The Availability of Sulphur for *Clostridium perfringens* and an Examination of Hydrogen Sulphide Production

BY ANNA-RIITTA FUCHS AND G. J. BONDE

The Institute of Hygiene, University of Copenhagen, Denmark

SUMMARY: The utilization of several inorganic and organic sulphur compounds by three strains of *Clostridium perfringens* and the production of hydrogen sulphide from these compounds was investigated. Sulphate, sulphite, thiosulphate and sulphide cannot supply sulphur to the organism, neither do they have any effect, when added in non-toxic concentrations, on the growth in media containing utilizable organic sulphur. Cystine or cysteine, which can be replaced by glutathione, were required by the three strains tested; no additional sulphur source was needed by two of the strains, the third requiring also methionine, which can be replaced by homocyst(e)ine. Hydrogen sulphide is produced by growing cultures from sulphite, thiosulphate, cystine, cysteine and glutathione, but not from methionine. Different enzyme systems are concerned with hydrogen sulphide production from cystine and sulphite, respectively. The sulphite-reducing capacity of some cultures was often decreased after several passages in laboratory media, the presence of nicotinamide being then required for this reduction. Sulphite was reduced to sulphide by suspensions of resting organisms. The presence of a hydrogenase system is suggested by preliminary experiments, the organisms being able to activate molecular hydrogen for reduction of methylene blue and sulphite. Other hydrogen donors are utilized by the organisms, in particular glucose. The addition of sulphites does not induce growth on substrates which do not support growth in sulphite-free media.

Hydrogen sulphide is produced by micro-organisms from compounds containing organic sulphur and from inorganic sulphur compounds, e.g. thiosulphate, sulphite, sulphate. Many different bacteria produce hydrogen sulphide from peptones, cystine and cysteine (Clarke, 1953; Stekol & Ransmeier, 1942), and some from thiourea (Tanner, 1917), but not from taurine (Sasaki & Otsuka, 1912; Bürger, 1914). A few produce H_2S from thiosulphate (Tanner, 1917; Tilley, 1923*a, b*) and from sulphite (Tilley, 1923*a, b*; Wilson, 1922-3; Tanner, 1917). Only a small group of sulphate-reducing bacteria (vibrios) has so far been described, first by Beijerinck (1895) and later studied more closely by several investigators.

Clostridium perfringens is capable of reducing sulphite in suitable media and forms colonies black with precipitated ferrous sulphide in iron sulphite agar (Wilson & Blair, 1924, 1927). The occurrence of *C. perfringens* in sewage before and after treatment in purification plants is used at this Institute as an indication of the effectiveness of purification, together with the coliform count. The isolation and counting is based on the sulphite-reducing capacity of these bacteria (Bonde & Hvid-Hansen, 1953). The mechanism of sulphite reduction, as well as that of sulphate and thiosulphate reduction, is still obscure, and it is also unknown what, if any, the significance of this reaction is to the bacterial metabolism. The aim of the present work is to investigate the conditions governing the reduction of sulphite by *C. perfringens*.

The studies of Roberts, Abelson, Cowie, Bolton & Britten (1955) on the sulphur metabolism of *Escherichia coli* showed that the total sulphur content of the bacterium depends in a large measure upon the sulphur compounds present in the chemically defined medium and that the utilization of sulphur by the organism from any particular sulphur source depends upon the chemical form of this sulphur compound and the type of organism used. The first part of the present paper reports the results of studies on the utilization of various sulphur compounds by *Clostridium perfringens*; the second part is concerned with hydrogen sulphide production from sulphite and other sulphur sources, and with the effect of the environment upon the reduction of sulphite.

METHODS

Organisms. All the strains used in this work were isolated from rotting tank sludge obtained from Municipal Sewage Works of Copenhagen. The general methods used for isolation, cultivation and the handling of inocula are described elsewhere (Fuchs & Bonde, 1957).

Standardization of suspensions. Amount of growth was measured as the turbidity of the cultures estimated by a Beckman colorimeter model C with a red filter, using uninoculated medium as a blank. It has previously been ascertained, that optical density of the cultures in the media used is, within certain limits, directly proportional to the nitrogen content of the suspension, as measured in a sample by the micro-Kjeldahl method (Fuchs & Bonde, 1957).

Medium. The composition of the media used is given in Table 1 of Fuchs & Bonde (1957, see p. 320 of this issue).

