml/hr)	Bile vol. (ml/hr)	% Radioactive dose recovered	
Time (hr)			Urine	Bile
1	0·60	1·03	16·9	1·2
2	0·19	1·15	9·8	0·8
3	0·23	1·20	10·0	0·8
4	0·23	1·37	4·3	0·7
5	0·26	1·29	3·2	0·6
6	0·22	1·51	1·2	0·6
0–6			45·4	4·7
Animal 2 (male)				
Time (hr)				
1	0·41	1·33	9·7	2·0
2	0·45	1·14	13·6	1·1
3	0·37	1·06	8·8	0·8
4	0·25	1·01	6·3	0·6
5	0·20	0·94	3·2	0·4
6	0·29	0·86	3·8	0·1
0–6			45·4	5·0

The small amount of labelled material appearing in the bile was not inorganic ^{35}S-sulphate. Thus the principal terminal fate of administered sulphide in rats is oxidation to sulphate and excretion in the urine, confirming the suggestions[18] and findings[1] of other workers. Attempts were next made to pinpoint the site(s) of oxidation by whole-body radioautography.

Whole-body radioautography

Young rats (4–6 weeks) were injected intraperitoneally with either sodium ^{35}S-sulphide (5 μmoles in 0·154 M phosphate buffer, pH 7·4, per 200 g body wt.) or sodium ^{35}S-sulphate (5 μmoles per 200 g body wt.) and killed at time intervals ranging from 3 min to 6 hr after injection. The results (Fig. 1) showed that both ^{35}S-sulphide and ^{35}S-sulphate became widely distributed and common areas of radioisotope accumulation include the gastrointestinal tract and cartilagenous tissues. The characteristic uptake of radioactivity in the bones (Figs. 1c and 1d) following both sulphide and sulphate administration suggests that sulphide is oxidized to sulphate *in vivo* prior to incorporation into mucopolysaccharides. The radioautograms obtained following sulphide administration were also characterized by accumulation of radioisotope in lung (Fig. 1a) and brain. The level of radioisotope in lung was considerably higher than that in blood and persisted up to 20 min after sulphide administration. In contrast, the level of isotope in brain was much less than in blood but was nevertheless clearly distinguishable and was more noticeable following the administration of a lethal dose of sodium ^{35}S-sulphide (Fig. 1e). Apart from the pattern of isotope in lung and brain, the gross distribution patterns of ^{35}S-sulphide and ^{35}S-sulphate were similar and did not pinpoint the tissue(s) responsible for sulphide oxidation. However, the persistence

