A Study of Vitamin B₁₂ Protection in Experimental Liver Injury to the Rat by Carbon Tetrachloride

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Recent work on the biochemical and histological changes after administration of carbon tetrachloride to rats has demonstrated that cell fractions from steatotic livers, particularly mitochondria, undergo morphological modifications (Dianzani, 1953; Dianzani & Bahr, 1954) accompanied by major biochemical alterations (Dianzani, 1954*a, b*, 1955, 1956, 1957; Dianzani & Dianzani, 1957; Dianzani & Viti, 1955) including uncoupling of oxidative phosphorylation (Dianzani, 1954*b*). Metabolic derangements would obviously follow the uncoupling of oxidative phosphorylation since this is the only major pathway linking the utilization of energy with its liberation by oxidative phosphorylation.

It has been generally observed that there is a spontaneous process of recovery after initial liver damage by carbon tetrachloride if the animals are given stock diet. More specifically, protection, as indicated by increased survival time, has been observed with methionine, choline, methyl purines, etc. (Hove, 1948), sulphur-containing amino acids, vitamin E (Hove, 1948; Hove & Hardin, 1952), vitamin B₁₂ (Popper, Koch-Weser & Szento, 1949) and folic acid (Hove & Hardin, 1951). The fundamental metabolic deviation causing fatty degeneration has, however, not been well understood. An attempt has been made here to show that a rapid rate of depletion of vitamin B₁₂ and of soluble sulphhydryl compounds are among the major derangements preceding liver injury by carbon tetrachloride. The possibility has been indicated that the protection afforded by vitamin B₁₂ may be due to its influence on sulphhydryl metabolism and on mitochondrial integrity. Observations on centrifugally separated cell fractions are included. A preliminary report on some of these observations has been published elsewhere (Kasbekar, Rege & Sreenivasan, 1956).

EXPERIMENTAL

Adult male albino rats (Wistar strain) weighing about 150 g. were used throughout the experiments. These were reared on the laboratory stock diet made up of (percentages) wheat flour 75, casein 12, whole-milk powder 2, dried yeast 2, arachis oil 4, vitaminized sesame oil 1, sodium chloride 2, calcium carbonate 2. When required, the

animals were injected intraperitoneally with 2 ml. of carbon tetrachloride/kg. body wt. The control animals were similarly given corresponding amounts of water. Where protection by vitamin B₁₂ was studied, the vitamin was given intraperitoneally (10 µg./animal) 3 hr. before the injection of carbon tetrachloride, since maximal protection by the vitamin against fat accumulation was observed under these conditions (Rege, 1953). At intervals of 6 and 48 hr. after administration of carbon tetrachloride, animals were killed by decapitation except when blood was required. Blood was withdrawn by venepuncture after ether anaesthesia, immediately citrated and a portion centrifuged for plasma. Livers were excised quickly, chilled in 0.25 M-sucrose for 5 min., dried with filter paper, weighed accurately and homogenized in a previously chilled Potter-Elvehjem glass homogenizer to 10% suspensions in cold 0.25 M-sucrose containing 0.0018 M-calcium chloride. Portions were used for fractionation according to the procedure of Schneider & Hogeboom (1950) as described by Kasbekar, Lavate, Rege & Sreenivasan (1959).

METHODS

Vitamin B₁₂. This was determined in liver and blood samples after papain liberation. Assays were carried out with *Lactobacillus leichmannii* by a turbidimetric adaptation of the U.S. Pharmacopoeia method (Kasbekar, Lavate, Rege & Sreenivasan 1959).

Soluble sulphhydryl compounds. These were estimated in cold 3% metaphosphoric acid extracts of liver and its fractions by the nitroprusside method of Grunert & Phillips (1951).

Total lipids and phospholipids. These were determined by the extraction procedure of Bloor (1943) as follows.

Portions of homogenates or fractions were refluxed twice with ethanol-ether mixture (3:1) for 30 min. The combined extracts and washings were evaporated to a small volume and rectified for ethanol-dissolved non-lipid substances by re-extraction with light petroleum (b.p. 40–60°), which specifically dissolves the lipids. The concentrates were gently boiled for 30 sec. with this solvent, the procedure was repeated and the combined extracts were centrifuged, evaporated to dryness and weighed as total lipids.

