Hydrogen Sulphide Production by Non-multiplying Organisms and its Inhibition by Antibiotics

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SUMMARY: Hydrogen sulphide production by washed bacterial suspensions in buffered substrate solutions was examined. The suspensions were tested for H₂S production from cysteine, cystine, homocystine, methionine, mercaptoacetate, sulphite, sulphate or thiosulphate. Some strains of the *Proteus*, *Klebsiella*, *Salmonella*, *Arizona* and *Bethesda* groups produced hydrogen sulphide when 10¹¹ or more washed organisms/ml. were incubated at 37° without an added source of sulphur. Generally tenfold greater quantities of bacteria were required to produce hydrogen sulphide within 24 hr. from homocystine, sulphite or thiosulphate as compared with cystine or cysteine. For the production of hydrogen sulphide from sodium mercaptoacetate, 10- and 100-fold larger quantities of bacteria were required. In the *Brucella* group all strains behaved alike, except two non-smooth strains which were spontaneously agglutinated in the buffer+substrate solutions and did not produce hydrogen sulphide. Among the *Enterobacteriaceae* the most active strains belonged to the *Proteus* group, the most inactive strains to the Large-Sachs group. All strains of the *Arizona* and *Bethesda* groups, and some *Klebsiella*, *Salmonella*, *Serratia* and *Providencia* strains multiplied on cysteine and cystine + buffer solutions, utilizing for growth the split products, pyruvic acid and ammonia.

Hydrogen sulphide production was inhibited by penicillin or streptomycin. Non-multiplying micro-organisms exposed to penicillin, streptomycin, aureomycin or chloramphenicol for 24 hr. at 37° remained viable, but lost their ability to produce hydrogen sulphide. This ability was regained on subculture. The hydrogen sulphide production of bacteria inactivated by penicillin was restored when the cells were removed by centrifugation, and residual penicillin destroyed by penicillinase. Addition of bacterial extracts heated at 50° or pyridoxal phosphate, separately or together to inactivated bacteria, reactivated the hydrogen sulphide production of *Proteus vulgaris*. Bacterial extracts heated at 100° exerted slight reactivating effects.

Hydrogen sulphide production by multiplying and non-multiplying bacteria has been described by many authors (see Clarke, 1953). Mention should be made of some investigations on factors which inhibit or stimulate the production of hydrogen sulphide. Beijerinck (1901) was the first to determine that many micro-organisms, among them *Escherichias* and *Klebsiellas*, were able to produce hydrogen sulphide from sulphite, thiosulphate or elementary sulphur, and that these reactions were more marked under anaerobic conditions. A further observation of Beijerinck (1904) was the 'indirect formation of sulphides' by *Escherichia* and *Klebsiella* spp., i.e. the biosynthesis of sulphur-containing organic compounds from sulphate as S source and the production of sulphides by their decomposition. Braun & Silberstein (1942) reported that hydrogen sulphide production from cystine, thiosulphate or elementary sulphur by *Enterobacteriaceae* was stimulated by the presence of glucose; the reaction

was inhibited by pyruvate. Kallio & Porter (1950) observed the anaerobic breakdown of cysteine to pyruvate, ammonia and hydrogen sulphide by *Proteus vulgaris* and described the reactivation of toluene-treated cells by the addition of boiled cell extract.

Kallio (1951) demonstrated the stimulation of the removal of H_2S from cysteine in *Proteus* sp. by pyridoxal phosphate, and Delwiche (1951) the stimulation of the same reaction in *Escherichia coli* by adenosine-5-phosphate, pyridoxal phosphate, biotin or α -ketoglutarate, and its inhibition by phosphoglyceric acid, serine, alanine or semicarbazide. Özek (1951) found that in the presence of different carbohydrates and alcohols different quantities of hydrogen sulphide were produced by the same strain. Skariton-Loewenthal, Rappaport & Olitzki (1953) found that hydrogen sulphide production by *Salmonella enteritidis* on Kligler's iron agar (Difco) was inhibited by sodium biselenite and penicillin in concentrations which did not damage the vital functions of the bacterial cells.

EXPERIMENTAL

The organisms were grown for 2 days in Roux bottles on nutrient agar, then removed with saline, washed twice by centrifugation and resuspended in saline; 0.5 ml. of the required bacterial suspension was added to 0.5 ml. of the buffer + substrate solution (0.15 % K_2HPO_4 ; 0.05 % KH_2PO_4 ; 0.5 % NaCl; substrate). Inorganic substrates were added in a concentration of 0.5 %; organic substrates in a concentration of 0.1 % or, if not soluble at this concentration, at saturation at 37°. The pH value of the mixtures was adjusted to 7.2. All experiments were carried out in 75 × 11 mm. test tubes firmly closed by rubber stoppers after insertion of lead acetate paper. The bacteria + substrate mixtures were incubated at 37° and the beginning of H_2S production noted when blackening of the paper started. Observations were continued for 48 hr., although strongly positive reactions appeared in less than 1 hr.

