METABOLISM OF SULPHUR AMINO ACIDS

I. THE UPTAKE OF CYSTEINE BY RAT LIVER

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Summary

Cysteine was administered to rats by stomach tube or by intraperitoneal injection. In some animals the concentration of glutathione in the liver was first lowered to 10 per cent. of the normal level by injecting the animals with bromobenzene. When 30 mg of cysteine were given by stomach tube, 75 per cent. of the cysteine taken up by the liver in normal rats was oxidized in 1 hr to products other than cystine. In animals treated with bromobenzene all the cysteine was converted to glutathione and no increase could be found in the oxidized sulphur fraction. In both groups of animals only traces of cysteine were present.

The accumulation of free cysteine in the liver could only be obtained if the cysteine was given intraperitoneally and if very large doses (150 mg) were used.

I. Introduction

In normal rat livers, cysteine contributes less than 1 per cent. to the total free amino acid pool (Wu 1954), whereas its concentration in protein molecules is much higher (Block and Bolling 1951). Much of the cysteine taken up by the liver is used for protein synthesis, and some is built up into glutathione (Anderson and Mosher 1951). In addition, the liver contains enzyme systems capable of oxidizing the sulphydryl group of cysteine to cystine and to other oxidation products (Singer and Kearney 1955).

In the present investigation, the conditions necessary for the accumulation of cysteine and the relative importance of the different metabolic pathways for the removal of cysteine were studied. It was found that cysteine conversion to glutathione could best be followed in animals where the concentration of glutathione in the liver had first been lowered. This is often done by feeding the rats a diet containing suboptimal amounts of sulphur amino acids (Barford and Eden 1956), but the glutathione concentration can be lowered more rapidly by administering a compound that will remove some of the cysteine present in the body. Halogenated hydrocarbons have been used for this purpose because they are detoxicated by conjugation with cysteine and the mercapturic acids thus formed are excreted in the urine (Koch-Weser *et al.* 1953). The effect of one of these compounds, bromobenzene, on the concentration of glutathione in the liver was investigated in some detail and the necessary conditions were established so that a single injection would lower the concentration of glutathione to 10 per cent. of its normal level. These animals were then used for the study of cysteine metabolism in the same manner as for normal rats.

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II. Methods

(*a*) *Animals*

Female albino rats of 150–250 g body weight were used. Bromobenzene treatment consisted of intraperitoneal injection of 75 mg (0·05 ml) of bromobenzene/100 g body weight (for discussion of bromobenzene treatment see Section III). Both injected and normal animals were fasted for 16 hr to eliminate any absorption of remaining food from the intestine.

In the early experiments the animals were dosed with glucose, but as this caused no increased accumulation of sulphur-containing compounds in the liver it was omitted in the later experiments. Only when cysteine was given by stomach tube was glucose added and in these experiments 30 mg of cysteine hydrochloride and 150 mg of glucose were dissolved in 1 ml of water. Although this solution was acid it was not neutralized because of the acid pH of the stomach. When cysteine was injected intraperitoneally the solution was adjusted to approximately pH 4 with sodium bicarbonate.

In some cases repeated samples of liver were removed from the same animals. The rats were anaesthetized with "Nembutal" (5 mg/100 g of body weight), a trachea cannula was inserted, and artificial respiration was applied when necessary. The abdomen was opened, a sample of liver (approx. 0·3 g) removed, and the bleeding stopped with gelatin sponge. Then the animal was dosed with cysteine by stomach tube and further samples of the liver were removed at 30-min intervals. With this technique the animals could be kept alive for approximately 2 hr.

(*b*) *Chemical Methods*

Samples of liver (0·67 g) were homogenized in trichloroacetic acid (5 per cent.) and the homogenates were made up to 10 ml. Trichloroacetic acid proved unsatisfactory when the enzymatic method was used for the determination of glutathione, and for these estimations the homogenates were prepared in sulphosalicylic acid (2 per cent.).

Total sulphur was determined after the digestion of organic sulphur by adding barium chloride and measuring the turbidity thus obtained (Denis and Reed 1926). The specific brucine method was used for the determination of cysteine (Hird and Springell 1954).

