XXVIII. THE ENZYMIC FORMATION OF HYDROGEN SULPHIDE BY CERTAIN HETEROTROPHIC BACTERIA. II.

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IN a previous communication [Tarr, 1933] the formation of hydrogen sulphide from organic sulphur compounds by certain heterotrophic bacteria was attributed to the activity of a specific enzyme present in their cells. Evidence was also obtained in support of the fact that different mechanisms are responsible for the formation of hydrogen sulphide from organic sulphur compounds and from thiosulphate by such bacteria. This evidence has been substantiated by the results obtained in an experiment described in the present paper. It is possible that the formation of hydrogen sulphide from inorganic sulphur compounds is also due to different mechanisms. Thus the sulphate bacteria produce hydrogen sulphide from sulphate, sulphite and thiosulphate [Stephenson and Stickland, 1931], and from hyposulphite and sulphur as well [Baars, 1930], while *P. vulgaris* cells only reduce thiosulphate and sulphur to hydrogen sulphide [Tarr, 1933]. The experimental work described in this paper has been almost entirely confined to the formation of hydrogen sulphide from organic compounds.

EXPERIMENTAL.

The technique employed was essentially the same as that previously described [Tarr, 1933]. Unless otherwise stated all experiments were made anaerobically in evacuated 75 ml. Thunberg tubes, each tube containing 5 ml. of bacterial suspension, 2 ml. of *M*/5 phosphate buffer, p_H 7·8, the required amount of substrate solution and a final fluid volume of 20 ml. Strictly aseptic precautions were only observed in experiments the duration of which was over 2 hours.

The homocystine used was kindly given to me by Prof. V. du Vigneaud, the dithiodihydracrylic acid (disulphide from β -thiolpropionic acid) by Dr A. Schöberl, and the *dl*-cysteine hydrochloride by Dr N. W. Pirie. Solutions of these compounds were prepared in accordance with the general procedure previously described [Tarr, 1933].

The formation of hydrogen sulphide from cysteine and from thiosulphate by P. vulgaris cells.

It was previously shown [Tarr, 1933] that hydrogen sulphide is formed from cysteine by cells of both *P. vulgaris* and *S. marcescens*, but from thiosulphate only by the former organism, thus indicating that two different systems capable

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of forming hydrogen sulphide from these substrates exist. The following experiment shows that *P. vulgaris* cells possess both systems.

Ten Thunberg tubes containing buffer and *P. vulgaris* cells as usual were prepared. One pair of tubes received 5 mg. of Na₂S₂O₃ per tube, a second pair 6 mg. of Na₂S₂O₃, a third pair 1 mg. of cysteine, and a fourth pair 5 mg. of Na₂S₂O₃ plus 1 mg. of cysteine. A fifth pair was retained as control. The tubes were evacuated as usual and placed in a water-bath at 37° for 3½ hours, and the amount of hydrogen sulphide formed was then determined. The results of this experiment, which are given in Table I, show that the addition of cysteine to

Table I. *The effect of the addition of cysteine on the production of H₂S by P. vulgaris cells saturated with respect to Na₂S₂O₃.*

Experiment	H ₂ S in γ after 3½ hours at 37°	
	Duplicates	Average
5 mg. Na ₂ S ₂ O ₃	67	68
	69	
6 mg. Na ₂ S ₂ O ₃	70	72
	74	
1 mg. cysteine	67	66
	65	
1 mg. cysteine + 5 mg. Na ₂ S ₂ O ₃	126	126
	126	

23 mg. dry weight of bacteria per exp. Controls formed no H₂S.

P. vulgaris cells which are saturated with respect to thiosulphate results in an increase in hydrogen sulphide formation almost equivalent (91 %) to the sum of the hydrogen sulphide formed from cysteine and thiosulphate alone. This may be considered practically conclusive proof that H₂S formation from thiosulphate and from cysteine by *P. vulgaris* is due to two entirely different mechanisms.

The action of P. vulgaris and S. marcescens cells on l-cysteine, dl-cysteine, homocystine and the disulphide from β-thiolpropionic acid.