The formation of soluble sulphides was tested by addition of NaOH and a reagent consisting of 0.1 % lead acetate, 0.15 % acetic acid and 0.25 % gum-arabic (Delwiche, 1951). This reagent occasionally gave weakly positive results when the reaction on lead acetate paper was completely negative. It did not give, however, satisfactory results in the presence of mercaptoacetate since by the mere addition of NaOH, hydrogen sulphide was liberated from this substance. The results were usually first read after 30 min. and then every 2 hr. until 6 hr. from the start of the experiment; 24 and 48 hr. readings were also taken. The velocity of the reaction was graded as follows: 3, positive reaction after a few hours; 2, positive at 24 hr.; 1, positive at 48 hr.; 0, negative reaction at 48 hr.

Another method used to measure relative activity of H_2S production was to dilute the original bacterial suspension containing 10^{11} organisms/ml. in two-fold dilutions and to determine the minimal dilution able to produce a positive reaction within 24 hr. The lowest limit found was 10^8 to 10^9 micro-organisms/ml. This method, however, revealed that certain Enterobacteriaceae were able to grow on cysteine or cystine + buffer solutions even with inocula of only 10 or

100 organisms/ml. Growth and H_2S production were manifested by an increasing turbidity of the medium and a slowly increasing blackening of the lead acetate paper.

In all experiments a control tube was added which contained buffer solution without any sulphur-containing substrate and an inoculum of 10^{11} organisms/ml. This control was necessary since heavy suspensions of certain strains of the *Proteus*, *Salmonella*, *Klebsiella* and paracolon groups evolved hydrogen sulphide even in the absence of an added sulphur-containing compound, by the decomposition of their own sulphur-containing organic substances, as observed by Beijerinck (1904).

RESULTS

Hydrogen sulphide production by Brucella spp.

Table 1 shows that smooth strains of all three species of *Brucella* produced hydrogen sulphide from cysteine, cystine, homocystine, mercaptoacetate, Na_2SO_3 and $Na_2S_2O_3$. On homocystine, tenfold greater inocula were required; smooth strains of *B. suis* produced H_2S on this substrate more readily than the other species. On Na_2SO_3 and $Na_2S_2O_3$ the smooth strains of *B. suis* and *B. abortus* produced H_2S more quickly than the smooth strain of *B. melitensis*. On meth-

Table 1. H_2S production by washed brucellas on six different substrates

Strains	Cystine and cysteine		Homocystine		Mercapto-acetate		Na_2SO_3 and $Na_2S_2O_3$		
	Size of inocula								
	3×10^{10}	3×10^9	3×10^{11}	3×10^{10}	3×10^{10}	3×10^9	3×10^{10}	3×10^9	3×10^8
	Relative H_2S production observed								
Smooth									
<i>B. melitensis</i> , 5	3	1-2	2	0	1	0	2	2	0-1
<i>B. melitensis</i> , 7	3	0-1	2	1	1	0	2	2	1
<i>B. suis</i> , 6	3	2	3	2	2	2	3	2	0
<i>B. suis</i> , 39	3	1-2	3	2	2	1	3	2	0-1
<i>B. abortus</i> , 19	2-3	0	2	0	2	1	3	2	0
<i>B. abortus</i> , 2308	2-3	0	2	0-1	2	1	3	2	0
Non-smooth									
<i>B. melitensis</i> , 7	0	0	0	0	2	0	2	1-2	0
<i>B. suis</i> , 6	2-3	1-2	2	2	2	1	3	2	0-1
<i>B. abortus</i> , 2308	3	1	2	1	2	1	3	1-2	0
Streptomycin resistant									
<i>B. melitensis</i> , 5	3	2	2	2	2	1	3	2	0
<i>B. suis</i> , 1*	0	0	0	0	0	0	0	0	0
<i>B. suis</i> , 2*	3	2	3	0	2	1	3	2	0
<i>B. suis</i> , 3*	2	0-1	2	0	2	1	2	1	0
<i>B. suis</i> , 4**	2	0	0	0	1	1	2	0	0
<i>B. abortus</i> , 19	3	2	2	1	2	1	3	1	0

3=strong H_2S production within 6 hr.; 2=moderate H_2S production within 24 hr.; 1=weak H_2S production 48 hr.; 0=after 48 hr. incubation H_2S not produced. *=derived from *B. suis* 6; **=derived from *B. suis* 39.

ionine only 4 strains, and on Na_2SO_4 only 3 strains out of 15 showed delayed weak reactions after 48 hr. when 3×10^{11} organisms/ml. were used. The non-smooth strains were strongly agglutinated in the buffer + substrate mixtures, particularly those which contained cysteine or homocystine. The non-smooth *B. melitensis* strain did not produce H_2S from these substrates. One of 4 streptomycin-resistant *B. suis* strains (non-smooth) lost completely its desulphhydrase activity for all substrates and another lost its activity for homocystine.

Hydrogen-sulphide production by Enterobacteriaceae

Tables 2 and 3 show that *Proteus* contains the most active H_2S -producing species. All four species (31 strains) produced H_2S from almost all substrates examined, and only relatively small inocula were needed to yield a positive reaction within 24 hr. No *Proteus* strains tested were able to grow on the substrate + buffer mixture alone.

