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THE DECOMPOSITION OF β-MERCAPTOPYRUVIC ACID BY ESCHERICHIA COLI AND ITS EXTRACTS

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Fromageot *et al.* (*1*, *7*) proposed a scheme for the decomposition of cysteine involving the enzymatic desulfhydration followed by spontaneous deamination. Recently Suda (*2*, *3*) Hanson and Mantel (*4*) and Tamiya (*5*) reported that in experiments with certain bacteria, the liberation of hydrogen sulphide from cysteine was preceded by the disappearance of cysteine and by liberation of ammonia; the reaction in these cases, therefore, essentially different from Fromageot's scheme of cysteine desulfhydrase.

One of the authors proposed the following reaction scheme in the previous paper (*6*);

$$\begin{array}{l} CH_2\text{-}SH \\ | \\ CH\text{-}NH_2 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CH_2\text{-}SH \\ | \\ CO \\ | \\ COOH \end{array} + H_2S + \text{other products}$$

A derivative of thiol keto acid was isolated from a reaction mixture in which liberation of hydrogen sulphide was inhibited by the addition of arsenite (*5*).

Meister reported that the enzymic decomposition of β-mercaptopyruvic acid was catalyzed by an enzyme from mammaliam liver (*8*). Garreau (*13*) and Hanson *et al.* (*4*) reported that this substance is decomposed also by suspensions of *Escherichia coli*. The present paper deals with the decomposition of β-mercaptopyruvic acid by cell suspensions and partially purified enzyme preparations from *Escherichia coli*.

EXPERIMENTAL

Cell Preparations—Escherichia coli strain Taki-2 was used throughout the experiments.

After cultivation at 37° on a normal agar slant for 18 hours or in a liquid medium under shaking at 30° for 12–15 hours (4 per cent peptone, 4 per cent meat extract pH 7.0), the cells of *E. coli* were collected and washed with water three times by centrigugation. In experiments carried out with intact cells, suspensions of washed cells in *M*/15 phosphate buffer at pH 7.0 were used. In some of the other experiments, freeze-dried cells were used; dried cells kept in vacuo in a refrigerator did not change their activity for several months.

Enzyme Preparations—Two g. of freeze-dried cells and 20 g. of alumina powder were kneaded with *M*/15 potassium dihydrogen phosphate, adjusted to pH 8.0 with *N* sodium hydroxide and ground in a machinery mill for 4 hours at room temperature. Then the mixture was adjusted to pH about 7.0 by the addition of 20 ml. of *M*/15 potassium dihydrogen phosphate and centrifuged at 4000 r.p.m. for 10 minutes. The supernatant was discarded; most of the enzyme protein was adsorbed on the alumina. The enzyme was extracted from the alumina precipitate by adding 50 ml. of *M*/15 sodium monohydrogen phosphate. After centrifugation at 16,000 r.p.m. for 10 minutes, a clear light yellow supernatant was obtained, which was dialyzed against flowing water overnight. The dialysate gave a white powder on freeze-drying. The enzyme prepation could be kept unchanged for several months and used as partially purified enzyme.

Conditions for the Reaction—Unless otherwise stated, the conditions for the reaction were as follows: All the reaction components were dissolved in *M*/15 phosphate buffer, pH 7.0. The reaction was carried out at 37° under continuous bubbling of air or nitrogen gas. This procedure insured not only aerobic or anaerobic conditions of the reaction medium, but also continuous removal of hydrogen sulphide produced by the reaction.

Determination of Hydrogen Sulphide—Hydrogen sulphide formation was determined by Szent Lorant's method (*9*). The hydrogen sulphide was passed with a stream of air into a zinc acetate solution. Upon the addition of an acid solution of iron alum and dimethyl-*p*-phenylene diamine, the sulphur of the zinc sulphide was incorporated into methylene blue molecule which was synthesized by this procedure. The colour developed was measured electrophotometrically at 610 mμ.

Determination of Free Sulphur—Free sulphur was estimated essentially by the method described by Smythe (*14*). Free sulphur was reduced to hydrogen sulphide by the addition of thioglycolic acid, and subjected to colorimetric analysis as described above.

