26. Metabolism of Sulphur

11. Further Investigation of the Enzymic Oxidation of Sulphur-containing Amino-acids*

By **Grace Medes**† and **Norman Floyd**, *From the Lankenau Hospital Research Institute, Philadelphia*

(*Received 2 February 1942*)

Of the enzymes which act on the S-containing amino-acids in the tissue, four have been described previously: (*a*) cytochrome oxidase, which oxidizes cysteine to cystine, (*b*) a cysteine oxidase, described by Pirie [1934], which gives rise to inorganic sulphate, (*c*) a sulphinic acid oxidase, reported by Medes [1939], also producing inorganic sulphate, and (*d*) a cysteine oxidase, reported by Bernheim & Bernheim [1939] and Medes [1939], which oxidizes cysteine to cysteic acid. Medes's results also suggested the direct oxidation of cystine, accompanied by decarboxylation. At that time it was not clear whether the action on cystine and on cysteine was due to the same or to different enzymes, or through what paths the oxidations proceeded. The series of observations reported here are attempts to elucidate some of these questions.

Studies with liver slices

A preliminary study was made of the action of liver slices on the S-containing amino-acids in order to identify the several end-products. Such a study should serve as a guide in determining whether all enzymes catalysing the oxidation of these substrates had been isolated. The slices were incubated in a constant temperature bath at 38° in phosphate or bicarbonate buffer at *p*H 7·6 with cysteine, cystine, methionine and cysteinesulphinic acid as substrates. The bicarbonate buffer was prepared as recommended by Krebs [1932] with substitution of $MgCl_2$ for $MgSO_4$ as Pirie [1934] suggested. When phosphate buffer was employed, its final concentration was 0·05 *M*. Volumetric analyses for the various end-products were carried out after 2 hr. incubation with O_2 bubbling through the phosphate buffer and an O_2-CO_2 mixture led through the bicarbonate buffer.

Methods of quantitative analyses. *Cysteine* and *cystine* were determined by the method of Shinohara & Padis [1935] with occasional checks by the Sullivan method.

For the determination of *methionine* added to tissue extract the precision method of Kolb & Toennies [1940] was adapted. An aliquot part of the brei containing about 0·1 m.mol. of methionine is placed in a 50 ml. centrifuge tube and is diluted with 20% trichloroacetic acid and H_2O to 50 ml. so that the resulting solution is about 0·002 *M* in methionine and contains approximately 4% trichloroacetic acid. All volumes are measured accurately. This mixture is stirred vigorously, allowed to stand for 5 min. to ensure adequate flocculation of the protein, and centrifuged. A 40 ml. aliquot part of the supernatant liquid is placed in a 100 ml. volumetric flask containing 36 ml. of 5 *M* $HClO_4$. To this are added 10 ml. of 2 *M* $HClO_4$ containing 0·06 *M* H_2O_2, and the mixture is then diluted to 100 ml. with H_2O. After 2 to 3 hr. aliquot parts are removed for iodimetric titration. Iodine is liberated by adding to each 20 ml. portion 20 ml. of a solution containing 5 m.mol. of KI and 0·05 m.mol. of ammonium molybdate and titrating with 0·025 *N* thiosulphate. Both the $HClO_4$-H_2O_2 and the

* Presented before the American Society of Biological Chemists, April, 1941.

† Aided in part by a grant from the American Philosophical Society, and in part by a grant from McNeil Laboratories, Inc., Philadelphia, Pa.

KI-$(NH_4)_2MoO_4$ mixtures are prepared fresh daily. A control in which the methionine is omitted gives the amount of H_2O_2 originally present. The value of this control is in complete agreement with a blank run on the $HClO_4$-H_2O_2 mixture substituting 40 ml. of H_2O for the supernatant liquid from the protein precipitation.

NH_2-N was determined by the manometric method of Van Slyke [1929] with KI added to the nitrite solution as suggested by Kendrick & Hanke [1940].

The carboxyl group of the amino-acids was estimated by the method of Van Slyke & Dillon [1938] in which Mason's [1938] procedure was slightly modified. 2 ml. of the deproteinized amino-acid solution were introduced into the combustion tube and a silica pebble added, followed by 1 g. of solid KH_2PO_4. CO_2 was expelled by boiling for 1 min., after which 30 mg. of ninhydrin were introduced and the combustion tube immediately attached to the condenser.

Later the method developed by Christensen *et al.* [1941] was substituted. Protein-free filtrates were obtained by means of salicylsulphonic acid. To 39 parts of water and 10 parts of the fluid to be tested was added 1 part of 50% salicylsulphonic acid. The salicylsulphonic acid had to be recrystallized from ether, as all commercial samples tested contained interfering impurities. By this method, recoveries of CO_2 in theoretical amounts were possible. Protein-free filtrates made with tungstic acid gave low results in the case of certain amino-acids, and trichloroacetic acid invariably decomposed with the production of too high results. The authors are indebted to Mary Stella Cammaroti for these determinations.

Sulphate determinations were carried out by the method of Medes & Stavers [1940]. Later, the following procedure was adopted. To 10 ml. of the solution to be tested, are added 2 ml. of 20% sulphate-free trichloroacetic acid in a 15 ml. centrifuge tube. The contents are stirred vigorously with a fine glass rod and centrifuged. 10 ml. of the supernatant liquid are transferred to a 50 ml. centrifuge tube, followed by 15 ml. of 0·5% benzidine in acetone. The contents are again stirred and the rod rinsed with a few drops of the benzidine in acetone solution. The tube is stoppered and allowed to stand for about 1 hr. in an ice bath, after which it is centrifuged and the supernatant liquid decanted. Washing of the precipitate and titration are carried out according to the method of Power & Wakefield [1938]. A blank determination on the reagents is subtracted from the values of the experimental solutions.