In the experiments concerning sulphur availability the minimal medium number 3 was generally used. The sulphur-containing amino acids were omitted when necessary. A sulphur-free medium, number 6, where the concentration of sulphate as impurities was minimized, was used to confirm the results obtained with the minimal medium about the effect of inorganic sulphur compounds on growth. In the medium 1 growth was heavier than in the minimal medium; it was therefore used to cultivate larger amounts of organisms and to confirm the results obtained with the minimal medium. Medium 2 (Fuchs & Bonde, 1957) was generally used in the experiments concerning sulphite reduction.

Medium was distributed in test tubes in 5 ml. amounts, autoclaved for 10 min. at 110° (with the ascorbic acid medium care must be taken not to exceed the temperature or time given), cooled, inoculated and incubated anaerobically according to the method of Prévot (see Lebert, 1949).

Sulphide detection. Numerous indicators are used to detect hydrogen sulphide production by micro-organisms, usually iron, lead, bismuth, nickel and cobalt salts. The sensitivity of these indicators is different in different media, and the results are not strictly comparable, as pointed out by Clarke (1953) and others (e.g. Hunter & Crecelius, 1938). In the present work two different methods were used.

(1) Iron salts. Ferrous ammonium sulphate or ferrous chloride were added in equimolar concentrations with the added sulphite. This method is not very

sensitive. In our media sulphide produced from cystine or other organic sulphur sources or from $1\mu\text{mole}$ sulphite/ml. was not detected by this means, the presence of $2\mu\text{mole}$ sulphite/ml. was necessary for the formation of the black ferrous sulphide precipitate.

(2) Lead acetate paper strips were generally used to detect sulphide formed during growth. Before turbidity determination the ampoules were punctured while holding a moist strip of lead acetate paper over the small opening. When the tubes were incubated in anaerobic jars, the strip was inserted in the mouth of the tube and held by a sterile cotton plug. It was noticed that uninoculated media containing mercaptoacetate liberated some hydrogen sulphide during incubation at 37° . When sulphide formation from unknown sulphur sources or by unknown cultures has to be detected, the media must not contain mercaptoacetate.

The lead acetate paper method is much more sensitive than the use of iron salts, strong blackening of the paper occurring with our media in cultures where no ferrous sulphide formation yet could be detected. ZoBell & Feltham (1934) reported, in the media used by them, a 10 to 100 times greater sensitivity of lead acetate as compared to iron and bismuth salts. By combining both methods it is possible to compare approximately the amounts of hydrogen sulphide formed without quantitative measurements. Such amounts of sulphide which cause a blackening of the lead acetate paper too intense to grade, will give rise to ferrous sulphide precipitate. With lower sulphide concentrations, below the sensitivity of iron salts, a rough grading of the degree of blackening of the lead acetate paper is possible.

Studies with washed bacterial suspensions. For studies of the bacterial enzymes which catalyse the reduction of sulphite, suspensions of resting organisms were prepared by cultivation in the medium 2 with added nicotinamide, in volumes of 100–300 ml., incubated in evacuated flasks for 16–20 hr., centrifuged, washed twice in 10–20 ml. of the buffered salt solution (1–5) of media 1 and 2 and resuspended in 10–20 ml. of this solution. These suspensions lost their reducing capacity rapidly, even when stored in the icebox under hydrogen or nitrogen and had to be used the day of preparation. In these experiments it was of great importance that the bacterial suspensions were prepared under as similar conditions as possible; the harvesting should be performed as soon as maximal growth is obtained.

The reducing capacity of the bacterial suspensions was studied by the Thunberg technique in evacuated ampoules, containing 1 ml. 0.00025M -methylene blue, 1 ml. 0.1M substrate, 1 ml. 0.05M -phosphate buffer (pH 7.0–7.2) and 1 ml. bacterial suspension.

Warburg vessels were used when methylene blue was replaced by sulphite; a strip of filter paper moistened with lead acetate was placed in the centre well.

RESULTS

Growth in sulphur-deficient media. The medium 1 (with omission of the sulphur-containing amino acids and mercaptoacetate; Fuchs & Bonde, 1957) did not support growth without added sulphur. A slight increase in the

bacterial cell mass occurred, just detectable by the colorimeter used, which corresponded to about 1 µg. N/ml. This increase must be ascribed to sulphur reserves of the organisms (see Roberts *et al.* (1955) with *Escherichia coli*), and not to impurities present in the reagents used, because added inorganic sulphur as sulphide, sulphite and sulphate did not improve growth.

