CCLIV. THE ENZYMIC FORMATION OF HYDROGEN SULPHIDE BY CERTAIN HETEROTROPHIC BACTERIA.

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None of the numerous investigators who have studied the formation of hydrogen sulphide by bacteria has thoroughly analysed the relation between the organic sulphur-containing substrate employed and the formation of this compound. Furthermore, the failure to eliminate bacterial multiplication may have obscured to some extent the simple formation of hydrogen sulphide.

Sasaki and Otsuka [1912] found that most of a large number of bacteria studied by them formed hydrogen sulphide when cultivated in Fränkel's artificial medium to which either cystine or sulphur had been added. Certain of the strains investigated formed this gas from thiosulphate, only a few formed it from sulphite, and none produced it from taurine or from sulphate. Bürger [1914] found that the bacteria which he studied formed hydrogen sulphide from cystine but not from taurine. Tanner [1917], employing Fränkel's medium, studied a very large number of cultures and found that most of these formed hydrogen sulphide from peptone and from cystine, some from thiosulphate and thiourea, and that none of the strains formed this gas from 2-thiohydantoin, sulphite or sulphate. Almy and James [1926] showed that, when *P. vulgaris* was grown in a peptone solution containing added cystine, all the sulphur of this amino-acid could be recovered as hydrogen sulphide. Hydrogen sulphide was formed in cultures of this organism under both anaerobic and aerobic conditions. Tarr [1933] showed that washed cells of *P. vulgaris* decomposed cystine completely under anaerobic conditions, with the formation of two molecules each of hydrogen sulphide, ammonia, acetic and formic acids.

In the present investigation it has been shown that the process of hydrogen sulphide formation by washed cells of certain heterotrophic bacteria is enzymic in nature, a relatively high degree of specificity existing between the structure of the organic molecule attacked and the production of hydrogen sulphide. A study of certain well-known bacterial species has been made in order to determine the distribution of the enzyme concerned; and one factor which stimulates its formation in the bacterial cell has been found.

Experimental.

Many of the substances used, *viz.* glutathione (oxidised and reduced forms), cystine, cysteine hydrochloride, glycylcysteine, *N*-acetylcysteine, *N*-acetylcysteine, methyl and propyl esters, *S*-ethylcysteine, *S*-benzylcysteine, methionine,

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glycylcysteine diketopiperazine, ergothioneine hydrochloride, 2-thio-4-methyl hydantoin and phthalimido-ethyl mercaptide were obtained through the kindness of Dr Pirie of this Department. All these compounds gave analytical figures in close agreement with those required by theory.

The sulphur compounds employed were dissolved in water to yield a concentration of 1 mg. or less per cc. The relatively insoluble compounds were dissolved in hot water. Where necessary the p_H of the solution was adjusted by titration to approximately 7·0 and the fluid sterilised by Seitz filtration. Cystine was dissolved in $N/100$ NaOH and the solution neutralised with sterile HCl after Seitz filtration. The sulphur used was autoclaved directly in the Thunberg tube employed.

Suspensions of *P. vulgaris* cells were prepared as described by Tarr [1933], with the exception that sterile Ringer solution was employed instead of sulphate solution. *Serratia marcescens* was cultivated for 36 hours at 28° on caseinogen digest agar, in other respects the technique employed in preparing cell suspensions of this organism was identical with that used in the case of *P. vulgaris*. Bacterial suspensions prepared in the above manner were stored aseptically at about 1° and were used within 5 days of preparation.

The following method was used in determining the formation of H_2S. 75 cc. Thunberg vacuum tubes, the necks of which were plugged with cotton-wool, were sterilised by autoclaving, the glass stoppers being wrapped and sterilised separately. 2 cc. of sterile $M/5$ phosphate buffer p_H 7·8, 5 cc. of bacterial suspension, and, except in control experiments, the required amount of substrate solution, were placed in each tube with aseptic precautions. The volume of liquid in each tube was made up to 20 cc. with sterile distilled water, the stopper, covered with sterile rubber grease, inserted and the tube evacuated. The evacuated tubes were incubated for 24 hours at 37° in experiments with *P. vulgaris* and at 28° in experiments with *S. marcescens*. All experiments were run in duplicate. At the conclusion of the incubation period the experimental fluid was acidified with 5 cc. of 5 % by volume H_2SO_4 and the H_2S aerated into 15 cc. of 2 % zinc acetate, suitable precautions being taken to avoid loss of the gas. The zinc acetate solution was employed for the colorimetric estimation of H_2S by the method of Almy [1925], care being taken to dissolve all traces of zinc sulphide in the acid dimethyl-*p*-phenylenediamine reagent.