In Table 1 is reported a series of experiments with cysteine as substrate. The sulphydryl reaction gradually disappeared and in all cases was lost at the close of the 2 hr. period. During this time the disulphide reaction was gradually developing but not in equivalent amounts. The difference could not be attributed to sulphate, which increased in insufficient quantities to account for the loss. NH_2-N was unaltered, whereas there was a slight disappearance of carboxyl. Iodimetric titration indicated that the end-product was a sulphonic acid.

When cystine was substituted for cysteine as substrate (Table 1), the reaction with phosphotungstic acid again disappeared to about the same extent as before. Since both sulphydryl and disulphide were present in the first series described above, and only disulphide in the second, it could not be determined by these experiments whether cystine alone or whether both cystine and cysteine underwent this type of oxidation. It may be seen from the table that sulphate did not account for the entire loss. NH_2-N, as in the previous series, remained unchanged, whereas the fall in carboxyl was more marked, suggesting that cystine rather than cysteine was the substrate undergoing decarboxylation.

Methionine (Table 2), in the presence of liver slices, appeared stable; a slight loss, as determined by the modified Kolb-Toennies H_2O_2-oxidation procedure was approximately equivalent to the sulphate formed. NH_2-N and carboxyl determinations were unaltered. The only changes in the sulphinic acid were an appearance of sulphate and a decrease of the iodine titre. No determinable change occurred in cysteic acid.

Table 1. *Incubation of liver slices in the presence of cysteine and cystine*

2 hr. incubation in 0·05 *M* phosphate and in bicarbonate buffers at *p*H 7·6. Concentrations are expressed in m.equiv. added per ml. of medium.

Buffer	Substrate m.equiv. × 10^{-3}	Dry wt. of tissue mg.	(Shinohara) m.equiv. × 10^{-3} SH before	(Shinohara) m.equiv. × 10^{-3} SS after	(Sullivan) m.equiv. × 10^{-3} SH before	(Sullivan) m.equiv. × 10^{-3} SS after	Sulphate m.equiv. × 10^{-4} Before	Sulphate m.equiv. × 10^{-4} After	Carboxyl m.equiv. × 10^{-3} Before	Carboxyl m.equiv. × 10^{-3} After	NH_2-N m.equiv. × 10^{-3} Before	NH_2-N m.equiv. × 10^{-3} After
					Cysteine							
Phosphate	8·00	50·2	8·02	6·58	8·00	5·89	0·9	0·7	8·19	8·00	8·00	8·02
	7·00	48·7	7·04	6·00	—	—	0·6	0·6	7·21	6·90	7·00	7·04
	7·00	49·3	6·86	5·29	6·40	4·32	0·5	0·7	7·06	6·88	7·02	6·98
	7·00	53·6	6·83	3·51	6·00	3·14	0·7	0·4	6·84	6·69	6·96	6·96
Carbonate	7·00	47·8	6·91	2·88	6·29	2·13	1·1	4·6	7·00	6·70	7·01	7·03
	7·00	45·9	7·16	5·10	—	—	0·8	4·8	6·94	6·72	7·04	6·97
Average	—	49·3	7·14	4·89	—	—	—	—	7·21	7·13	7·17	7·18
					Cystine (as Na-cystinate)							
Phosphate	4·50	50·1	4·38	2·64	4·11	2·30	—	—	4·33	3·80	—	—
	7·00	50·9	7·10	3·60	5·95	3·91	—	—	6·80	6·22	—	—
	7·00	47·3	7·07	4·36	7·13	4·51	—	—	6·97	5·82	—	—
	7·00	53·6	6·93	6·35	6·67	3·53	—	—	6·91	6·62	—	—
Carbonate	7·00	48·5	6·86	5·32	6·95	4·72	0·0	3·3	6·87	6·45	—	—
	7·00	49·4	6·93	5·33	7·17	3·59	1·2	4·1	7·32	6·88	—	—
	7·00	46·8	6·90	4·73	6·75	4·61	0·7	4·5	7·10	4·20	—	—
Average	—	49·5	6·71	4·66	—	—	—	—	6·61	5·71	—	—

Table 2. *Action of liver slices on methionine, cysteinesulphinic acid and cysteic acid*

2 hr. incubation in 0·05 *M* phosphate or bicarbonate buffer at *p*H 7·6. Concentrations are expressed in m.equiv. per total volume.

Buffer	Substrate m.equiv. × 10^{-3}	Sulphate m.equiv. × 10^{-4} Before	Sulphate m.equiv. × 10^{-4} After	Carboxyl m.equiv. × 10^{-3} Before	Carboxyl m.equiv. × 10^{-3} After	NH_2-N m.equiv. × 10^{-3} Before	NH_2-N m.equiv. × 10^{-3} After	Kolb-Toennies m.equiv. × 10^{-3} Before	Kolb-Toennies m.equiv. × 10^{-3} After	I_2 titration Before	I_2 titration After
					Methionine						
Phosphate	21·92	—	—	—	—	—	—	22·00	22·00	—	—
	1·42	—	—	1·36	1·43	—	—	1·40	1·40	—	—
Carbonate	6·00	0·85	3·00	6·02	6·05	5·97	6·00	6·65	6·45	—	—
	4·96	0·40	3·30	4·91	4·84	5·02	4·91	4·93	4·76	—	—
	4·96	0·34	2·91	4·95	4·95	4·97	4·97	4·96	4·76	—	—
					Cysteinesulphinic acid						
Phosphate	24·00	—	—	24·3	23·7	24·1	23·9	—	—	23·71	23·71
Carbonate	24·00	1·2	6·7	23·41	23·17	23·4	23·4	—	—	22·75	20·85
					Cysteic acid						
Phosphate	24·00	—	—	24·3	23·7	24·1	23·9	—	—	—	—
	24·00	—	—	24·4	24·5	24·0	23·9	—	—	—	—
	24·00	0	0	24·3	24·1	23·9	23·7	—	—	—	—

Conclusion. From these experiments it was confirmed that besides cytochrome oxidase and the enzymes which give rise to inorganic sulphate from cysteine and cysteinesulphinic acid, enzymes are present which oxidize cysteine and cystine and which decarboxylate cystine. The succeeding studies were undertaken for the purpose of further characterizing these enzymes.

