REDUCTION OF SULPHITES BY CERTAIN BACTERIA IN MEDIA CONTAINING A FERMENTABLE CARBOHYDRATE AND METALLIC SALTS.

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It has long been known that the majority of bacteria possess the power of forming sulphuretted hydrogen in organic media. An account of the work of Rubner, Petri and Maasen, Nencki and others is given in Kruse's textbook (1910). Nearly all investigators consider that cystine is the source from which the sulphuretted hydrogen is derived. Thus Wells (1914) writes: "Most, if not all of the sulphur in the protein molecule seems to be contained in the amino-acid cystine which has the following composition:

$$\begin{array}{l} S\text{—}CH_2\text{—}CHNH_2\text{—}COOH \\ | \\ S\text{—}CH_2\text{—}CHNH_2\text{—}COOH. \end{array}$$

From this is formed the hydrogen sulphide of the intestinal gases, of which about 0·058–0·066 gram is present in each one hundred grams of normal colon contents."

Sasaki and Otsuka (1912) found that in protein-free media containing cystine most intestinal bacteria form sulphide of hydrogen. Somewhat similar results were obtained when free sulphur or sodium thiosulphate were employed instead of cystine, but with sodium sulphate and taurin the results were negative.

A tabular statement of their results indicates that they found a trace of sulphuretted hydrogen formed from sodium sulphite by *B. coli communis*, *B. paratyphosus* B, *B. dysenteriae* Shiga-Kruse, *B. dysenteriae* Flexner and *Vibrio cholerae* but none by *B. typhosus*, *Proteus vulgaris* and *B. paratyphosus* A. Bürger (1914) reported that several varieties of bacteria form sulphuretted hydrogen from cystine and none from taurin.

Tanner (1918) found the ability of bacteria to form hydrogen sulphide from peptone and cystine to be a widespread characteristic. No hydrogen sulphide was formed from taurin and magnesium sulphate, both of which contain sulphur in a highly oxidised form. It is particularly interesting to note that under the conditions of his experiments, Tanner found no bacterial strain capable of reducing sodium sulphite.

Myers (1920) found that a considerable number of bacteria were able to produce hydrogen sulphide from Witte's peptone and not from Difco or Fairchild's preparation. He thought that this result might possibly be due

to differences in the cystin-content. The presence of glucose or lactose had no effect on hydrogen sulphide formation. Of the micro-organisms examined by him, Myers found that *B. paratyphosus* B, *B. typhosus*, *B. enteritidis* and *B. proteus vulgaris* proved active formers of sulphuretted hydrogen, whilst *B. paratyphosus* A, and the dysentery bacilli of Shiga and Flexner were inert. From cystin, but not from taurin or magnesium sulphate, sulphuretted hydrogen could be split by numerous micro-organisms.

With regard to water examination, Myers concluded that the hydrogen sulphide test was too delicate for use in examination of water for detection of faecal contamination of any type, that all contaminated waters examined were positive to this test and that many were positive which gave no evidence of contamination by the usual criteria.

The investigators that I have so far cited determined the presence of hydrogen sulphide by means of strips of lead acetate paper suspended over the fluid cultures and their results were usually read after several days' or several weeks' incubation. The method was not very suitable for the differentiation of bacteria. Assistance in the differentiation of *B. paratyphosus* B, *B. paratyphosus* A, *B. typhosus* and *B. dysenteriae* was afforded by the addition of salts of lead and iron to peptone media as practised by Orlowski (1897), Sacquépée and Chevrel (1905), Burnet and Weissenbach (1915), Hollande and Beauverie (1915), Jordan and Victorson (1917), Kligler (1917), Grosso (1917), Tribondeau (1918), Morishima (1918).

The chief result of this work was to show that when a tube of nutrient agar containing a small amount of lead acetate was inoculated by stabbing between the agar and the glass, browning occurred along the track of the inoculation in the case of *B. typhosus* and *B. paratyphosus* B, but not in the case of *B. paratyphosus* A and *B. dysenteriae*.