In Table I the production of H_2S by *P. vulgaris* and *S. marcescens* cells from a large number of organic sulphur compounds is given. The results obtained permit the following conclusions to be drawn.

(1) Compounds containing the α-amino-β-thiolcarboxylic acid structure (cysteine), or its —S—S— form, yield over 75 % of their sulphur as H_2S.

(2) The substitution of the amino-group, carboxyl group, or both groups of cysteine inhibits the formation of H_2S enormously.

(3) The substitution of the sulphur of cysteine completely inhibits H_2S formation.

(4) α-Thiolcarboxylic-acids yield only very small amounts of H_2S.

(5) All the remaining organic sulphur compounds studied in which the S is not linked as in cysteine yield no H_2S.

It is concluded from the above data that H_2S formation from organic sulphur-containing substrates by the organisms investigated is specific inasmuch as, of the large number of compounds studied, only those which possess a cysteine or potential cysteine group are attacked to any significant extent.

Of the inorganic sulphur substrates studied sulphur was reduced to H_2S by both the bacteria, the comparative insolubility of this substance in water

Table I. *The anaerobic formation of H_2S by* P. vulgaris *and* S. marcescens.

Compound	Weight used in mg.	Theoretical amount H_2S mg.	H_2S recovered: *P. vulgaris* mg.	*P. vulgaris* %*	*S. marcescens* mg.	*S. marcescens* %*
Control†	0·0	0·0	0·004‡ 0·004	—	0·004 0·004	—
Cysteine	0·5	0·141	0·112‡ 0·120	79	0·120 0·115	80
Cystine	0·5	0·142	0·120‡ 0·123	83	0·120 0·115	80
Glutathione (reduced form)	1·0	0·111	0·095‡ 0·095	82	0·091 0·091	78
Glutathione (oxidised form)	1·0	0·111	0·098‡ 0·092	82	0·090 0·094	79
Glycylcysteine	0·5	0·095	0·080 0·084	82	0·074 0·078	76
N-Acetylcysteine	0·5	0·104	0·020 0·020	15	0·006 0·006	2
N-Acetylcysteine methyl ester	0·5	0·096	0·010 0·010	6	0·006 0·006	2
N-Acetylcysteine propyl ester	0·5	0·083	0·006 0·006	2	0·017 0·017	16
Thiolacetic acid	0·25	0·093	0·006 0·006	2	0·010 0·009	6
α-Thiolpropionic acid	0·5	0·159	0·006 0·006	2	0·004§ 0·004	0
S-Ethylcysteine	0·5	0·114	0·004‡ 0·004	0	0·004 0·004	0
S-Benzylcysteine	0·5	0·086	0·004‡ 0·004	0	0·004 0·004	0
Methionine	0·5	0·114	0·004‡ 0·004	0	0·004 0·004	0
Glycylcysteine diketopiperazine	0·5	0·089	0·004 0·004	0	0·004 0·004	0
Ergothioneine	1·0	0·113	0·002 0·002	0	0·002 0·002	0
Phthalimido-ethyl mercaptide	0·5	0·093	0·004 0·004	0	0·004 0·004	0
Thiourea	0·25	0·112	0·004‡ 0·004	0	0·004 0·004	0
Monophenylthiourea	0·5	0·112	0·004‡ 0·004	0	0·004 0·004	0
Allylthiourea	0·5	0·146	0·002 0·002	0	0·004 0·004	0
2-Thio-4-methylhydantoin	0·5	0·131	0·002 0·002	0	0·004 0·004	0
Sodium thiosulphate	0·5	0·108	0·087 0·087	78	0·004§ 0·004	0
Sodium sulphite	0·5	0·135	0·004 0·004	0	0·004§ 0·004	0
Sodium sulphate	0·5	0·120	0·004 0·004	0	0·004§ 0·004	0
Sulphur	1·0	1·062	0·026 0·029	2	0·029§ 0·025	2

* Calculated on the theoretical value after subtracting the control.