Determination of Pyruvic Acid—One ml. of 10 per cent mercuirc sulphate solution in 3.6 *N* sulphuric acid was added to one ml. of reaction mixture. Thiol compounds and protein were precipitated by this procedure. After centrifugation, pyruvic acid content in the supernatant was determined by the method of Friedemann and Haugen (*10*).

Determination of Other Compounds—Carbon dioxide was determined by Warburg's manometric method. Volatile organic acids were measured titrimetrically after distillation of the reaction mixture in microacetyl determinating apparatus.

Chemicals—Ammonium β-mercaptopyruvic acid was prepared as described by

Parrod (*11, 12*). The ammonium salt was used throughout these experiments. The results obtained with ammonium salt were similar to those obtained with sodium salt, which was prepared by passing a saturated aqueous solution of ammonium salt through a Amberlite IRC-120(Na-form)column.

RESULTS

Fundamental Conditions for the Decomposition of β-Mercaptopyruvic Acid by Cell Suspensions of Escherichia coli—Some preliminary experiments were carried out to find out the optimal conditions for the reaction. In these experiments only hydrogen sulphide production was followed. In none of the experiments, hydrogen sulphide was produced in the absence of the substrate.

β-Mercaptopyruvic acid is fairly unstable in aqueous solution and decomposes slowly producing a small amount of hydrogen sulphide. However, with added *E. coli* suspension, the decomposition of β-mercaptopyruvic acid was greatly stimulated. The rate of hydrogen sulphide production from β-mercaptopyruvic acid was much more rapid than that from cysteine under similar conditions. (Fig. 1)

With cell suspensions, the production of hydrogen sulphide from β-mercaptopyruvic acid was accompanied by the production of carbon

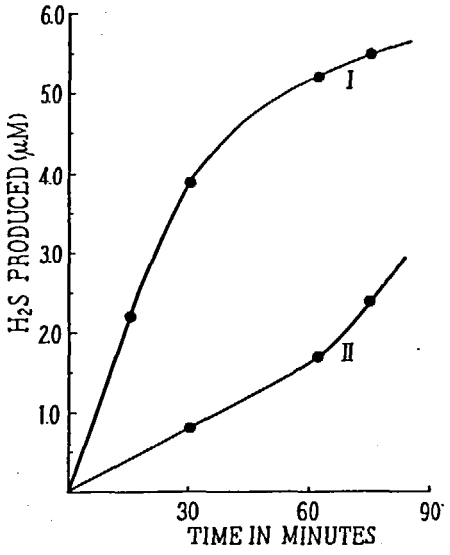


FIG. 1. Hydrogen sulphide production from β-mercaptopyruvic acid and cysteine.

Each reaction tube contained 20 μ moles of substrate (I: β-mercaptopyruvic acid, II: cysteine), 40 mg. of lyophylized cells of Escherichia coli, and phosphate buffer, pH 7.0 (final *M*/15) in total five ml. Reaction temperature 37°. Gas phase: air.

dioxide. The hydrogen sulphide production did not proceed more than 50–60 percent of the amount calculated from substrate concentration. With cell suspensions there was no accumulation of free sulphur, as was observed in experiments with partially purified enzyme.

Effect of Addition of Various Substances on the Enzymic Decomposition of β-Mercaptopyruvic Acid—In the previous paper (*6*), one of the authors reported that the decomposition of cysteine by *Escherichia coli* suspensions was markedly stimulated by the addition of various respiratory substrates such as glucose or organic acids. We therefore studied the stimulating effect of these substances on the decomposition of β-mercaptopyruvic acid. The addition of glucose had no effect on the reaction rate. Potassium cyanide, which showed inhibiting effect on the formation of both ammonia and hydrogen sulphide from cysteine in the presence of *Escherichia coli* suspension, exhibited 50 percent inhibition at the concentration of 5.4×10^{-3} *M*. Arsenite, which showed an inhibitory effect on the formation of hydrogen sulphide from cysteine without affecting ammonia production from it, showed a strong inhibitory effect on the formation of hydrogen sulphide from β-mercaptopyruvic acid.