Isolation experiments

Cysteine oxidase. The first series of isolation experiments was carried out with cysteine oxidase. The enzyme was prepared by a modification of the method of Bernheim & Bernheim [1939]. After perfusion with physiological salt solution, the liver was ground with sand in 75 ml. of 0·01 *M* phosphate buffer at *p*H 6·7. The insoluble protein was centrifuged down at about 3000 r.p.m. for 30 min., the supernatant liquid discarded and

the washing repeated. All operations, including the centrifuging, were carried out in the cold.

Determinations of O_2 uptake were made in the Warburg respirometer. 1·30 ml. of 0·0769 *M* phosphate buffer at the desired *p*H were introduced into the main chamber of the Warburg vessel by means of a burette with a curved capillary tip. 0·30 ml. of the neutral substrate was then measured into the side bulb. 0·40 ml. of the enzyme suspension was added to the buffer in the main chamber, and in those cases in which CO_2 evolution was to be determined, the KOH paper was introduced into the central cup. As an additional check, retention of CO_2 was tested by transferring 1·70 ml. of the fluid to a fresh vessel, 0·30 ml. of saturated oxalic acid solution was introduced into the side bulb, and any retained CO_2 determined. In the experiments with cysteine derivatives, cysteine was treated similarly in an additional set of chambers, to check the activity of the enzyme. If oxidation of cysteine did not approximate the theoretical value, the results were discarded. All experiments were carried out in duplicate.

This enzyme, according to Bernheim & Bernheim, acts on thiolacetic acid as well as on cysteine, but does not effect the oxidation of glutathione; ethyl mercaptan is oxidized slowly, but they could not demonstrate a definite end-point.

Table 3. *Oxidation of cysteine and chemically related compounds by cysteine oxidase as shown by O_2 uptake in the Warburg respirometer*

Temp. 38·0°. Col. 3 gives the time in min. required for the reaction to reach completion. In all other cases the reaction was stopped at the close of 2 hr.

Substrate	Amount used μmol.	Time for completion min.	O_2 uptake Consumed μl.	O_2 uptake Theoretical μl.	% of theoretical
Cysteine	1·86	70	63·0	62·6	101
	1·88	70	63·5	63·3	100
	1·82	90	66·0	61·3	107
	1·73	110	58·0	58·3	100
	1·84	80	64·0	62·4	103
Methionine	3·47	—	13·5	116·6	12
	3·47	—	10·0	116·6	9
Na cysteinesulphinate	5·98	—	0	67·0	—
	3·53	—	0	39·6	—
Cysteic acid (Na salt)	12·00	—	0	0	—
	12·00	—	0	0	—
Homocysteine	2·24	110	97·6	75·3	130
	1·70	—	55·5	57·1	97
dl-Cysteine	1·99	80	60·7	66·7	91
	1·43	90	60	48	125
	2·00	—	67·1	56·5	84
n-Butyl mercaptan	2·13	—	17·8	71·6	25
*iso*Amyl mercaptan	2·04	—	11·3	68·6	16
Methionine sulphoxide	5·59	—	0	125·3	—
	5·59	—	0	125·3	—
Glutathione	2·25	—	0	75·6	—
S-Ethyl cysteine	2·16	—	0	—	—
Thiolacetic acid	2·38	160	90·2	80·0	112

Its behaviour towards a number of chemically related substrates is reported in Table 3. In general, the concentrations employed were computed on the basis of a theoretical oxidation to the sulphonic acid stage; i.e. the molecular concentration of sulphinic acid used was three times that of cysteine, since the theoretical O_2 uptakes are in the ratio of 1 : 3.

The results previously reported for cysteine were confirmed—oxidation, as shown in the Warburg apparatus, was carried to the sulphonic acid stage. In spite of uniformity

in the method of preparation of the enzyme, considerable differences in the O_2 uptake of the controls were shown from day to day, varying from 10 to 20μl. over the 2 hr. period. Fig. 1 depicts the oxidation of 1·865 m.mol. of cysteine. The mol. % of O_2 consumed, after subtraction of the O_2 uptake of the control, are plotted against the time in min. As may be seen, 150 mol. % O_2 were absorbed during approximately 1 hr., representing oxidation to the sulphonic acid stage. There was no evidence of CO_2 evolution. When tissue slices were substituted in the Warburg apparatus for the enzyme preparation, similar results in general were obtained. However, the results were more difficult of interpretation, as cytochrome oxidase, present in the slices, was slowly giving rise to cystine, which, as will be shown, may also serve as substrate for enzyme action. The high evolution of CO_2 by the slices left some doubt as to whether it was being derived from the added substrate as well as from respiratory processes.

Either increasing or decreasing the length of the carbon chain causes a slowing of the rate of oxidation as shown in Fig. 1 by homocysteine and thiolacetic acid, although the same theoretical uptake of O_2 is finally attained. *dl*-Cysteine is oxidized at approximately the same rate, as shown in Table 3.

