XLII. THE FORMATION OF SULPHATE FROM CYSTEINE AND METHIONINE BY TISSUES *IN VITRO*.

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In spite of the very large amount of work that has been done on the variations in the rate of excretion of sulphate by animals and men after the administration of various sulphur compounds, it seems that no one has shown that either an organ or a tissue preparation can make sulphate from an unoxidised sulphur compound *in vitro*. Rothera [1905] could find no sulphate when he incubated cystine with liver mince or when he perfused the liver with cystine, and it seems probable that several other workers have been similarly unsuccessful. The formation of sulphate from sulphuric esters by minced mammalian organs has been studied by Neuberg and Simon [1925], but this process, although perhaps connected with the type of oxidation which forms the subject of this paper, is obviously relatively simple.

The organ slice technique of Warburg [1923] is admirably suited to this work, for it causes little or no damage to the cells, and a series of experiments can be carried out under exactly comparable conditions, using parts of the same liver. By this means the troubles caused by the extreme individual variations between animals are avoided.

In this paper are described some preliminary experiments on the formation of sulphate by slices of kidney and liver. Later it is hoped that a larger group of substances will be investigated, as well as the probable intermediate stages in the oxidation and the extremely fragile enzyme systems by which these oxidations are carried out.

Experimental.

Rat tissue was used in all the experiments recorded; the animal was stunned and the required organs removed immediately and sliced as soon as possible. 30–80 mg. (dry weight) of slices were put in a vessel, of the type described by Krebs [1933], containing 8 ml. of bicarbonate Ringer solution. The latter was made up according to Krebs's directions [1932], except that magnesium chloride was used in place of magnesium sulphate. The vessels were filled with a gas mixture consisting of 95 % O_2 and 5 % CO_2 and shaken at 37°, generally for two hours. At the end of this time the flasks were taken out of the bath, rinsed on the outside with distilled water, dried round the joint with filter-paper and opened. The organ slices were removed with forceps, as much Ringer as possible was drained from them, and they were dried for 2–3 hours at 100° before being weighed. 2 ml. of 20 % trichloroacetic acid were added to the fluid in each flask, the mixture was poured into a 10 ml. pointed centrifuge-tube and the

protein precipitate centrifuged down. As a general rule the supernatant fluid was perfectly clear, but it sometimes contained small particles of tissue which floated because of entangled bubbles of CO_2. If the tube were shaken gently these could be centrifuged down.

Estimation of sulphate.

The method used for the estimation of sulphate is essentially that of Cuthbertson and Tompsett [1931]. In this the sulphate is precipitated in 70 % acetone with benzidine, the precipitate is washed thoroughly, and then the benzidine is estimated by diazotisation and coupling with thymol. Cuthbertson and Tompsett use a centrifuge throughout but very much more consistent results are obtained by using a sintered glass funnel (Schott 13f G4) of the type used by Pregl for the estimation of sulphur. The filter, mounted on a rubber bung, is placed inside a glass tube about 2 inches long and an inch wide, into the lower end of which the bung fits. This bung also carries a draining tube and an inlet tube for compressed air. The top of the wide glass tube can be closed firmly by another bung; fluid that is in the filter may then be forced through by opening the compressed air tube and closing the draining tube.

2 ml. of the centrifugate are added to 5 ml. of a 0·5 % solution of benzidine in acetone. The mixture is left in a stoppered tube for at least two hours on ice, it is then filtered by suction through the sintered glass funnel, and the tube is rinsed out on to the filter with acetone. The benzidine sulphate is washed by filling up the funnel (which holds 3 ml.) three times with acetone while it is connected with the pump. Care must be taken to wash the lip of the funnel during this process. The funnel is filled a fourth time, and the acetone is forced through by air pressure, the stem of the funnel being washed thoroughly with water during this last filtration. Two 3 ml. lots of boiling $N/3$ hydrochloric acid are used to transfer the benzidine sulphate to a test-tube; the fluid is forced through the filter by air pressure in each case. After sucking a little more $N/3$ HCl through the filter it is once more ready for use. The whole cycle of operations takes 6–10 minutes and the filter requires cleaning, by sucking nitric acid through it in the reverse direction, after about 10 estimations. At longer intervals it, like all the apparatus used in this work, is heated with cleaning mixture.