Kliger (1917) suggested a simple method for the differentiation of *B. paratyphosus* A and B, *B. typhosus* and *B. dysenteriae* based on a double sugar medium similar to that of Russell, containing lead acetate. Jordan and Victorson (1917) found that in lead acetate media 20 strains of hog-cholera bacilli (*B. suipestifer*) and 20 strains of *B. paratyphosus* A were negative, whereas six strains of *B. enteritidis* (Gaertner) like those of *B. paratyphosus* B were positive. Krumwiede, Kohn, and Valentine (1918) found the lead acetate media useful in their study of the Paratyphoid-enteritidis Group, but they observed that some strains of *B. paratyphosus* B were exceptions in that no browning occurred with them, and that browning sometimes occurred with certain strains of *B. paratyphosus* A. As a rule hog-cholera strains were negative in the test but they remark "with one *B. cholerae suis* strain the two fractions gave different results. Apparently the lead acetate reaction is susceptible to variation and may lose its differential value when strains have been in cultivation for some time."

L. S. Thompson (1921) using anaerobic lead agar plates found that *B. typhosus*, *B. paratyphosus* B, *B. enteritidis* (Gaertner), *Proteus vulgaris*

and *Erythrobacillus prodigiosus* produced dark colonies, whilst there was little or no colour with *B. coli*, *B. aerogenes*, *B. alcaligenes*, *B. paratyphosus* A, and the various strains of *B. dysenteriae*.

He also concluded (1) that the production of hydrogen sulphide and lack of ability to act on lactose seem to go together, (2) that the finding of hydrogen-sulphide-producing bacteria (nearly all Methyl Red +) in water, is evidence that it receives human or animal excreta; and (3) that the cultural, morphological, and agglutinogenic characters possessed by these hydrogen sulphide bacteria indicate that they belong to a closely related group. Contrary to Myers it was found by Thompson that greater blackening occurred with the use of Difco peptone than with that of Witte.

A consideration of the writings of investigators on the metabolism of sulphur by bacteria, appears to indicate that a differentiation of bacterial types would not readily be obtained by their action on the complex molecules present in organic compounds, but that their action on simple inorganic salts of sulphur was worthy of study.

Beyerinck (1895) isolated from earth and river mud his *Spirillum desulphuricans* which is capable of reducing sulphates to sulphides. Stockvis and Saltet (1900) cultivated from sewage a micro-organism (*B. desulphuricans*) which reduced sulphates to a less strongly oxidised body, probably a sulphite, which in its turn was further reduced to sulphides by other unisolated species of bacteria. Van Delden (1903) experimented with a micro-organism (*Spirillum aertuarii*) which reduced sulphates to sulphides.

As we have already seen, Sasaki and Otsuka, when dealing with pure cultures of bacteria, obtained a doubtful reduction of sulphites and Tanner no reduction at all.

Author's Investigations.

The chief result of my investigations is to show that many bacteria of the Typhoid and Paratyphoid-Enteritidis group in the presence of a fermentable sugar are able rapidly to reduce sulphites to sulphides and that this test can be usefully employed for their isolation and identification.

The media which I have employed are as follows:

I. Glucose agar containing sodium sulphite and an iron salt.

Nutrient agar (100 c.c.) containing 1 per cent. of glucose is melted and to it are added 1 c.c. of an 8·1 per cent. solution of ferric chloride and 0·5 c.c. of a 12 per cent. solution of sodium hydrate and finally 5 c.c. of a 20 per cent. solution of anhydrous sodium sulphite in distilled water. A stock solution of sodium sulphite is filtered and kept.

II. Glucose agar containing sodium sulphites and lead salts.

Nutrient agar (100 c.c.) containing 1 per cent. of glucose is melted and to it are added 5 c.c. of a 26 per cent. solution of neutral lead acetate in distilled water and 10 c.c. of a 20 per cent. solution of sodium sulphite. The medium is of an opaque milk-white appearance.

III. Media similar to I and II, but containing in addition bile salt (sodium taurocholate). When the ferric chloride and caustic soda solutions are added to MacConkey's bile-salt-agar, precipitates occur, but on the addition of the sodium sulphite a nearly transparent medium is obtained.

IV. *MacConkey's bile-salt-glucose-broth* to each 100 c.c. of which have been added 1 c.c. of a 0·81 per cent. solution of ferric chloride and 0·5 c.c. of a 1·2 per cent. solution of sodium hydrate and then 8 c.c. of a 20 per cent. solution of sodium sulphite. The iron hydrate in suspension is converted by the growth of sulphite-reducing bacteria to iron sulphide. The medium becomes ink-like in appearance and a black deposit forms. The supernatant fluid and deposit may become yellowish again after a few days' incubation.