Growth response to inorganic sulphur as sole sulphur source. Inorganic sulphur as ammonium sulphate, sodium sulphite, sodium thiosulphate, or sodium sulphide was added in varying concentrations to the sulphur-free media 1 or 6; no growth was obtained with any concentration of these salts. Thus *Clostridium perfringens* is unable to utilize these compounds as sole source of sulphur.

Table 1. *Growth response to organic sulphur-containing compounds of three different strains of Clostridium perfringens*

Sulphur compound	<i>C. perfringens</i>		
	Strain A	Strain B	Strain C
Thioacetamide	—	—	—
Mercaptoacetate	+	+	+
Cysteine	++	++	+(+)
Cystine	++	++	+(+)
Methionine	Trace	—	+
Homocysteine	—	—	+
Homocystine	—	Trace	+
Glutathione	+	—	+(+)
Taurocholate	—	—	—

Growth response to organic sulphur compounds. The sulphur-containing substances listed in Table 1 were tested as possible sulphur sources for three different strains of *Clostridium perfringens*. Some differences were observed in the response to different sulphur-containing amino acids among these strains; the results are summarized in Table 1. Strain A was kept in laboratory media for over a month before the experiments, strain B was a newly-isolated strain, and C had been maintained in laboratory media for over three months. Cystine, cysteine and glutathione were nearly equally good as a sole source of sulphur for all strains. Mercaptoacetate alone could also support some growth, while thioacetamide was unable to supply sulphur. Methionine, homocysteine and homocystine failed to support growth of strains A and B. With strain C, which requires both cystine and methionine for optimal growth, suboptimal growth occurred also with homocysteine or homocystine. The effect of increasing concentration of cysteine and cystine is shown in Table 2.

Effect of additional sulphur compounds on growth in cystine-containing media. When sulphate, sulphite or sulphide was added in concentrations varying from 0.1 to 500 µg. S/ml., no increase in growth could be obtained over that occurring in the controls containing cystine only. Sulphate did not have any inhibitory effect in the concentrations used, but sulphite and sulphide both depressed growth when the concentration was increased to 100 µg. S/ml. and more. Table 3 shows the effect of increasing concentrations of sulphate,

sulphite and sulphide on growth in cystine media. When adequate amounts of cystine were present the addition of cysteine, mercaptoacetate or thioacetamide was without effect on growth. Methionine had a stimulatory effect with strain A, little effect with strain B, but with strain C only very poor growth was obtained in the absence of methionine.

Table 2. *Growth response of Clostridium perfringens (strain A) to increasing amounts of cysteine and cystine*

Concn. of cysteine ($\mu\text{g./ml.}$)	Optical density (scale units)	Concn. of cystine ($\mu\text{g./ml.}$)	Optical density (scale units)
0.1	0.0	0.1	2.5
1.0	0.0	0.5	5.4
10.0	14.1	10.0	29.5
100.0	35.9	100.0	25.2
1000.0	0.8		

Table 3. *Effect of increasing concentrations of sulphate, sulphite and sulphide on growth of Clostridium perfringens (strain A)*

Sulphur added ($\mu\text{g. S/ml.}$)	Sulphur added as		
	Na_2SO_4	Na_2SO_3	Na_2S
	Optical density (scale units)		
0.0	21.2	21.2	21.2
0.1	.	18.1	21.6
1.0	18.1	21.6	19.4
5.0	19.4	.	.
10.0	21.1	21.6	21.2
50.0	22.3	19.6	.
100.0	20.8	10.6	16.2
200.0	21.6	.	.
500.0	23.8	1.3	0.0

The addition of homocystine to strains A and B in cystine medium had an inhibitory effect. With the methionine-requiring strain C, the addition of homocystine or homocysteine to cystine medium improved growth to nearly the same extent as methionine, but they could not replace cystine in methionine-containing medium.

Apparently methionine is synthesized from cystine (cysteine) by *Clostridium perfringens*, but not the reverse. Homocysteine and homocystine serve as intermediates or, more probably, they are transformed in some way into the real intermediates. The reaction $\text{cyst(e)ine} \rightarrow \text{R} \rightarrow \text{homocyst(e)ine}$ is the one blocked in the methionine-requiring strain. Tables 4 and 5 show the growth response of the strains A, B and C to the above-mentioned organic sulphur compounds.