† No appreciable variation was found in the control values for different suspensions of the same organism.

‡ 31 mg. dry weight of bacteria per exp. Exps. in the same column without asterisk had 34 mg. dry weight per exp.

§ 66 mg. dry weight of bacteria per exp. Exps. in the same column without asterisk had 49 mg. dry weight per exp.

probably limiting the amount reduced. Neither sulphite nor sulphate was reduced. The fact that thiosulphate was strongly attacked with the formation of H_2S by *P. vulgaris* and was not attacked by *S. marcescens*, and that cysteine was attacked by both organisms, suggests that H_2S formation from thiosulphate and from cysteine is due to two distinct mechanisms.

Distribution of the enzyme forming H_2S from cysteine.

In order to determine the distribution of the enzyme among different bacterial species a study was made of aerobic, facultative anaerobic and anaerobic bacteria. Washed cell suspensions of *B. subtilis*, *B. megatherium*, *Ps. aeruginosa*, *E. coli* and *A. faecalis* were prepared by the method used in obtaining *P. vulgaris* cells. *S. lutea* was cultivated for 48 hours at 28°, the technique of preparing cell suspensions of this organism being in other respects identical with that used in preparing *P. vulgaris* cells. *C. sporogenes* was grown anaerobically for 44 hours on caseinogen digest broth, the cells being washed and suspended in Ringer solution by the method already referred to. Duplicate experiments for determining the amount of H_2S produced from 0·5 mg. of cystine, together with the corresponding controls, were run for all the organisms studied, the technique employed being identical with that already described. The experimental solu-

Table II. *The anaerobic formation of H_2S from 0·5 mg. of cystine by different bacteria.*

Organism	Dry wt. of bacteria per experiment mg.	Experiment	H_2S recovered (theory for 0·5 mg. cystine = 0·142 mg.) mg.	% of theoretical
A. faecalis	26	Controls	0·002	
			0·002	
		Cystine	0·013	8
			0·013	
B. subtilis	23	Controls	0·000	
			0·000	
		Cystine	0·018	11
			0·014	
B. megatherium	16	Controls	0·000	
			0·000	
		Cystine	0·002	1
			0·002	
E. coli	21	Controls	0·002	
			0·002	
		Cystine	0·111	78
			0·111	
Ps. aeruginosa	33	Controls	0·004	
			0·004	
		Cystine	0·012	6
			0·014	
S. lutea	55	Controls	0·000	
			0·001	
		Cystine	0·001	0
			0·001	
C. sporogenes	4	Controls	0·004	
			0·004	
		Cystine	0·065	43
			0·065	

tions were incubated for 24 hours, at 28° in the case of *S. lutea*, and at 37° in the case of all other bacteria studied.

The results of these experiments are given in Table II. From this Table it is evident that the power of various bacteria to produce H_2S from cysteine varies considerably. Thus *S. lutea* and *B. megatherium* are inactive or nearly so, while *E. coli* and *C. sporogenes* are very active. It is interesting to note that the enzyme is present both in cells of such aerobic bacteria as *B. subtilis* and *A. faecalis*, and in the strict anaerobe *C. sporogenes*.