The concentration of sulphydryl groups was estimated by the nitroprusside test (Grunert and Phillips 1951). Cystine was determined by reaction with a concentrated cyanide solution followed by estimation of the cysteine sulphydryl groups produced. The reaction was carried out by incubating 0·5 ml of the protein-free extract with 0·5 ml of a solution containing potassium cyanide (5 per cent.) in potassium carbonate (28 per cent.). The cyanide reaction yields approximately one thiol group for each disulphide and with each set of estimations a standard cystine solution was included from which the extent of reaction could be calculated. The amount of oxidized sulphur was calculated by subtracting the value obtained for sulphydryl sulphur from that of total sulphur.

Glutathione was determined by means of the specific glutathione reductase method. All sulphydryl groups were oxidized with hydrogen peroxide to the disulphide form, then glutathione reductase was added for the regeneration of glutathione. Other disulphide compounds are not reduced by this enzyme (Racker 1954). The actual procedure used was as follows: to 1·5 ml of tissue extract in 2 per cent. sulphosalicylic acid was added 0·2 ml of tris(hydroxymethyl)amino-methane buffer (0·5M, pH 7·4), 0·1 ml of disodium ethylenediaminetetra-acetate (5×10^{-3}M), and 0·1 ml of hydrogen peroxide (0·3M) and the tube kept at 37°C for 5 min. The solution was then adjusted to pH 7·4 with sodium hydroxide and the reduction system was added: triphosphopyridine nucleotide (0·1 ml, 3×10^{-4}M), the sodium salt of glucose-6-phosphate (0·1 ml, 6×10^{-2}M), and 0·5 ml of pea enzyme extract. This extract contained the glutathione reductase and also the glucose-6-phosphate dehydrogenase which was used to regenerate the triphosphopyridine nucleotide. The enzyme extract was prepared according to Mapson and Goddard (1951). The test system was made up to 2·7 ml with water and was incubated for 30 min at 37°C. The reaction was then stopped with 0·3 ml of 20 per cent. sulphosalicylic acid and an aliquot of the supernatant was used for the nitroprusside test. In normal tissue extracts the recoveries of added glutathione were always over 90 per cent.; however, large amounts of cysteine interfered with the determination of oxidized glutathione.

(c) *Chromatography*

Paper chromatography was used to confirm the results obtained by the chemical methods. Before the extracts were chromatographed the trichloroacetic acid was removed by extracting an aliquot (2 ml) twice with equal volumes of peroxide-free ether. The sulphydryl compounds were then coupled with *N*-ethyl maleimide (0·1 ml of a 0·05M solution) in order to prevent their oxidation during chromatography (Smith and Tuller 1955), the extracts were adjusted to pH 6 with sodium hydroxide and were dried overnight in a vacuum desiccator. The residue was dissolved in 0·2 ml of water and 30 μl was spotted on to Whatman No. 1 paper. For most work unidimensional chromatography was satisfactory and the solvent used was generally butanol–acetic acid–water (4 : 1 : 1). The papers were sprayed either with ninhydrin (Williams and Kirby 1948), or with an azide-iodine solution for the detection of sulphur amino acids (Chargaff, Levine, and Green 1948).

III. Results

(a) *The Depletion of Liver Glutathione*

In preliminary experiments, administration of bromobenzene was as described by Koch-Weser *et al.* (1953). The animals were fasted 24 hr before injection and then were injected intraperitoneally with 0·1 ml of bromobenzene/100 g body weight; they were killed 48 hr later. This treatment lowered the concentration of glutathione in the liver to about 10 per cent. of the normal level. A milder and simpler procedure proved to be equally satisfactory; the dose was halved and the initial period of fasting was eliminated. Table 1 shows that, with this treatment, after 16 hr the concentration of glutathione had decreased from 21 to 2 mg of

glutathione sulphur/100 g of liver and remained at this low level for at least another 8 hr. With increased length of time there was a gradual restoration of the glutathione concentration, presumably by the mobilization of the body stores of cysteine. The time of this recovery was variable, in some animals being complete by 48 hr. The

TABLE 1

CHANGES IN THE SULPHUR-CONTAINING COMPOUNDS OF THE LIVER AFTER INJECTION WITH BROMOBENZENE

Each value represents the average of five animals. The rats were injected intraperitoneally with 0·05 ml of bromobenzene/100 g body weight

Time (hr)	Glutathione Sulphur*	Oxidized Sulphur*
0	21	24
16	2	22
20	2	28
24	3	21

* Mg sulphur in protein-free extract/100 g of liver.

experiments on the uptake of cysteine were of short duration, generally 2 hr or less, and hence complete before the onset of this mobilization. No changes were observed in the oxidized sulphur fraction of the liver.