Table 2. H_2S production by different species

Organism	No. of strains examined	Inoculum: 10^{11} organisms/ml. No. of strains producing H_2S within 24 hr. on				
		Cysteine and cystine	Homo-cystine	Mercapto-acetate	Na_2SO_3	$\text{Na}_2\text{S}_2\text{O}_3$
<i>Proteus vulgaris</i>	15	15	15	7	15	15
<i>P. mirabilis</i>	6	6	6	4	6	6
<i>P. rettgeri</i>	5	5	2	1	5	1
<i>P. morganii</i>	5	5	5	2	5	2
Providence group	6	6	6	6	5	6
<i>Klebsiella</i> spp.	12	12	8	9	12	12
<i>Salmonella</i> spp.	12	12	4	6	9	10
Arizona group	5	5	2	2	5	2
Bethesda-Ballerup	6	6	2	2	3	2
<i>Serratia</i> spp.	2	2	2	0	2	2
<i>Escherichia</i> spp.	10	10	0	0	8	8
<i>Alkalescens-dispar</i>	6	6	6	2	6	6
<i>Shigella flexneri</i>	6	6	0	0	0	2
<i>S. boydii</i>	8	8	3	0	2	3
<i>S. sonnei</i>	3	3	2	0	2	2
<i>S. dysenteriae</i> type 2 (Schmitz)	2	2	1	0	0	1
<i>S. dysenteriae</i> type 1 (Shiga)	5	5	2	0	0	0
Large-Sachs group	4	4	0	0	0	0

Another group of organisms also proved to be very active, but these were able to grow on cysteine or cystine. All strains of the Arizona and Bethesda-Ballerup groups grew when inocula of *c.* 10 organisms/ml. were used; other groups (*Providence*, *Salmonella*, *Klebsiella*, *Serratia*) contained some strains which were able to grow on cystine or cysteine. Among the salmonellas four organisms grew on cystine or cysteine as sole source of nitrogen, sulphur and energy, namely *Salmonella paratyphi-B*, *S. cholerae-suis*, *S. typhi-murium* and *S. enteritidis*. All these organisms were also able to grow on plain agar plates to which were added only pyruvate and ammonium sulphate. According to

Kallio & Porter, pyruvate, ammonia and H_2S are the enzymic split-products of cysteine. It seems, therefore, that these organisms were able to decompose cystine and cysteine and to use the products for growth. The *Proteus* strains did not grow in cysteine + buffer solution or in pyruvate + ammonium agar, but they grew when 1.0 μg . nicotinamide/100 ml. was also added.

Table 3. *Minimal inocula required for hydrogen sulphide formation within 24 hr.*

Organism	Substrate				
	Cysteine and cystine	Homocystine	Mercaptoacetate	Na_2SO_3	$Na_2S_2O_3$
	Minimal inocula (cells/ml.)				
<i>Proteus vulgaris</i>	2.5×10^8	2×10^9	10^{10}	2×10^9	3×10^9
<i>P. mirabilis</i>	1.5×10^8	5×10^8	10^{10}	2×10^9	4×10^9
<i>P. rettgeri</i>	2.5×10^8	4×10^9	10^{10}	3×10^9	1×10^{10}
<i>P. morgani</i>	3.5×10^8	2×10^9	10^{10}	5×10^9	1×10^{10}
Providence	G or 2.5×10^8	4×10^9	10^{10}	1×10^{10}	6×10^9
<i>Klebsiella</i> spp.	G or 2.5×10^8	1×10^{10}	10^{10}	8×10^9	9×10^9
<i>Salmonella</i> spp.	G or 6.0×10^9	1×10^{10}	10^{11}	4×10^{10}	2×10^{10}
Arizona	G	1×10^{10}	10^{10}	1×10^{10}	1×10^{10}
Bethesda-Ballerup	G	6×10^{10}	10^{10}	1×10^{10}	1×10^{10}
<i>Serratia</i> spp.	G or 1.0×10^9	1×10^{10}	N	1×10^{11}	1×10^{10}
<i>Escherichia</i> spp.	7.0×10^8	N	N	3×10^9	4×10^9
Alkalescens-dispar	1.0×10^9	1×10^{10}	10^{11}	4×10^{10}	1×10^{10}
<i>Shigella boydii</i>	4.5×10^{10}	1×10^{11}	N	1×10^{11}	1×10^{11}
<i>S. sonnei</i>	7.5×10^8	1×10^{11}	N	1×10^{11}	5×10^{10}
<i>S. flexneri</i>	1.0×10^{11}	N	N	N	1×10^{11}
<i>S. dysenteriae</i> , type 2	1.0×10^9	1×10^{11}	N	N	1×10^{11}
<i>S. dysenteriae</i> , type 1	7.0×10^9	1×10^{11}	N	N	N
Large-Sachs	7.5×10^{10}	N	N	N	N

G = growth on the substrate + buffer solution;

N = H_2S formation not observed after an incubation of 48 hr. with an inoculum of 10^{11} micro-organisms.