The velocity of H_2S formation from cysteine by washed cells of P. vulgaris *obtained from an identical medium with and without added cysteine.*

P. vulgaris was grown in the usual manner on caseinogen digest agar to which 0·1 % of cysteine (as the neutral hydrochloride solution sterilised by Seitz filtration) had been added prior to solidification of the medium, and on an identical medium without added cysteine. Washed cell suspensions of both these types of bacteria prepared as usual were stored at 1° and were used within 12 hours of preparation. H_2S formation from 0·5 mg. of cysteine was determined as already described, a number of experiments being run in the case of each suspension. The velocity of H_2S formation was determined by incubating the Thunberg tubes in a water-bath at 37° and withdrawing them at suitable intervals for the estimation of this gas. In Fig. 1 the results of these experi-

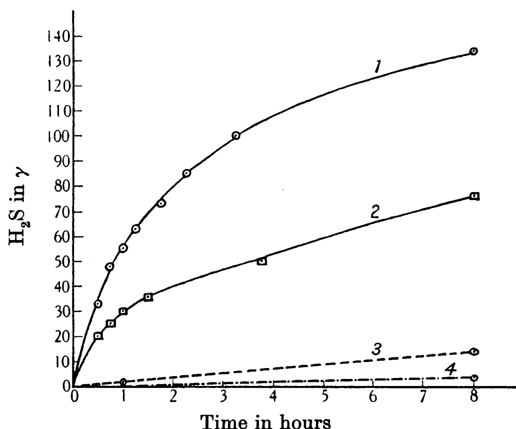

Fig. 1. 1. ⊙—⊙ H_2S formed from cysteine by *P. vulgaris* cells obtained from medium with 0·1 % added cysteine; 2. ⊡—⊡ H_2S formed from cysteine by *P. vulgaris* cells obtained from medium without added cysteine; 3. ——— H_2S formed in absence of cysteine by *P. vulgaris* cells obtained from medium with 0·1 % added cysteine; 4. —•—•— H_2S formed in absence of cysteine by *P. vulgaris* cells obtained from medium without added cysteine.

1 and 3. 14 mg. dry wt. of bacteria per exp. 2 and 4. 24 mg. dry wt. of bacteria per exp.

ments are plotted graphically. It is clear from this diagram that H_2S is formed with much greater rapidity by cells cultivated in the presence of 0·1 % cysteine than by cells cultivated on an identical medium in the absence of added cysteine. Suspensions of cells cultivated in the medium rich in cysteine also give off more H_2S when incubated in the absence of cysteine than do suspensions obtained from the medium without added cysteine. The formation of H_2S from cysteine takes place with greatest velocity at the commencement of the experiment. After 8 hours 85 % of the cysteine added was recovered as H_2S in the

experiment in which cells from the medium rich in cysteine were employed, while in the case of the other suspension only 51 % of the cysteine sulphur was recovered as H_2S. Calculation showed that, in this experiment, 10 mg. (dry weight) of bacteria from the medium rich in cysteine would be capable of forming 85γ of H_2S in 8 hours, while, under similar experimental conditions, 10 mg. (dry weight) of bacterial cells from the medium with no added cysteine would only form 38γ of H_2S in 8 hours.

SUMMARY.

1. The formation of hydrogen sulphide from a variety of organic and inorganic sulphur compounds has been studied employing washed cells of *P. vulgaris* and *S. marcescens*.

2. Of the organic sulphur compounds studied only cysteine, cystine or those containing either of these molecules yield over 75 % of their sulphur as H_2S.

3. Substituted cysteine compounds and α-thiolcarboxylic acids, when attacked, only yield very small amounts of H_2S, and all other organic sulphur compounds studied yield no H_2S.

4. Of the inorganic sulphur compounds studied sulphur is reduced to H_2S by both bacteria, while neither sulphite nor sulphate is reduced with the formation of H_2S by either strain.

5. Thiosulphate is reduced with the formation of H_2S by *P. vulgaris*, but not by *S. marcescens*.

6. The formation of H_2S from organic sulphur compounds appears, therefore, to require the presence of a specific enzyme in the bacterial cell.

7. This enzyme has not been found in the cells of all bacteria studied, but has been found in the cells of aerobes, facultative anaerobes and in one strict anaerobe.

8. Cysteine added to the medium employed for producing the bacterial cells stimulates the formation of the enzyme.

This investigation was largely made possible by the kindness of Dr N. W. Pirie of this Department in supplying me with most of the organic sulphur compounds employed. My thanks are due to Dr Spooner of the Department of Pathology for giving me certain of the cultures used. To Sir F. G. Hopkins I am indebted for his constant interest and encouragement.

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