Protein-Catalyzed Isotopic Exchange Reaction between Cysteine and Sulfide in Spinach Leaves

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Sulfide, Cysteine, O-Acetyl-L-serine, Cysteine Synthase

A protein has been isolated from spinach leaves which catalyzes the following isotopic exchange reaction:

$$\text{Cys-SH} + \text{H}_2{}^{35}\text{S} \longleftrightarrow \text{Cys-}{}^{35}\text{SH} + \text{H}_2\text{S}\,.$$

This enzyme has an pH-optimum above 9.0; its molecular weight has been estimated on a Sephadex-G-100 column to be around 64 000 daltons. During purification this exchange reaction activity follows cysteine synthase activity on DEAE-cellulose and Sephadex-G-100 column chromatography; however this enzyme fraction has not been purified to homogeneity to prove that both activities are catalyzed by the same protein.

The apparent K_m for a) H_2S has been determined to be 0.86 mM using cysteine as substrate and 0.6 mM using O-acetylserine as substrate; b) for cysteine was found to be 3.3 mM; and c) for O-acetylserine to be 3.3 mM. For catalysis no metal ion is required and the reaction proceeds without addition of pyridoxalphosphat. This exchange reaction might prove to be a simple method to prepare labelled cysteine from non-labelled cysteine and labelled H_2S. The exchange reaction was found too in *Chlorella pyrenoidosa* and in *Rhodospirillum rubrum*.

Introduction

Sulfur chemistry was been difficult because side reactions might occur, which could jeopardize results obtained with biological material. In this connection the isotopic exchange reactions with "Bunte salts" and free sulfite might be mentioned here as one example causing trouble.

In this publication evidence is presented that spinach leaves contain an enzymatic activity which is capable to exchange the sulfur of cysteine with free H_2S, which might cause difficulties in analyzing data of sulfur metabolism using labelled substrates.

Materials and Methods

Protein preparation

Spinacia oleracea L var. "Vital R" was grown in the botanical garden of this university. Plants were homogenized in 0.02 M Tris-HCl buffer pH 8.0 containing 2 mM mercaptoethanol and 10 mM $MgCl_2$. The crude extract was clarified by centrifugation for 15 min at $10\,000 \times g$. The supernatant was fractioned by solid ammoniumsulfate and the fraction between 35% and 65% saturation was collected and dialyzed over night against the buffer mentioned above. The dialyzed extract was applied on a DEAE-cellulose column (2×10 cm) and developed with a linear gradient of 0 to 0.5 M NaCl in 0.02 M Tris-HCl pH 8.0; 200 ml were used for each reservoir and fractions of 5.1 ml were collected. Active fractions were pooled, concentrated by ammoniumsulfate precipitation (to 70%) and after dialysis rerun on a second DEAE-cellulose column under the same conditions. Fig. 1 shows the elution profile. Active fractions (26–32) were pooled, concentrated as described above and subjected to a Sephadex-G-100 column (1.8×75 cm) equilibrated with 0.02 M Tris-HCl pH 8.0 containing 0.1 M KCl. Fractions of 3.1 ml were collected. Active fractions (28–32) were pooled and stored frozen at -18 °C. Under these conditions the enzyme fraction can be stored for a longer period without loss of activity.

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Protein determination

Protein concentrations were determined by TCA-precipitation and measurement of the density after 30 sec at 436 nm using bovine albumin as reference [1].

Measurement of the cysteine synthase activity

The formation of cysteine from H_2S and O-acetyl-L-serine was followed. Cysteine was determined as the red ninhydrin-complex as described by Peniazek *et al.* [2].