The rate of reaction is still further reduced by substituting a simple carbon chain for those with amino and carboxyl groups attached. Compare, for instance, the curves

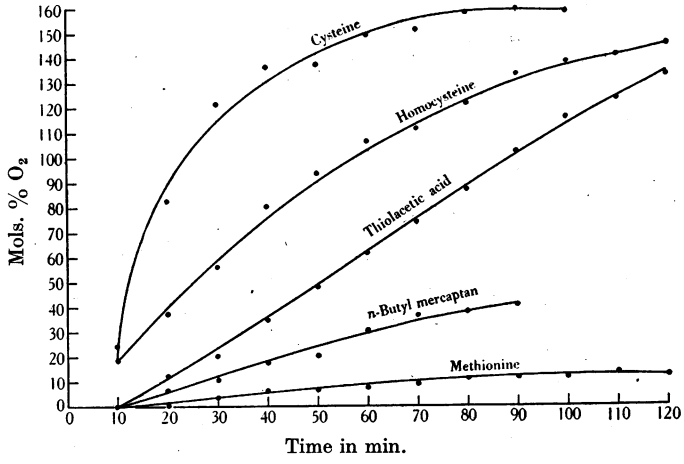


Fig. 1. Rate of oxidation of cysteine and chemically related compounds by cysteine oxidase as determined in the Warburg apparatus. Cysteine, 1·84 μmol.; homocysteine, 1·70 μmol.; thiolacetic acid, 1·87 μmol.; *n*-butyl mercaptan, 2·13 μmol.; methionine, 3·47 μmol. Final concentration of phosphate buffer, 0·05 *M* at *p*H 6·7. Temp. 38°.

representing oxidation of homocysteine and of *n*-butyl mercaptan (Fig. 1). The latter compound, and *iso*amyl mercaptan, as well as ethyl mercaptan described by Bernheim & Bernheim, oxidized so slowly that no definite end-point could be demonstrated.

When an S-methyl group replaced the sulphydryl group, i.e. methionine (see Fig. 1) as contrasted with homocysteine, the rate of reaction was even more retarded. With substitution of an ethyl group, i.e. S-ethyl cysteine, oxidation was completely inhibited.

That disulphide groups are present in the enzyme itself, and play an important part in its activity, seems probable from the fact that in the presence of reduced glutathione, the activity is considerably decreased. On the other hand, when metals in certain combinations are present, oxidation of the enzyme itself, as well as of the substrate, occurs with great rapidity. This phase of the problem is under further investigation.

Action of cysteine oxidase on cystine. The enzyme preparation which oxidizes cysteine also oxidizes cystine; i.e. it is contained in the insoluble residue obtained by extracting

ground liver with 0·01 *M* phosphate buffer at *p*H 6·7, centrifuging and washing the precipitate in additional buffer.

The rate of oxidation of cystine is always much slower than that of cysteine; usually when cysteine has reached the cysteic acid state, i.e. has taken up its theoretical amount of O_2, cystine has consumed about 50–70 % of its theoretical amount. Although the absolute rates of oxidation varied considerably with the different preparations, there was always a general parallelism between the two oxidative reactions, both being high or low in the same specimen.

Attempts to separate two enzymes have been unsuccessful. Repeatedly washing in the dilute phosphate buffer reduced both activities about equally (Fig. 2) as did other methods of extraction, i.e. using different concentrations of phosphate buffer, altering their *p*H from 5·2 to 8, or extracting in various salt mixtures.

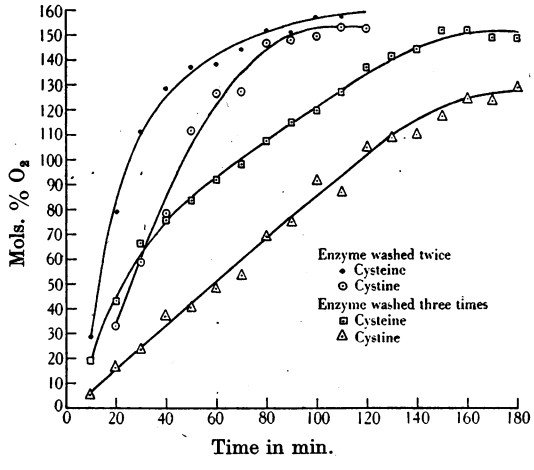


Fig. 2. Effect of repeated washings on activity of the oxidase which acts on cysteine and cystine with production of cysteic acid.

Considerable difficulty was experienced in determining the end-point of this reaction. On account of the slow rate of oxidation, the activity of the enzymes was often very slight before the end-point was reached and tests for S-S were usually still positive even after 2 or 3 hr. of incubation. For this reason it was difficult to decide whether sulphinic acid or cysteic acid is the end-product. Four procedures were employed. (*a*) Iodimetric titrations with deduction of the S-S remaining, as determined by the Shinohara method, were inconclusive. (*b*) Since sulphinic acid is readily oxidized by an enzyme to be described below, with production of inorganic sulphate, it was thought that by using a combination of these two enzymes it could be determined whether sulphate is increased following incubation with cystine. If so it could be concluded that sulphinic acid had been formed, but on account of various technical difficulties, this method also proved inconclusive. (*c*) A colorimetric test for sulphinic acid, devised by Dr Lavine (unpublished data), indicated that sulphinic acid was not formed, but in this case also, technical difficulties somewhat obscured the results. (*d*) A final method, also based on Dr Lavine's studies of sulphinic acid, gave clear-cut results. He showed [Lavine, 1937] that cysteine, in the presence of sulphinic acid with KI as catalyst, is quantitatively oxidized to cystine, while sulphinic acid is reduced according to the equation, $3RSH + RSO_2H \xrightarrow{KI} 2RSSR + 2H_2O$.

Since disappearance of cysteine may be followed with ease by means of the Shinohara method, the following procedure was employed. Sodium cystinate, 30 mg., were added to 30 ml. of an enzyme suspension in 0·05 phosphate buffer at *p*H 6·7. After 2½ hr. 1·5 ml. were removed and the S-S present was determined. It was found to be reduced from a theoretical 0·0033 *M* to a 0·0007 *M* solution of S-S. To the remaining 28·5 ml. of the enzyme suspension, 3 ml. of 50 % trichloroacetic acid were added and the precipitate centrifuged down. The filtrate measured 26 ml. and its acidity was 0·381 *N*. 1·5 ml. of 10 *N* HCl were added, bringing the volume to 27·5 ml. and the acidity to approximately 1 *N*.

