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Effect of Sulfur-Containing Compounds on Growth and Hydrogen Sulfide Production by *Bacterium tularense.**

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The discovery of the growth-promoting property of cysteine and cystine for *Bacterium tularense* by Francis[1] was of great importance, and made the cultivation of this organism simple and practical. The manner in which cystine exerts its action is not, however, even today clearly understood. Francis found the amino acids tryptophane, tyrosine, histidine dihydrochloride, phenylalanine, leucine, lysine dihydrochloride, and glutamic acid hydrochloride ineffective, as were inorganic sulfur compounds such as sodium sulfite, sodium bisulfite, sodium thiosulfate, ammonium sulfate, magnesium sulfate, and also sublimed and precipitated sulfur. Later work has shown that glutathione[2] and sodium thioglycollate[3] when added to appropriate culture media are capable of supporting growth of *Bact. tularense,* so that attention became focussed on the free or potential —SH group in these substances as the possible active radical. Brigham and Rettger[4] observed that hydrogen sulfide was produced by *Bact. tularense* when growing on slants of blood glucose cystine beef infusion agar. The present

TABLE I.
Compounds Studied for Effect on Growth and Hydrogen Sulfide Production by *Bacterium tularense.*

Substance	Source of reference to method of synthesis	Amounts used per 100 cc of medium, mg	Effect: Growth	Effect: H_2S
Thioglycollic acid	Eastman Kodak Co.	66	+	+
l-Cysteine hydrochloride	Eastman Kodak Co.	168	+++	+++
S.-Benzyl-*l*-cysteine	Synthetic[5]	174	0	0
N-Benzoyl-*l*-cysteine	Synthetic[6]	184	0	0
Cysteic acid	Synthetic[7]	138	0	0
l-Cystine	Eastman Kodak Co.	100	+++	+++
N,N-Dibenzoyl-*l*-cystine	Synthetic[6]	188	0	0
dl-Homocystine	S.M.A. Corporation	112	0	0
dl-Methionine	S.M.A. Corporation	102	0	0
S-Benzyl-*dl*-homocysteine	Synthetic[8]	**184**	**0**	**0**
S-Benzyl-glutathione	Synthetic[9]	326	0	0
Dibenzyl bisulfide	Eastman Kodak Co.	104	0	0

+ = slight. +++ = moderate to fairly good. ++++ = Maximal observed on blood glucose cystine agar.

* The studies referred to herein were conducted in part under the auspices of the International Health Division of the Rockefeller Foundation by the Department of Preventive Medicine and Public Health and the Nutrition Unit of the Department of Biochemistry and the Department of Medicine of Vanderbilt University School of Medicine, Nashville, Tenn.

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3 Ransmeier, J. C., and Schaub, I. G., *Arch. Int. Med.,* 1941, **68**, 747.

4 Brigham, G. D., and Rettger, L. F., *J. Infect. Dis.,* 1935, **56**, 225.

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7 Gortner, R. A., and Hoffman, W. F., *J. Biol. Chem.,* 1927, **72**, 433.

8 du Vigneaud, V., and Patterson, W. I., *J. Biol. Chem.,* 1935, **109**, 97.

9 Stekol, J. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 108.

studies were undertaken to investigate the possible correlation between growth and the production of hydrogen sulfide by this organism in the presence of various sulfur-containing compounds.

Methods. Analytically pure compounds used in this study and the concentrations employed are shown in Table I. In these amounts the substances were equivalent in sulfur content to 0.1% cystine. The compounds were dissolved in M/10 phosphate buffer pH 7.2 with calculated amounts of dilute NaOH required to convert them to sodium salts, 1 g of glucose added, and the volume made up to 100 cc with molten beef infusion agar. The mixtures were autoclaved for 12½ min at 15 lb. pressure and 120°C. After cooling to 60°C, 5% of sterile rabbit serum was added, the pH was adjusted colorimetrically to 7.1-7.2, and the media were tubed and slanted. The slants were incubated for about 3 days until the water of syneresis had almost all evaporated, and examined for sterility. They were then inoculated heavily with a loopful of growth from a 24-hr blood glucose cystine agar culture of *Bact. tularense.* Strips of lead acetate paper sterilized by heating 10 min at 160°C were inserted in the tops of the tubes, and the slants were incubated at 37°C for one week. When growth appeared, attempts were made to transfer it on similar media. If there was no definite growth at the end of one week a bit of the heavy inoculum remaining on the original slant was transferred to a fresh slant of the same composition to see whether any adaptation to the new medium was developed. When traces of growth appeared only on the first slant these were considered possibly due to substances carried over with the inoculum. Control slants of all media were streaked with a sterile loop and incubated with lead acetate paper, but no spontaneous evolution of hydrogen sulfide was ever observed.

Five strains of *Bact. tularense* were used. "B-38" was obtained through the kindness of Dr. Edward Francis of the National Institute of Health, who isolated it from a patient in 1920 and maintained it by transfer on blood glucose cystine agar. It is now considered non-virulent. "V-13" was recovered by Dr. G. John Buddingh from the spleen of a patient dying of tularemia at Vanderbilt University Hospital in February 1940. It was virulent when the first studies were made, but during the course of our work it lost the ability to kill mice and chick embryos as described elsewhere.[10] The "Conn" and "Pack" strains were likewise obtained from Francis, and the former was found highly virulent for mice and chick embryos, as was the "Bishop" strain, which was isolated by one of us in culture from pleural fluid of a non-fatal case of tularemia at Vanderbilt University Hospital, March 18, 1942. The "Pack" strain has not been subjected to recent virulence tests.