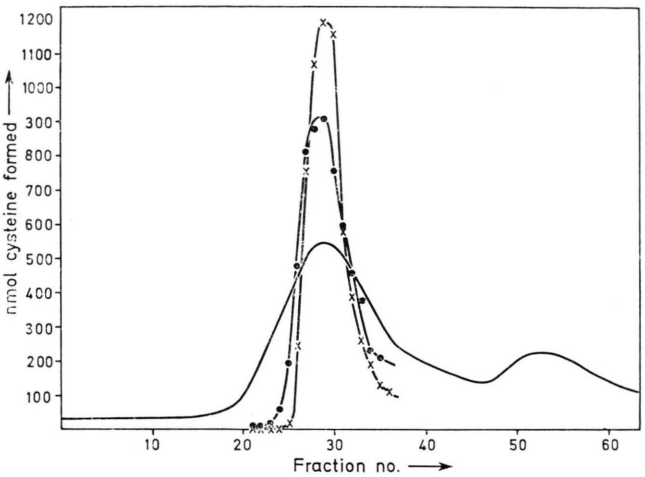

Fig. 1. Chromatography of cysteine synthase and isotopic exchange protein on DEAE-cellulose. Column conditions are described in "Materials and Methods". Assay conditions (in μmol) a) for cysteine synthase: Tris-HCl pH 7.5: 100; O-acetyl-L-serine: 5; H_2S: 5; and 0.05 ml of each fraction in a total volume of 1 ml. Incubation for 10 min at 37 °C. b) for the isotopic exchange reaction: Tris-HCl pH 8.0: 100; dithioerythritol: 10; cysteine: 5; H_2S: 2 (1 μmol = 63.400 cpm); and 0.02 ml of each fraction in a total volume of 1 ml. Incubation for 1 hour at 37 °C under N_2. —, optical density at 259 nm; ×—×, cysteine synthase activity; ●—●, exchange activity.

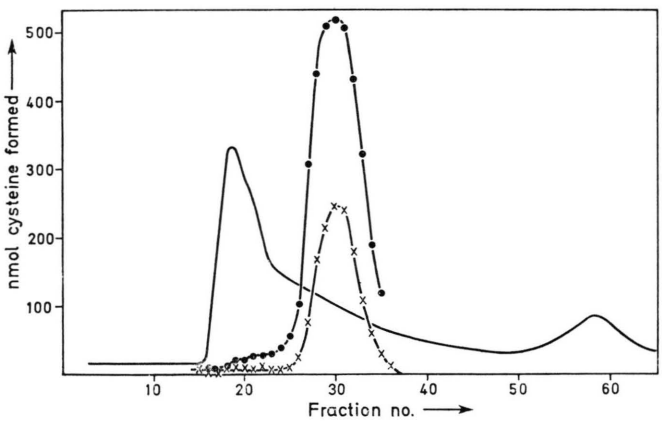

Fig. 2. Chromatography of cysteine synthase and isotopic exchange protein on a Sephadex-G-100 column. The same conditions as in Fig. 1, however 0.005 ml of each fraction was used as protein source.

Measurement of the isotopic exchange reaction

This activity was analyzed by incubating non-labelled cysteine with labelled H_2S. H_2S and cysteine were separated on a small (2.5 ml) Dowex-50 column in the following way: After heating the sample was applied to the Dowex-column and H_2S was removed by washing of the column with 25 ml of water. Cysteine was eluted with 3 N NH_4OH and the radioactivity was determined. The product was identified by paper electrophoresis as described by Schmidt *et al.* [3].

Determination of radioactivity

Radioactivity was determined in a liquid scintillation counter Beckmann model LS 100 using the scintillation coctail according to Patterson and Greene [4].

Chemicals

Labelled H_2S was purchased from Buchler (Ammersham); O-acetyl-L-serine, DL-homocysteine-thiolacton, ninhydrin, DEAE-cellulose and Dowex-50 WX 8, 100 – 200 mesh, H^+-form were obtained from Serva (Heidelberg); O-phospho-L-serine was obtained from Sigma and O-phospho-L-homoserine was a generous gift of Dr. Schnyder, Wanda-AG Bern. Sephadex-G-100 was obtained from Pharmacia (Sweden) and all other chemicals used were from Merck (Darmstadt). DL-homocysteine-thiolacton was brought to free homocysteine by acid hydrolysis following the appearence of free SH-groups with the Ellman-reagent (3,5′-dithio-bis(2-nitrobenzoic acid)) which was supplied together with the protein standards by Boehringer (Mannheim).