If all the S-S lost had appeared as sulphinic acid, the latter should now be 0·00313 *M*. Three times this molar equivalent of cysteine, or 46·95 mg. of cysteine-HCl were added. The SH content was 0·0102 *M* (theoretical 0·0104). Approximately 0·2 g. of KI was added, and the mixture was incubated in a water bath at 80° for 15 min. (Previous

experiments had shown this time to be sufficient for an equilibrium between RSH and RSO$_2$H to be established.) SH was now determined and found to be unchanged. Incubation was repeated for a second 15 min. period, the SH redetermined and again found the same. Hence sulphinic acid could not have been present. As a further demonstration, the theoretical equivalent of sulphinic acid (one-third of the molar equivalent of cysteine), was now added, and the solution incubated for a third 15 min. period. The SH reaction had now completely disappeared. From these experiments it was concluded that cysteic acid rather than sulphinic acid had been formed as a result of the action of the enzyme on cystine.

Production of taurine. Preliminary experiments [Medes, 1939] indicated that cystine is decarboxylated by an enzyme in liver. In those experiments volumetric methods of analysis were employed. The further investigation of the problem reported here was carried out with a Warburg respirometer. The liver enzyme was prepared similarly to the cysteine oxidase—centrifuging and subsequent washing of the insoluble protein of liver brei in a dilute phosphate buffer at pH 6·7. Determinations of O_2 uptake and CO_2 evolution were carried out in the usual manner, with customary checks in identifying the gases involved. Tests with lead acetate paper were negative and NH_2-N, as previously reported, was unchanged.

When cystine was employed as substrate, evolution of CO_2 and absorption of O_2 occurred simultaneously. The relative amounts varied widely, the CO_2 evolution ranging from 60 to 100 mol. % and the O_2 uptake, from 100 to 200 mol. %. No indication of decarboxylation was found when homocystine, di-formyl cystine, dithio-dilactic acid, or cysteic acid was employed as substrate. The reaction seems to be highly specific for cystine. As for oxidation, among the other disulphides tried, dithio-dilactic acid (prepared by the method of Westerman & Rose [1928] was the only one which gave evidence of O_2 absorption, homocystine, di-formylcystine and cystamine being negative. Hence free amino groups are not essential for the oxidation of the disulphide, although certain radicals, i.e. the formyl radical, $-C\overset{O}{\diagdown\!\!\diagup}H$, can inhibit the reaction, possibly by forming irreversible addition compounds with groups that are necessary in the oxidation, and thus modifying the reactivity of the radical attached to its S atom.

A third compound, cystamine disulphoxide, was oxidized with great ease. The amounts of O_2 absorbed varied widely in these experiments with crude enzyme preparations and ranged from 75 to 225 mol. %. Theoretical O_2 uptake for conversion of cystamine disulphoxide into the sulphonic acid stage would be 150 mol. % assuming that hydrolysis first occurs. This ready oxidation of cystamine disulphoxide, as contrasted with the complete resistance of cystamine, opens the question of whether the disulphoxide may not be an essential stage in the conversion of cystine into taurine. In this case, possibly two enzymes are present, one converting cystine into the disulphoxide, and one continuing the oxidation. Since cysteic acid cannot be decarboxylated to taurine, elimination of CO_2 must occur at some previous stage, either the disulphide or possibly the disulphoxide. The ready oxidation of cystamine disulphoxide presents the case for the latter alternative as an intriguing possibility. Unfortunately, the instability and limited solubility of cystine disulphoxide offered too great technical difficulties for its employment as substrate in gaining further light on this question.

Sulphate-producing enzymes. Pirie [1934], in describing the enzyme which oxidizes the sulphur of cysteine to inorganic sulphate, stated that he was unable to extract it from tissues. Medes [1939] encountered the same difficulties, but further investigation has shown the following method to be effective. The tissue is ground with sand in the physiological salt mixture, filtered through linen, and centrifuged to separate the soluble from the insoluble portion. The greater portion of the activity emanates from the lower layer. Two enzymes productive of inorganic sulphate are found, the one, described by Pirie,

acting on cysteine, and a second, identified by Medes, requiring sulphinic acid as substrate and shown by her to be a separate enzyme.

In Table 4 are recorded the results of a series of experiments with various substrates. Cysteine and methionine are oxidized possibly by the same enzyme, since no separation could be effected. Cystine, as shown by Pirie, gives rise to sulphate more slowly. He demonstrated the presence of SH groups and believed reduction of SS to SH occurred.

Table 4. *Sulphate formation by enzymes of rat's liver*

$8{\cdot}26 \times 10^{-2}$ m.equiv. of substrate added for each 10 ml. of the medium. Determinations were carried out after 2 hr. incubation in phosphate buffers at *p*H 6·7 and 7·4 or bicarbonate buffer at *p*H 7·3. Results are expressed in m.equiv./10 ml. of the medium.

	Liver slices							Liver brei			
	Phosphate buffer *p*H 6·7			Phosphate buffer *p*H 7·4		Bicarbonate buffer *p*H 7·3		Phosphate buffer *p*H 7·4		Bicarbonate buffer *p*H 7·3	
Substrate	SH m.eq. $\times 10^{-2}$	S-S m.eq. $\times 10^{-2}$	SO_4 m.eq. $\times 10^{-3}$	S-S m.eq. $\times 10^{-2}$	SO_4 m.eq. $\times 10^{-3}$	S-S m.eq. $\times 10^{-2}$	SO_4 m.eq. $\times 10^{-3}$	S-S m.eq. $\times 10^{-2}$	SO_4 m.eq. $\times 10^{-3}$	S-S m.eq. $\times 10^{-2}$	SO_4 m.eq. $\times 10^{-3}$
Control	—	—	0·32	—	1·70	—	0·22	—	0·84	—	0·62
Cysteine	3·77	3·12	1·32	5·90	5·48	6·66	7·09	2·30	11·80	2·85	12·66
Cystine	—	5·76	0·97	7·17	7·40	5·48	3·19	2·61	3·33	2·26	5·52
Sulphinic acid	—	—	0·97	—	6·76	—	6·14	—	15·35	—	62·92
Cysteic acid	—	—	—	—	—	—	0·23	—	—	—	—
Diamino-diethylene disulphoxide	—	—	—	—	2·05	—	—	—	—	—	—
Methionine	—	—	—	—	—	—	—	—	2·17	—	0·71
Methionine sulphoxide	—	—	—	—	—	—	—	—	0·81	—	2·91