For phospholipid estimations, the weighed lipid residue was redissolved in light petroleum (b.p. 40–60°) and the solution was evaporated to a small volume. Measured portions were transferred to centrifuge cups, acetone was added to fill the tubes and this was followed by 4–5 drops of 4.5% magnesium chloride solution in ethanol. The precipitate was centrifuged, taken up in chloroform and a portion evaporated to dryness. The residue was digested by 2 ml. of 30% sulphuric acid over a microburner to a clear solution.

A drop of nitric acid was added to facilitate digestion. The solution was cooled, made up to a convenient volume and phosphorus was determined by the colorimetric method of Fiske & Subbarow (1925). Phospholipids were calculated by using 22.7 as conversion factor.

Methionine. This was determined by the method of Horn, Jones & Blum (1946). The colour was read in a Klett-Summerson photoelectric colorimeter at 540 m μ . Blanks were run and due allowances made.

Glycogen. This was determined according to the method of Reinecke (1942) after ethanol precipitation and hydrolysis with N-sulphuric acid (Walaas & Walaas, 1950). After oxidation with potassium ferricyanide, the ferrocyanide left over was read in a Klett-Summerson photoelectric colorimeter at 660 m μ against a blank similarly treated.

Nucleic acids. These were extracted with trichloroacetic acid essentially as recommended by Schneider (1946). Suitable portions were used for the determination of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

For RNA determinations, the method of Albaum & Umbreit (1947) was used with modifications, as adopted by Rege & Sreenivasan (1954). RNA (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was used as standard. The values were computed from a graph with standard material.

The diphenylamine method of Dische (1930) was used for the estimation of DNA. To a suitable portion of the extract made to 2 ml. with water, 4 ml. of Dische reagent was added and the tubes, after mixing, were kept in vigorously boiling water for 5 min. The tubes were immediately cooled under the tap and allowed to stand at room temperature for exactly 1 hr., as the colour deepens on standing. The intensity of the colour was measured in a photoelectric colorimeter at 660 m μ . DNA (Schwarz Laboratories Inc., N.Y., U.S.A.) was used as standard.

Total protein. This was determined by the method of Osborn, Felts & Chaikoff (1953) after trichloroacetic acid precipitation.

All results reported represent averages of at least four independent determinations.

RESULTS

Suitable dosage of carbon tetrachloride and the period of maximum damage were determined in preliminary experiments. With a single intraperitoneal injection of 2 ml. of carbon tetrachloride/kg. body wt., maximum increase in total liver lipids was seen within 48 hr. (Table 1). The treatment was not fatal; the animals received a complete ration and the liver tended to return to normal after 48 hr.

Changes in vitamin B₁₂. Within 6 hr. of administration of carbon tetrachloride there was a marked fall in liver vitamin B₁₂ levels (Table 2). This depletion was reflected in the lowering of the vitamin reserves in the particulate fractions of liver cells. No major change was observable in blood at 6 hr., although a further study of the distribution of vitamin B₁₂ revealed that there was a considerable increase in plasma vitamin levels with a concomitant fall in the erythrocyte vitamin B₁₂. These

Table 1. *Optimum dosage of carbon tetrachloride and time of maximum liver damage*

Rats on the laboratory stock diet (see text), averaging 150 g. wt., were used. Carbon tetrachloride was administered intraperitoneally.

CCl ₄ (ml./kg. body wt.)	Time (hr.)	Liver wt. (g./100 g. body wt.)	Lipids (mg./g. of fresh liver)
1.0	24	5.6	30.6
	48	6.0	41.3
	72	5.3	40.8
1.5	24	5.3	37.5
	48	6.7	40.9
	72	6.7	40.1
2.0	24	6.2	44.7
	48	8.0	48.9
	72	8.4	50.7
Nil	—	4.4	23.4

observations suggest that the steatotic poison probably acts by provoking a temporary release of the vitamin from cellular particles to plasma.

At 48 hr. after administration of the steatogen, a partial regeneration of vitamin B₁₂ levels in liver was apparent though there was no improvement in the mitochondrial vitamin B₁₂ content. The plasma vitamin B₁₂ continued to be high but was slightly lower than that at 6 hr. A corresponding improved trend was noticed in erythrocyte vitamin B₁₂.