Results

It will be shown in this publication that spinach leaves contain a protein catalyzing an exchange reaction between cysteine and H_2S. To study this reaction in some detail it was tried to purify the protein involved and a 130-fold purification was achieved using the procedure described in "Materials and Methods". It should be indicated here that cysteine synthase activity and the isotopic exchange protein were not separated with the fractionating methods used; thus both activities were present in the so far purified enzyme fraction and therefore both activities were compared during this investigation. With this so far purified protein extract the following experiments were performed.

Substrate specificity

Different derivatives of amino acids tested for the ability to form bound H_2O (retained on a Dowex-50 column) with the purified enzyme fraction. As can be seen from the data of Table I, there is a good incorporation of H_2S when O-acetylserine is used

Table I. Substrate specificity of the isotopic exchange reaction.

Substrate	H_2S incorporated [cpm$\times 10^{-4}$]	[nmol]
none	1.14	9.2
L-serine	1.08	8.7
O-phospho-L-serine	1.23	9.9
O-acetyl-L-serine	58.56	473.2
L-cysteine	45.87	370.7
DL-homocysteine	1.82	14.7
O-phospho-L-homoserine	1.32	10.7

Conditions (in μmol): Tris-HCl pH 8.0: 100; DTE: 10; H_2S: 2 (1 nmol = 1230 cpm); protein: 2.8 μg of purified enzyme; aminoacids: 5 when indicated in a total volume of 1 ml. Incubation for 1 hour under N_2 at 37° C.

as a substrate; serine and phosphoserine are nearly ineffective as judged from the control without added substrate. However, the addition of cysteine leads to an incorporation which is about 2/3 of the rate obtained with O-acetylserine (and the exchange rate may be higher, since the specific activity of H_2S is diluted by non-labelled H_2S derived from cysteine). Homocysteine and O-phosphohomoserine do show some effect, however, this rates are only slightly higher than the background and less than 5% of the rates obtained with cysteine or O-acetylserine.

Molecular weight determination

The separation of cysteine synthase and isotopic exchange activity has been analyzed on a Sephadex-G-100 column (2 × 55 cm) and from references with bovin albumin (MW = 67 000); egg albumin (MW = 45 000); chemotrypsinogen A (MW = 25 000); and cytochrom C (MW = 12 500) an apparent molecular weight of 64 000 daltons has been found using the method of Andrews [5], which is shown in Fig. 3.

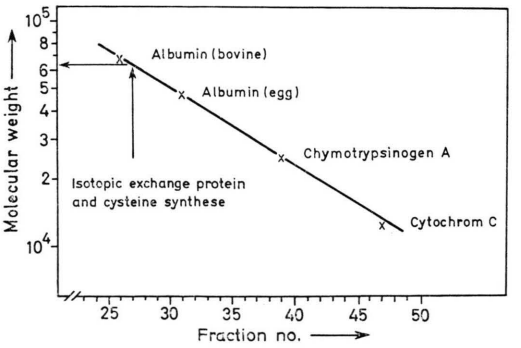

Fig. 3. Determination of the molecular weight of the protein catalyzing the isotopic exchange reaction on a Sephadex-G-100 column. Cysteine synthase and isotopic exchange reaction were determined according to Fig. 1, the Sephadex-column was equilibrated with 0.02 M Tris-HCl pH 8.0 containing 0.1 M KCl. Fractions of 3.1 ml were collected.

Heat inactivation

Since the protein catalyzing the isotopic exchange reaction and the cysteine synthase were not separated by the methods used it was tried to see if both activities are inactivated by heat treatment to the same extent (see Fig. 4). As can be seen, both activities are inactivated by treatment of ten min above 60 °C, thus demonstrating the protein-catalyzed nature of both reactions. The inactivation pattern does not distinguish between cysteine synthase and isotopic exchange reaction, which could be used as an indication that both activities are catalyzed by the same protein.