It should be mentioned that H_2S production by the bacteria on the substrate + buffer solution was not paralleled by their ability to produce H_2S when grown in Kligler's iron agar (Difco). *Salmonella paratyphi-A* showed almost the same activity on the buffer + substrate solution as did *S. paratyphi-B*, but on Kligler's iron agar only *S. paratyphi-B* yielded a positive H_2S reaction. A similar observation was made with three streptomycin-resistant strains of *S. typhi*: on Kligler's iron agar they lost completely their ability to produce hydrogen sulphide (cf. Seligmann & Wassermann, 1947) but in buffer + cystine, cysteine, mercaptoacetate, sulphite or thiosulphite they were almost as active as their streptomycin-sensitive parent strain.

With the *Escherichia* and *alkalescens-dispar* groups and the species *Shigella flexneri*, *S. boydii*, *S. sonnei* and *S. dysenteriae* type 2, relatively large inocula were required to produce H_2S from cystine or cysteine; no strain grew on these compounds, few strains produced H_2S from homocystine (inoculum of 10^{11} organisms/ml.), and only two strains produced H_2S from mercaptoacetate.

The least active organisms were *Shigella dysenteriae* type 1 (Shiga's bacillus) and the strains belonging to the Large-Sachs group. None produced H_2S from sulphite, thiosulphate or mercaptoacetate; two strains formed H_2S from homocystine. For H_2S production from cysteine or cystine *c.* 10^{11} organisms/ml. were required. None of the organisms belonging to the *Shigella* group produced H_2S on Kligler's iron agar.

Penicillin inhibition of H_2S formation by non-multiplying cells in the presence of substrate

Non-multiplying cells from three *Brucella* species were incubated, each with four different substrates and four different concentrations of penicillin; the results of this experiment are summarized in Table 4. In the presence of

Table 4. *Inhibition by penicillin of H_2S production by brucellas*

Strain	Substrates	Penicillin (u./ml.)	Reaction observed with inoculum of			
			1×10^{10}	5×10^9	2×10^9	1×10^9
<i>B. melitensis</i>	C	None	3	2	2	0-1
	S		3	2	2	2
	C	200	3	2	0-1	0-1
	S		3	2	2	1
	C	2,000	2	0-1	0	0
	S		2	0-1	0	0
	C	20,000	0	0	0	0
	S		0-1	0	0	0
<i>B. suis</i>	C	None	3	2-3	2-3	2
	S		3	2	2	1-2
	C	200	3	2-3	2	2
	S		3	2	2	2
	C	2,000	3	1-2	0-1	0
	S		2	2	2	0-1
	C	20,000	0	0	0	0
	S		0-1	0	0	0
<i>B. abortus</i>	C	None	2-3	1-2	1	0
	S		3	2	2	1-2
	C	200	2	1-2	1-2	0
	S		3	2	2	1-2
	C	2,000	1-2	0	0	0
	S		3	2	1-2	0
	C	20,000	0	0	0	0
	S		0	0	0	0

C=cystine or cysteine; S= Na_2SO_3 or $Na_2S_2O_3$. 3, 2, 1, 0 for relative H_2S production have the same significance as in Table 1.

20,000 units penicillin/ml. H_2S production from cysteine, cystine, sulphite or thiosulphate was completely inhibited. In the presence of 2000 units penicillin/ml. a marked decrease of H_2S production was observed when smaller inocula were used. This inhibition by penicillin was more marked with cysteine or cystine than with thiosulphate or sulphite.

After incubating two suspensions (10^{10} and 5×10^9 organisms/ml.) with

20,000 units penicillin/ml. and the above-mentioned substrates for 48 hr. the cells were removed by centrifugation, washed in buffer solution and re-suspended in the original substrate + buffer solutions without penicillin. All three organisms (*Brucella melitensis*, *B. suis*, *B. abortus*) with both sizes of inoculum showed H_2S production from cysteine or cystine but not from sulphite or thiosulphate. It seemed, therefore, that some enzyme or enzymes which acted on the latter substrates had been destroyed by penicillin, while the enzymes concerned with cysteine and cystine were only inhibited by penicillin and restored to activity when it was removed. The viability of the cells was not damaged by the exposure to penicillin.

Effect of streptomycin on H_2S production

One strain, *Brucella abortus* 2308, was found in which the cysteine desulphhydrase appeared to be more sensitive to streptomycin than the enzymes which produced H_2S from the other substrates tested. The cells were exposed to different concentrations of streptomycin and the required substrate. H_2S production from sulphite or thiosulphate remained active even in the presence of 100,000 $\mu g.$ streptomycin/ml., but H_2S production from cystine or cysteine ceased in the presence of 1000 $\mu g.$ /ml. (Table 5). The cells were allowed to

Table 5. *Inhibition of H_2S production by Brucella abortus 2308 by streptomycin*