The technique employed was identical with that previously used in studying H₂S formation from different compounds [Tarr, 1933], and the results of the experiment are given in Table II. The action on *l*-cysteine was studied in order that the results obtained might be strictly comparable with those recorded in the previous paper. Although the disulphide from β-thiolpropionic acid is reduced to the —SH form by both organisms, as is evidenced by a strong nitroprusside reaction, neither of them forms H₂S from it. On the other hand homocystine is reduced by both bacteria, with simultaneous formation of H₂S in small amounts. *S. marcescens* forms H₂S from *l*-cysteine and from *dl*-cysteine equally well, but *P. vulgaris* exhibits greater activity in this respect toward the former compound. The facts that most of the sulphur of cysteine can be recovered as H₂S, while none of the sulphur of the corresponding acid which lacks an amino-group yields H₂S, make it appear highly probable that the presence of an amino-group, and consequently deamination, is necessary to the successful course of the reaction. Thus homocystine which contains an amino-group yields H₂S under the experimental conditions in spite of the fact that the —SH group is on the γ-carbon atom.

Table II. *The anaerobic formation of H₂S from l-cysteine, dl-cysteine, homocystine and the disulphide from β-thiolpropionic acid by washed cells of P. vulgaris and S. marcescens.*

Compound	Weight used mg.	Theoretical amount of H ₂ S in mg.	H ₂ S recovered			
			<i>P. vulgaris</i>		<i>S. marcescens</i>	
			mg.	%*	mg.	%*
l-Cysteine	0.5	0.141	0.120	79	0.120	79
			0.115		0.112	
dl-Cysteine	0.5	0.141	0.073	49	0.115	80
			0.075		0.120	
Homocystine	0.5	0.126	0.033	23	0.018	12
			0.038		0.020	
Disulphide from β-thiolpropionic acid	0.5	0.162	0.006	0	0.004	0
			0.006		0.004	
Controls	0.0	0.0	0.006	0	0.004	0
			0.006		0.004	

* Calculated on the theoretical value after subtracting the control.

P. vulgaris 28 mg. dry weight of bacteria per exp.

S. marcescens 54 " " "

The extraction from P. vulgaris cells of a cell-free preparation of the enzyme forming H₂S from cysteine.

The procedure to be described was conducted under as nearly aseptic conditions as possible in order to avoid the presence of contaminating bacteria in the final preparations. The suspensions of *P. vulgaris* cells were prepared as usual, the cells being suspended in distilled water instead of Ringer solution, so that a suspension containing about 50 mg. dry weight of bacteria in 10 ml. was obtained. Suspensions prepared in this manner were run slowly, and with rapid stirring, into 10 volumes of acetone. The resulting flocculent precipitate was permitted to settle and the supernatant liquid carefully syphoned off, and a second 10 volumes of acetone were added. The precipitate was then collected on a small Büchner funnel, pressed between sterile filter-papers and dried for 2-3 hours at 28°. The resulting dry brittle mass was used within 2 days of preparation.

The acetone-dried bacteria were ground to a fine powder in a small mortar and mixed with *M*/20 phosphate buffer (*p*_H 7.8), 10 ml. being used for each 100 mg. of bacteria. The resulting suspension (A) was incubated for 2 hours at 37° and then centrifuged for 1 hour at high speed. The faintly opalescent, pale yellow supernatant liquid (B) was decanted, and the precipitate (C) was resuspended in distilled water and made up to a volume equivalent to that of the original suspension. Some of liquid B was filtered through a layer of kieselguhr, and some through a Seitz asbestos filter, and the resulting almost water-clear solutions labelled (D) and (E) respectively. A portion of the kieselguhr filtrate was heated to 80° for 2 minutes (F). The ability of these various fractions to form H₂S from 10 mg. of cysteine was determined, corresponding controls without cysteine being run in each case. The results, which are given in Table III, show that suspensions of acetone-treated bacteria form small amounts of H₂S, though calculation has shown that such preparations possess only 2-3 % of the original activity of the freshly prepared cells. On centrifuging suspensions of acetone-bacteria about one-half of the active principle remains in the supernatant liquid, while the remaining half is to be

found in the precipitate. Filtering the supernatant liquid through kieselguhr yields a clear, cell-free fluid, which retains the activity of the original liquid in full. On the other hand all the activity is lost by filtering the solution through a Seitz filter. Heating to 80° for 2 minutes renders kieselguhr filtrates inactive.