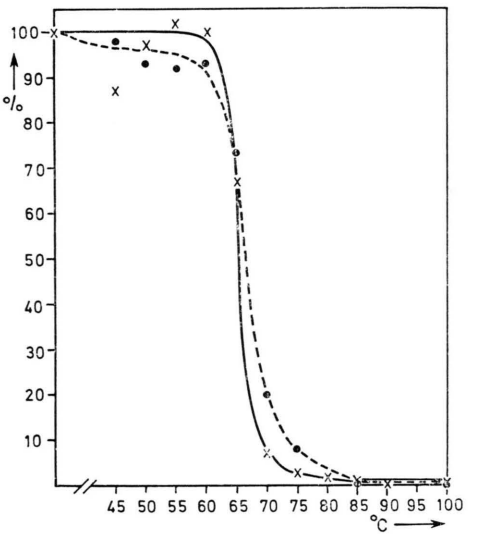

Fig. 4. Heat inactivation of cysteine synthase and isotopic exchange activity. Conditions as in Fig. 1, however 2.8 μg of protein was used for the assay after heating. 100% are: 154 nmol cysteine formed from O-acetylserine; and 0.645 μmol H_2S exchanged with cysteine. × — ×, cysteine synthase activity; ● — ●, exchange activity.

Protein- and time dependencies

Figs 5 to 8 demonstrate that these reactions are linear with the protein concentration over the range of 1 to 10 μg of the so far purified enzyme fraction using either O-acetylserine or cysteine as substrate. Therefore all experiments were run with 1 to 5 μg of protein. Time dependence of both reactions shows, that the isotopic exchange reaction proceeds nearly linear over a period of 90 min, therefore

routine measurements were done with an incubation time of 1 hour. Cysteine formation from O-acetylserine stopped between 30 and 45 min, therefore cysteine formation was routinely assayed after ten min.

pH-optimum

The pH-optimum was measured with the isotopic exchange reaction. As can be seen from Fig. 9, the exchange rate drastically increases with higher pH, beeing highest at the pH of 9.5; however, there are no further measurements above this pH. So the optimal pH for the exchange reaction might still be higher. For the cysteine synthase reaction it is difficult to determine a pH-optimum since above 7.5 O-acetylserine is converted to N-acetylserine therefore no measurements are possible at alkaline conditions and below a pH of 7 the activity of the cysteine synthase ceased, so that the cysteine synthase activity was determined at a pH of 7.5 whereas the exchange reaction was normally measured at a pH of 8.0 to have data close to the pH of the cysteine synthase.

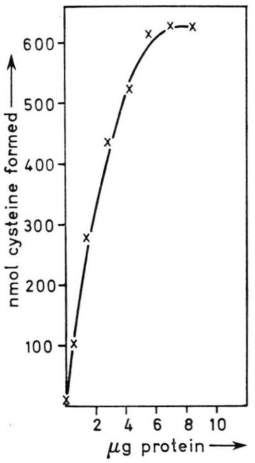

Fig. 5. Protein dependence of the isotopic exchange reaction. Conditions as in Fig. 1, however a higher specific activity of H_2S was used (1 µmol = 792,000 cpm).

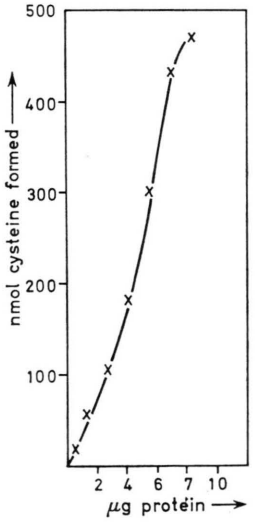

Fig. 6. Protein dependence of the cysteine synthase reaction. Conditions as in Fig. 1.

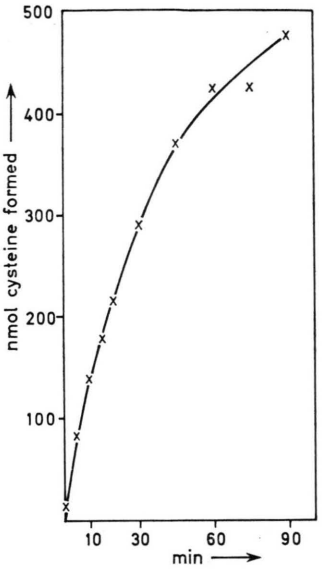

Fig. 7. Time dependence of the isotopic exchange reaction. Conditions as in Fig. 5, however 2.8 µg of protein was used.

