THE OXIDATION OF CYSTEINESULFINIC AND CYSTEIC ACIDS IN *PROTEUS VULGARIS*[*]

by

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Definitive knowledge of the pathway and the enzymes involved in the oxidation of sulfur-containing amino acids has been scanty. Besides its oxidation to cystine, cysteine has been shown to undergo extensive oxidation in particulate liver preparations, with the possible formation of cysteic acid, as judged from O_2 yield[2]. PIRIE[3] has demonstrated the formation of inorganic sulfate from cysteine, cystine, and methionine in slices of rat kidney and liver and has postulated that both cysteine and cystine are oxidized to cysteinesulfinic acid, with the intermediate formation of cysteinesulfenic acid. The sulfinic acid was thought to be the point of desulfination and the inorganic sulfite the precursor of sulfate, since cysteic acid did not form sulfate under these conditions. MEDES[4] extended these experiments and showed that, of a series of sulfur-containing amino acids, cysteinesulfinate was the best substrate for sulfate formation in tissue slices. MEDES[4] and MEDES AND FLOYD[5] provided support for the intermediate role of cysteinesulfinic acid in the oxidative metabolism of cysteine and cystine by the finding that the sulfinic acid is rapidly utilized in homogenates and extracts of rat tissues and gives rise to inorganic sulfate. FROMAGEOT, CHATAGNER AND BERGERET[6,7] extracted an enzyme system from rabbit liver which transformed the sulfinic acid to alanine and inorganic sulfite. These findings are in accord with the pathway earlier postulated by PIRIE[3], but MEDES AND FLOYD believed that cysteine and cystine may also be oxidized by other routes to cysteic acid and taurine, respectively.

For a closer delineation of the oxidative fate of sulfur-containing amino acids it seemed desirable to isolate and study each step in the overall process, one by one, and to characterize the enzymes responsible for these transformations. As an effort in that direction, the intermediary metabolism of cysteinesulfinic and cysteic acids in a microorganism is described in this series of papers. The oxidative metabolism of these amino acids in animal tissues will be the subject of other reports. It is hoped to extend these investigations to the reactions responsible for the formation of cysteinesulfinic acid from cysteine and cystine.

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[**] Fellows of the John Simon Guggenheim Memorial Foundation, 1951–52. Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. (U.S.A.). A preliminary account of this work has appeared[1] and a summary was presented at the Second International Congress of Biochemistry, in Paris, July, 1952.

References p. 275.

MATERIALS AND METHODS

Proteus vulgaris, strain OX-19 (from the collection of the Pasteur Institute) was grown on a beef infusion-agar medium, containing 0.5% added tryptone and 0.5% NaCl. After 18–20 hours at 37° the harvested cells were thrice washed with distilled water.

L- and DL-cysteinesulfinic acids were synthesized by the method of LAVINE[8] and their purity was established iodometrically, by paper chromatography, and by titration with $KMnO_4$. L-cysteic acid was purchased from the Light Chemical Co.

RESULTS

Characteristics of the oxidation of cysteinesulfinate by resting cells. Thoroughly washed, resting cells of *P. vulgaris*, suspended in *tris*(hydroxymethyl)amino methane buffer (TRIS buffer), pH 8.4, oxidize L-cysteinesulfinic acid vigorously. Under these conditions, and with high substrate concentration, Q_{O_2} values of 30–40 are obtained at 35°, after subtraction of the small endogenous respiration. As shown in Fig. 1, a definite lag period in the oxidation of L-cysteinesulfinate can be observed with thoroughly washed organisms. After about 10 minutes, the rate becomes linear and is maintained for several hours, provided that the substrate is not exhausted.

The oxidation proceeds optimally in the range of pH 8.0 to 8.6. A relatively high initial substrate concentration is required (Fig. 1); the rate increases up to about 0.05 *M* initial L-cysteinesulfinate. The oxidation proceeds equally well in phosphate and TRIS buffers.