Growth and hydrogen sulphide formation

Organic sulphur compounds. Growth of *Clostridium perfringens* in cystine-containing medium always showed sulphide production, a very common reaction in bacterial metabolism. Other sulphur-containing organic substances giving rise to hydrogen sulphide production by *C. perfringens* are mercapto-

acetate, cysteine; and in the methionine-requiring strain C homocystine and homocysteine, although to a lesser extent than cyst(e)ine; but not methionine. Glutathione did not give hydrogen sulphide. Apparently free cyst(e)ine must be present, before hydrogen sulphide can be formed.

Inorganic sulphur compounds. Although no growth occurred with sulphite or thiosulphate as sole sulphur source, these ions are nevertheless attacked by a growing culture of *Clostridium perfringens* with reduction to hydrogen sulphide. *C. perfringens* did not reduce sulphate.

Table 4. *Effect of cysteine and methionine on growth of three strains of Clostridium perfringens in cystine-free medium and in cystine medium*

Sulphur compound added	<i>Clostridium perfringens</i>		
	Strain A	Strain B	Strain C
	Amount of growth (optical density scale units)		
Methionine	3.8	4.1	0.5
Cystine	30.3	27.6	10.5
Cysteine	21.0	15.0	.
Mercaptoacetate + methionine	9.7	.	.
Cystine + methionine	43.9	26.7	38.0
Cystine + methionine + SO ₄	40.0	.	.
Cystine + cysteine	22.3	30.0	.
Cysteine + methionine	30.0	31.0	.

Table 5. *Effect of homocystine and homocysteine on growth of a methionine-requiring strain of Clostridium perfringens*

Sulphur compounds added	Strain C
	(optical density scale units)
Methionine + cystine	53.3
Methionine + homocysteine	10.3
Methionine + homocystine	14.7
Cystine + homocysteine	42.0
Cystine + homocystine	47.0
Homocysteine + homocystine	15.0

Conditions for sulphite reduction. A newly-isolated strain will grow abundantly on medium 2 and in the presence of sulphite and ferrous-iron produce a black precipitate of ferrous sulphide. The minimal concentration of sulphite required for the formation of this precipitate is 2 μ mole/ml., maximally 10 μ mole/ml. After several successive transfers in peptone broth the threshold concentration of sulphite needed for the ferrous sulphide formation is increased, and eventually the ability to form ferrous sulphide in this medium is often lost, though not with all the strains.

In meat extract + peptone agar with added sulphite and ferrous iron, black colonies were formed even by strains which no longer produced any detectable ferrous sulphide in the medium 2. Apparently a factor which is present in meat-extract agar is needed for the reduction of sulphite. This factor was found to be nicotinamide. When nicotinamide was added to medium 2 a

ferrous sulphide precipitate was again formed with the same threshold concentration of sulphite as for a newly-isolated strain. With strain A growth was also improved by the addition of nicotinamide; with strain C growth was unaltered. The tolerance for sulphite was also increased by the addition of nicotinamide with strains which required it for sulphite reduction. In Table 6 is shown the effect of nicotinamide on growth and ferrous sulphide formation by a nicotinamide-requiring strain (A) and by a strain (C) not requiring this factor. It is evident from the results in Table 6 that nicotinamide alone was responsible for the effect with strain A, the addition of various other growth factors being without effect.

Table 6. *Effect of nicotinamide on growth of Clostridium perfringens and ferrous sulphide formation in the presence of varying concentrations of sulphite in medium 2 in strains A and C*

Substance added:	—		Nic. amide		Nic. amide + various growth factors		—		Nic. amide	
Concn. of sulphite (μ mole/ml.)	Strain A		Strain A		Strain A		Strain C		Strain C	
	μ g. N/ml.	FeS	μ g. N/ml.	FeS	μ g. N/ml.	FeS	μ g. N/ml.	FeS	μ g. N/ml.	FeS
÷	39.9	÷	67.0	÷	51.9	÷	73.0	÷	78.0	÷
1	24.9	÷	67.1	(+)	51.9	(+)	77.0	÷	76.0	÷
2	24.0	(+)	61.3	++	51.9	+++	62.0	+	60	+
4	22.5	(+)	51.3	+++	47.1	+++	63.0	+++	61	+++
6	7.8	+	38.0	+++	18.4	+++	.	.	.	.
8	1.8	÷	39.0	+	.	.	.	.	.	.
10	0.0	÷	13.8	+	.	.	27.0	+++	61	+++
20	.	.	.	.	.	.	0.0	++	24.1	+++

Growth is expressed in μ g. N/ml.; ÷ denotes no ferrous sulphide formation, (+), +, ++, +++ denotes increasing degrees of blackening of the medium.