Effect of Thioglycolic Acid on the Reaction—The addition of thioglycolic acid increased the yield of hydrogen sulphide from β-mercaptopyruvic acid by *Escherichia coli* suspensions (see Fig. 2), but it inhibited the carbon dioxide evolution at the same time. (Fig. 3)

As described below, one of the primary decomposition products from β-mercaptopyruvic acid is pyruvic acid. Thioglycolic acid, therefore, seems to inhibit further decomposition of pyruvic acid and thus inhibits the carbon dioxide evolution (Fig. 4).

The Decomposition of β-Mercaptopyruvic Acid by Partially Purified Enzyme Preparation—Enzyme preparations made as described in experimental part showed a high activity in β-mercaptopyruvic acid decomposition. In this case also, the hydrogen sulphide or pyruvic acid formation in the presence of thioglycolic acid did not proceed more than 45–50 per cent of added substrate. These preparations had no cysteine desulfhydrase activity. The activity of the crude preparation, *i.e.*, the supernatant of alumina ground material, remained unchanged after heating in a boiling water bath for 10 minutes.

The enzyme was decomposed more rapidly by heating after further purification by means of adsorbtion and elution on alumina and fractionation with acetone or ammonium sulphate. The enzyme activity was also destroyed by acid or pepsin hydrolysis (Fig. 5).

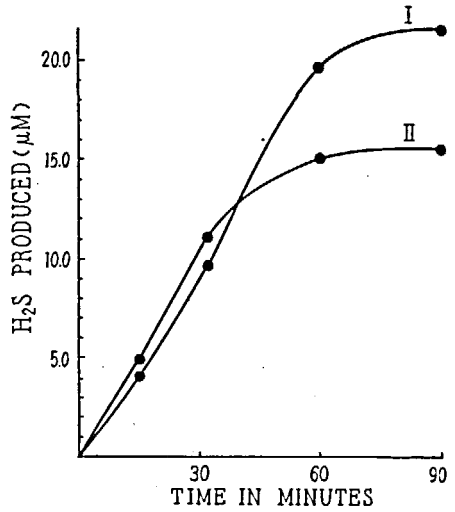


FIG. 2. Effect of the addition of thioglycolic acid Each reaction tube contained 40 μ moles of mercaptopyruvic acid, 20 mg. of lyophylized cells, and phosphate buffer, pH 7.0 (final *M*/15). To tube I, 40 μ moles of thioglycolic acid was added.

Total volume: 5 ml. Temperature: 37°. Gas phase: air.

Further Purification of the Enzyme—The enzyme prepared as described in the experimental part was subjected to further purification by ammonium sulphate fractionation. Dried enzyme preparation was dissolved in 10 volumes of water and fractionated by the addition of solid ammonium sulphate. The activity was concentrated in a fraction of 0.4–0.8 ammonium sulphate saturation.

Effect of Oxygen upon the Reaction—The enzymatic decomposition of β-mercaptopyruvic acid proceeded both in the presence and absence of oxygen. However, in experiments with cell suspension, the reaction velocity was greater when air was aspirated than when nitrogen was used. In an experiment with partially purified enzyme, the reaction rate was almost equal under both conditions (Figs. 6 and 7).

Identification of Reaction Products—Pyruvic acid: 500 mg. of the partially purified enzyme and 2 millimoles of β-mercaptopyruvic acid were dissolved in 150 ml. of *M*/15 phosphate buffer (pH 7.0). After two hours' incubation, under continuous aspiration of nitrogen gas at 37°, an equal volume of 10 per cent mercuric sulphate in 3.6 *N* sulphuric

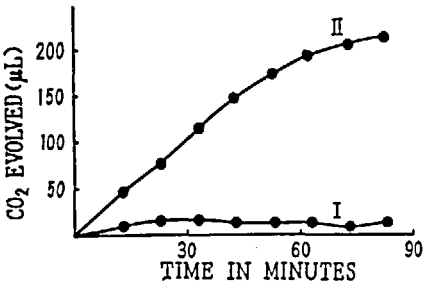


FIG. 3. The effect of thioglycolic acid addition on the evolution of carbon dioxide.