There are noteworthy differences in the characteristics of the oxidation of L- and of DL-cysteinesulfinate. The latter substrate is oxidized somewhat more rapidly than the former and its oxidation shows no lag period (Fig. 1). However, the initial high rate is not maintained, as is the case in the oxidation of the L-isomer. Furthermore,

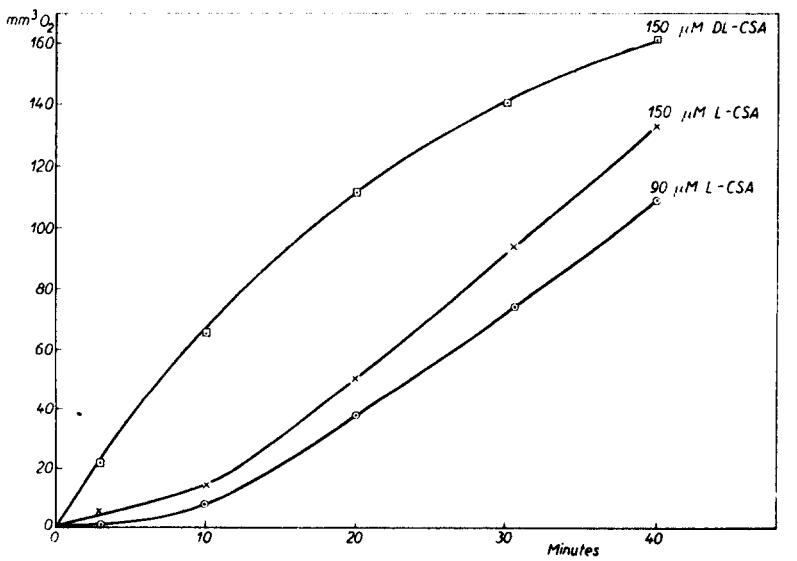


Fig. 1. Characteristics of the oxidation of L- and DL-cysteinesulfinic acid (CSA). Each Warburg vessel contained 1 ml 0.1 *M* TRIS buffer, pH 8.4, 12.4 mg washed cells, and substrate in a total volume of 3 ml temp., 35° C. An equilibration period of 30 min was allowed prior to tipping in the substrate, in order to minimize the endogenous respiration. The latter has been subtracted from the values given on the ordinate.

References p. 275.

the substrate requirements for the oxidation of the racemic amino acid are lower than for the L-component (Figs. 1 and 2). It appears, therefore, that the initial steps in the oxidative metabolism of L- and D-cysteinesulfinate are catalyzed by different enzymes.

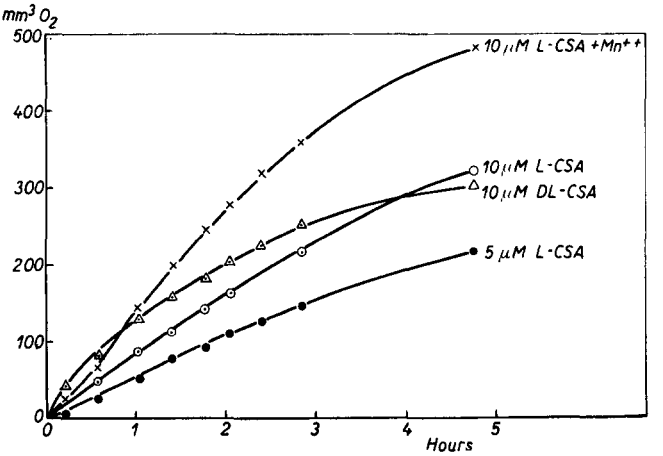


Fig. 2. Comparison of the rates of oxidation of L- and DL-cysteinesulfinate at low substrate concentration. The effect of added Mn^{++}. Conditions as in Fig. 1, except that 15.3 mg dry weight of bacteria were used per vessel. $MnCl_2$, at a final concentration of $2 \cdot 10^{-3}$ *M*, was present, where indicated.

It may be further noted in Fig. 2 that the addition of $2 \cdot 10^{-3}$ *M* Mn^{++} markedly iucreases the rate of O_2 uptake, without abolishing the initial lag period. This effect of Mn^{++} has been repeatedly observed and it becomes more accentuated the more thoroughly the bacteria are washed. Delayed addition of Mn^{++} to exhaustively washed cells oxidizing L-cysteinesulfinate has the same effect as its incorporation in the initial reaction mixture. Fig. 3 shows that, after 1 atom of O_2 is consumed per mole of the amino acid, addition of Mn^{++} causes over a 2-fold increase in O_2 uptake. This is in harmony with the observation that Mn^{++} fails to abolish the lag period and indicates that the cation is concerned with the oxidation of a *product* arising from L-cysteinesulfinate.

