terium lacks the mechanism for effective splitting of benzyl and benzoyl radicals from the substituted amino acids. 6. Since homocystine did not permit growth or production of hydrogen sulfide by *Bact. tularense* while cystine was highly effective in both these respects, it is apparent that the length of the carbon chain as well as the availability of amino and —SH or S-S groups is of importance in the reactions involved. 7. It is likely that the enzymic potentialities for metabolism by *Bact. tularense* are relatively quite limited. Its tendency to intracellular growth and its biological position of almost if not entirely obligate parasitism suggest that it has come to depend upon its hosts to supply it with certain essential and possibly quite specific utilizable substances, among them cysteine or derivatives thereof. This is in contrast to many other pathogenic bacteria which, while able to attack cysteine with production of hydrogen sulfide, can grow equally well in the absence of this substance. Investigations with a number of such organisms are reported in the next paper.

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Production of Hydrogen Sulfide from Sulfur-Containing Compounds by Various Bacteria. I. Experiments with Beef Infusion Agar Basic Medium.*

JAKOB A. STEKOL AND JOHN C. RANSMEIER.

From the Department of Biochemistry, and the Department of Preventive Medicine and Public Health, Vanderbilt University School of Medicine, Nashville, Tenn.

Earlier work has shown that many widely differing microörganisms can produce hydrogen sulfide from cystine or cysteine.[1,2,3] On the other hand, taurine,[1] methionine, and various sulfhydryl, amino, and carboxyl substitution products of cysteine proved to be unavailable for this process.[3] In a previous communication we reported that cystine, cysteine, or to a lesser extent thioglycollic acid appeared to be essential for growth and hydrogen sulfide production by *Bacterium tularense in vitro*. Several substitution products of cystine and cysteine, as well as methionine, were found ineffective in this respect.[4] The present report deals with a similar study extended to other bacteria, some of which have not been thus investigated previously.

Methods. The compounds studied and the sources thereof were the same as those described in the previous paper,[4] and the concentrations used are shown in Table I. The compounds were dissolved in m/10 phosphate buffer pH 7.2 containing dilute NaOH as before, the volume made up to 100 cc with molten beef infusion agar, and autoclaved 12½ min at 15 lb. pressure and 120°C. After cooling to 60°C, the pH was adjusted colorimetrically to 7.1-7.2, and slants prepared. Strips of sterile lead acetate paper inserted in the tops of the tubes were used to indicate hydrogen sulfide production. The bacteria studied are listed in Table I. All cultures were incubated at 37°C for 1 week.

Some of the strains used were from stock collections of Vanderbilt University, many

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1 Bürger, Max, *Arch. f. Hyg.*, 1914, **82**, 201.

2 Tarr, H. L., *Biochem. J.*, 1933, **27**, 759.

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TABLE I.
H_2S Production by Various Bacteria from Sulfur-containing Compounds Added to Beef Infusion Agar. Results at 24 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	Beef infusion agar control
Concentration (mg%)	168	174	184	138	100	186	51	92	56	
Bacteria studied										
*E. coli**	4+	4+ Y‡	tr	0	3+	tr	tr?	tr	tr?	tr
E. coli†	4+	3+ Y-Br	tr?	tr?	4+	0	tr?	tr?	0	tr?
Proteus X-19†	4+	4+ Y-Br	2+	1+	4+	tr	1+	1+	2+	tr
Proteus OX-2†	4+	3+ Y-Br	2+	1+	4+	1+	1+	1+	2+	1+
K. pneumoniæ, type B*	2+	3+ Y	2+	0	2+	0	0	tr	0	0
E. typhosa (Brown)*	4+	3+ Y	0	0	3+	0	0	0	0	0
E. typhosa (58)†	4+	2+ Y	0	0	4+	0	0	0	0	0
E. typhosa (H-901)†	4+	3+ Y	0	0	4+	0	0	0	0	0
E. typhosa (O-901)†	4+	2+ Y	0	0	4+	0	0	0	0	0
*S. paratyphi**	4+	2+ Y	0	0	4+	0	0	0	0	0
S. paratyphi (NIH)†	4+	3+ Y	0	0	4+	0	0	0	0	0
S. paratyphi (Dilliard)†	4+	3+ Y	0	0	4+	0	0	0	0	0
S. schottmuelleri (NIH)†	4+	3+ Y-B	0	0	4+	0	0	tr	tr	0
S. enteritidis†	4+	2+ Y	0	0	4+	0	Not studied			0
S. ambigua (Quarles)*	3+	4+ Y-Br	0	0	2+	0	0	0	0	0
S. newcastle†	3+	1+ Y	0	0	3+	0	0	0	0	0
B. abortus (456)*	1+	2+ Br	1+	tr	1+	1+	1+	1+	tr	1+
*M. tuberculosis**	4+	tr Br	tr?	0	4+	0	Not studied			0
*B. subtilis**	4+	3+ Y-B	tr	0	4+	tr	0	tr	tr	0
*S. aureus**	1+	0	1+	0	1+	0	0	0	0	0
*S. aureus**	1+	tr Br	2+	0	1+	0	Not studied			0
*S. lutea**	2+	1+ Br	0	0	2+	0	0	0	0	0
Uninoculated controls	0	0	0	0	0	0	0	0	0	0