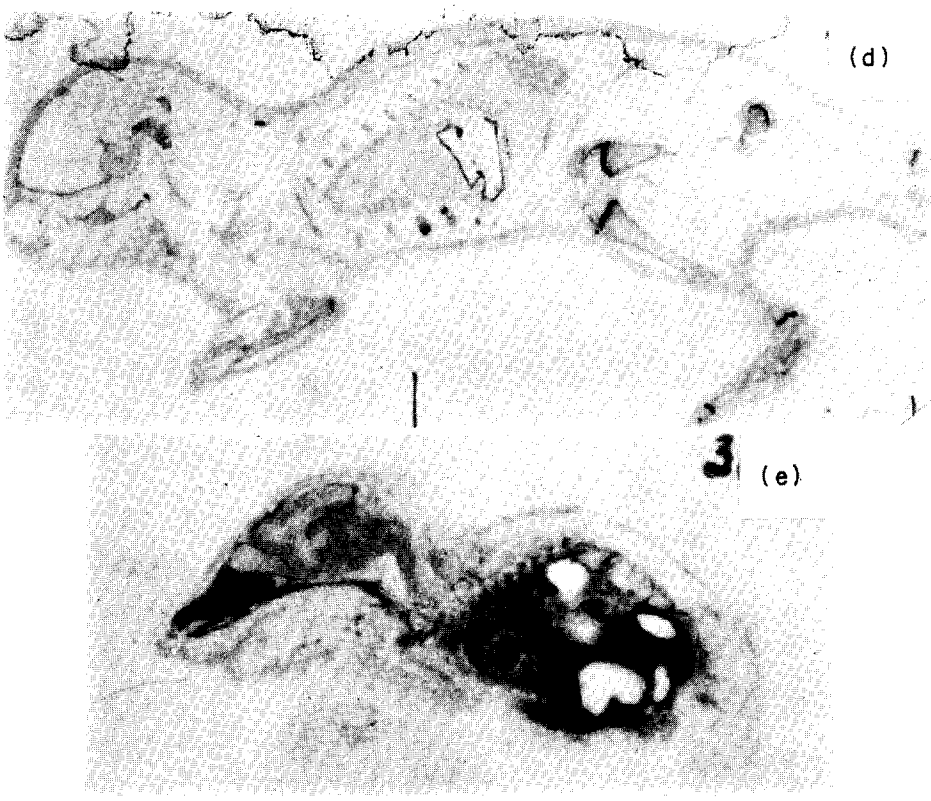
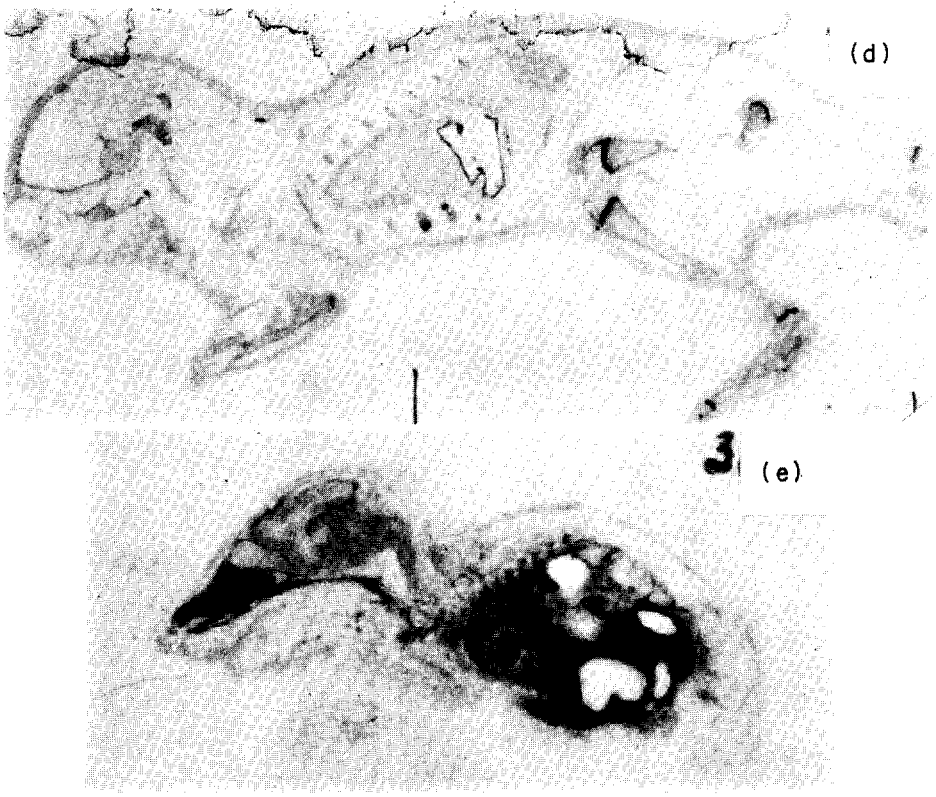


FIG. 1. (d and e)

FIG. 1. Whole-body radioautograms obtained from young rats receiving sodium ^{35}S-sulphide (1 μmole per 40 g body wt.) and sodium ^{35}S-sulphate (1 μmole per 40 g body wt.). Animals were killed; (a) 30 min after $Na_2{}^{35}S$ administration; (b), 30 min after $Na_2{}^{35}SO_4$; (c), 6 hr after $Na_2{}^{35}S$; (d), 3 hr after $Na_2{}^{35}SO_4$ and (e), after a lethal dose of $Na_2{}^{35}S$.



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of radioactivity in the blood coupled with the conflicting reports of the role of the plasma and red-cells in the detoxication and oxidation of sulphide, indicated that a more detailed study of the fate of sulphide in the blood was necessary.