Streptomycin ($\mu g.$ /ml.)	Substrates: cysteine and cystine.		
	Inoculum size (cells/ml.)		
	10 ¹⁰	5 × 10 ⁹	2 × 10 ⁹
	Relative H_2S production		
None	3	3	2
10	3	3	2
100	2	2	2
1,000	1	0	0
10,000	0	0	0
100,000	0	0	0

remain in contact with substrate + 100,000 $\mu g.$ streptomycin/ml. for 48 hr., then removed by centrifugation, washed in buffer solution and resuspended in the substrate-buffer solution; H_2S was then no longer produced. A similar experiment was carried out with *Salmonella typhi* O901 and three streptomycin-resistant mutants derived from it. The results, summarized in Table 6, showed that washed suspensions of strain O901 and its three streptomycin-resistant mutants produced H_2S from cysteine; on Kligler's iron agar, only the original strain produced H_2S , while the streptomycin-dependent mutants failed to do so. The desulphhydrase activity of all four strains was markedly inhibited by 20,000 $\mu g.$ streptomycin/ml. When the cells were removed by centrifugation, washed and again resuspended in the cysteine + buffer solution, H_2S production was not restored, while in a control abundant H_2S production occurred.

Table 6. *Inhibition by streptomycin of H₂S production by Salmonella typhi* O901 and three streptomycin-resistant mutants

		Substrate: cysteine.					
		Inoculum size (cells/ml.)					
Streptomycin (μ g./ml.)	Strain	Relative H ₂ S production					
		2×10^{11}	1×10^{11}	5×10^{10}	2×10^{10}	1×10^{10}	5×10^9
None	O901 R1	3	2	2	1	0	0
	O901 R2	3	2	2	1	0	0
	O901 R3	3	2	2	0	0	0
	O901 Orig.	3	3	3	3	2	1
2,000	O901 R1	2	1	0	0	0	0
	O901 R2	2	1	1	0	0	0
	O901 R3	2	2	0	0	0	0
	O901 Orig.	3	3	3	3	0	0
20,000	O901 R1	1	0	0	0	0	0
	O901 R2	2	1	1	0	0	0
	O901 R3	1	0	0	0	0	0
	O901 Orig.	3	3	2	0	0	0
20,000; after 48 hr. cells washed and resuspended in cysteine buffer	O901 R1	0	0	0	0	0	0
	O901 R2	1	1	0	0	0	0
	O901 R3	1	1	0	0	0	0
	O901 Orig.	1	0	0	0	0	0

Inactivation of the hydrogen sulphide-producing enzymes by exposure

In the earlier experiments bacteria had been brought in contact with sulphur-containing substrates and antibiotics simultaneously. It was therefore not clear whether the antibiotics inactivated the H₂S-producing enzymes or whether the sulphur-containing substrates prevented the action of the antibiotics. To exclude any mutual influence the technique in the following experiments was changed. The bacteria were exposed for 24 hr. at 37° to different concentrations of antibiotics, washed twice in saline and then resuspended in the buffer + substrate solution. The experiments presented in Table 7 show that an exposure to 20,000 units penicillin/ml. inactivated the H₂S-producing enzymes of all strains under experiment. Aureomycin (1.0 mg./ml.) acted in the same way, but did not prevent hydrogen sulphide production from thio-sulphate by *Brucella suis*. Chloramphenicol (1.0 mg./ml.) or streptomycin (0.1 mg./ml.) prevented H₂S-production by *B. melitensis* and two *Salmonella typhi* strains, while *Brucella abortus* and *B. suis* remained active.

Reactivation of H₂S-producing enzymes by subcultivation

The experiment presented in Table 8 was designed to show whether this inactivation was permanent or whether the H₂S-producing enzyme could be reactivated by subcultivation of the inactivated bacteria. The bacteria (two *Salmonella typhi* strains) were exposed to inactivating concentrations of antibiotics and then subcultured on plain agar, on casein hydrolysed by acid with or without 0.01 % cysteine, or with 0.05 % sodium thiosulphate. After one or

two subcultures the H_2S -producing activity was again restored. Before the subcultures were started, all bacterial suspensions were counted by plating. Suspensions of *S. typhi* strains O901 and Ty2 contained almost the same number of viable organisms whether or not they had been kept in buffer

Table 7. *Inactivation of hydrogen sulphide producing enzymes by exposure of bacteria to antibiotics*

Antibiotic	Concentration (mg./ml.)	Substrate									
		Cysteine					Na ₂ S ₂ O ₃				
		Strain									
		A	B	C	D	E	A	B	C	D	E
		Relative H ₂ S production									
Aureomycin	1.0	0	0	0	0	0	0	3	0	0	0
	0.1	0	3	3	0	3	0	3	3	0	2
Chloramphenicol	1.0	0	3	3	0	0	0	3	3	0	0
	0.1	0	3	3	0	2	0	3	3	0	0
Streptomycin	0.1	0	3	3	0	0	0	3	3	0	0
	0.01	3	3	3	3	3	3	3	3	0	2
	0.001	3	3	3	3	3	3	3	3	3	3
Penicillin	(u./ml.)										
	20,000	0	0	0	0	0	0	0	0	0	0
	2,000	2	0	0	3	3	0	3	3	0	0
	200	3	3	2	3	3	3	3	3	3	3

Bacteria = 10^{11} ml. A = *B. melitensis*; B = *B. suis*; C = *B. abortus*; D = *S. typhi* (O901); E = *S. typhi* (Ty2).

solution or exposed to antibiotics; the respective counts for the two strains were 6.5×10^{11} and 8.0×10^{11} organisms/ml. This experiment proved, therefore, that although the antibiotics did not decrease the viability of resting cells, they damaged their H_2S -producing enzymes.