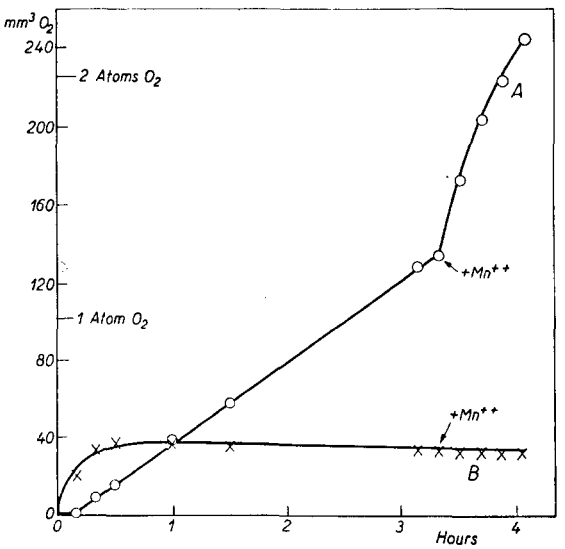


Fig. 3. The effect of *delayed* addition of Mn^{++} on the oxidation of limited quantities of cysteinesulfinate (Curve A) and cysteate (Curve B). Conditions as in Fig. 1, except that 5.2 mg dry weight of exhaustively washed cells were present in each vessel in order to magnify the effect of added Mn^{++}. Substrate concentration, 10 μM per 3 ml $2 \cdot 10^{-3}$ *M* $MnCl_2$ added to each vessel at the time indicated.

Oxidation of L-*cysteate by resting cells.* Suspensions of *P. vulgaris* in TRIS buffer, pH 8.4, oxidize 0.05 *M* L-cysteate at about the same rate as L-cysteinesulfinate, while at low substrate concentration (0.003 *M*) the oxidation of the sulfonate exceeds that of the sulfinate (Fig. 3). When L-cysteate is used as substrate, no lag period is observed, and addition of Mn^{++} affects neither the rate nor the total O_2 uptake (Fig. 3).

References p. 275.

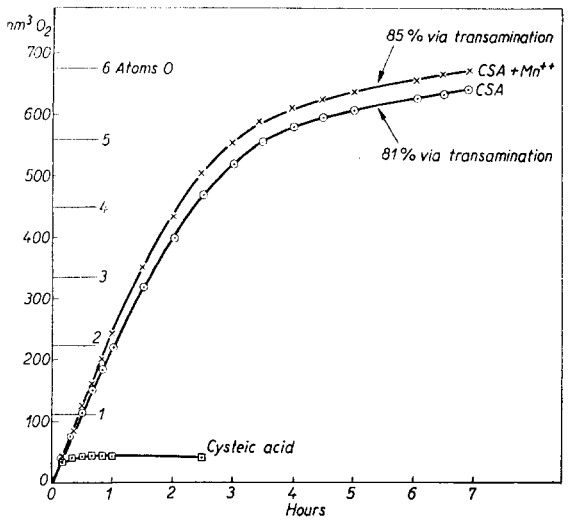
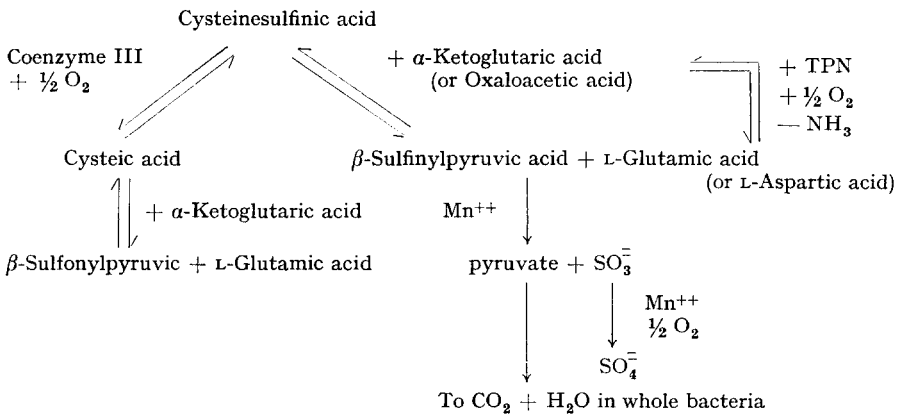


Fig. 4. Extent of oxidation of limited quantities of cysteinesulfinate and cysteate. Conditions as in Fig. 1 except that 24.9 mg dry weight of organisms were present in each vessel. Substrate concentration, 10 μM per 3 ml; $MnCl_2$ concentration, where indicated, $2 \cdot 10^{-3}$ M.

Extent of oxidation of L-*cysteinesulfinate and* L-*cysteate.* The oxidation of limited amounts of the sulfonic acid ceases before the uptake of 1 atom of O_2 per mole (Figs. 3 and 4). In the presence of the sulfinic acid, on the other hand, O_2 uptake continues until about 6 atoms are consumed per mole of substrate (Fig. 4). Since the organisms are rich in catalase and no H_2O_2 accumulates, complete oxidation of cysteinesulfinic acid to CO_2, water, and sulfate would require 7 atoms of O_2. Addition of Mn^{++} increases the total O_2 consumption to a small, but significant extent.