This enzyme is highly unstable, and shows greatly reduced activity after the insoluble protein is washed with physiological salt, either buffered or unbuffered. The supernatant liquid is totally inactive. The enzyme oxidizing sulphinic acid is more stable, and retains considerable activity after the insoluble protein is recentrifuged in one or two washings of physiological salt solution or after salting out with $MgSO_4$ and subsequent dialysis.

Both enzymes, as shown by Table 4, are more active in phosphate buffer at *p*H 7·4 than at *p*H 6·7, and still more active in bicarbonate than in phosphate medium. Cysteic acid and methionine sulphoxide cannot serve as substrates for either enzyme, whereas diamino-diethylene disulphoxide, under the action of cysteine oxidase, apparently gives rise to small amounts of sulphate. This appearance of sulphate from the disulphoxide, in contrast to the failure of its formation from the sulphoxide, may possibly be ascribed to the fact that the former is more readily reduced to the disulphide than is the latter to methionine.

Decarboxylating enzyme. An enzyme which decarboxylates cystine is present in liver brei. It was prepared by grinding the tissue with sand in 20 ml. of 0·05 *M* phosphate buffer and filtering through fine cloth. 0·25 m.equiv. of the substrate was incubated in 7·50 ml. of water, 2·50 ml. of buffer and 2·50 ml. of liver brei. The incubation period was usually 24 hr. A 5 ml. sample was removed before, and a second 5 ml. portion, at the close of the incubation period. Each 5 ml. sample was added to 19·50 ml. of H_2O and 0·50 ml. of 50 % salicylsulphonic acid. The mixture was centrifuged for 20 min., the supernatant liquid filtered and 3·00 ml. were used for CO_2 determinations. Each sample analysed thus contained 0·0040 m.mol. per ml. All determinations were carried out in duplicate.

In addition to cystine as substrate, the sodium salt of cysteic acid was employed to verify our previous evidence that this amino-acid is not decarboxylated. Leucine and histidine were also used, the latter because its decarboxylation by liver and kidney tissue had been reported by Holz & Heise [1937]. Three typical experiments are recorded in Table 5, the first two experiments in phosphate buffer at *p*H 7·4 and the third in a similar

Table 5. *Decarboxylation of various amino-acids by liver brei*

3·0 ml. of liver brei in a volume of 12·5 ml. contained 0·25 m.equiv. of substrate. 2×10^{-3} m.equiv. of the amino-acid was present in each ml. of the sample employed. The first sample of 3 ml. was removed before incubation, and the second 3 ml. after an incubation of 24 hr. Results are expressed in m.equiv. $\times 10^{-3}$ of the amino-acid present in each ml.

	Found		Control subtracted		
	Before	After	Before	After	Substrate consumed
Control	3·22	3·59			
Na cystinate	7·24	6·65	4·02	3·06	0·96
Leucine	7·29	7·18	4·07	3·59	0·48
Histidine	7·29	4·93	4·07	1·34	2·73
Control	3·22	3·59			
Na cystinate	7·24	6·65	4·02	3·06	0·96
Cysteic acid	7·29	7·67	4·07	4·08	0·00
Leucine	7·29	7·24	4·07	3·65	0·42
Histidine	7·29	5·41	4·07	1·82	2·25
Control	3·22	3·59			
Na cystinate	7·24	6·70	4·02	3·11	0·91
Cysteic acid	7·29	7·61	4·07	4·02	0·05
Leucine	7·24	7·29	4·02	3·70	0·32
Histidine	7·24	5·47	4·02	1·88	2·14

buffer at *p*H 6·0. In all cases decarboxylation of histidine was most rapid, cysteic acid was unchanged, cystine was intermediate, usually about 25 % undergoing decarboxylation. Leucine was usually acted upon only slightly.

Experiments carried out at *p*H 8·0 showed even greater decomposition of the histidine, while breakdown of cystine was somewhat less marked. Dialysing the enzyme only slightly reduces its activity. It may be concentrated by precipitation with 3·5 *M* sodium sulphate followed by dialysis.

Action of coenzymes. No evidence for the necessity of cytochrome or cozymase could be found in the cases of the oxidase which gives rise to cysteic acid, but that coenzyme plays a part in the oxidation of cysteine and sulphinic acid to sulphate is indicated in the following experiment: The perfused liver of an albino rat was ground with sand in physiological salt, filtered through cloth and dialysed for 24 hr. against repeated changes of 0·9 % salt solution. In each of five tubes were placed 2 ml. of the brei, 8 ml. of the Krebs salt mixture (NaCl, $CaCl_2$, KCl, $MgCl_2$) and 1·50 ml. 0·154 *M* $NaHCO_3$. To the five tubes were added respectively: *a*, 0; *b*, 10·0 mg. cysteine; *c*, 12·3 mg. sulphinic acid; *d*, 10·0 mg. cysteine plus 0·50 ml. cozymase; *e*, 12·3 mg. sulphinic acid plus 0·50 ml. cozymase. In *c* and *d*, 0·50 ml. of the salt mixture replaced the coenzyme. The sulphur appeared as sulphate to the following extents: *a*, 0; *b*, 0; *c*, 25·3 %; *d*, 1·3 %; *e*, 46·3 %. Although the activity of the cysteine oxidase was nearly lost during the dialysis, a slight restoration was effected through addition of the coenzyme. In the case of the sulphinic acid oxidase sulphate formation was approximately doubled.