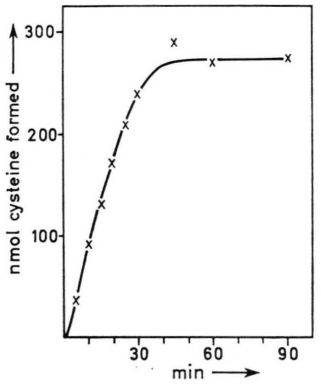

Fig. 8. Time dependence of the cysteine synthase reaction. Conditions as in Fig. 1; 2.8 µ of protein was used for the assay.

K_m-determination

This so far purified enzyme fraction was used to determine the apparent K_m-data for H_2S, cysteine,

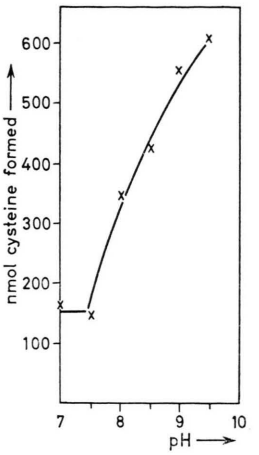

Fig. 9. pH-optimum for the isotopic exchange reaction. Conditions as in Fig. 7.

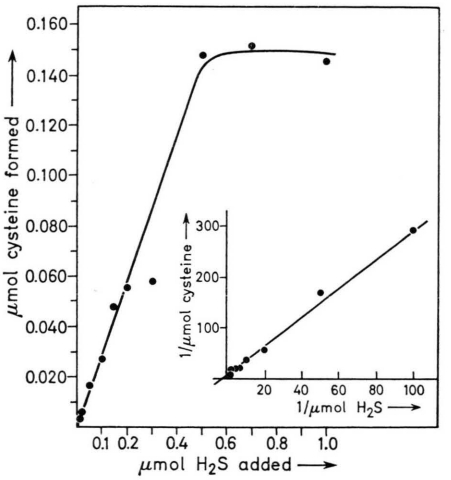

Fig. 10. K_m-determination for H_2S in the cysteine synthase reaction. Conditions as in Fig. 1; 2.8 μg of protein was used; H_2S was varied as indicated.

and O-acetylserine. These data are shown in Figs 10 to 13. The apparent K_m for H_2S using O-acetylserine as substrate was found to be 0.6 mM; higher concentrations above 2.5 mM seem to inhibit color formation of cysteine with the ninhydrin reagent, so the experiments were kept in the range of 0.1 to 1 mM H_2S for K_m-determinations. The apparent K_m of H_2S for the isotopic exchange reaction with cysteine as substrate was determined to be 0.87 mM at a pH of 8.0, which is in the same range as the K_m for H_2S with O-acetylserine as substrate. It should be pointed out here that the data for the isotopic exchange reaction are not corrected for

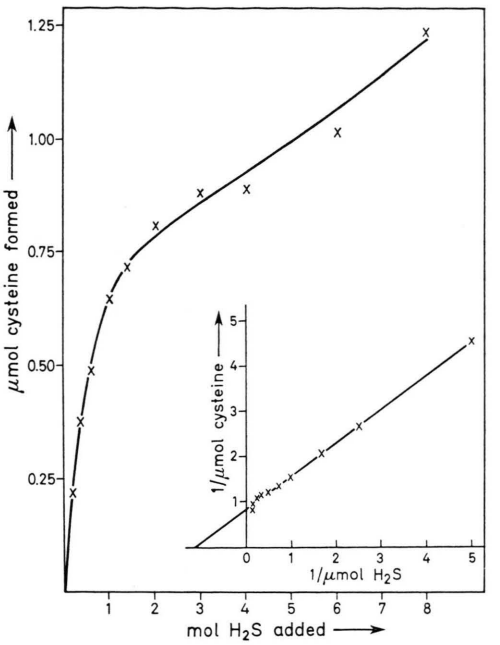

Fig. 11. K_m-determination for H_2S in the isotopic exchange reaction. Conditions as in Fig. 5, however 5.6 μg of protein was used and H_2S was varied as indicated.