The benzidine is diazotised and coupled with thymol according to the directions of Cuthbertson and Tompsett except for the omission of the 15 % NaOH recommended by them[1]. The standard used was 0·3 ml. of a 0·053 % solution of benzidine hydrochloride in N HCl; this corresponds with 20γ of sulphur in the form of sulphate. The colorimeter cup containing the unknown solution is set at 20 on the scale, and the standard cup is adjusted to match; the standard scale now reads directly in thousandths of a mg. of sulphur in the form of sulphate in the 2 ml. sample of Ringer originally taken. On multiplying by five the figure that is recorded in the tables in this paper is obtained. All the estimations were done in duplicate, and the figure given is the mean of duplicates that did not differ by more than 1γ.

The cysteine used in this work was prepared by adding pyridine to an alcoholic solution of cysteine hydrochloride and washing the crystalline precipitate very thoroughly with alcohol. The glutathione, methionine and ergothioneine were prepared by the methods already published [Pirie, 1930; 1933, 1]. *S*-Ethylcysteine was prepared by the method of Clarke and Inouye [1931].

[1] I am very grateful to Miss E. Watchorn, of this laboratory, for pointing out the advantages of this modification.

Oxidation of cysteine and glutathione.

The results of a number of experiments on the oxidation of cysteine by liver slices are given in Table I. In each case the sliced liver, of which the dry weight is

Table I.

No. of rat	Wt. of cysteine in 8 ml. Ringer (mg.)	Dry wt. of liver (mg.)	Wt. of S found in the form of SO_4 (γ)
14	1·3	40	20
14	5·0	52	60
14	5·0	12	20
14	10·4	39	60
15	7	41	87 (72)
17	7	39	80
20	8	96	100
20	8 cystine	92	50
26	10	72	100
30	8	62	70
31	8	98	104 (100)
31	8 cystine	110	50
32	8	87	160
32	8	87	163

given, was suspended in 8 ml. of bicarbonate-Ringer containing the amount of cysteine specified. The flask was filled with the gas mixture and shaken for two hours at 37°. The figures in the fourth column are simply the amount of sulphur that was found in the flask in the form of sulphate at the end of the experiment; a series of blank experiments with cysteine alone, or with liver but no cysteine, or with liver slices that have been inactivated by heating to 60° or by prolonged freezing gave values ranging from 10 to 15γ. It is clear therefore that the sulphate concentration can be increased 5 or 10-fold by the oxidation of cysteine in this way. The reliability of the method used for the estimation of sulphate is also tested by the experiments done in nitrogen and by those in which different organs were used; these are described later.

The quantity of sulphate formed is too small to be estimated accurately by precipitation as barium sulphate followed by weighing. In a few cases, however, this has been attempted. A 4 ml. sample of the trichloroacetic acid centrifugate was used, and the weight of sulphur corresponding with the observed weight of barium sulphate was multiplied by 10/4; the figure so obtained is recorded in brackets in column 4 of certain of the tables. The two sets of figures are in sufficiently good agreement to make it certain that it is sulphate that is being estimated by the colorimetric method. It is necessary to emphasise this point, because the benzidine methods for the estimation of sulphate have often been criticised on account of their lack of specificity. Furthermore, some substances, cystine for example, are precipitated by the high concentrations of acetone used in the estimation. I find, however, that so long as sufficient trichloroacetic acid has been added in the first place, these precipitates can easily be washed free from adsorbed benzidine; they do not therefore interfere with the estimation. Small amounts of phosphate do not matter, but if it is present in large amounts sulphate estimations become impossible.

Table II contains the results of experiments in which cysteine is oxidised by kidney. Liver and kidney have much the same oxidising capacity, but the blood, testis, spleen, heart and lung of the rat seem to be quite inactive under these

Table II.