In preliminary experiments it was observed that vitamin B₁₂ could afford maximum protection, as judged from liver lipids, at a dosage of 10 μ g./animal only when administered 3 hr. before injection of carbon tetrachloride. In subsequent work on this protective effect these conditions were therefore used.

In the animals protected with vitamin B₁₂, near-normal levels of the vitamin were found in liver particulate fractions 6 hr. after administration of the steatogen. The levels of plasma and the supernatant liver fraction showed a rise. At 48 hr., the cell fractions were replete with the vitamin and the plasma levels showed a tendency to return to normal (Table 2). The protection afforded by vitamin B₁₂ against toxicity of carbon tetrachloride could therefore be related to this restoration of vitamin B₁₂ levels.

Sulphydryl compounds. The lowering of liver vitamin B₁₂ levels was closely followed by changes in the sulphydryl reserves of the livers in carbon tetrachloride injury (Table 3). The liver sulphydryl compounds reached minimum levels within 6 hr. of the administration of the steatogen (cf. Patwardhan, Ramalingaswami, Sriramachari & Patwardhan, 1953). The nuclear glutathione remained steady, and the depletion was limited entirely to the soluble fraction; the mitochondrial fraction was

Table 2. *Intracellular distribution of vitamin B₁₂*

Rats about 150 g. were reared on stock diet (see text). Carbon tetrachloride (0.3 ml.) was administered intraperitoneally. Vitamin B₁₂ was also given intraperitoneally (10 µg./animal) 3 hr. before administration of carbon tetrachloride. Methods of fractionation and vitamin B₁₂ assay are as described in the text.

Time after administration of carbon tetrachloride (hr.)	Untreated control (stock diet)	6		48	
		Without vitamin B ₁₂	Vitamin B ₁₂ -protected	Without vitamin B ₁₂	Vitamin B ₁₂ -protected
		(µmg./g. of fresh liver)			
Liver	105.0 ± 8.5	67.5 ± 10.0	122.0 ± 11.0	90.0 ± 7.0	120.0 ± 5.0
Nuclei	35.5 ± 4.5	20.0 ± 5.0	37.5 ± 2.5	36.0 ± 6.6	48.0 ± 2.5
Mitochondria	19.1 ± 2.9	11.3 ± 3.5	16.6 ± 4.0	12.0 ± 3.4	18.5 ± 2.6
Supernatant	37.5 ± 5.2	32.5 ± 4.3	55.5 ± 5.0	40.0 ± 6.0	48.0 ± 4.0
		(µmg./ml.)			
Blood	1.60 ± 0.15	1.45 ± 0.20	1.85 ± 0.19	1.45 ± 0.15	1.70 ± 0.30
Plasma	0.85 ± 0.10	1.10 ± 0.10	1.25 ± 0.15	1.00 ± 0.05	0.95 ± 0.26
Erythrocytes (by difference)	0.75	0.35	0.60	0.45	0.75

Table 3. *Concentrations of liver sulphydryl compounds*

See Table 2 and text for details.

Group	Liver	Nuclei	Supernatant
	(mg./100 g. of fresh liver)		
Control	141 ± 29	18 ± 2	113 ± 17
6 hr. after CCl ₄	41 ± 20	17 ± 3	23 ± 14
6 hr. after CCl ₄ ; vitamin B ₁₂ -protected	134 ± 23	18 ± 2	106 ± 11
48 hr. after CCl ₄	122 ± 14	16 ± 2	100 ± 16
48 hr. after CCl ₄ ; vitamin B ₁₂ -protected	173 ± 10	19 ± 3	149 ± 11

Table 4. *Incorporation of cysteine into liver sulphydryl compounds*

For details of diet, grouping and methods see text and Table 2. Cysteine hydrochloride (30 mg. in 1 ml. of neutral solution/animal) was administered intraperitoneally to all groups and the animals were killed 3 hr. later.

Time after carbon tetrachloride administration (hr.)