Inactivation of the anti-desulphhydrase activity of penicillin by penicillinase

Penicillin (25,000 units/ml.) was left in contact for different periods with different concentrations of penicillinase (Nutritional Biochemicals Corporation, Cleveland, Ohio). Bacterial suspensions of 10^{11} organisms/ml. were exposed for 24 hr. to the products of this reaction. The organisms were removed from the penicillin + penicillinase mixture by centrifugation, washed in buffer solution and resuspended in cysteine + buffer solution. The results obtained with *Proteus vulgaris* and *Brucella abortus* (strain 19) are summarized in Table 9 which shows that a contact of 25,000 units penicillin/ml. with 0.2 % penicillinase for 2 hr. was long enough to inactivate the anti-desulphhydrase activity against *Proteus vulgaris*, while for *Brucella abortus* even 0.1 % penicillinase was sufficient. No further changes occurred when the activity of the penicillin and penicillinase mixtures was tested after 4 hr. contact.

Table 8. *Reactivation of hydrogen sulphide production by subculture on antibiotic-free medium*

Strain tested	Antibiotic (concentration/ml.)	Effect of 24 hr. exposure, H ₂ S production on		No. of subcultures required for reactivation on medium							
		Cyst-eine (C)	Na ₂ S ₂ O ₃ (T)	I		II		III		IV	
				C	T	C	T	C	T	C	T
<i>S. typhi</i> O901	Penicillin 20,000 u.	0*	0*	1	1	1	2	1	1	1	1
	Streptomycin 0.2 mg.	0	0	1	2	2	2	2	2	2	1
	0.02 mg.	2	0	.	2	.	1	.	1	.	1
	Aureomycin 1.0 mg.	0	0	1	2	2	2	2	2	2	2
	0.1 mg.	0	0	1	2	1	2	1	2	2	2
	Chloramphenicol 1.0 mg.	0	0	1	2	1	2	1	1	1	1
	Control (Buffer)	3	3	.	.	.	.	.	.	.	.
	Penicillin 20,000 u.	0	0	1	1	1	2	1	1	1	1
	Streptomycin 0.2 mg.	0	0	1	2	2	2	2	2	2	2
<i>S. typhi</i> Ty2	Aureomycin 1.0 mg.	0	0	1	2	2	2	2	2	2	2
	0.1 mg.	0	0	1	2	1	2	1	2	1	1
	Chloromycetin 1.0 mg.	0	0	1	2	1	2	1	1	1	1
	Control (Buffer)	3	3	.	.	.	.	.	.	.	.
	Penicillin 20,000 u.	0	0	1	1	1	2	1	1	1	1
	Streptomycin 0.2 mg.	0	0	1	2	2	2	2	2	2	2
	Aureomycin 1.0 mg.	0	0	1	2	2	2	2	2	2	2

Medium I=plain agar; medium II=acid hydrolysed casein; medium III=as II with cysteine 0.01 %; medium IV=as II with Na₂S₂O₃ 0.05 %.

* Figures in these columns have the same significance as in Table 1.

C=cysteine as substrate; T=thiosulphate as substrate.

Table 9. *Inactivation of anti-desulphhydrase activity of penicillin by penicillinase*

Test performed with *B. abortus* and *P. vulgaris* on cysteine (10¹¹ cells/ml.).

Time of reaction (hr.)	Test strain	Concentration of penicillinase				
		0.5 %	0.2 %	0.1 %	0.05 %	0 (control)
		Relative H ₂ S production after contact with the product of the penicillin-penicillinase reaction				
2	<i>P. vulgaris</i>	2	2	0	0	0
	<i>B. abortus</i>	2	2	2	0	0
4	<i>P. vulgaris</i>	2	2	0	0	0
	<i>B. abortus</i>	2	2	2	0	0

The minimal time of contact with penicillin required for the inactivation of bacterial desulphhydrases

Organisms (10¹¹/ml.) were exposed for different times to 25,000 units penicillin/ml. at 37°, centrifuged, washed in buffer solution, resuspended in substrate+buffer solution and examined for H₂S production. The results,

summarized in Table 10, show that time intervals from 5 to 20 hr. had to elapse before complete inactivation was reached. After an exposure to penicillin for 20 hr. all five organisms were completely inactivated.

Table 10. *Inactivation of H₂S formation by bacterial suspensions exposed to penicillin*

Suspensions: 10¹¹ cells/ml. Penicillin 25,000 u./ml. Temp. 37°.