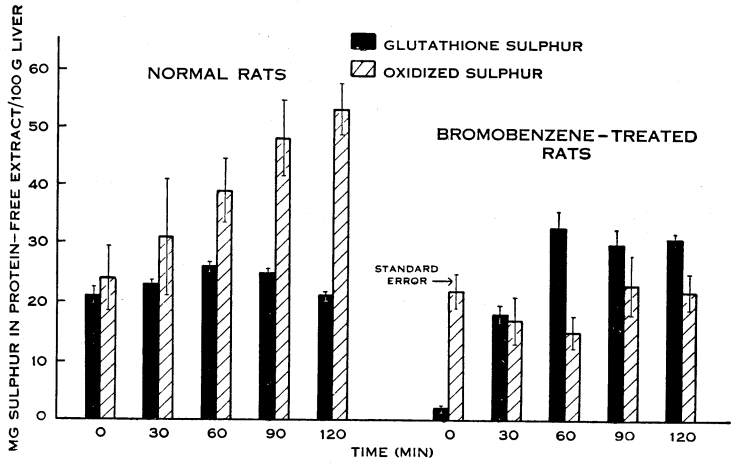

Fig. 1.—Changes in the sulphur-containing compounds of the liver after oral administration of cysteine (30 mg).

Although it has been claimed that bromobenzene depletes only the cysteine stores of the liver, it was considered advisable to check some other components. After the injection of bromobenzene no changes were observed either in the total α-amino nitrogen or in the protein concentration of the liver.

(*b*) *The Uptake of Cysteine by the Liver*

Initially, samples of liver were removed from an anaesthetized animal before and at various time intervals after dosing with cysteine. Cysteine uptake and glutathione synthesis were observed in such animals. However, this technique was tedious and it was decided to use a large number of animals and take only a single liver sample from each.

TABLE 2

DETERMINATION OF THE NATURE OF SULPHYDRYL-CONTAINING COMPOUNDS IN THE LIVER

Total sulphydryl concentration was estimated by the nitroprusside method, and the glutathione by the glutathione reductase method. Cysteine hydrochloride (30 mg) and glucose (150 mg) were dissolved in 1 ml of water and given by stomach tube at time 0

Time (min)	Normal Rats		Rats Deficient in Gluthathione	
	Total Sulphydryl Sulphur*	Glutathione Sulphur*	Total Sulphydryl Sulphur*	Glutathione Sulphur*
0	18·1	18·4	3·3	3·1
60	25·2	24·7	26·8	26·8
120	22·7	22·9	28·8	28·3

* Mg sulphur in protein-free extract/100 g of liver.

Figure 1 shows that in normal livers the concentration of glutathione and oxidized sulphur was 21 and 24 mg sulphur/100 g of tissue respectively. The concentration of cysteine was too small to be estimated (<1 mg sulphur/100 g of liver). No accumulation of cysteine in the liver could be demonstrated after dosing the animals with 30 mg of cysteine. While there was only a slight increase in the concentration of glutathione, the concentration of oxidized sulphur was more than doubled. In a few animals investigated the concentration of oxidized sulphur was still above normal at 8 hr after dosing but had returned to the base line by 24 hr.

When cysteine was given to those animals in which the concentration of glutathione in the liver had been lowered beforehand, a marked and rapid increase in the concentration of sulphydryl groups occurred. This increase was not due to cysteine but could be accounted for by an increase in glutathione (Table 2). No increase, on the other hand, was observed in the oxidized sulphur fraction (Fig. 1).

(*c*) *The Accumulation of Cysteine in the Liver*

In order to obtain an increase in the concentration of cysteine it was necessary to raise the dose to 150 mg and to use the intraperitoneal route. Figure 2 shows the results obtained when cysteine was injected into rats deficient in glutathione. An increase in the concentration of glutathione occurred as before. The cysteine

concentration increased at first from <1 to 16 mg of cysteine sulphur/100 g of liver, but this increase was only temporary and had decreased to 8 mg by 60 min and was down still further (4 mg) after 120 min. With these high doses, cysteine also accumulated in plasma and in red blood cells (Eden, unpublished data). A concomitant increase of the oxidized sulphur fraction was also obtained in the tissues (Fig. 2). The same degree of cysteine accumulation was observed when normal animals were injected with these high doses.