Control	Without vitamin B ₁₂	6		48	
		Without vitamin B ₁₂	Vitamin B ₁₂ -protected	Without vitamin B ₁₂	Vitamin B ₁₂ -protected
		(mg./100 g. of fresh liver)			
Liver	251.1 ± 21.0	46.7 ± 12.3	186.5 ± 26.2	258.7 ± 24.1	248.9 ± 18.8
Nuclei	23.5 ± 3.6	15.4 ± 2.1	21.1 ± 4.0	24.5 ± 2.9	25.5 ± 1.8
Supernatant	230.8 ± 17.4	28.1 ± 9.8	156.8 ± 20.1	231.4 ± 21.4	219.6 ± 14.3

Table 5. *Alterations in certain liver constituents 6 and 48 hr. after injection with carbon tetrachloride*

Animals (150 g.) fed on stock diet were used. Vitamin B₁₂ (10 µg.), where given, preceded administration of carbon tetrachloride (0.3 ml.) by 3 hr. Animals were killed 6 or 48 hr. after the last injection. For methods see text.

	Control	Control + vitamin B ₁₂	CCl ₄ treated		CCl ₄ treated + vitamin B ₁₂	
			6 hr.	48 hr.	6 hr.	48 hr.
			(mg./g. of fresh liver)			
Lipids	23.60 ± 1.51	22.78 ± 1.61	29.14 ± 1.24	48.79 ± 2.51	28.22 ± 1.09	34.91 ± 1.52
Phospholipids	15.14 ± 0.20	15.32 ± 0.11	15.06 ± 0.32	10.60 ± 0.16	15.51 ± 0.23	14.64 ± 0.21
RNA	10.64 ± 0.61	10.78 ± 0.72	8.81 ± 0.64	6.91 ± 0.91	9.44 ± 0.57	10.32 ± 0.33
DNA	3.81 ± 0.36	3.76 ± 0.42	3.64 ± 0.30	3.65 ± 0.28	3.72 ± 0.34	3.61 ± 0.32
Protein	196.54 ± 10.71	197.21 ± 11.94	181.27 ± 8.41	164.66 ± 9.62	197.94 ± 13.60	190.18 ± 12.16
Glycogen	3.34 ± 0.51	3.52 ± 0.41	1.03 ± 0.22	0.71 ± 0.24	1.51 ± 0.38	0.64 ± 0.40
Methionine	5.01 ± 0.49	5.12 ± 0.29	4.90 ± 0.53	4.91 ± 0.33	5.09 ± 0.41	4.97 ± 1.04

Table 6. *Fractionwise distribution of liver constituents 6 and 48 hr. after injection with carbon tetrachloride*

For details and methods see Table 5 and text. N, Nuclei; M, mitochondria; S, supernatant.

Group	Liver fraction	Lipids	Phospholipids (mg./g. of fresh liver)	RNA	DNA	Proteins
Control	N	4.68	2.71	2.50	3.84	59.6
	M	6.15	5.23	1.60	0	34.1
	S	13.08	7.39	6.71	0	101.8
Control + vitamin B ₁₂	N	4.56	2.88	2.52	3.79	60.5
	M	6.00	5.30	1.64	0	33.9
	S	12.89	7.44	6.80	0	102.5
6 hr. after CCl ₄	N	4.91	2.64	2.41	3.51	57.5
	M	8.54	5.01	1.52	0	32.1
	S	15.85	7.13	4.97	0	92.1
48 hr. after CCl ₄	N	8.51	2.19	2.22	3.39	55.6
	M	9.97	4.41	1.00	0	30.4
	S	29.89	3.94	3.75	0	77.8
6 hr. after CCl ₄ + vitamin B ₁₂	N	4.79	2.60	2.58	3.77	58.9
	M	8.19	5.24	1.62	0	32.9
	S	15.12	7.29	5.36	0	104.6
48 hr. after CCl ₄ + vitamin B ₁₂	N	5.23	2.41	2.46	3.54	57.6
	M	8.86	4.89	1.52	0	33.1
	S	20.74	7.32	6.57	0	98.2

practically devoid of glutathione. Prior administration of vitamin B₁₂ afforded practically complete protection against this sulphydryl depletion.

In the absence of protection by vitamin B₁₂ the sulphydryl level was restored to within normal limits about 48 hr. after carbon tetrachloride administration. There was also a marked regeneration of liver vitamin B₁₂ levels during this period (Table 2).