Medium No. I is probably most suitable for general use. It is of a clear sherry colour and in shake or plate cultures prepared from it, deep colonies of reducing micro-organisms appear as black spots surrounded by a dark zone. Stab cultures show a dark line along the track of inoculation. The agar is sloped and the inoculation is best made through the condensation fluid. Gas appears in the condensation fluid when the micro-organism is a gas-producer and this serves to differentiate the typhoid bacillus from *B. paratyphosus* B. The important point is that the blackening appears early even after a few hours' incubation in the case of stab cultures.

I have studied the effects on this medium of cultures of various types of micro-organisms obtained from the National Collection of Type Cultures, with the following results: Definite blackening indicating reduction occurred with all four strains of *B. typhosus* examined, *i.e.* ("Howard," "Lister," "Rawlings," and "York-Trotter"), *B. paratyphosus* B (Tidy), *B. paratyphosus* C (Hirschfeld) and *B. enteritidis* Gaertner (Bainbridge). Of other members of the Salmonella group and referred to mainly as *B. suipestifer* in the Catalogue, the types "Arkansas," "Binns," Mutton," "Newport," "Reading," "Stanley," *B. suis* (Salmonella, type Hirschfeld) and "*B. caviae*" were strongly positive whilst *B. suipestifer* Uhlenhuth (Salmonella, type Hirschfeld), *B. suipestifer* (Salmonella, type G), *B. abortivo-equinus* "626 Foal," *B. voldagsen* Wegener (Salmonella, type Hirschfeld), *B. gallinarum*, *B. pullorum* and *B. sanguinarium* were negative. Similarly no blackening occurred with *B. paratyphosus* A (strains "Schottmüller," "Haddon," and American Museum type strain "No. 16").

Other micro-organisms which were examined and showed no formation of iron sulphide in medium No. I were *B. avisepticus*, *B. bovisepticus*, *B. bronchisepticus*, *B. murisepticus*, *B. ovisepticus*, *B. pseudotuberculosis rodentium*, *B. suisepticus*, *B. pestis* (Parel), *B. dysenteriae* Flexner types "W" and "Y," *B. dysenteriae* Shiga ("Wynne"), *B. dysenteriae* Sonne, type II (Thjøtta III), *Vibrio cholerae* (Liston), *Vibrio Finckler-Prior*, *B. proteus mirabilis*, *B. proteus vulgaris*, *B. proteus zenkeri*, *B. proteus vulgaris* X 19, *B. proteus anindologenes* (Elders), *B. pneumoniae* Friedländer, *B. lactis aerogenes*, *B. pyocyaneus*, *B. anthracis*, *B. Morgan* No. 1, *B. mycoides*, *B. neapolitanus*, *B. coli communior*, *B. coli* (six strains isolated from cases of cystitis), *B. acidi lactici*, *B. mallei*, *B. melitensis*, *Corynebacterium diphtheriae* ("Park's No. 8"), *Streptococcus faecalis*, *Staphylococcus albus*, *Staphylococcus aureus*. A few micro-organisms failed to grow on the medium which appeared to restrain Pneumococci and Streptococci occurring in swabs from throats.

It was found that the medium would not be suitable for the direct isolation

of typhoid or paratyphoid bacilli from stools as dark colonies developed when normal stools were planted out and proved to be composed of lactose-fermenting bacilli—apparently varieties of *B. coli*. Whether these reducing strains of *B. coli* form a serological type is being investigated. Cultivation of the faeces in sulphite-glucose-Bile-Salt-broth and subsequent planting out on Brilliant-Green-lactose-Bile-Salt plates has given promising results, as under these conditions there is an enrichment of the growth of the pathogenic micro-organisms.

The sulphite-glucose-Bile-Salt broth is also useful for attempting to isolate typhoid or paratyphoid B bacilli from water. By its use I have isolated from a public water supply a bacillus apparently belonging to the Paratyphosus B group. The medium is not suitable for routine water examination as I have found that six samples of wells which were undoubtedly polluted (containing coliform bacilli in 0·1 c.c.) yielded no sulphite-reducing bacteria even in 30 c.c. The distribution of sulphite-reducing bacteria in soil, excreta, milk and water supplies is being investigated. Coliform bacilli possessing this power have been found in the dejecta of cattle as well as of man, but only in comparatively small numbers. Their presence in large numbers in milk in my opinion indicates gross pollution.