Peptone broth is rich in nicotinamide and apparently on subcultivation in this medium a 'negative' adaptation occurs, with resulting loss of ability to synthesize nicotinamide. The loss of this capacity did not affect the hydrogen sulphide formation from cystine in the medium, which continued unimpaired in the laboratory-maintained strains without adding nicotinamide to the defined medium 1. The hydrogen sulphide production from cyst(e)ine is thus independent of the reduction of sulphite; and the hydrogen sulphide formation from cystine and sulphite is additive, as estimated by the lead acetate and iron salt method. A black precipitate was not formed in medium which contained 3μ mole S/ml. as cystine, while the addition of 2μ mole S/ml. as sulphite resulted in the precipitation of ferrous sulphide both in the presence of 0.3μ mole and 3μ mole S/ml. as cystine. Therefore two distinct enzyme systems are concerned in the hydrogen sulphide production; this is also the case in *Proteus* (Tarr, 1934). The strains tested could not be 'trained' to dismiss one system with added nicotinamide by successive transfers in sulphite-containing medium 2, but their tolerance for sulphite was increased after subcultivation in sulphite-containing medium. Also the colony count in meat extract + peptone agar of the sulphite-adapted strain was much increased compared with the strain maintained on peptone water.

Growth experiments in the presence of atmospheric oxygen. It was shown by Quastel & Stephenson (1925) that certain facultative anaerobes grew anaerobically in the presence of nitrate in media which, in the absence of nitrate, could only support aerobic growth; the nitrate was reduced to nitrite. What is the significance of a corresponding reaction, the reduction of sulphite, in an anaerobic organism such as *Clostridium perfringens*? This organism was reported by Kligler & Guggenheim (1938) to grow aerobically in the presence of large amounts of ascorbic acid (0.02 %). The addition of sulphite to our culture media did not allow 'aerobic' growth of *C. perfringens* in the medium 1 or 2.

Growth experiments in the presence of various carbon compounds and sulphite. In the defined medium *Clostridium perfringens*, like many anaerobes, can obtain the energy needed for growth only from carbohydrates (see Fuchs & Bonde, 1957). A series of experiments was therefore carried out to investigate whether *C. perfringens* could utilize other carbon compounds, which serve as substrates in aerobic life, as energy sources when sulphite was serving as hydrogen acceptor. A number of substrates which support aerobic growth in a variety of species, but not the anaerobic growth of *C. perfringens*, were tested in the presence of sulphite and nicotinamide and in absence of glucose. Of the substances tested the following did not support growth: formate, acetate, propionate, butyrate, succinate, succinate + fumarate, tartrate, citrate, pyruvate, lactate, gluconate, mannitol and ethanol. Slight growth occurred in glycerol, glycerophosphate and acetone.

Dehydrogenase activity in Clostridium perfringens

Different substances were investigated with methylene blue as hydrogen acceptor. Glucose, α -ketoglutarate, succinate, pyruvate, glycerophosphate and glycollate, and of amino acids, histidine and glutamic acid, were active in reducing methylene blue in the presence of washed suspensions of *Clostridium perfringens*. Lactate, propionate, butyrate, fumarate, malonate, arginine, phenylalanine, isoleucine, valine, aspartic acid, methionine and threonine were inactive. It was very difficult to obtain uniform suspensions, the rate of methylene blue reduction with the same substrate differed considerably in different preparations; therefore, reduction rates, to be comparable, should be obtained with the same suspension. Glucose was very rapidly dehydrogenated. Of the other substrates tested, only histidine reduced methylene blue at almost the same rate, α -ketoglutarate, succinate, glycerophosphate, glycollate, and glutamate were dehydrogenated by the suspensions at a much slower rate than glucose. Nevertheless, besides glucose, only glycerophosphate can support anaerobic growth and then very poorly. Of the amino acids reported by Stickland (1934) to serve as hydrogen donors in the reaction by which *C. sporogenes* obtains its energy, leucine, histidine and glutamic acid were also dehydrogenated by *C. perfringens* suspensions in the presence of methylene blue. Neutral red and Nile blue could also serve as hydrogen acceptors in these reactions.