A similar trend was observed in the incorporation of intraperitoneally administered cysteine into liver sulphydryl compounds of animals poisoned with carbon tetrachloride (Table 4). In other experiments, a maximal increase in sulphydryl compounds of liver in normal animals was observed 3 hr. after administration of 30 mg. of cysteine (Kasbekar, Lavate, Rege & Sreenivasan, 1959). In the animals with steatosis, which were given similar doses of cysteine 3 hr. after carbon tetrachloride administration, no increase in liver sulphydryl compounds was noticed after a further 3 hr. Prior administration of vitamin B₁₂ had a considerable influence on this derangement. At 48 hr., however, there was nearly normal incorporation and vitamin B₁₂ treatment had little stimulatory effect. While the major increase in the sulphydryl compounds was limited to the soluble fraction, the nuclear sulphydryl compounds also showed some rise (Table 4).

The depletion of liver vitamin B₁₂ and impairment in sulphydryl metabolism considerably precede the general derangement in liver functions, as is indicated by observations reported in Tables 5 and 6.

Total lipids. As a result of carbon tetrachloride

treatment, the total lipid content of the liver showed a slight increase at 6 hr. At 48 hr., there was a nearly twofold increase in the fat content of liver. The major portion of the lipids was in the supernatant. The lipid found in the supernatant fraction seems to be an artifact due to homogenization and centrifuging, and its exact location in the intracellular structures is not understood from the experiments described. A general rise was observable in the particulate fractions.

Phospholipids. A slight decrease in liver phospholipids at 6 hr. was further magnified at 48 hr. Since a general morphological damage to the particulate fractions is observed in steatosis (Dianzani, 1953; Dianzani & Bahr, 1954), a lowering of phospholipids in the fractions might be expected. As with neutral lipids, the decrease in phospholipids observed was reflected most in the supernatant fraction.

Nucleic acids. Whereas the DNA content of liver was unaltered in carbon tetrachloride poisoning, there was considerable depletion in RNA. During 48 hr. of injury the RNA in the supernatant was reduced by nearly half.

Proteins. A slight but significant decrease in total proteins was evident both at 6 and 48 hr. after injury.

Methionine. Methionine content of the liver was unaffected throughout.

Glycogen. A marked drop in liver glycogen immediately after carbon tetrachloride poisoning persisted even beyond 48 hr.

Activities of liver choline oxidase, D-amino acid oxidase and xanthine dehydrogenase were studied at 48 hr. after administration of the steatogen. No

appreciable change was, however, noticeable when results were expressed per unit of dry liver.

The protective action of vitamin B₁₂ against carbon tetrachloride injury is evident from the data in Tables 5 and 6. Prior treatment with the vitamin (10 µg./animal) afforded protection as seen from restoration of almost all the constituents studied. There was, however, no effect on glycogen depletion within the 48 hr. period of study.

DISCUSSION

Treatment with carbon tetrachloride results in a depletion of liver vitamin B₁₂. The simultaneous increase in plasma vitamin level suggests the possibility that the steatogen provokes a loss of cellular vitamin B₁₂ to the soluble fractions and plasma. The possibility exists that a part at least of this increase in plasma vitamin B₁₂ might arise from damage to erythrocytes. The observations of Yamamoto, Okuda & Chow (1957), that an increased urinary excretion of vitamin B₁₂ accompanies the increase in plasma B₁₂ levels after carbon tetrachloride administration, supports this view. This temporary depletion of the vitamin is manifested early and hence probably initiates a series of degenerative processes in the liver and its cell fractions, particularly the mitochondria.

A major change that accompanies the lowering of liver vitamin B₁₂ is the remarkable depletion of liver reserves of sulphhydryl compounds. The lowering of glutathione in blood and liver in vitamin B₁₂ deficiency has also been observed by others (Ling & Chow, 1954; Register, 1954). Possibly, this depletion of sulphhydryl compounds which accompanies the lowering of liver vitamin B₁₂ precedes other changes observed in carbon tetrachloride injury, since prior administration of vitamin B₁₂ counteracts appreciably the disappearance of stores of soluble sulphhydryl compounds in liver and affords protection against subsequent damage.