Substrate tested	Exposure (hr.)	Relative H ₂ S production observed with strain				
		A	B	C	D	E
Cysteine	2.5	2	3	3	3	3
	5.0	1	3	3	3	1
	10.0	0	3	3	3	0
	15.0	0	2	2	0	0
	20.0	0	0	0	0	0
	Control not exposed	3	3	3	3	3
Sodium thiosulphate	2.5	0	2	2	3	3
	5.0	0	2	0	3	3
	10.0	0	0	0	3	3
	15.0	0	0	0	0	0
	20.0	0	0	0	0	0
	Control not exposed	1	3	2	3	3

A=*S. typhi*; B=*P. vulgaris*; C=*P. morganii*; D=*B. abortus* (strain 19); E=*B. abortus* (strain 2308).

Reactivation of the desulphhydrase activity by penicillinase

The experiments described above showed that under the conditions used, the anti-desulphhydrase action of penicillin was destroyed by penicillinase within 2–4 hr. An attempt was made to determine the minimal time of contact between penicillin and bacteria required to inactivate the desulphhydrase, by addition of penicillinase at different times after mixing penicillin and bacteria. The addition of the penicillinase effected a reactivation of the bacterial desulphhydrases while washing left the bacteria inactive.

Table 11 shows that the bacterial desulphhydrase activity was not reactivated by addition of the substrate to the penicillin + bacteria mixture or by washing and resuspension in the substrate + buffer solution. However, when substrate + penicillinase were added, some traces of hydrogen sulphide

Table 11. *Penicillinase reactivation of penicillin-inactivated suspensions*

Suspension: 10¹¹ bacteria/ml. exposed 20 hr. to 25,000 u. penicillin/ml.

Treatment of the bacteria after 24 hr. exposure to penicillin	Relative H ₂ S production of		
	<i>S. typhi</i>	<i>P. vulgaris</i>	<i>B. abortus</i>
(1) Cysteine and penicillinase (0.5 %) added	1	1	1
(2) Cysteine added	0	0	0
(3) Bacteria washed, then cysteine and penicillinase (0.5 %) added	2	2	2
(4) Bacteria washed, then cysteine added	0	0	0

appeared after 48 hr. When the bacteria were washed and the substrate and penicillinase added, the reactivation was complete and within 24 hr. hydrogen sulphide was produced. It seems, therefore, that centrifugation and washing alone did not remove all traces of penicillin. On the other hand, washing followed by penicillinase treatment reactivated the bacterial cells, proving that the changes exerted by penicillin were reversible. Washing alone, even repeated five times, did not exert this effect.

Since the penicillinase solutions were partly inactivated by filtration through Seitz filters, we compared in the following experiments the reactivating action of different concentrations of both filtered and non-filtered penicillinase solutions. Table 12 shows that with 0.2 and 0.1 % non-filtered, and with 0.5 and 0.2 % filtered solutions of penicillinase no reactivation of cysteine desulphhydrase was obtained unless the bacteria were washed and then brought in contact with penicillinase + cysteine. Similar results were obtained with sodium thiosulphate instead of cysteine. Higher concentrations of non-filtered penicillinase solutions were not employed, since higher concentrations of this enzyme preparation alone when brought in contact with cysteine themselves produced hydrogen sulphide after 48 hr. In order to exclude this source of error the maximal concentration used was 0.2 %.

Table 12. *Reactivating effect of different types of penicillinase solutions*

Suspensions 10^{11} bacteria/ml. inactivated by 25,000 u. penicillin/ml.

Treatment after inactivation	Penicillinase		Relative H ₂ S production by strain				
	Type	Concentration (%)					
			A	B	C	D	E
Penicillinase added	Not filtered	0.2	0	0	1	0	0
		0.1	0	0	0	0	0
	Filtered	0.5	0	0	0	0	0
		0.2	0	0	0	0	0
Bacteria washed and penicillinase added	Not filtered	0.2	2	2	2	2	2
		0.1	1	2	2	2	1
	Filtered	0.5	0	2	2	2	0
		0.2	0	2	1	2	0

A = *S. typhi*; B = *P. vulgaris*; C = *P. morganii*; D = *E. coli*; E = *B. abortus* (strain 19).

Reactivation of the desulphhydrase activity by bacterial extracts

Two bacterial extracts, using McIlwain's (1948) method, were prepared from *Proteus vulgaris* and *Brucella abortus* (strain 19). The respective protein content of the extracts was 6.8 and 5.6 mg./ml. When varying quantities of each extract were brought in contact with cysteine or sodium thiosulphate, hydrogen sulphide was produced as follows:

Extract concentration (%)	...	50	20	10	5	2
Proteus extract						
Cysteine		3	1	1	1	0
Sodium thiosulphate		3	1	0	0	0
Brucella extract						
Cysteine		1	1	1	1	0
Sodium thiosulphate		0	0	0	0	0

In order to avoid error from this source of hydrogen sulphide production, the extracts were added to the inactivated and washed bacteria in 1 % concentrations on cysteine, and 10–20 % on sodium thiosulphate.

As in the earlier experiments bacterial suspensions containing 10^{11} organisms/ml. were left in contact at 37° with 25,000 units penicillin/ml. for 24 hr. The bacteria were then centrifuged, washed and resuspended in the substrate + buffer solutions, and bacterial extracts were added.

