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Production of Hydrogen Sulfide from Sulfur-Containing Compounds by Various Bacteria. II. Experiments with Synthetic Medium.

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In the first paper of this series[1] the production of hydrogen sulfide by various bacteria from several sulfur-containing compounds added to beef infusion agar was investigated. Because traces of hydrogen sulfide were often formed by many of these organisms from beef infusion agar without supplements, it appeared desirable to extend these studies further, employing synthetic medium of known composition and purity. Such an approach would eliminate the interfering influences of substances present in the beef infusion agar, and permit an unequivocal interpretation of the results obtained.

Methods. The compounds employed were the same as those described previously[1,2] and the concentrations used are shown in Table I. The composition of the basic medium was as follows: asparagine, 3.0 g; glucose, 1 g; K_2HPO_4, 0.5 g; $MgCl_2$, 0.5 g; KCl, 0.2 g, and distilled water, 1000 cc. The pH was adjusted to 7.8, and the solution autoclaved 15 min at 15 lb. pressure and 120°C. The weighed substances were added to the medium which was then heated in an Arnold sterilizer under nitrogen atmosphere for 1 hr. After cooling, 3 cc of sterile phosphate buffer pH 7.8 were added to each 100 cc of supplemented medium. Six cc of the buffer were used in medium containing cysteine hydrochloride. The bacteria employed were 3 of the Vanderbilt University stock strains used in the previous study,[1] namely *Escherichia coli, Klebsiella pneumoniae* type B, *Bacillus subtilis,* and a pigment-forming laboratory strain of *Pseudomonas aeruginosa.* All grew readily in the unbuffered, unsupplemented synthetic medium, and were carried through

TABLE I.
Effect of Sulfur-Containing Compounds on Bacterial H_2S Production in Synthetic Medium. Results at 72 Hours.*

	l-Cysteine hydrochloride	S-Benzyl-*l*-cysteine	N-Benzoyl-*l*-cysteine	Cysteic acid	*l*-Cystine	N,N-Dibenzoyl-*l*-cystine	*dl*-Methionine	S-Benzyl-*dl*-homocysteine	*dl*-Homocystine	Control medium with buffer
Concentration (mg%)	84	87	92	69	50	94	51	92	56	
Bacteria studied										
E. coli	2+	0	0	0	4+	0	0	0	0	0
K. pneumoniæ, type B	4+	0	3+	0	4+	tr	0	0	0	0
B. subtilis	4+	0	0	†	4+	0	0	0	0	0
P. æruginosa	tr	0	0	†	tr	0	0	0	0	0

*tr = trace, 1+ = slight, 2+ = moderate, 3+ = marked, and 4+ = maximal H_2S production.
† = not studied.

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1 Stekol, J. A., and Ransmeier, J. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 88.

2 Ransmeier, J. C., and Stekol, J. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 85.

from 12 to 35 serial transfers prior to inoculation of the test media. The inoculum consisted of 0.1 to 0.2 cc of 24 hr culture introduced into 5 cc volumes of test media distributed in 50 cc Erlenmeyer flasks so as to expose a maximum surface to the air. Sterile strips of lead acetate paper were inserted in the tops of the flasks, the latter incubated at 37°C and examined daily for a period of 5 days. In all cases similar flasks uninoculated failed to show evolution of hydrogen sulfide except with cysteine hydrochloride. Slight but perceptible darkening of the lead acetate paper was consistently noticed with the cysteine controls, suggesting partial decomposition of cysteine under these conditions. All of the uninoculated test media remained sterile. Hydrogen sulfide was never produced by any of the organisms from the buffered unsupplemented basic synthetic medium.

Results. Media containing cysteine or cystine yielded moderate to maximal amounts of hydrogen sulfide with *E. coli, K. pneumoniae,* and *B. subtilis,* as shown in Table I. *P. aeruginosa,* gave rise to traces of the sulfide from these substances. Evolution of hydrogen sulfide was noted at 24 hr, reaching the maximal observed reading often by 48 and always by 72 hr.

Only *K. pneumoniae* produced hydrogen sulfide from N-benzoyl-cysteine, and faint traces from N,N-dibenzoyl-cystine. None of the other bacteria studied were able to liberate sulfide under these conditions.

None of the organisms formed hydrogen sulfide or mercaptan from S-benzyl-cysteine, in contrast to the results obtained when this substance was added to beef infusion agar.[1] It appears that the conditions prevailing in the beef infusion agar in some way permit desulfurization of S-benzyl-cysteine with mercaptan formation. Whether this is due to the presence in the beef infusion agar of a specific catalyst lacking in the synthetic medium cannot definitely be asserted at the present time. The possible role of the vitamins in this reaction invites investigation.