Hydrogenase activity in Clostridium perfringens. In the first series of experiments methylene blue reduction in the absence and in the presence of gaseous hydrogen was compared, with and without added nicotinamide. The results obtained with three different strains are presented in Table 7. Clear acceleration of the reduction rate was observed in the presence of gaseous hydrogen, although the results were variable. Only with strain B was the reduction rapid, *C. perfringens* thus being able to activate molecular hydrogen.

The presence of a hydrogenase in a non-multiplying suspension of *C. perfringens* thus having been demonstrated, a second series of experiments was made to see whether sulphite could serve as hydrogen acceptor for molecular hydrogen.

Table 7. *Hydrogenase activity in Clostridium perfringens*

Reduction of methylene blue by non-multiplying suspensions of three strains of *C. perfringens* in the presence of molecular hydrogen.

Strain	Substrate	15 min.	30 min.	45 min.	60 min.	18 hr.	48 hr.
Degree % reduction methylene blue							
B	.	.	.	.	.	100 %	.
B	H ₂	80 %	95 %	95 %	95 %	100 %	.
C	.	.	.	.	.	.	.
C	H ₂	Some	Some	.	.	50 %	.
A	.	.	.	.	.	.	100 %
A	H ₂	.	.	.	.	100 %	.

B: newly isolated strain, grown in absence of nicotinamide. A and C: laboratory strains, grown in presence of nicotinamide.

Table 8. *Hydrogenase activity in Clostridium perfringens*

Reduction of sulphite by non-multiplying suspensions of two strains in the absence and presence of molecular hydrogen or glucose as hydrogen donors measured as blackening of lead acetate paper.

Strain	Substrate	Time of observation (hr.)				
		0.5	1	1.5	18	48
		Estimated degree of H ₂ S evolved				
A	Na ₂ SO ₃	—	—	—	—	+
A	Na ₂ SO ₃ + w.e.	—	—	+	.	+
A	Na ₂ SO ₃ + n.a.	—	—	+	.	++
A	Na ₂ SO ₃ + H ₂	+	+	+	.	++++
A	Na ₂ SO ₃ + H ₂ + w.e.	—	—	+	.	++++
A	Na ₂ SO ₃ + glucose	—	+	.	.	++++
A	Na ₂ SO ₃ + H ₂ + n.a.	—	—	—	.	—
A	Na ₂ SO ₃ + glucose + w.e.	—	—	—	.	+++
A	Na ₂ SO ₃ + glucose + n.a.	—	—	—	.	+++
B	Na ₂ SO ₃ + H ₂	—	—	—	++	.
B	Na ₂ SO ₃ + H ₂ + w.e.	+	.	.	++	.
B	Na ₂ SO ₃ + glucose	+	++	++	+++	.
B	Na ₂ SO ₃ + glucose + w.e.	+	.	.	+++	.
Boiled suspensions	Na ₂ SO ₃ + H ₂	—	—	—	—	—

w.e.: boiled water extract of the washed cells

n.a.: nicotinamide

The organisms were grown in the presence of nicotinamide and the rate of reduction was estimated by the degree of blackening of the lead acetate paper strip immersed in the centre well of the Warburg vessel used in the experiments. The results are presented in Table 8. As with methylene blue as hydrogen acceptor for hydrogenase, the results differed with different preparations, and the importance of extreme care and uniformity in the preparation of the bacterial suspension must be stressed.

In the presence of boiled organisms no hydrogen sulphide was produced from sulphite. The bacteria are able to utilize some internal compounds as hydrogen donors for sulphite reduction, although the reaction proceeds at a very slow rate in the absence of any added hydrogen donor. A water extract of washed organisms + nicotinamide accelerated this reaction, although it remains to be seen, whether they act really as carriers (or co-factors of another kind) or merely provide the organism with a hydrogen donor.

In the presence of molecular hydrogen, sulphite was reduced at much greater speed and much more hydrogen sulphide was produced (as judged by the degree of blackening of the lead acetate paper) than in its absence. When glucose was present, the hydrogen sulphide production was also rapid. From the preliminary experiments it may be concluded, that *Clostridium perfringens* possesses a hydrogenase system and is able to use molecular hydrogen for the reduction of sulphite, as well as other hydrogen-donors. Further experiments are planned to investigate the properties of these enzymes and to study quantitatively the reduction of sulphite to sulphide.