Each Warburg vessel contained: in the main compartment, 20 μ moles of mercaptopyruvic acid, and phosphate buffer pH 7.0 (final *M*/15) in a total amount of 2.5 ml., to tube I 20 μ moles of thioglycolic acid was added; in the side arm, 0.5 ml. of lyophylized cell suspension (40 mg/ml), in the center well, 0.2 ml. of lead acetate (2 *M*). The reaction was initiated by the addition of the content of side arm.

Temperature: 37°. Gas phase: air.

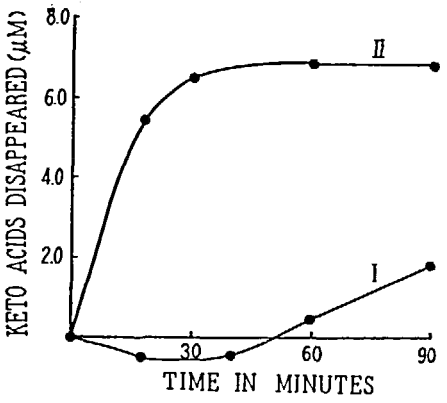


FIG. 4. The effect of addition of thioglycolic acid on the disappearance of keto acids.

Each reaction tube contained 40 μ moles of mercaptopyruvic acid, 20 mg. of lyophylized cells and phosphate buffer pH 7.0 (final *M*/15) in a total amount of 5 ml. to tube I, 40 μ moles of thioglycolic acid was added.

Temperature: 37°. Gas phase: air.

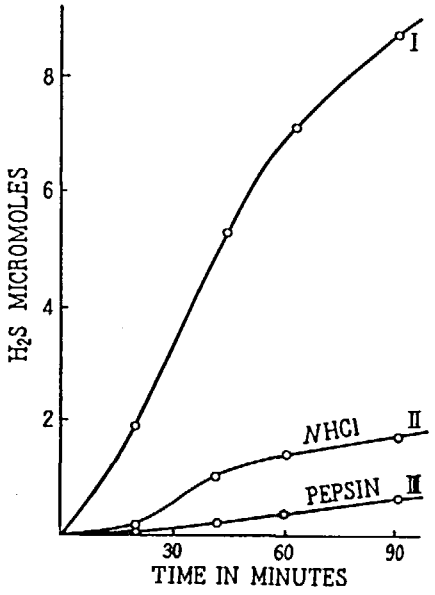


FIG. 5. Inactivation of Enzyme by hydrolysis and digestion.

Each reaction tube contained 40 μ moles of mercaptopyruvic acid, 40 μ moles of thioglycolic acid, 1 ml. of enzyme solution (43 mg. acetone powder of purified enzyme by acetone fractionation) or hydrolysed material, and phosphate buffer pH 7.0 (final *M*/15) in a total amount of 5 ml.

Temperature 37°. Gas phase: air.

The hydrolysed or digested material was prepared as follows: 43 mg. of enzyme preparation was dissolved in 2 ml. of distilled water.

N·HCl: 1 ml. of 2 *N* HCl was added to 1 ml. of this enzyme solution. It was boiled on boiling water for 10 minutes. After cooling it was used as hydrolysed material.

Pepsin: 1 ml. of 0.12 *N* HCl was added to 1 ml. of the enzyme solution and 5 mg of crystal pepsin (2 times recrystalized from alcohol) was added.

After incubation for 1 hour, this reaction mixture was used as pepsin digest.

I: Control (enzyme without treatment).

II: *N* HCl (*N* HCl hydrolysate in place of enzyme).

III: Pepsin (pepsin digest in place of enzyme).