No. of rat	Wt. of cysteine mg.	Dry wt. of kidney mg.	Wt. of S found in the form of SO_4 (γ)
16	8	26	55
20	8	42	90
27	7	48	100
28	7	63	100
30	8	36	70
31	8	34	50
31	8	38	60 (50)

conditions. The study of the kinetics of sulphate formation from cysteine is complicated by the fact that under the conditions of these experiments the cysteine is being rapidly oxidised to cystine; this separates as minute crystals if the concentration exceeds about 1 mg. per ml. Although cystine at lower concentrations than this stays in solution it cannot be directly oxidised to sulphate. If cystine is shaken with tissue slices, some sulphate is indeed formed, but this is probably due to a preliminary reduction to cysteine by the systems which Hopkins and Elliott [1931] and Mann [1932] have described. Two experiments of this type are recorded in Table I. The cystine, dissolved in the minimum amount of dilute hydrochloric acid, was added to Ringer solution containing a corresponding amount of NaOH. No separation of free cystine could be observed.

If a number of flasks all containing the same quantities of cysteine and liver are set up, and the amount of sulphate present is estimated after various intervals, it is found that no more sulphate is formed after the nitroprusside reaction has disappeared. With 7–8 mg. of cysteine and 60–80 mg. dry weight of liver this generally takes from 60–90 minutes, but the time is variable, although the relationship is constant. The following experiment is more definite. 12 mg. of cysteine and 140 mg. dry weight of liver were shaken for an hour at 37° in 16 ml. of Ringer. The flask was then opened, 8 ml. of the fluid were withdrawn for sulphate estimation, and half the slices were taken out and placed immediately into another flask containing 6 mg. of cysteine in 8 ml. of Ringer, *i.e.* the same concentration of cysteine that the first flask, which now contains only cystine, had contained initially. Both flasks were then closed, refilled with gas mixture and shaken for an hour at 37°. The sulphate contents of each flask and of the fluid withdrawn from the first flask after one hour were estimated. Within the limits of the method the sulphate content of a 2 ml. sample was the same in each case. Clearly therefore no sulphate was formed during the second hour in the flask from which all the cysteine had disappeared, but a part of the same group of slices was very well able to make sulphate when supplied with fresh cysteine. This conclusion, that disulphides are first reduced to mercaptans before undergoing oxidation to sulphate, has already been reached by Lewis *et al.* [1924] and others from feeding experiments on the intact animal. Furthermore, cysteine but not cystine can undergo the further stage of oxidation by the disulphide form of thiocarbamide which I have already described [Pirie, 1933, 2].

Hele and Pirie [1931] and Schelling [1932] have found that the intact animal can oxidise glutathione to sulphate even if it is given subcutaneously. The figures in Table III show that glutathione is also oxidised by tissue slices. It is clear that kidney has a much greater capacity to oxidise glutathione than liver. This fact makes it probable that the first step is the hydrolysis of the tripeptide into its constituent amino-acids, for it is well known that kidney undergoes

Table III.

No. of rat	Wt. of glutathione mg.	Dry wt. of tissue mg.	Wt. of S oxidised to SO_4 (γ)
19	24	60 liver	30
19	15	60 liver	20
19	54	48 liver	65
19	54	43 kidney	105
19	9	43 kidney	68
21	30	64 liver	45
21	30	45 kidney	75
21	15	52 kidney	75
21	30	22 kidney	57
25	30	69 liver	40
25	30	29 kidney	50
25	60	30 kidney	65
25	20	44 kidney	65
25	10	44 kidney	55
29	21	56 liver	45

autolysis more rapidly than most tissues, and it is the only tissue in which Bierich and Kalle [1928] were able to find free cysteine. Confirmatory evidence can be got by applying Sullivan's test to the fluid in which liver or kidney has been shaken with glutathione. The former gives no trace of cysteine colour, *i.e.* there is less than 0·03 mg. in a 2 ml. sample, whereas the latter contains about 0·7 mg. The most probable explanation of this would seem to be that the peptide-splitting enzymes of kidney make cysteine more rapidly than the cysteine-oxidising enzymes can destroy it, whereas in the case of liver this is not so. Needless to say the fluid in which kidney has lain for two hours without the addition of glutathione gives no detectable Sullivan reaction.

Oxidation of methionine.