Our sincere thanks are due to Professor P. Bonnevie, M.D., Director of this Institute, for valuable support and encouragement in the work.

REFERENCES

- BEIJERINCK, W. M. (1895). Über *Spirillum desulfuricans* als Ursache von Sulfat-reduktion. *Z. Bakt.* **1**, 1.
- BONDE, G. J. & HVID-HANSEN, N. (1953). Om mikrobiologisk kontrol med spildevand og spildevandsrecipienter. *Nord. hyg. Tidsskr.* **11-12**, 221.
- BÜRGER, M. (1914). Über Schwefelwasserstoffbildung aus Zystin durch Bakterien. *Arch. Hyg., Berl.* **82**, 201.
- CLARKE, P. H. (1953). Hydrogen sulphide production by bacteria. *J. gen. Microbiol.* **8**, 397.
- FUCHS, A.-R. & BONDE, G. J. (1957). Nutritional requirements of *Clostridium perfringens*. *J. gen. Microbiol.* **16**, 317.
- HUNTER, C. A. & CRECELIUS, H. G. (1938). Hydrogen sulphide studies. I. Detection of hydrogen sulphide in cultures. *J. Bact.* **35**, 185.
- KLIGLER, I. J. & GUGGENHEIM, K. (1938). The influence of vitamin C on the growth of anaerobes in the presence of air, with special reference to the relative significance of E_h and O₂ in the growth of anaerobes. *J. Bact.* **35**, 141.
- LEBERT, F. (1949). *Technique actuelle d'isolement et de détermination des bactéries anaérobies*. Paris: 'Pachomy', Sarl Éditeurs.
- QUASTEL, J. H. & STEPHENSON, M. (1925). Further observations on the anaerobic growth of bacteria. *Biochem. J.* **19**, 660.
- ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. T. & BRITTEN, R. J. (1955). Studies of biosynthesis in *Escherichia coli*. *Publ. Carneg. Inst.* no. 607.

- SASAKI, T. & OTSUKA, I. (1912). Experimentelle Untersuchungen über die Schwefelwasserstoffentwicklung der Bakterien aus Zystin und sonstigen Schwefelverbindungen. *Biochem. Z.* **39**, 208.
- STEKOL, J. A. & RANSMEIER, J. C. (1942). Production of hydrogen sulphide from sulphur containing compounds by various bacteria. I. Experiments with beef-infusion agar basic medium. *Proc. Soc. exp. Biol., N.Y.* **51**, 88.
- STICKLAND, L. H. (1934). Studies in the metabolism of strict anaerobes (genus *Clostridium*). I. The chemical reactions by which *Cl. sporogenes* obtains its energy. *Biochem. J.* **28**, 1746.
- TANNER, F. W. (1917). Studies of the bacterial metabolism of sulphur. I. Formation of hydrogen sulphide from certain sulphur compounds under aerobic conditions. *J. Bact.* **2**, 585.
- TARR, H. L. A. (1934). The enzymic formation of hydrogen sulphide by certain heterotrophic bacteria. II. *Biochem. J.* **28**, 192.
- TILLEY, F. W. (1923*a*). Variations in hydrogen sulphide production by bacteria. *J. Bact.* **8**, 115.
- TILLEY, F. W. (1923*b*). The relation between chemical composition of peptones and hydrogen sulphide production by bacteria. *J. Bact.* **8**, 287.
- WILSON, W. J. (1922-3). Reduction of sulphites by certain bacteria in media containing a fermentable carbohydrate and metallic salts. *J. Hyg., Camb.* **21**, 392.
- WILSON, W. J. & BLAIR, E. M. McV. (1924). The application of a sulphite glucose iron agar medium to the quantitative estimation of *B. welchii* and other reducing bacteria in water supplies. *J. Path. Bact.* **27**, 119.
- WILSON, W. J. & BLAIR, E. M. McV. (1927). Use of glucose bismuth sulphite iron medium for the isolation of *B. typhosus* and *B. proteus*. *J. Hyg., Camb.* **26**, 374.
- ZOBELL, C. E. & FELTHAM, C. B. (1934). A comparison of lead, bismuth, and iron as detectors of hydrogen sulphide produced by bacteria. *J. Bact.* **28**, 169.

(Received 9 August 1956)