Table III. *The formation of H_2S by extracts of acetone-dried P. vulgaris cells.*

Preparation (10 ml. per exp.)	H_2S in γ produced from 10 mg. of cysteine in 4 hours at 37°
A	45
B	20
C	23
D	19
E	0
F	0

Controls gave no H_2S . Bacterial examinations showed that all solutions prepared under such conditions are sterile or nearly so.

All attempts to obtain active cell-free enzyme preparations by autolysing *P. vulgaris* cells for a week at 37° in phosphate buffer of p_H 7.8 or by drying cells *in vacuo* and extracting them with phosphate buffer have proved unsuccessful.

The properties of the enzyme forming H_2S from cysteine as determined by the use of intact P. vulgaris cells.

The effect of p_H on the velocity of H_2S formation from cysteine was determined. Phthalate-NaOH and KH_2PO_4 -NaOH buffers (Clark and Lubs), and a glycine-NaCl-NaOH buffer (Sørensen) were employed. (Unpublished experiments have shown that *P. vulgaris* cells do not decompose glycine under anaerobic conditions.) The amount of H_2S formed from 1 mg. of cysteine in 4 hours at 37° was estimated at various p_H values, 2 ml. of the required buffer solution being employed in each experiment. The H_2S formed in the absence of cysteine under identical conditions was subtracted in each case. The results of this experiment are shown in Fig. 1. A study of this figure indicates that the

Fig. 1. The p_H -activity curve of the enzyme forming H_2S from cysteine.

- Phthalate-NaOH buffer. 34 mg. dry weight of bacteria per experiment.
- KH_2PO_4 -NaOH buffer.
- △ Glycine-NaCl-NaOH buffer.

reaction proceeds with maximum velocity between p_H 7.8 and 9.0, there being a rapid decline in activity at higher and lower p_H values respectively. The enzyme is inactive at p_H 5.5. Curiously enough the enzyme is still active at p_H 12, although no H_2S is obtained from cysteine alone at this p_H under the

experimental conditions. Additional experiments have shown that if washed *P. vulgaris* cells are held at 37° for 5 minutes in *M*/20 glycine-NaCl-NaOH buffer of p_H 12 and the p_H then adjusted to approximately 7.8, about 37 % of the original activity of the cells remains. Thus in one experiment untreated cells formed 80 γ of H_2S from 10 mg. of cysteine in 1 hour at 37°, and cells treated as above described formed 30 γ of H_2S under similar conditions.

In Fig. 2 the effect of temperature on the amount of H_2S formed from 1 mg. of cysteine in 4 hours is shown. It is evident from this figure that the reaction has a relatively sharp optimum temperature of about 40°, and that an extremely rapid decline in activity is exhibited at higher or lower temperatures. The fact that the enzyme is inactive at 50° indicates that it is fairly sensitive to heat.

The influence of aerobic and anaerobic conditions on the velocity of H_2S formation from 10 mg. of cysteine in $\frac{1}{2}$ hour at 37° was determined. In one experiment the Thunberg tubes were evacuated in the usual manner, in a second a rapid stream of nitrogen washed thoroughly through alkaline pyrogallol solution was bubbled through the solutions during the course of the reaction, and in a third a stream of air was used in place of nitrogen. In the last two of these experiments the H_2S formed was absorbed in zinc acetate solution during the course of the reaction. In all experiments after half an hour the experimental solution was acidified with H_2SO_4 in the usual manner, and the H_2S present aerated into the zinc acetate solution. The results of these experiments, recorded in Table IV, indicate that H_2S is formed much more rapidly from cysteine under aerobic than under anaerobic conditions, this result being in agreement with the data obtained by Almy and James [1926] for *P. vulgaris* grown in a cystine-containing medium. In addition it is evident from Table IV that the velocity of H_2S formation is greater under the anaerobic

Fig. 2. The effect of temperature on the velocity of H_2S formation from cysteine by *P. vulgaris* cells. 26 mg. dry weight of bacteria per experiment.

Table IV. The effect of aerobic and anaerobic conditions on the formation of H_2S from cysteine by *P. vulgaris*.

Conditions of experiment	H ₂ S in γ after $\frac{1}{2}$ hour at 37°	
	Duplicates	Average
Aeration with air	141	141
	141	
Nitrogen passed through the solutions	80	80
	80	
Evacuated tubes	60	59
	58	

Controls without cysteine gave no H_2S . 22 mg. of dry weight of bacteria per exp.

conditions created by passing a stream of nitrogen through the experimental liquid than under similar conditions created by a partial vacuum. It is possible that this last-named effect is due to the fact that in experiments in which

nitrogen is employed the reaction product (H₂S) is more rapidly removed from the seat of the reaction than in experiments *in vacuo*.