* = Vanderbilt University, † = Tenn. State Health Dept. Lab. Stock strains, NIH = originally from National Institute of Health, ‡Y = yellow, Br = brown, B = black color on lead acetate paper.

having been isolated from patients in the hospital. Others were obtained through the kindness of the Tennessee State Health Department Laboratory. The example of *Mycobacterium tuberculosis* studied was an old laboratory strain of human origin received from Dr. Roy C. Avery of the Department of Pathology. Five per cent glycerin was added to the media for its cultivation, resulting in rapid and abundant growth. Control slants of all media were streaked with a sterile loop and incubated with lead acetate paper, but spontaneous evolution of hydrogen sulfide was never observed, even after liquification and acidification with strong HCl.

Results. Excellent growth occurred on all slants. The results observed after 24 hr incubation are shown in Table I.† It will be noted that moderate to large amounts of hydrogen sulfide were produced from cysteine and cystine by all organisms with the exception of *Brucella abortus* and the two strains of *Staphylococcus aureus*. Maximal hydrogen sulfide production occurred within the first 24 hr in most cases, although with *Klebsiella*

† The readings indicated for *M. tuberculosis* were made on the 5th day, after maximum growth had been obtained.

pneumoniae further increase was noted at 48 hr, and with *Shigella ambigua* and *Shigella newcastle* maximal amounts were recorded after 1 week. In fact, one of the most interesting things about this reaction is the great rapidity with which it is initiated. Definite evidence of the evolution of hydrogen sulfide was noted in several instances,‡ for example, at room temperature 1 to 2 hr after inoculation of the slants. Similar observations with *Proteus vulgaris* were reported by Tarr.[2]

Only slight further increase in the amount of hydrogen sulfide produced from cysteine and cystine by *B. abortus* was noted up to 1 week, and with *S. aureus* no additional change occurred after 24 hr. These organisms thus appeared to differ from the rest in producing relatively small quantities of hydrogen sulfide under such conditions, while *Sarcina lutea* was only slightly more active. Nevertheless it is important to note that hydrogen sulfide was formed from cysteine and cystine slants by all bacteria tested.

A different type of reaction was noted on slants containing S-benzyl-cysteine. During the first 24 hr most of the organisms tested formed in the presence of this compound considerable amounts of a volatile substance which turned the lead acetate paper a bright yellow color. On further incubation the extent of this discoloration usually increased, with part of the paper becoming brown or black. Simultaneously the agar slants with S-benzyl-cysteine became milky and opaque in degree roughly paralleling the amount of discoloration observed on the lead acetate paper. Uninoculated control slants of the same medium invariably remained clear. At the end of 1 week this reaction was quite marked or maximal in the case of all bacteria studied except *M. tuberculosis* and 2 strains of *S. aureus*. These did not produce a definite yellow color, but caused a slight brownish discoloration of the lead acetate paper, while the slants on which they were growing remained clear and translucent. Pending identification, it would be premature to indicate the nature of the volatile product liberated from S-benzyl-cysteine, although *a priori* it seems likely that it may be benzyl mercaptan.

Few organisms produced appreciable amounts of hydrogen sulfide from slants containing N-benzoyl-cysteine. Only in the case of the 2 *Proteus* strains, the 2 strains of *S. aureus*, and *K. pneumoniae* was this definitely in excess of that observed with the beef infusion agar control. With *S. aureus* and *Proteus* the reaction did not progress beyond the point indicated at 24 hr, but with *K. pneumoniae* the amount of hydrogen sulfide produced increased somewhat up to 48 hr and was equal to that formed by this organism from slants containing cysteine or cystine. On the other hand, none of the bacteria tested liberated significant amounts of hydrogen sulfide from N,N-dibenzoyl-cystine slants.