Quantitative and qualitative examination of blood of rats following intravenous or oral administration of sodium ^{35}S-sulphide

Adult male rats were injected intravenously (5 animals) and orally (5 animals) with sodium ^{35}S-sulphide (5 μmoles in 0·5 ml 0·154 M phosphate buffer, pH 7·4, per 200 g body wt.) while under light ether anaesthesia. Animals were killed by exsanguination under ether at time intervals ranging from 5 min to 6 hr after injection and the blood (approx. 6 ml from each rat) was added directly to heparin (2 mg). Samples of the heparinized blood and plasma from each animal were assayed for total and trichloroacetate-insoluble ^{35}S. Further portions of blood (0·5 ml) were added to solutions of *N*-ethylmaleimide (5 μmoles in 50 μl of phosphate buffer, pH 7·4) at room temperature and portions (5–10 μl) were removed after 30 min and subjected to paper electrophoresis. Assuming a blood volume of 9 ml per 200 g rat, it was calculated that 5 min after intravenous administration of ^{35}S-sulphide only 10·7 per cent of the dose remained in the blood. Animals sacrificed after longer time periods showed progressively less radioactivity in the blood (Table 3). The corresponding results obtained after oral administration (Table 3) showed even less radioactivity in the blood. In both sets of animals the major portion (up to 94 per cent) of the ^{35}S in the blood was associated with the plasma rather than the cells. Scans and radioautograms of blood samples showed that after oral administration of ^{35}S-sulphide over 95 per cent of the ^{35}S present was there as inorganic ^{35}S-sulphate. After intravenous administration two radioactive components could be detected in the blood up to 1 hr after administration. The major peak corresponded to inorganic ^{35}S-sulphate while the other peak remained at the origin and accounted for 17 and 5 per cent of the total ^{35}S in blood after 5 min and 1 hr respectively. The analysis of trichloroacetate-insoluble ^{35}S in the blood and plasma suggested that the radioactive component at the origin could be attributed in part to the binding of ^{35}S to blood proteins.

TABLE 3. DISTRIBUTION OF ^{35}S IN BLOOD FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION OF SODIUM ^{35}S-SULPHIDE (5 μMOLES PER 200 g BODY WT.) TO RATS

Route of administration	Time	Total recovery in blood (% of injected dose)	Distribution of ^{35}S in blood (% of total ^{35}S in blood)	
			Plasma	Cells
Oral	5 min	3·6	93·3	6·7
	30 min	3·6	88·1	11·9
	60 min	4·7	90·5	9·5
	120 min	2·6	79·2	20·8
	360 min	2·0	88·1	11·9
Intravenous	5 min	10·7	94·7	5·3
	30 min	7·8	91·7	8·3
	60 min	5·4	87·0	13·0
	120 min	3·3	78·2	21·8
	360 min	1·2	78·9	21·1

The ability of ^{35}S-sulphide to bind to plasma proteins was confirmed *in vitro* when samples (2 μl) of a plasma–sodium ^{35}S-sulphide mixture (after incubating for 3 hr at 37°) were subjected to electrophoresis on cellulose acetate membranes. The resulting electrophoretograms were placed in contact with X-ray film for 28 days after which time the films were developed and the electrophoretograms stained for protein with Ponceau S. Both the stained electrophoretograms and the radioautograms were analysed by densitometric measurement. The results showed that ^{35}S-labelled material was bound to all the major plasma proteins with approximately 50 per cent being associated with the albumin fraction.

Thus, sodium ^{35}S-sulphide has only a transient existence in blood and even 5 min after intravenous administration the bulk of the radioactivity in the blood corresponds to inorganic ^{35}S-sulphate. The question then arises as to the contribution of the blood to the overall oxidation of administered sulphide.

The fate of sodium ^{35}S-sulphide in rat blood in vitro

Heparinized rat blood (85 ml) was equilibrated for 5 min at 37° with sodium ^{35}S-sulphide (50 μmoles in 5 ml of 0·154 M-phosphate buffer, pH 7·4) after which time samples were removed and; (a) assayed for total and trichloroacetate–insoluble ^{35}S; (b) added to *N*-ethylmaleimide (5 μmoles in 50 μl phosphate buffer, pH 7·4) and subjected to paper electrophoresis; (c) the plasma was separated as quickly as possible and assayed for total and trichloroacetate-insoluble ^{35}S and; (d) the haematocrit was determined. After the 5 min equilibration period the blood–^{35}S-sulphide mixture was circulated and oxygenated at 37° in an apparatus designed for the perfusion of isolated organs[19] and fitted with suitable zinc–acetate traps to absorb volatile sulphide. Further samples were subsequently removed after 30 min, 1, 3 and 6 hr and assayed in the same way. The distribution of ^{35}S in blood (Table 4) showed that after 5 min, 55·0 per cent of total ^{35}S in the blood was associated with the plasma and 44·3 per cent with the cells. In plasma alone 51·5 per cent of total ^{35}S was precipitated by trichloroacetate and the corresponding figure for cells showed that 70·6 per cent of total ^{35}S in cells was trichloroacetate–insoluble. Subsequent circulation and oxygenation of the blood–^{35}S-sulphide mixture resulted in a loss (as volatile free sulphide) of 36·6 per cent of total ^{35}S in the blood after 30 min, predominantly from the cells. Scans of the electrophoretograms obtained over the 6 hr period showed the appearance of five radioactive peaks (Fig. 2). The bulk of the radioactivity remained at the origin and inorganic ^{35}S-sulphate appeared as a minor component only.