Interpretation of the observations on the oxidation of cysteinesulfinate and cysteate by intact cells. The authors have previously suggested[1] on the basis of experiments on intact cells and enzymes extracted from this organism that the following dual pathway represents the metabolism of L-cysteinesulfinate in *P. vulgaris*:



Evidence for the detailed reactions, as they occur in cell-free extracts, will be presented in the following two papers[9,10]. The observations described above lend some support to this scheme and suggest that it describes the metabolic events in whole cells, as well as in enzyme systems isolated from the bacteria.

According to this scheme, the initial reactions of L-cysteinesulfinic acid are as follows: oxidation to L-cysteic acid (pathway A) and transamination to yield β-sulfinylpyruvic acid (pathway B). With soluble enzymes, both of these primary reactions proceed best at a somewhat alkaline pH, and both require high substrate concentrations for saturation[9,10]. This parallels the alkaline pH optimum and high substrate concentration needed for rapid oxidation by whole cells. The desulfination of β-sulfinylpyruvate

References p. 275.

to sulfite and pyruvate and the oxidation of sulfite to sulfate are Mn^{++}-catalyzed reactions in extracts[1,9]. The clear-cut effect of added Mn^{++} on the oxidation of the amino acid by *Proteus* cells suggests that the same may be true in the intact organism. It is difficult to decide, however, whether the effect of added Mn^{++} on the respiration of the cells is one of rapid non-enzymic catalysis, as in the extracts, or whether Mn^{++}-requiring enzymes are involved.

The lag in the initial O_2 uptake by whole cells is in accord with the postulated transamination, which precedes the actual oxidation steps (reoxidation of the glutamate and aspartate formed by transamination and oxidation of sulfite and of pyruvic acid). The supply of the α-keto acids needed for this transamination is undoubtedly limited in well-washed cells and the lag period may be associated with their formation by endogenous respiration.

Pathway B, the transaminative route, is the more important of the two competing pathways of cysteinesulfinate metabolism in whole cells. This is indicated by the relative magnitudes of the lag period and Mn^{++} effect (which are associated only with pathway B). Proof for the contention that oxidation via cysteic acid (pathway A) cannot be the preponderant route may be adduced from the fact that under the experimental conditions L-cysteic acid takes up only about 0.5 atom of O_2 per mole, while L-cysteinesulfinic acid consumes about 6. As a matter of fact, by comparison of the O_2 yields of the sulfinic and sulfonic acids in Fig. 4, it can be calculated that 81% of the metabolism of the former occurred by way of pathway B, and 19% via pathway A. Addition of Mn^{++} appears to shift the oxidation in favor of pathway B to a limited extent; with added Mn^{++} 85% of the oxidation is routed via initial transamination. The reason for this small but regular shift may be that the accumulation of the end products of transaminase action in Mn^{++}-deficient organisms slows down that reaction rate sufficiently to render the alternate route to cysteic acid a more effective competitor for the limiting amount of cysteinesulfinate present.

It may be added that under anaerobic conditions no decarboxylation of L-cysteinesulfinate was detected with whole cells. This is in contrast to its known decarboxylation in liver[7].

Concerning the fate of D-cysteinesulfinate, it seems quite probable that at least one enzyme is present in the organisms which fails to act on the L-antipode. Possibly the D-amino acid oxidase studied by STUMPF AND GREEN[11] in extracts of another strain of *P. vulgaris* is associated with the primary reaction. If that should be the case, the pathways of oxidation of the D- and L-isomers would be identical, except for the initial step. We hope to establish this point by the use of pure D-cysteinesulfinate.

We are greatly indebted to Mlle. F. CHATAGNER for the gift of samples of L-cysteinesulfinic acid and to Professor C. FROMAGEOT for his hospitality and sincere interest and for valuable discussions.

SUMMARY

1. Thoroughly washed, resting cells of *P. vulgaris* OX-19 rapidly oxidize L- and D-cysteinesulfinic and L-cysteic acids.

2. Optimal oxidation of L-cysteinesulfinate occurs at pH 8–8.6 at 0.05 *M* (or higher) substrate concentration. An initial lag in O_2 uptake is regularly observed, which appears to be associated with a transaminative reaction. Addition of $2 \cdot 10^{-3}$ *M* Mn^{++} increases the oxidation of the L-amino acid markedly, but fails to decrease the lag period. Mn^{++} is concerned with the removal of the primary reaction product of L-cysteinesulfinate.

References p. 275.