Cysteic acid, methionine, and S-benzyl-homocysteine failed to give rise to hydrogen sulfide or mercaptan with any of the organisms studied.

The two *Proteus* strains were the only organisms which produced small but definite quantities of hydrogen sulfide from homocystine slants. Although the amounts formed appeared greater than with controls, the ability of these organisms to liberate hydrogen sulfide from beef infusion agar alone makes interpretation of this observation difficult.

After 48 hr incubation most of the bacteria studied produced minimal to slight darkening of the lead acetate paper from the control slants of beef infusion agar. At the end of 1 week the paper was unchanged only with *S. ambigua, S. newcastle, M. tuberculosis,* 1 strain of *S. aureus,* and *S. lutea.* Similar small amounts of hydrogen sulfide were often formed by many of the bacteria on prolonged incubation from slants containing compounds for which the reaction is recorded as negative or slight at the end of 24 hr. The *Proteus* strains and *B. abortus* were able to liberate a little hydrogen sulfide from all slants, even within the first 24 hr. It seems likely in such cases, except where marked excess is noted

‡ With *Eberthella typhosa* (58), (H-901), and (O-901), *Salmonella paratyphi* (NIH) and (Dilliard), *S. schottmuelleri* (NIH), and *S. enteritidis, Proteus* X-19 and OX-2.

on certain slants, that the hydrogen sulfide was formed from substances present in the beef infusion agar medium rather than from the compounds added. In order to avoid possible interference from this factor, further studies have been undertaken with synthetic media and will be reported in the next communication.

Discussion and Summary. 1. None of the 22 strains of bacteria studied failed to produce hydrogen sulfide from beef infusion agar slants supplemented with cystine and cysteine. The latter may have been partially oxidized to cystine during autoclaving and incubation. From these data we cannot state whether the reduced or oxidized form of cystine is the immediate precursor for hydrogen sulfide formation. 2. The benzylation of the sulfhydryl group of cysteine did not prevent the desulfurization of the molecule by these bacteria, with the possible exception of *M. tuberculosis* and the two strains of *S. aureus*. The volatile decomposition product of S-benzyl-cysteine which reacted with the lead acetate paper was possibly benzyl mercaptan. 3. Hydrogen sulfide was produced from N-benzoyl-cysteine slants only by *Proteus X-19* and *OX-2*, *K. pneumoniae*, type B, and the two strains of *S. aureus*, while none of the bacteria was able to metabolize N,N-dibenzoyl-cystine with hydrogen sulfide formation. It appears, therefore, that benzoylation of the alpha amino groups prevents the evolution of hydrogen sulfide from cystine. If as proposed by Desnuelle for *Escherichia coli*,[5] the reduction of cystine to cysteine is a necessary step for the production of hydrogen sulfide, it may be that blocking of the amino groups of cystine prevents the conversion of the disulfide to the sulfhydryl form. This would be contrary to the results of experiments with animals by Lewis, Updegraff, and McGinty[6] who administered N,N-dibenzoyl-cystine and recovered N-benzoyl-cysteine from the urine. Some bacteria are apparently able to use the sulfhydryl group of the benzoylated cysteine for hydrogen sulfide production.

4. None of the bacteria studied utilized the sulfur of cysteic acid, methionine, or S-benzyl-homocysteine for hydrogen sulfide or mercaptan formation. It seems that methionine is not converted to cystine or cysteine by these organisms prior to its possible utilization. Similar conclusions were reached in studies with *Bact. tularense*.[4]

5. The failure of homocystine or S-benzyl-homocysteine to yield hydrogen sulfide or mercaptan with any of the organisms excepting possibly the two *Proteus* strains is in contrast to the results obtained with cystine and S-benzyl-cysteine, and suggests that the length of the carbon chain of the sulfur-containing amino acids is one of the determining factors governing the mechanism of desulfurization.

[5] Desnuelle, P., *Enzymologia*, 1939, **6**, 80, 242, 387.

[6] Lewis, H. B., Updegraff, H., and McGinty, D. A., *J. Biol. Chem.*, 1924, **59**, 59.