The low concentrations of extract only caused a slight reactivation of the hydrogen sulphide production by penicillin-inactivated bacteria (Table 13). This desulphhydrase activity was eliminated by heating extracts at 50° for 30 min. while their reactivating power on inactivated bacteria was still intact. $10\text{ }\mu\text{g./ml.}$ of pyridoxal phosphate also exerted a slight reactivating activity.

Table 13. *The reactivation of penicillin-inactivated suspensions*

Suspensions 10^{11} /ml. inactivated by 25,000 u./ml.

Extracts from	Concentra- tion (%)	Substrate tested	Relative H_2S production by strains			
			A	B	C	D
<i>B. abortus</i>	1.0	Cy.	0	0	0	1
	20.0	T.	0	0	1	3
<i>P. vulgaris</i>	1.0	Cy.	0	0	1	1
	10.0	T.	1	0	1	1
Controls without extract		Cy.	0	0	0	0
		T.	0	0	0	0

A = *P. vulgaris*; B = *P. morganii*; C = *E. coli*; D = *B. abortus* (strain 19). Cy. = cysteine; T. = Sodium thiosulphate.

These two activating factors, extracts heated at 50° for 30 min. and pyridoxal phosphate, were employed in the following experiments. Since the desulphhydrase activity of the extract was inactivated, 20 % concentrations were used for the reactivation of the bacteria. The bacterial inocula and the concentration of penicillin employed for inactivation were the same as in the experiment shown in Table 13. Table 14 summarizes an experiment of this type. Pyridoxal phosphate exerted only a slight reactivating effect on the bacteria tested on cysteine. In comparison with this weak action, the reactivation by the bacterial extract was much stronger and in the presence of both extract and pyridoxal phosphate, hydrogen sulphide production was accelerated.

The next experiment was carried out with *Proteus vulgaris*. Extract was prepared from *Brucella abortus* and was heated at 50° for 1 hr. The reactivation was tested on cysteine and different inocula and different concentrations of the two reactivating factors were employed. The inactivation of the bacteria was carried out as in the earlier experiments.

A control showed that the extract and pyridoxal phosphate either separately or together did not produce hydrogen sulphide on cysteine + buffer solution without the inactivated bacteria. The experiment summarized in Table 15 showed that inocula of 10^{11} and 10^{10} organisms/ml. were reactivated by

2.5–20 $\mu\text{g./ml.}$ of pyridoxal phosphate, while this effect was not produced with an inoculum of 10^9 organisms/ml.

Table 14. *The reactivating effect of heated proteus extract and of pyridoxal phosphate*

Suspensions 10^{11} /ml. inactivated by 25,000 u. penicillin/ml.			
Quantities added			Relative H_2S production by strain
Heated <i>P. vulgaris</i> extract (%)	Pyridoxal phosphate ($\mu\text{g./ml.}$)	Substrate tested	
20	0	Cy.	3
20	10	Cy.	3
20	0	T.	2
20	10	T.	3
0	0	Cy.	0
0	10	Cy.	1
0	0	T.	0
0	10	T.	0

Strains A–D: the same as in Table 13. Cy. = cysteine; T. = sodium thiosulphate.

Table 15. *Reactivation of inactivated P. vulgaris suspensions by pyridoxal phosphate and B. abortus extract*

Concentrations of the brucella extract (%)	<i>Proteus vulgaris</i> suspension (cells/ml.)					
	10^{11}		10^{10}		10^9	
	P.	NP.	P.	NP.	P.	NP.
	Relative H_2S production observed					
20	3	2	3	2	3	3
10	3	2	2	2	3	2
5	2	1	2	1	2	1
2	2	1	2	1	2	1
1	2	0	2	0	2	1
Control without extract	2	0	2	0	0	0

P. = 2.5–20 $\mu\text{g./ml.}$ pyridoxal phosphate added; NP. = pyridoxal phosphate not added.

In higher concentrations of the bacterial extract and in the absence of pyridoxal phosphate, all inocula produced hydrogen sulphide within 24 hr., while the lower concentrations were relatively inactive and did not exert much effect. It seems, therefore, that for a complete reactivation of small inocula of *Proteus vulgaris* (10^9 organisms/ml.) two different methods may be employed: high concentrations of bacterial extracts or low concentrations of bacterial extracts simultaneously with pyridoxal phosphate. However, the reactivation of suspensions containing 10^{10} – 10^{11} organisms/ml. can also be effected by pyridoxal phosphate alone.

Suspensions containing 10^{11} organisms/ml. were kept in the ice-box for 14 days to autolyse, then heated at 100° for 1 hr., and centrifuged until the supernatant fluid became completely clear. 50% concentrations of these

extracts exerted some reactivating effects. An extract prepared from *Brucella abortus* reactivated *Proteus morganii* and *Escherichia coli* on cysteine and sodium thiosulphate, but failed to reactivate *Salmonella typhi* and *Brucella abortus*. While these reactivating effects were observed, the extracted bacterial suspensions failed to exert such an effect.

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