3. The differences in the characteristic features in the initial oxidation of L- and of DL-cysteinesulfinate suggest that the primary enzymic reactions of the two optical antipodes are catalyzed by separate enzymes and may be different reactions.

4. The initially rapid oxidation of L-cysteate comes to a halt after the uptake of less than 1 atom of O_2 per mole of substrate, while the oxidation of L-cysteinesulfinate continues until about 6 atoms of O_2 are utilized.

5. The experimental observations have been interpreted in terms of a dual pathway for L-cysteinesulfinate metabolism. It involves an initial oxidation to cysteate by one route and a transamination with an α-keto acid by the other route; β-sulfinylpyruvic acid is then desulfinated to yield pyruvic acid and sulfite, and the latter yield CO_2, water, and sulfate.

RÉSUMÉ

1. Des cellules de *P. vulgaris* OX-19, au repos et soigneusement lavées, oxydent rapidement les acides L- et D-cystéinesulfiniques et L-cystéique.

2. Le pH optimum de l'oxydation du L-cystéinesulfinate est de 8–8.6, pour une concentration en substrat égale ou supérieure à 0.05 *M*. La consommation d'oxygène présente toujours un temps de latence, qui semble être lié à une réaction transaminative. L'addition de Mn^{++} à la concentration $2 \cdot 10^{-3}$ *M* augmente nettement l'oxydation du L-aminoacide, mais ne diminue pas le temps de latence. Mn^{++} agit sur le transfert du premier produit de réaction du L-cystéinesulfinate.

3. Les différences caractéristiques entre l'oxydation du L-cystéinesulfinate et celle du DL-cystéinesulfinate suggèrent que les premières réactions enzymatiques des deux antipodes optiques sont catalysées par des enzymes différents et ne sont peut-être pas les mêmes.

4. L'oxydation du L-cystéate, d'abord rapide, s'arrête alors que la consommation en O_2 est inférieure à un atome par molécule de substrat tandis que dans l'oxydation du L-cystéinesulfinate la consommation en O_2 atteint 6 atomes par molécule.

5. Les résultats expérimentaux peuvent s'interpréter à l'aide d'un schéma à deux voies pour le métabolisme du L-cystéinesulfinate. Ce schéma fait intervenir une oxydation initiale en cystéate ou une transamination avec un α-cétoacide; l'acide β-sulfinylpyruvique est ensuite désulfiné pour donner de l'acide pyruvique et du sulfite, qui donne à son tour du CO_2, de l'eau et du sulfate.

ZUSAMMENFASSUNG

1. Sorgfältig gewaschene, sich im Ruhestadium befindende Zellen von *P. vulgaris* OX-19 oxydieren schnell L- und D-Cysteinsulfinsäure und L-Cysteinsäure.

2. Die optimale Oxydation des L-Cysteinsulfinats liegt bei einem pH von 8–8.6 und einer Substratkonzentration von 0.05 *M* (oder häher). Eine anf·ngliche Verzögerung in der Sauerstoffaufnahme kann regelmässig beobachtet werden, sie scheint mit einer Transaminierung verbunden zu sein. Ein Hinzufügen von $2 \cdot 10^{-3}$ *M* Mn^{++} steigert die Oxydation der L-Cysteinsäure bemerkenswert, die Zeit der scheinbaren Hemmung zu Beginn der Reaktion wird dadurch aber nicht verkleinert. Das Mn^{++} beteiligt sich an der Entfernung des primären Reaktionsproduktes des L-Cysteinsulfinats.

3. Die charakteristischen Unterschiede bei Beginn der Oxydation des L- und des DL-Cysteinsulfinats lassen vermuten, dass die primären enzymatischen Reaktionen der 2 optischen Antipoden durch verschiedene Enzyme katalysiert werden und zwei verschiedene Reaktionen sein könnten.

4. Die anfänglich schnelle Oxydation des L-Cysteates kommt nach Aufnahme von weniger als 1 Atom Sauerstoff per Mol Substrat zum Stillstand, während die Oxydation des L-Cysteinsulfinates weitergeht, bis ungefähr 6 Atome Sauerstoff verbraucht sind.

5. Die experimentellen Beobachtungen wurden in einer zwei Möglichkeiten offenlassenden Weise der L-Cysteinsulfinatumwandlung interpretiert. Sie besteht aus einer anfänglichen Oxydation zum Cysteat auf dem einen Weg und einer Transaminierung mit einer α-Ketosäure auf dem anderen Weg; β-Sulnfiylbrenztraubensäure wird dann desulfiniert zu Brenztraubensäure und Sulfit, welche CO_2, Wasser und Sulfat geben.

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