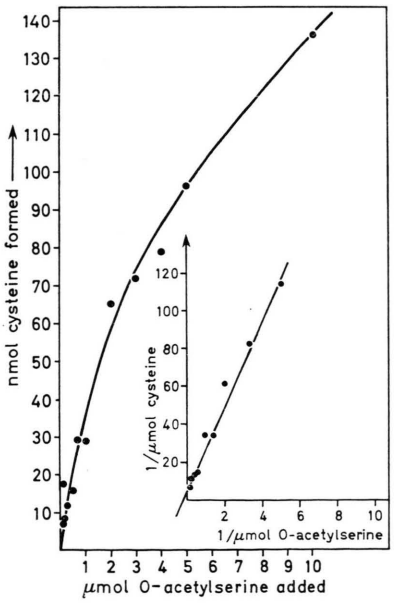

Fig. 12. K_m-determination for O-acetylserine. Conditions as in Fig. 1, O-acetylserine varied as indicated; 5.8 μg of protein was used.

the dilution of the specific activity of H_2S, therefore the actual K_m of H_2S for the isotopic exchange reaction might be lower.

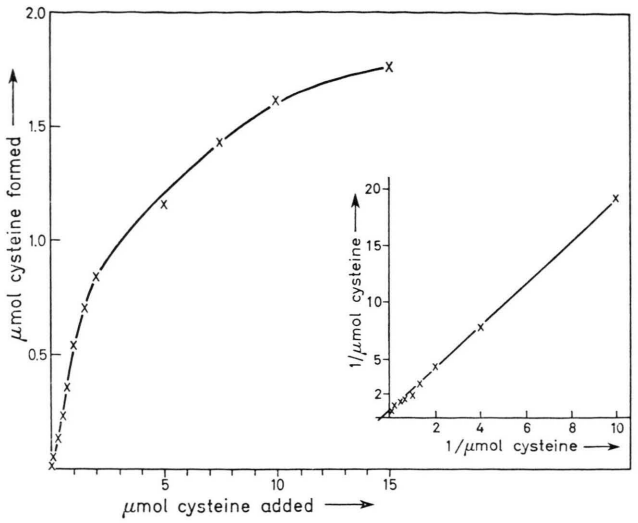

Fig. 13. K_m-determination for cysteine in the isotopic exchange reaction. Conditions as in Fig. 11, however, cysteine was varied as indicated.

The apparent K_m for O-acetylserine was found to be 3.3 mM and for cysteine the apparent K_m for the isotopic exchange reaction was also determined to be 3.3 mM. Thus both K_m-data for these reactions are comparable.

Discussion

Incorporation of H_2S into the amino acid cysteine has been studied by Schlossmann and Lynen [6, 7] who found a protein which catalyzed the reaction of serin with H_2S to form cysteine; according to Greenberg this enzymatic activity will be named L-serine-hydrolase [8]. This protein was purified about 50-fold from yeast [7]; for activity the addition of pyridoxalphosphate was required. This L-serine-hydrolase could be demonstrated in bacteria, animals, and spinach [9]. Later it was demonstrated that a second pathway exists which forms cysteine from O-acetylserine and H_2S and according to Greenberg [8] this activity will be named here cysteine synthase. Cysteine synthase is found in bacteria and plants [8]. In higher plants it has been analyzed in spinach [10–12]; in rape seeds [13]; in rape leaves [14]; and rape roots [15]; in *Phaseolus vulgaris* [16, 17]; in onions [18, 19]; as well as in 12 other plant species [20]. Evidence has been published that purified cysteine synthase contains bound pyridoxal phosphate with sources from *Salmonella* [21] and from rape [14].