TABLE 4. DISTRIBUTION OF ^{35}S IN RAT BLOOD FOLLOWING THE ADDITION OF SODIUM ^{35}S-SULPHIDE

Time after addition of Na_2 ^{35}S	Distribution of ^{35}S in blood % of administered dose			
	Plasma		Cells	
	Total	T.C.A. insoluble	Total	T.C.A. insoluble
5 min	55·0	28·3	44·3	31·3
30 min	54·4	32·0	8·3	7·1
1 hr	54·7	35·8	7·3	4·3
3 hr	58·7	41·8	9·5	5·5
6 hr	54·4	39·7	11·1	8·2

Oxidation of sodium ^{35}S-sulphide by the isolated, perfused rat liver

The isolated rat liver was perfused with heparinized whole blood[17] (90 ml) at a rate of 7·5–10 ml/min and sodium ^{35}S-sulphide (50 μmoles in 5 ml 0·15 M phosphate buffer, pH 7·4) was added. Samples were subsequently withdrawn after 9, 30 min, 1, 3 and 6 hr respectively and analysed as described for the control experiment in the absence of the liver (see preceding section).

The quantitative distribution of ^{35}S in the blood was different in one major respect from that observed in the control experiment (Table 5). When the liver was included

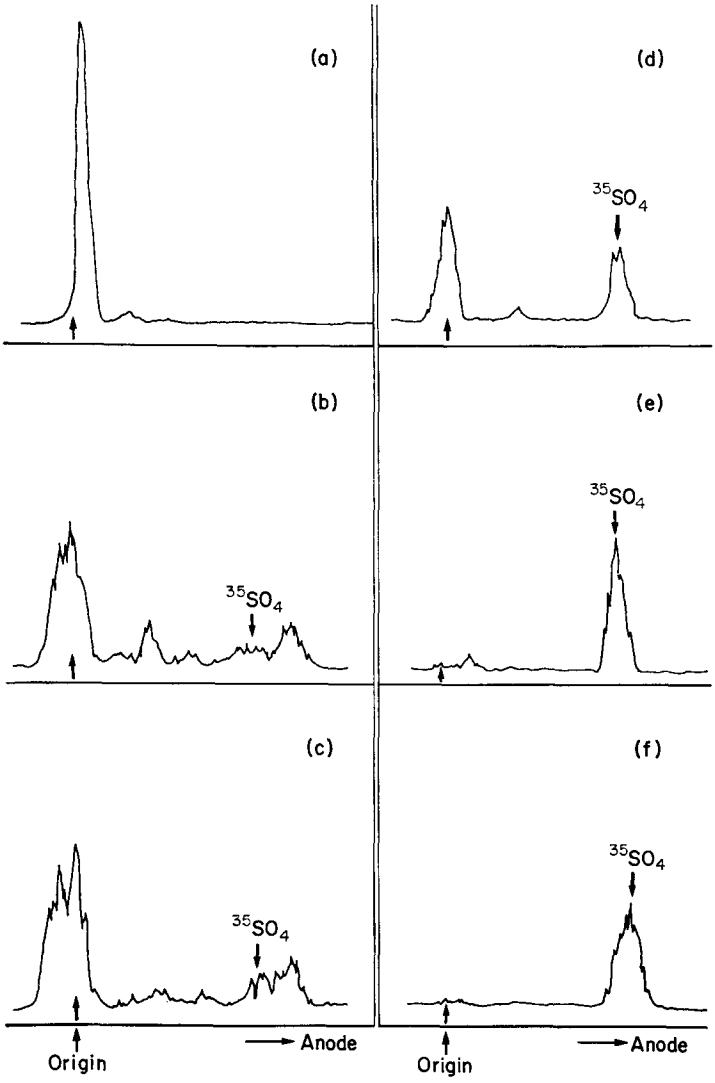

FIG. 2. Distribution of ^{35}S in blood following the addition of sodium ^{35}S-sulphide. Samples were subjected to electrophoresis 5 min; (a), 1 hr (b), 6 hr (c), following the addition of sodium ^{35}S-sulphide to whole blood. Samples (d), (e) and (f) were obtained 9 min, 1 and 6 hr respectively after the addition of sodium ^{35}S-sulphide to the isolated perfused rat liver system.

in the system, the amount of ^{35}S in the plasma which was precipitated by trichloroacetate decreased to 3 per cent of the administered dose after 6 hr perfusion. In the control experiment the amount of ^{35}S in this fraction increased with time in the absence of the liver and accounted for approximately 40 per cent of the administered dose. The quantitative redistribution of ^{35}S in blood during liver perfusion was accompanied by qualitative changes. Scans of the electrophoretograms of the perfusing blood (Fig. 2) showed that sodium ^{35}S-sulphide was rapidly oxidized to inorganic ^{35}S-sulphate and after 1 hr perfusion, only trace amounts of other ^{35}S-labeled compounds could be detected.

In a similar experiment, sodium ^{35}S-sulphide (50 μmoles in 5 ml 0·15 M phosphate buffer, pH 7·4) was preincubated with whole rat blood (30 ml) at 37° for 1·5 hr and then added to an isolated liver preparation which was perfused with normal rat blood (80 ml). Electrophoretograms of the blood again showed rapid oxidation to inorganic ^{35}S-sulphate. Thus, the ability of ^{35}S-sulphide to bind to blood proteins does not prevent subsequent oxidation to ^{35}S-sulphate by the isolated perfused liver.