Discussion

Pirie [1934] demonstrated the appearance of sulphate in tissues from various S-containing amino-acids. He concluded from his results that the path of sulphate formation proceeded through the oxidation of SH groups, to which cystine and methionine were previously enzymically reduced. The first compound formed in the oxidation of cysteine would be the sulphenic acid, which dismutes spontaneously into sulphinic acid and cysteine, according to the following equation: $2R\text{SOH} \rightarrow R\text{SO}_2\text{H} + R\text{SH}$. Pirie postulated that the sulphinic acid then proceeds by enzyme action to sulphite and eventually to sulphate.

Medes [1939] established the existence of an enzyme which oxidizes sulphinic acid to sulphate, the resulting yield of sulphate being much greater than that from either cysteine

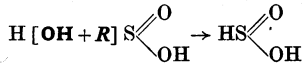
or cystine. She also demonstrated that cysteine *in vivo* gives rise to sulphate more rapidly than does cystine and that cysteine disappears more readily than cystine *in vitro*.

In view of these facts and the results presented in this paper, it seems possible that sulphate formation in the body takes place along the following path. Cystine, acting as a reservoir, is first hydrolysed to cysteine and cysteinesulphenic acid [see also Simonsen, 1931]. Pirie postulated an enzymic reduction at this stage, but this investigation has failed to find evidence for the existence of an enzyme functioning thus. As long as the two resultants, cysteine and cysteinesulphenic acid, are present, an equilibrium exists which strongly favours the reverse reaction. But sulphenic acid is also being formed by enzymic oxidation of cysteine, probably by the addition of HOH and the subsequent removal of two H atoms. This is possibly the step performed by the 'sulphate-producing' cysteine oxidase.

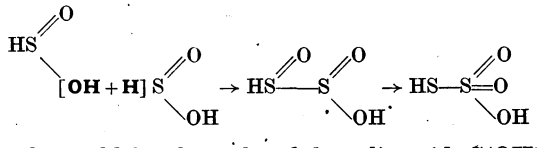
The sulphenic acid then dismutes spontaneously to cysteinesulphinic acid and cysteine. Since the formation of sulphate both *in vivo* and *in vitro* is less rapid with cystine than with cysteine, and since the oxidation of cystine as compared with cysteine in the Warburg respirometer is also slower, it follows that the rate of hydrolysis is much lower than the rate of oxidation of cysteine to cysteinesulphenic acid.

Cysteinesulphinic acid is not acted upon by the enzymes which, according to Bernheim & Bernheim [1939] and Medes [1939] produce cysteic acid from cysteine. Cysteinesulphinic acid does, however, rapidly give rise to inorganic sulphate by enzyme action. It seems highly probable that removal of the *R* group is the essential step performed by this enzyme and that the sulphur acid which results goes spontaneously to sulphate.

The exact nature of the acid produced by elimination of the *R* group has not been determined, but we can point out one or two paths that this reaction may follow. Clarke & Inouye [1930] demonstrated that the alkaline plumbite decomposition of cystine leads to thiosulphate formation. This can be derived theoretically from sulphinic acid by the following reaction:



The acid could then react with a second molecule like itself with production of a thiosulphate which could easily pass to sulphate:



Another possible path would be through sulphoxylic acid, $S(OH)_2$, which in alkaline solution produces disulphoxy-acid as shown by Bassett & Durante [1927]:

$$2S(OH)_2 \xrightarrow{\text{alk.}} HO—S—O—S—OH + H_2O.$$

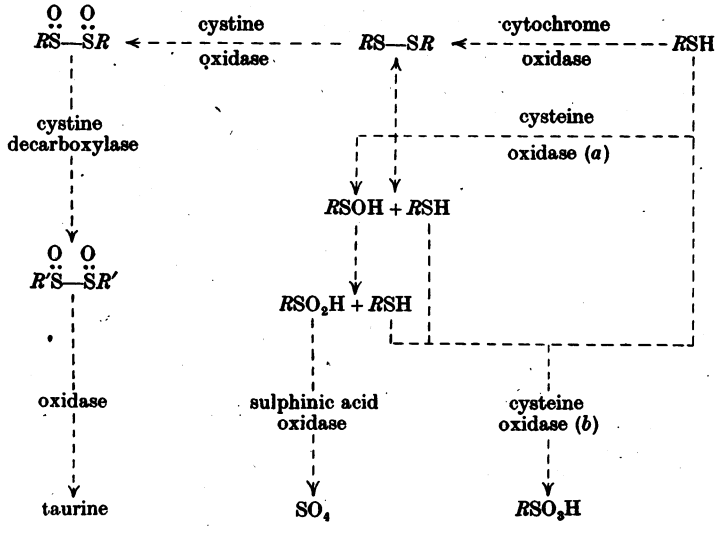
The disulphoxy-acid then reacts with another molecule of sulphoxy-acid to yield pyrosulphurous acid:

$$HO—S—O—S—OH + S(OH)_2 \rightarrow H_2S + H_2S_2O_5.$$

Pyrosulphurous acid easily goes to sulphate, and this latter path may be the preferred one under certain conditions as it offers a possible explanation for the formation of H_2S observed by Smythe [1941].

The enzyme which gives rise to cysteic acid has no place in this scheme, since cysteic acid has not been shown to give rise to sulphate. As no separation of the cysteic acid-producing enzymes acting on cystine and cysteine could be effected, it seems permissible to postulate that here also a non-enzymic hydrolysis of disulphide to sulphydryl and sulphenic acid must be the first step when cystine serves as substrate. The enzyme would

then act on cystine. This hypothesis accords with the slow production of cysteic acid from cystine. Conditions in the medium tending to decrease the dissociation would delay or might even totally prevent cysteic acid production—a fact which might explain the failure of Bernheim & Bernheim [private communication] to find consistent evidence for the ability of cystine to serve as substrate in their experiments.