In this paper an enzymatic activity is described which catalyzes the formation of labelled cysteine from cysteine and labelled H_2S. Such an exchange reaction has been demonstrated with purified cysteine synthase from *Salmonella* [21] and crude extracts from *Lemna minor* [22] and *Brassica rapa* [12]. It might be speculated here that this exchange activity could be catalyzed by the L-serine-hydrolase, since the reaction with serine and H_2S was reported to be reversible [23]. However, the exchange reaction described in this paper is independent of added pyridoxal phosphate and it will not accept serine as substrate which clearly distinguishes it from L-serine-hydrolase. Whether this exchange reaction is part of the cysteine synthase, or whether it is catalyzed by a distinct protein can not be decided at the moment, however, K_m-data for H_2S and the other substrates cysteine and O-acetylserine are about equal and treatment with ammoniumsulfate, DEAE-cellulose, Sephadex-G-100 or heat treatment will not distinguish between the exchange reaction and cysteine synthase, which seems to open the possibility that both reactions could be catalyzed by the same protein.

Cook and Wedding [21] reported for the cysteine synthase from *Salmonella* that the reaction is catalyzed by a Bi Bi Ping Pong mechanism and they suggest the formation of an aminoacrylic acid in shiff base linkage with pyridoxal phosphate. It is suggested here that the spinach cysteine synthase reacts with either cysteine or O-acetylserine to form an aminoacryl-intermediate with bound pyridoxal phosphate, and H_2S acts as an attack on the double bound thus forming cysteine, which is released from the protein. The peculiarity of the system described seems to be that cysteine – the product – and O-acetylserine can enter the active site (assuming the exchange reaction to be catalyzed by the cysteine synthase) which might be of significance in relation to O-acetylserine. It suggests that O-acetylserine and cysteine are antagonists; and this could be of significance, if the concentration of cysteine is high compared to O-acetylserine. This would explain why cysteine formation *in vitro* will stop after longer incubation time, which was noticed in the experiments described in this paper using O-acetylserine as substrate. Cook and Wedding [21] noticed an inhibition of cysteine formation from O-acetylserine by cysteine; which could be explained by this reaction. If such an inhibition could be of importance *in vivo*

is not known. Furthermore, striking differences in the K_m-data for the cysteine synthase from rape were reported. Ngo and Shargool[13] found a K_m of 1.74×10^{-6} for O-acetylserine, working with the H_2S-ion specific electrode, whereas Masada *et al.*[14] found a K_m for O-acetylserine of 6.1 mM, which is close to the K_m-data found from spinach (3.3 mM, this paper) or *Salmonella* (7 mM[24]). These differences might be due to the measurements applied, since colorimetric determination of cysteine needs reasonable amounts of cysteine formed; and these amounts might already be in competition with the substrate O-acetylserine. In this respect it is of interest that Castric and Conn[25] reported the formation of β-cyanoalanin by the cysteine synthase from *Bacillus megaterium*, and this formation of β-cyanoalanine was catalyzed either by O-acetylserine or by cysteine, however no activity was found with serine.

It was noticed that the pH-optimum of the exchange reaction showed a strong increase toward alkaline conditions; best exchange rates were obtained at a pH of 9.5. This pH-dependence can not be found with O-acetylserine as a substrate, since above 8.0 O-acetylserine is converted to N-acetylserine; thus the broad optimum of the cysteine synthase about pH of 7.5 could be explained by this fact towards alkaline conditions. Use of this exchange reaction could be made by preparing labelled cysteine from labelled H_2S and non-labelled cysteine.

It should be pointed out here that isotopic exchange reactions of this type (between H_2S and cysteine) may give rise to erroneus conclusions. For instance the appearence of free H_2S within a leaf can not be demonstrated by trapping radioactivity from labelled precursers with non-labelled H_2S[26, 27] since due to the exchange reaction one will find H_2S originally bound in cysteine. On the other hand feeding experiments with labelled H_2S are not conclusive to demonstrate that cysteine formation from appropriate precursers is operative[28] since the actual incorporation of the label could be due to isotopic exchange reactions as an alternative possibility to the cysteine synthase reaction.

These possibilities should be concerned when discussing problems of sulfur metabolism.

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