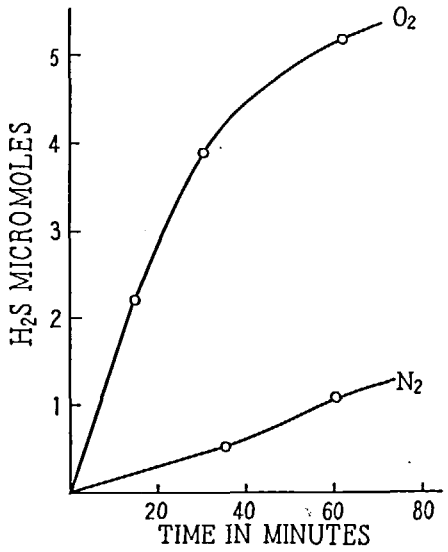


FIG. 6. Effect of presence of oxygen on hydrogen sulphide production by lyophilized cells.

Each reaction tube contained 20 μ moles of mercaptopyruvic acid, 1 ml. of cell suspension (1.03 mg./ml.), and phosphate buffer pH 7.0 (*M*/15) in a total amount of 5 ml.

Temperature: 37°. Gas phase: air or nitrogen gas.

acid was added to remove protein and unreacted substrate (Meister *et al.* (*8*)). The yellow precipitate was filtered off and concentrated hydrochloric acid was added to the filtrate to bring the final concentration up to 2 *N*. 80 ml. of 0.5 per cent 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid was then added. After standing for 20 minutes at room temperature, yellow precipitate of hydrazone was obtained. The hydrazone was extracted into ethyl acetate together with excess hydrazine, and dried *in vacuo*. The orange yellow material obtained here was dissolved in a small amount of ethyl acetate and added to a celite column, buffered at pH 6.8. Ether was passed down the column and the material separated into three bands. A band which showed the R value of 0.3–0.4 was extracted in sodium bicarbonate solution, re-extracted into ether and treated with calcium chloride and dried in vacuo. 2,4-dinitrophenylhydrazone obtained here was identified as that of pyruvic acid

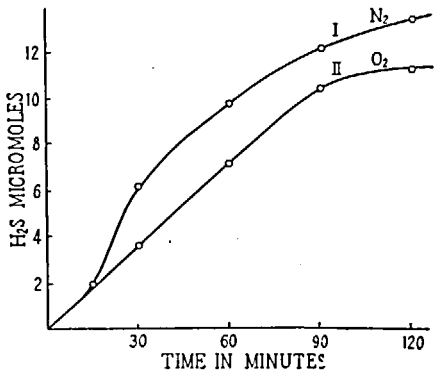


FIG. 7. Effect of presence of oxygen on hydrogen sulphide production by enzyme preparation.

Each reaction tube contained 40 μ moles of mercaptopyruvic acid, 40 μ moles of thioglycolic acid, 1 ml. of dialysed enzyme preparation, and phosphate buffer pH 7.0 (*M*/15) in a total amount of 5 ml. Temperature:37°. Gas phase: I: nitrogen gas. II: air.

by measurement of mixed melting point.

hydrazone isolated m.p.=217°
pyruvic acid 2,4-dinitrophenylhydrazone m.p.=221°
mix test m.p.=219°

Sulphur: Meister *et al.* reported that a decomposition product of β-mercaptopyruvic acid in the presence of a mammalian liver enzyme preparation was sulphur. In experiments with partially purified enzyme from *Escherichia coli*, we were able to show similar fact, *i.e.*, the formation of free sulphur. In the experiments with cell suspensions, however, this substance was not detected. This is expected because in living cells sulphur is readily reduced to hydrogen sulphide.

The decomposition of β-mercaptopyruvic acid by partially purified enzyme was accompanied by increase in turbidity of the reaction medium, although considerable evolution of hydrogen sulphide took place at the same time. This precipitate was treated by essentially the same method as described by Meister *et al.* (*8*). The amount of sulphur in the precipitate was estimated by the addition of thioglycolic acid, followed by St. Lorant's colorimetric method.

These results showed that the decomposition products from β-mercaptopyruvic acid were pyruvic acid, free sulphur and hydrogen

sulphide. The molar ratio of formation of free sulphur and hydrogen sulphide was variable but the total amount of these two substances showed a stoichiometric relation to pyruvic acid production (Table I).