A tentative scheme embodying the results of this investigation is presented in the accompanying diagram. Oxidation of cystine involves a non-enzymic sulphinic acid production by hydrolysis and dismutation. In test-tube experiments this series of reactions proceeds slowly, even in the presence of Cu and other catalysts of tissue juice; but in this case we have powerful enzymes removing the end-products and hence speeding the reaction. Sulphinic acid is acted upon rapidly by sulphinic acid oxidase, while cysteine, the other product at each stage, may be seized by any one of three enzymes—cytochrome oxidase, which oxidizes it to cystine, cysteine oxidase, which oxidizes it to the sulphenic acid, and a second cysteine oxidase which converts it into cysteic acid.

The only reactions which could be observed in these experiments as possibly leading to the production of taurine were through the disulphoxide of cystine. Although the oxidation of cystine to the disulphoxide is enzymic, the reverse reaction may occur non-enzymically on account of the reducing properties of the tissues. That this may take place in the body under certain special conditions was concluded by Bennett [1937], who showed that cystine disulphoxide could replace cystine in the diets of cystine-deficient rats. Beyond this stage, the reaction would be irreversible, cystamine disulphoxide and taurine being incapable of contributing to sulphate formation.

Although all these reactions become irreversible as the end-products are approached, an equilibrium may be said to exist, in that the presence of excess amounts of taurine or of cysteic acid may prevent their further elaboration by their respective enzymes, and all the extra cystine or cysteine supplied would then be shunted to sulphate. But if the store of either taurine or cysteic acid becomes lowered or exhausted, activity in that direction would be resumed.

Reasoning along this line might explain the observation of Virtue [1939] that cysteic acid given orally gives rise to taurine. According to the accompanying diagram, once there is no demand for the elaboration of cysteic acid, the fate of the disulphide is left to two possible channels, i.e. sulphate or taurine formation. Since the cholic acid fed creates a demand for taurine, possibly by rapidly uniting with it and in effect removing it from

the reaction mixture, the further oxidation of disulphide proceeds to this stage. When cysteine sulphinic acid is injected subcutaneously and cholic acid fed, an increase in taurocholic acid is again demonstrated [Virtue, 1939]. In this case also, the removal by the cholic acid of any taurine already present would keep this pathway open for the oxidation of all available disulphide.

The validity of this system could be tested by feeding radioactive compounds. Thus if radioactive cysteic acid or sulphinic acid is fed simultaneously with cholic acid, then the lack of radioactivity in the taurocholic acid produced would demonstrate the validity of this theory, but if the taurocholic acid showed radioactive properties, alternate paths would have to be considered.

The possible formation of taurine through other channels in which the S-containing molecule has united with cholic acid or some precursor of cholic acid before its oxidation, is not excluded by this investigation. A few experiments attempted along this line were negative, but since conditions are exceedingly complex the significance of negative results should be considered with caution.

The fate of the cysteic acid is unsolved. It does not give rise to sulphate in the liver, and no mechanism for its conversion into taurine has been found. A suggestion as to its fate has been presented by Cohen [1940] who found that it takes part in the transamination processes in muscle tissue. Since the resulting sulphopyruvic acid would seem to be labile, it may prove a possible pathway to sulphate formation, otherwise it may be excreted unchanged. The authors are now investigating these questions.

REFERENCES

Bassett, H. & Durrant, R. G. [1927]. *J. chem. Soc.* 1401.
Bennett, M. A. [1939]. *Biochem. J.* **33**, 885.
Bernheim, F. & Bernheim, L. C. [1939]. *J. biol. Chem.* **127**, 695.
Cohen, P. P. [1940]. *J. biol. Chem.* **136**, 565.
Christensen, B. E., West, E. S. & Dimick, K. P. [1941]. *J. biol. Chem.* **137**, 735.
Clarke, H. T. & Inouye, J. M. [1930]. *J. biol. Chem.* **89**, 399.
Holz, P. & Heise, R. [1937]. *Naturwissenschaften*, **25**, 201.
Kendrick, A. B. & Hanke, M. E. [1940]. *J. biol. Chem.* **132**, 739.
Kolb, J. J. & Toennies, G. [1940]. *Industr. Engng Chem.* (Anal. Ed.), **12**, 723.
Krebs, H. A. [1932]. *Hoppe-Seyl. Z.* **210**, 33.
Lavine, T. F. [1937]. *J. biol. Chem.* **117**, 309.
Mason, M. F. [1938]. *Biochem. J.* **32**, 719.
Medes, G. [1939]. *Biochem. J.* **33**, 1559.
Medes, G. & Stavers, E. [1940]. *J. Lab. clin. Med.* **25**, 624.
Pirie, N. W. [1934]. *Biochem. J.* **28**, 305.
Power, M. H. & Wakefield, E. G. [1938]. *J. biol. Chem.* **123**, 665.
Shinohara, K. & Padis, K. E. [1935]. *J. biol. Chem.* **112**, 697.
Simonsen, D. G. [1931]. *J. biol. Chem.* **94**, 323.
Smythe, C. V. [1941]. *Proc. Amer. Soc. biol. Chem.* **35**, cxviii.
Van Slyke, D. D. [1929]. *J. biol. Chem.* **83**, 425.
Van Slyke, D. D. & Dillon, R. T. [1938]. *C.R. Trav. Lab. Carlsberg*, **22**, 480.
Virtue, R. W. [1939]. *J. biol. Chem.* **127**, 431.
Westerman, B. D. & Rose, W. C. [1928]. *J. biol. Chem.* **79**, 413.