Both liver and kidney can oxidise methionine to very much the same extent. In both cases, however, the rate of oxidation is very much smaller than the corresponding rate in the case of a similar concentration of cysteine. These points are illustrated in Tables IV and V. It is clear that methionine has to be present

Table IV.

No. of rat	Wt. of methionine mg.	Dry wt. of liver mg.	Wt. of S found in the form of SO_4 (γ)
4	40	90	60
4	80	70	30
8	40	60	90
8	20	59	50
9	10	40	50
9	20	32	55
9	30	55	75
9	40	47	47
10	40	70	95
13	24	45	60
15	40	55	60
17	40	54	60
24	30	52	52
30	30	74	97
32	24	91	52
32	24	78	50

Table V.

No. of rat	Wt. of methionine mg.	Dry wt. of kidney mg.	Wt. of S found in the form of SO_4 (γ)
8	40	31	60
10	30	41	85
10	50	35	75
10	80	35	80
30	30	30	62

in twice or three times the concentration that is necessary in the case of cysteine to get an equal rate of formation of sulphate. Individual variation between rats makes comparisons difficult, but it would seem from the figures for rats 4 and 9 in Table IV that there is quite a definite optimum concentration for sulphate production from methionine. Evidence of this sort could not be obtained in the case of cysteine on account of the formation of cystine.

I have found it impossible to get consistent results in the presence of toluene or other disinfectants. It is exceedingly unlikely that the sulphate formation is due to bacterial action, and the relative constancy of the sulphate production in different experiments makes it still less probable. The figures in Table VI show that the amount of sulphate formed during the third hour of an experiment is the same as that formed during the first and second hours. This fact entirely excludes the possibility of bacterial action.

Table VI.

No. of rat	Wt. of methionine mg.	Dry wt. of liver mg.	Duration of experiment (hr.)	Wt. of S found in the form of SO_4 (γ)
12	30	87	0·5	20 (25)
12	30	57	1·0	36
12	30	52	2·0	60 (50)
12	30	49	3·0	85 (90)

Chase and Lewis [1933] have found in the urine of rats receiving *dl*-methionine a substance which gives a red colour with sodium nitroprusside in the presence of sodium cyanide. I have tested the fluid in which tissue slices have been shaken with methionine and find in all cases a faint but definite nitroprusside reaction. This reaction is given neither by fluid to which no methionine has been added nor by methionine solutions shaken without tissue. The possible significance of this will be discussed later.

Table VII.

No. of rat	Wt. of *S*-ethyl-cysteine (mg.)	Dry wt. of tissue (mg.)	Wt. of S found in the form of SO_4 (γ)
16	10	64 liver	15
16	40	76 liver	35
16	80	71 liver	57
16	40	32 kidney	37

In Table VII the results of an experiment with *S*-ethylcysteine are given. At a concentration at which cysteine would be very rapidly oxidised and methionine would be oxidised at an appreciable though not optimum rate this substance is hardly oxidised at all. It is only in relatively concentrated solutions that a definite formation of sulphate can be shown. The three substances cysteine, methionine and *S*-ethylcysteine do, however, form a series so far as *in vitro* oxidations are concerned which is very unlike their behaviour in the intact dog [Pirie, 1932]. There cysteine and methionine resembled one another while *S*-ethylcysteine was dissimilar. It is likely that this difference is due to

the fact that the tissue slice experiments are not complicated by the excretory mechanism, whereby an intact animal gets rid of foreign compounds into the urine.

Miscellaneous experiments.

Neither liver nor kidney can oxidise ergothioneine to sulphate under these conditions. This is somewhat unexpected since the sulphur atom in a thiol-glyoxaline is very much more readily oxidised to sulphate by chemical means than that in cysteine or methionine. Ergothioneine has been tested as a catalyst in the oxidation of the other sulphur compounds; here also no effect could be found. Thiocarbamide also was not oxidised by liver slices when present in low concentration, nor did it catalyse the oxidation of cysteine. It seems therefore that this oxidation cannot be directly compared with the oxidation by H_2O_2 [Pirie, 1933, 2]. The extract obtained by freezing and grinding liver and then centrifuging out the particles was also tested both as a substrate and as an activator; it was inactive. In this connection it should be recorded that the tissues of a rat from which food had been withheld for 24 hours had the usual oxidising power; rat 30 in Tables I, II, IV and V illustrates this.