TABLE I

Experiment No.	Formation of pyruvic acid	Evolution of hydrogen sulphide	Formation of free sulphur	H_2S+S
	μ moles	*μ moles*	*μ moles*	*μ moles*
1	8.32	1.78	6.50	8.28
2	7.27	3.69	3.35	7.04
3	2.73	1.64	0.96	2.60

Formation of pyruvic acid, hydrogen sulphide and free sulphur. Each reaction tube contained: 20 μ moles of β-mercaptopyruvic acid, 1 ml. of enzyme preparation and phosphate buffer (pH 7.0) in total 5 ml. Temperature: 37° Gas phase: air. The figure in the table are expressed in μ moles of substances formed in 80 minutes.

The stimulating effect of hydrogen sulphide production by the addition of thioglycolic acid can be explained as being due to the reduction of free sulphur by this substance. In the presence of this substance, equimolecular amount of hydrogen sulphide and pyruvic acid were formed.

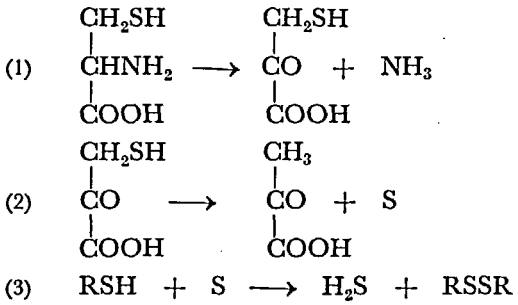
Other Porducts: The decomposition of β-mercaptopyruvic acid into pyruvic acid and free sulphur did not proceed more than 45–50 per cent under our experimental conditions. This was not due to the inactivation of enzyme because the second addition of freshly prepared enzyme solution did not effect further production of hydrogen sulphide or pyruvic acid. It is clear, therefore, that β-mercaptopyruvic acid was converted into some inactive substance or substances (*8*). Among the products which were tested for only glyoxylic acid was detected, which corresponded to two percent of added β-mercaptopyruvic acid.

Formaldehyde, Acetaldehyde, other keto-compounds, formic acid, and acetic acid were not detected. Fresh β-mercaptopyruvic acid was kept at room temperature for 7–8 hours or aerated for 30 minutes, decomposed and the keto-group of the acid can no longer be detected. With such a solution, no hydrogen sulphide production was observed upon the addition of enzyme preparation together with thioglycolic acid. The low yield of hydrogen sulphide from the enzymic reaction

may thus be due to this non-enzymic decomposition.

DISCUSSION

In the previous paper (*6*) one of the authors proposed a scheme of cysteine decomposition by *E. coli* suspensions which involved the intermediary formation of β-mercaptopyruvic acid. The results presented in this paper shows that this acid is further decomposed into pyruvic acid and free sulphur, a part of which is reduced to hydrogen sulphide. In the presence of added thiol compound or with intact cell suspensions, all of the sulphur is reduced to hydrogen sulphide, while in the absence of it only a part of sulphur is reduced to hydrogen sulphide by thiol group of substrate itself. Now we can visualize the whole scheme of cysteine decomposition by *Escherichia coli* under aerobic conditions as follows:



The first step, which was studied in the previous paper, requires the presence of oxygen or nitrate and is inhibited by potassium cyanide. The second step is specifically inhibited by arsenite. With intact cell suspensions in the presence of thiol compounds, the free sulphur produced by the second reaction is reduced to hydrogen sulphide.

SUMMARY

1. β-Mercaptopyruvic acid is decomposed by an enzyme prepared from *E. coli.*

2. Products of this reaction are pyruvic acid and elemental sulfur. But in the case of reaction with intact cell β-mercaptopyruvic acid is further decomposed to carbon dioxide and hydrogen sulfide.

3. This enzyme is stable against heat in crude state. However, in

pure state this thermal stability is lost.

4. The probability of this compound to be in the metabolic path of cysteine is discussed.

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