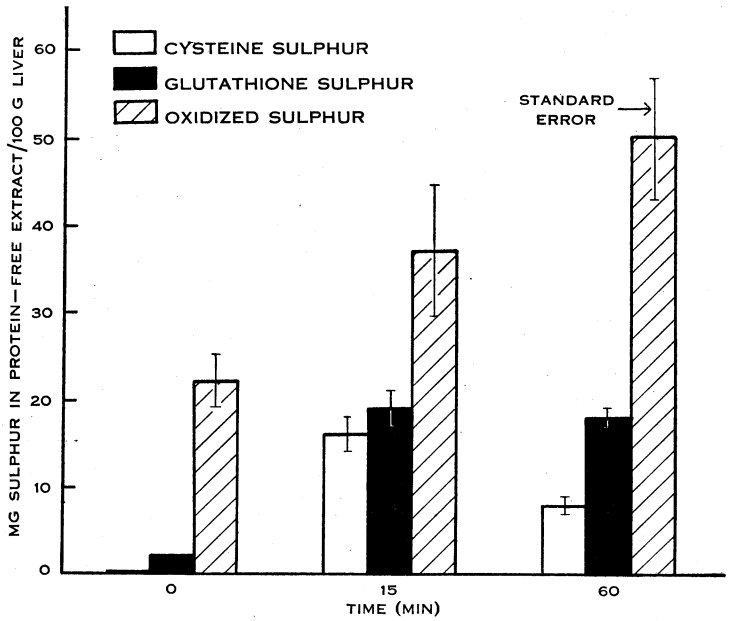

Fig. 2.—Changes in the sulphur-containing compounds of the liver after intraperitoneal injection of cysteine (150 mg).

IV. Discussion

No accumulation of free cysteine occurred in the liver when 30 mg of cysteine was given by stomach tube. This amount is more than the daily intake of cysteine even on a high protein diet. Only when very high doses (150 mg) were injected intraperitoneally did cysteine accumulate temporarily. The presence of cysteine in the liver has also been observed by Awapara (1953). In these experiments likewise large amounts of cysteine were injected intraperitoneally.

When 30 mg cysteine was given by stomach tube, more than half of the sulphur was recovered in the liver of all animals dosed. In those animals in which the concentration of glutathione in the liver had first been lowered to 10 per cent. of the normal level, all the cysteine taken up was converted to glutathione. The concentration of glutathione was restored in less than 1 hr, hence the rate of synthesis was higher than in normal animals where the turnover has been estimated to be about 3 hr (Waelsch and Rittenberg 1942).

In normal animals, nearly all the cysteine taken up was oxidized. In these livers no cystine could be found although it is known that isolated liver mitochondria can oxidize cysteine to cystine (Lang and Keller 1955). Thus the increase in the concentration of oxidized sulphur was caused by the accumulation of other oxidation products of cysteine. The oxidation of cysteine to taurine and to inorganic sulphate involves a large number of intermediary products, but only taurine normally accumulates in the liver (Awapara 1953) and is eventually excreted in the bile (Portman and Mann 1955) and in the urine (Awapara 1956). When the intake of sulphur amino acids is high as with a high protein diet, more taurine (Evered 1956) and more sulphate (Hawk, Oser, and Summerson 1954) are excreted than when the intake is low. As cysteine is not excreted to any considerable degree in the urine, the oxidation of cysteine in the liver seems to be the mechanism by which cysteine not required for anabolic reactions by the body is removed. This may be compared to the role of the liver in converting the nitrogen of the amino acids to urea.

The results obtained in the present investigation suggest that when cysteine is taken up, it is rapidly converted to glutathione, and once the glutathione level is saturated the excess cysteine is oxidized. Only when both the synthesizing and the oxidizing systems are overloaded, by giving quite unphysiological amounts of cysteine, can free cysteine be detected in the liver.

In contrast to cysteine, glutathione makes up a considerable proportion (over 10 per cent.) of the amino acids present in the protein-free extracts of the liver (Ferrari and Harkness 1954). Several steps in the oxidation of cysteine to inorganic sulphate in the liver are not reversible to any significant extent (Singer and Kearney 1955); on the other hand, glutathione, if oxidized, is readily reduced by glutathione reductase present in the liver (Rall and Lehninger 1952). The rapid conversion of cysteine to glutathione may prevent the irreversible oxidation of cysteine in the cell and at the same time provide a readily available source of cysteine for protein synthesis.

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VI. References

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