TABLE 5. DISTRIBUTION OF ^{35}S IN BLOOD DURING THE PERFUSION OF ISOLATED RAT LIVER WITH BLOOD TO WHICH HAD BEEN ADDED SODIUM ^{35}S-SULPHIDE

Time after onset of perfusion	Distribution of ^{35}S in blood % of administered dose			
	Plasma		Cells	
	Total	T.C.A. insoluble	Total	T.C.A. insoluble
9 min	44·3	16·2	7·2	6·7
30 min	46·0	10·6	4·3	2·3
1 hr	45·6	7·9	5·2	1·2
3 hr	42·0	4·9	5·6	0·5
6 hr		3·0		1·1

DISCUSSION

The very high toxicity of H_2S is due to its direct action on nerve centres[3] and, in the past, the lack of an accumulative effect of sulphide poisoning has been explained in terms of rapid detoxication in either plasma or red-blood cells. The *in vitro* studies described above support this view, since ^{35}S-sulphide detoxication occurs in both plasma and red-blood cells, primarily as a result of rapid binding to blood proteins. The mechanism of sulphide binding has not been established with certainty, but preliminary studies have shown that thiols such as cysteine and reduced glutathione effect the release of ^{35}S from plasma proteins labeled by reaction with sodium ^{35}S-sulphide. These findings suggest the possibility that ^{35}S-sulphide binds to blood proteins by the reduction of disulphide bridges and the production of persulphide (R-S ^{35}SH) groups.[20] *In vitro* studies also show that the rate of oxidation of $^{35}S^{2-}$ ions to $^{35}SO_4^{2-}$ ions by the blood is too low to account for the rate of oxidation of administered ^{35}S-sulphide in the whole animal. Indeed oxidation to $^{35}SO_4^{2-}$ ions is much more rapid in phosphate buffer alone and in this respect the blood reduces the rate of inorganic ^{35}S-sulphate formation. However, *in vivo* ,^{35}S-sulphide has only a transient existence in blood and is rapidly oxidised to inorganic ^{35}S-sulphate in the

tissues. The isolated perfused liver is effective in oxidizing sulphide to sulphate although this is likely to be a property of many other cell types. The ability of other organs to oxidise sulphide is now under investigation and preliminary studies with the isolated perfused rat lung indicate that this tissue does not possess these oxidizing properties.

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