None of the bacteria tested produced hydrogen sulfide from cysteic acid, methionine, S-benzyl-homocysteine, or homocystine. These results are similar to those obtained previously with beef infusion agar.[1]

Moderate to excellent growth was observed with all the supplemented media. It appears that methionine, homocystine, and N,N-dibenzoyl-cystine stimulated growth somewhat, but lack of quantitative criteria prevents definite conclusions in this respect. It is evident that these organisms grow abundantly in synthetic medium with or without the sulfur-containing amino acids or their derivatives, and that the reactions leading to hydrogen sulfide production are not necessarily an expression of the rapidity or extent of growth. This is in contrast to the results obtained with *Bacterium tularense* where cystine, cysteine, or to a much lesser extent thioglycollic acid were found essential for growth *in vitro,* and hydrogen sulfide production always paralleled the extent of growth.[2]

A few observations on pigment formation by *P. aeruginosa* may be noted. Little or no color was produced in buffered or unbuffered unsupplemented synthetic medium, or in the presence of S-benzyl-homocysteine or choline (51 mg %). A moderate muddy brown coloration was observed in medium containing N-benzoyl-cysteine, and none or very pale greenish-yellow tint resulted with N,N - dibenzoyl - cystine. S-benzyl-cysteine yielded moderate yellow-green color. Deep bluish-green pigment was produced with cysteine, cystine, methionine, and homocystine. Apparently benzylation of homocysteine and not of cysteine prevented pigment formation. Further study is needed to elucidate the role of the sulfur-containing amino acids and their substitution products in the mechanism of pigment production by *P. aeruginosa.*

Another observation of interest is that after 34 transfers over a period of 2 months in the basic synthetic medium, *K. pneumoniae* continued to produce prominent capsules, thus confirming the findings of Hoogerheide.[3] It also retained high virulence, killing all of 27 white mice injected subcutaneously with 1 cc of 48-hr culture diluted

[3] Hoogerheide, J. C., *J. Bact.,* 1939, **38**, 367.

from 10^{-2} to 10^{-5}, 6 out of 10 receiving the 10^{-6} dilution, and 7 out of 8 receiving the 10^{-7} dilution. The latter dose contained approximately 8 organisms.

Summary. 1. Production of hydrogen sulfide by *E. coli, K. pneumoniae, B. subtilis,* and *P. aeruginosa* in synthetic media containing cysteine or methionine and their derivatives was studied. 2. With the exception of *P. aeruginosa,* all these organisms formed moderate to maximal amounts of hydrogen sulfide from cysteine and cystine. *P. aeruginosa* produced only traces.

3. Only *K. pncumoniae* produced hydrogen sulfide from N-benzoyl-cysteine. This confirms previous observations made with beef infusion agar basic medium.[1] Benzoylation of the alpha amino group of cysteine does not interfere with desulfurization by this organism, although it prevents the reaction in the case of other bacteria studied. This indicates fundamental metabolic differences prevailing in the organisms investigated with respect to this particular reaction. Additional strains of *K. pneumoniae* and other organisms should be tested under similar conditions.

4. None of the organisms formed hydrogen sulfide or mercaptan from S-benzyl-cysteine, in contrast to the results obtained when this substance was added to beef infusion agar. Hydrogen sulfide was not produced by any of the bacteria from cysteic acid, methionine, S-benzyl-homocysteine, or homocystine. 5. A limited number of observations on pigment formation by *P. aeruginosa* in the presence of cysteine, methionine, and their derivatives was made.

6. A strain of *K. pneumoniae* continued to produce large capsules and maintained high virulence for mice after many transfers in a simple synthetic medium.

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A Rapid Method for the Determination of Races of *Shigella dysenteriae* Flexner.

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Several investigators have already isolated the polysaccharide fraction of *Shigella dysenteriae.* Kurauchi[1] and Meyer and Morgan[2] isolated specific polysaccharide from the Shiga organism; Kurauchi[1] from the Flexner and Sonne. Spassky and Dannenfeldt[3] used the precipitin test for the diagnosis of *Sh. dysenteriae* infection, but failed to note the specificity of the precipitin reaction, basing their failure on the extreme complexity of the antigenic apparatus of the dysenteric bacillus.

We have tried to develop a practical method for the typing of races of *Shigella dysenteriae* Flexner by using type specific antibodies capable of reacting with the polysaccharide. Antiserum made with the classical Oxford strains did not give clear cut results. However, when freshly isolated strains were utilized, specific reaction was obtained.

Freshly isolated cultures of *Shigella dysenteriae* Flexner strains, classified as W, V, and Z, were utilized. Rabbits were immunized by intravenous injections of saline suspensions of the organisms killed by formalin. A 3-week course of graded doses of dysenteric bacilli was then given to each rabbit. Injections were applied on 3 consecutive days, followed by a rest period of 4 days before starting the next 3 doses. Eight days after the last inoculation, the ani-

[1] Kurauchi (Report of K. Ando), *J. Immunol.,* 1929, **17**, 555.

[2] Meyer, K., and Morgan, W. T. J., *J. Exp. Path.,* 1935, **16**, 476.

[3] Spassky, N. M., and Dannenfeldt, L. A., *Bul. Biol. et Méd. Exp.,* U. S. S. R., 1939, **7**, 202.