The influence of cyanide on the velocity of H₂S formation was studied, 10 mg. of cysteine being employed in each experiment. All experiments were carried out at 37°, H₂S being estimated after $\frac{1}{2}$ and 1 hour. 5 ml. of *M*/5 phosphate buffer of *p*_H 7.8 were used instead of the usual 2 ml. The effect of cyanide was investigated by placing a small tube containing 1 ml. of KCN solution of appropriate concentration in the fluid in the Thunberg tube employed and mixing the liquids subsequent to evacuation. It is evident from the results given in Table V that KCN in the concentrations employed causes only a partial inhibition of the reaction. The inhibition due to a given cyanide concentration decreases with time, and the reaction is, therefore, reversible.

Table V. *The effect of KCN on the velocity of H₂S formation from cysteine by P. vulgaris.*

Experiment	H ₂ S formed in γ from 10 mg. cysteine			
	$\frac{1}{2}$ hour	% inhibition	1 hour	% inhibition
Controls (no KCN)	84	—	120	—
	84		126	
<i>M</i> /1000 KCN	60	29	100	20
	60		95	
<i>M</i> /500 KCN	36	56	76	37
	38		78	

24 mg. dry weight of bacteria per exp. Controls without cysteine gave no H₂S.

A study was also made of the effect of toluene treatment, 5 % ethylurethane and phenylurethane (saturated solution) on the velocity of H₂S formation from cysteine. The toluene-treated bacteria were prepared according to the method of Quastel and Wooldridge [1928], and all attempts to obtain growth of bacteria from such suspensions on caseinogen digest agar were unsuccessful. The results of these experiments (Table VI) show that phenylurethane causes no inhibition

Table VI. *The effect of toluene treatment, phenylurethane and ethylurethane on the velocity of H₂S formation from cysteine by P. vulgaris.*

Experiment	H ₂ S in γ from 10 mg. cysteine in 1 hour at 37°		% inhibition
Controls	97		0
	97		
Toluene-treated cells	54		45
	54		
5 % ethylurethane	36		62
	38		
Saturated phenylurethane	95		0
	100		

34 mg. dry weight of bacteria per exp. Control exps. without cysteine gave no H₂S.

of the reaction, while 5 % ethylurethane inhibits the reaction by about 60 %. It is interesting to note that toluene-treated *P. vulgaris* cells, which are "non-viable" as far as ability to reproduce on culture media is concerned, still retain over 50 % of their ability to form H₂S.

SUMMARY.

1. Hydrogen sulphide formation from cysteine and from thiosulphate by washed *P. vulgaris* cells is due to two different mechanisms. The formation of H_2S from organic sulphur compounds by this bacterium is due to the activity of a specific enzyme present in its cells.

2. Sterile, cell-free extracts containing small amounts of this enzyme can be obtained by extracting acetone-dried *P. vulgaris* cells with phosphate buffer, centrifuging and filtering the resulting supernatant liquid through kieselguhr. Such extracts exhibit only 2-3 % of the original activity of the cells from which they are prepared.

3. This enzyme as it exists in intact *P. vulgaris* and *S. marcescens* cells apparently requires the presence of an amino-group before it can become active. Thus H_2S is formed from α -amino- β -thiolpropionic acid (cysteine) but not from β -thiolpropionic acid, while α -amino- γ -thiolbutyric acid (homocysteine) gives rise to small amounts of H_2S .

4. The enzyme as it exists in intact *P. vulgaris* cells is inactive at p_H 5.5, is most active at p_H 7.8-9.0, and retains nearly 20 % of its optimum activity at p_H 12. It has an optimum temperature of 40° and is inactive at 50° . More H_2S is formed in a given time by the enzyme under aerobic than under anaerobic conditions. Non-viable, toluene-treated bacteria retain over 50 % of their activity with respect to H_2S formation. Phenylurethane does not inhibit H_2S formation from cysteine, while 5 % ethylurethane causes some 60 % inhibition of this reaction. Cyanide only partially inhibits the reaction, and this inhibition is reversible.

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