Table VIII.

No. of rat	Wt. of substrate (mg.)	Dry wt. of liver (mg.)	Gas mixture	Wt. of S found in the form of SO_4 (γ)
17	None	53	95 % N_2 and 5 % CO_2	6 (20)
17	40 methionine	41		10
17	8 cysteine	49		15 (20)
17	30 methionine	46	95 % O_2 and 5 % CO_2	45
17	40 methionine	54		60
17	8 cysteine	39		80
17	30 methionine	53	Air	25
17	30 methionine	40	97·5 % O_2 and 2·5 % CO_2	45

Table VIII relates to the effect of different atmospheres on the oxidation by liver. The results call for little comment; as might be expected the oxidation does not proceed anaerobically. The oxidation is slow in air and there is no appreciable difference between the results obtained when a gas mixture containing 5 % CO_2 (p_H of Ringer 7·3) or one containing only 2·5 % CO_2 (p_H of Ringer 7·6) was used.

Little attempt has yet been made to investigate either the stability of this enzyme system or the effect of inhibitors upon it. Cooling to about $-5°$ generally inactivates the slices, and heating for 20 minutes at 60° does so with certainty, but short periods of freezing give erratic results. Finely ground tissue is inactive. The action of inhibitory substances is complicated by their effect on the autoxidation of the cysteine; a description of the effects observed will be published shortly.

Discussion.

Lawrie [1932] found that a rat, of the strain used in this laboratory, weighing about 240 g. excreted 10 mg. of sulphur in the form of sulphate per day when eating a synthetic diet. By feeding cystine the sulphate excretion could be raised to 20 mg. S per day, but at this level there was a definite rise in the neutral sulphur also. Now the liver and kidneys of a 240 g. rat weigh together about 12 g.; their dry weight is therefore 2·4 g., and this quantity of tissue will seldom be called on to oxidise more than 20 mg. of sulphur to sulphate in 24 hours. 50 mg. dry weight of tissue should on this basis oxidise 35γ of sulphur to sulphate in 2 hours. The figures in Tables I and II show that tissue slices can make sulphate from cysteine at a rate considerably in excess of this if the cysteine

concentration is raised to unphysiologically high levels. Since, however, the sulphate-forming mechanism, when working *in vitro*, has to compete with the autoxidation processes this agreement is fairly satisfactory. The figures in Tables IV and V are more important, for methionine is the principal sulphur-containing constituent of most diets. When we consider the very high concentrations of methionine that are being used and also the fact that the action is proceeding in 95 % oxygen rather than in air, it is clear that there is little quantitative relation between the rates at which rat organs can oxidise methionine *in vitro* and *in vivo*.

It would be premature to put forward a theory of the mechanism of this oxidation with any degree of confidence. As a working hypothesis, however, the following scheme is suggested:

$$\begin{matrix} \text{R.SS.R} \\ \text{or} \\ \text{2R.SCH}_3 \end{matrix} \rightarrow 2\text{R.SH} \rightarrow 2\text{R.SOH} \rightarrow \text{R.SH} + \text{R.SO}_2\text{H} \rightarrow \text{H}_2\text{SO}_3 \rightarrow \text{H}_2\text{SO}_4$$

Of these processes the first and second are probably enzymic, the third proceeds spontaneously at body p_H [Pirie, 1933, 2], the fourth is enzymic and the fifth need not necessarily be so. The relatively slow rate at which sulphate is formed from methionine, taken in conjunction with the occurrence of disulphides in the fluid in which methionine is being oxidised, is at least consistent with the theory that this substance has to be demethylated before it can be oxidised.

Summary.

The oxidation of cysteine and methionine to sulphate by slices of rat liver and kidney has been studied.

Cystine can only be oxidised after undergoing reduction and glutathione only after hydrolysis.

Ethylcysteine is oxidised slowly and ergothioneine not at all.

A modification in the technique of micro-sulphate estimation is described.

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