Impairment of mitochondrial integrity could either arise from or be the cause of depletion in liver vitamin B₁₂ and glutathione. A role for the sulphhydryl group and glutathione in the protection of cellular morphology has been postulated by other workers (Labarre, 1945; Tapley & Cooper, 1956). The intense fatty degeneration of liver and its depletion of pyridine nucleotides has been suggested to result from a lowered concentration of adenosine triphosphate as a result of impaired mitochondrial integrity (Dianzani, 1955). The depletion of RNA, phospholipids, proteins, etc. appears to reflect in a general manner the impaired production of energy-rich phosphate esters in damaged mitochondria. It has been observed that in mitochondria from steatotic livers there is a decreased ability to retain nucleotides and other

cofactors of oxidative phosphorylation (Kasbekar & Sreenivasan, 1956). A similar behaviour is seen with mitochondria from livers of rats rendered thyrotoxic (Kasbekar, Rege & Sreenivasan, 1959). There is a striking similarity in the mechanism of protection by vitamin B₁₂ against apparently unrelated metabolic stress conditions caused by experimental thyrotoxicosis (Kasbekar, Lavate, Rege & Sreenivasan, 1959) or poisoning with carbon tetrachloride.

The severe depletion of glycogen reserves in the initial stage of liver injury may be caused by the necessity to compensate for the loss of synthesis of adenosine triphosphate and also to help counteract the effects of the steatogen. Increased glycolytic activity is also observable in tumour tissues in which impairment to mitochondrial structure occurs (Potter & Siekevitz, 1952).

In other work from this laboratory (Nadkarni, Wagle & Sreenivasan, 1957) it has been observed that vitamin B₁₂ affords protection against depletion of liver pyridine nucleotides and the consequent reduction in the ratio of oxidized to reduced pyridine nucleotides in carbon tetrachloride injury. Since the synthesis of adenosine triphosphate is impaired as a result of structural damage to mitochondria (Kielley & Kielley, 1951), the observations are explicable on the basis that vitamin B₁₂ may aid in the maintenance of mitochondrial integrity and hence of pyridine nucleotide levels.

The protection by vitamin B₁₂ when administered before carbon tetrachloride injury is in sharp contrast with the meagre effect observed when the vitamin is administered after carbon tetrachloride poisoning (Rege, 1953). The capacity of the liver to store the vitamin may thus be diminished immediately after administration of the steatogen. The phenomenon of vitamin B₁₂ protection in carbon tetrachloride injury therefore appears to be a general one arising from its effect on mitochondrial integrity rather than particular constituent in liver. Protection by vitamin E in dietary necrotic liver degeneration has been similarly correlated to mitochondrial function (Chernick, Moe, Rodnan & Schwarz, 1955; Rodnan, Chernick & Schwarz, 1956).

SUMMARY

1. The effects of carbon tetrachloride poisoning and of the protection afforded by vitamin B₁₂ were studied. The steatogen appears to act by provoking a loss of vitamin B₁₂ from liver and its particulate fractions.

2. A simultaneous impairment in the metabolism of sulphhydryl compounds in liver is observed.

3. These changes are followed by a depletion of liver ribonucleic acid, phospholipids and proteins

and an accumulation of fat. Methionine levels are unaffected. Depletion of liver glycogen is pronounced and lasting and vitamin B₁₂ apparently does not protect against this metabolic disturbance.

4. Protection by vitamin B₁₂ is non-specific and may be due to an effect on the maintenance of mitochondrial integrity.

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Biosynthesis of Glutathione by Rat Erythrocytes

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Studies on the turnover of reduced glutathione in the blood of several species *in vivo* have indicated that this tripeptide is in a dynamic state (Burwell, Brickley & Finch, 1953; Dimant, Landsberg & London, 1955; Grinstein, Kamen & Moore, 1949; Jope, 1946; London, Shemin, West & Rittenberg, 1949; Mortensen, Haley & Elder, 1956; Shemin & Rittenberg, 1946). The half-life of glutathione, as determined from a disappearance of the label in

glutathione after a single injection of [¹⁵N]glycine, has been shown to be much shorter in comparison with the time for 50 % survival of the erythrocytes, as evaluated from the ¹⁵N concentration in haemin (Dimant *et al.* 1955). These observations raised speculation about the origin of glutathione in mammalian erythrocytes.

Anderson & Mosher (1951) detected ³⁵S in erythrocyte glutathione after administration of