Results. Using the "B-38" and "V-13" strains, no growth was obtained on media containing amounts of cysteic acid, N,N-dibenzoyl-cystine, methionine, S-benzyl-homocysteine, S-benzyl-glutathione, and dibenzyl disulfide equivalent in sulfur content to 0.1% cystine. With S-benzyl-cysteine, N-benzol-cysteine, and homocystine there was very questionable slight growth about which it was difficult to be sure owing to the heaviness of inoculum used. Only with slants containing S-benzyl-cysteine was very faint darkening of the lead acetate paper observed, which may have been due either to hydrogen sulfide or benzyl mercaptan. Growth could not be transferred on these media. Moderate to fairly good growth was observed on slants containing cystine which, however, never equalled in luxuriance that yielded by the best blood glucose cystine agar, but was maintained without diminution through 4 transfers. Cysteine hydrochloride permitted moderate and thioglycollic acid poor growth, which could nevertheless be transferred on the same media.† Hydrogen sulfide was always formed from cystine, cysteine hydrochloride, and thioglycollic acid in quantities roughly parallel to the amount of growth.

[10] Ransmeier, J. C., unpublished data.

† In a limited number of experiments poor growth and slight hydrogen sulfide production were obtained on media with 64 mg % of sodium sulfide.

Additional media were prepared containing double quantities of cystine (0.2%), cysteine hydrochloride, and thioglycollic acid dissolved in double the former amount of phosphate buffer. In such concentration some of the cystine crystallized out from the finished medium. Growth occurred on these slants but it was somewhat inferior to that noted above. The hydrogen sulfide production again paralleled the growth.

Since Sahyun *et al.*[11] observed optimal growth of *E. coli* in synthetic medium containing cysteine in concentrations of 50 mg % and noted inhibition with 100 mg % or more, it was decided to test lower concentrations of some of the substances studied. Accordingly media were prepared containing half the amounts of methionine, homocystine, and S-benzyl-homocysteine shown in the table. Media with equivalent amounts of cystine (0.05%) and cysteine hydrochloride were heated for 1 hr in an Arnold sterilizer under nitrogen atmosphere to avoid possible oxidation or partial decomposition of these compounds during autoclaving. Using 5 strains of *Bact. tularense,* by far the best growth and hydrogen sulfide production were observed on the cystine and cysteine slants, "V-13" showing somewhat less vigorous performance than the others. Questionable to slight growth appeared with methionine, homocystine, and S-benzyl-homocysteine, but it could not be transferred after 1 week's incubation. No significant amounts of hydrogen sulfide were formed by any of the strains on media containing these three substances.

Summary and Conclusions. 1. Of all the substances studied, cystine, cysteine, and to a much lesser extent thioglycollic acid were the only compounds effective in promoting growth of *Bact. tularense.* 2. Under the conditions described it is confirmed that cystine or cysteine is an essential component of media for cultivation of *Bact. tularense,* as was found previously.[1,4] The effectiveness of glutathione could be ascribed to the cysteine moiety of the tripeptide, while that of thioglycollic acid may not be due to the utilization of the acid *per se* but to some intermediary metabolic product common to cystine and cysteine. 3. Inasmuch as practically no stimulation of growth of *Bact. tularense* by methionine and homocystine was observed, it appears justifiable to conclude that cystine and cysteine were not elaborated from these compounds. It is of interest to note that this is in contrast to results reported with animals.[12-18]

4. Whenever definite growth of *Bact. tularense* was observed hydrogen sulfide was formed. It is not possible at present to state the paths of the reactions leading to hydrogen sulfide production from cystine and cysteine by this organism. It may be that the same mechanism prevails as was postulated by Fromageot[19] and Desnuelle[20] for other bacteria and Smythe[21] for liver tissue. 5. Benzylation of the —SH radical of cysteine and glutathione or benzoylation of the alpha amino groups of cysteine or cystine prevents the utilization of these amino acids for purposes both of growth and hydrogen sulfide production by *Bact. tularense.* These results parallel those obtained in metabolic experiments with animals.[22] Apparently this bac-

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19 Fromageot, C., and Moubasher, R., *Enzymologia,* 1937, **2**, 121.

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21 Smythe, C. V., *J. Biol. Chem.,* 1942, **142**, 387.

22 Magnus-Levy, A., *Biochem. Z.,* 1907, **6**, 541; Shiple, G. J., Rose, A. R., and Sherwin, C. P., PROC. SOC. EXP. BIOL. AND MED., 1922, **20**, 360; Lewis, H. B., Updegraff, H., and McGinty, D. A., *J. Biol. Chem.,* 1924, **59**, 59; Jones, J. H., Andrews, K. C., and Andrews, J. C., *J. Biol. Chem.,* 1935, **109**, xlvii.

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.*

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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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