Medium No. II containing Lead Salts does not afford as clear a differentiation of bacteria as medium No. I. In nutrient glucose agar containing lead sulphite, a browning occurs with very many micro-organisms which show no darkening in the iron medium. Why this is so I have not discovered; it may be that it is more sensitive and detects sulphuretted hydrogen formed from organic matter or mercaptan. It is stated that mercaptan causes a browning of lead salts and no darkening of iron salts. Many micro-organisms produce either no effect or a mere trace of browning whilst the Typhoid-Enteritidis group cause the medium to become jet black.

The tests which I have performed have been carried out as stab, surface or deep plate cultures under partial aerobic conditions. Typhoid and other reducing bacteria under anaerobic conditions yield a black surface growth. Sodium sulphite is stated by Ciani (1920) to allow the growth of obligatory anaerobes in open plate cultures.

In many cases, in the sulphite-glucose-broth, the bacterial growth is of a slimy, tenacious mucoid consistence. It is well known that sodium sulphite affects the products of fermentation by yeast and that instead of 3 per cent. of glycerine being formed the percentage can be increased up to 30 per cent. Whether bacteria are affected in the same way I have not ascertained. In my media I have found the presence of a fermentable carbohydrate to be indispensable. It probably supplies the necessary energy to the bacteria and also the acid formed brings into solution the iron or lead salts and so facilitates the reaction concerned in the formation of insoluble sulphides. Mannite can take the place of glucose. A sulphite-reducing *B. coli* which ferments lactose blackens mediums No. I or No. II when they contain lactose instead of glucose whereas typhoid bacilli and the Paratyphoid-Enteritidis group cause no

darkening. I have however found that the symbiosis of a lactose-fermenting non-reducing *B. coli* with a reducer such as *B. typhosus* or *B. paratyphosus* B in sulphite-lactose-bile-salt-iron-broth (medium IV) enables the latter to cause reduction. This would seem to indicate that the acid formed by the former enabled the reaction to occur.

When films are examined black dots evidently of lead sulphide are sometimes found inside the bacilli, often near their poles. The blackening by the growth of Typhoid and Paratyphoid B bacilli which occurs in peptone-agar containing lead salts varies with the brand of peptone employed. It is not improbable that it may be partly due to the reduction of sulphites which occur as impurities in the medium. Peptone and media made from it when tested for the presence of sulphites by means of zinc and dilute sulphuric acid yield considerable amounts of sulphuretted hydrogen.

Although my experiments indicated that the hydrogen sulphide produced by the reducing bacteria was derived from the sulphites added to the media —thus no darkening occurred in the absence of the sulphite or in the absence of a fermentable sugar—still the conclusive evidence could only be obtained by demonstrating reduction in a protein-free simple synthetic medium where the only sulphur present was in the sulphite salt.

The medium I employed was a modification of that of Braun and Cohn-Bronner (1921). It consisted of 5 grms. sodium chloride, 2 grms. potassium phosphate, 6 grms. ammonium lactate, 10 grms. glucose and distilled water 1 litre. Reaction was made slightly alkaline to litmus by addition of $N/1$ caustic soda. In this medium the Paratyphoid B group of bacteria can develop and when sodium sulphite and iron or lead salts were added, dark deposits were formed.

A solid peptone-free medium which is useful for demonstrating reduction of sulphites by the Paratyphoid B-Enteritidis group, and which, for the purpose of this paper, may be termed medium No. V, is prepared as follows: Make 6 grms. of dried powdered agar-agar into a thin paste and dissolve in 100 c.c. of distilled water. Boil until a clear jelly is obtained and to it add an equal volume of a solution containing 1 grm. sodium chloride, 0·4 grm. potassium phosphate, 1·2 c.c. ammonium lactate and 2 grms. glucose. Boil the 200 c.c. of resulting medium and make it slightly alkaline to litmus paper by the addition of $N/1$ caustic soda.

Whilst it is still hot, there is added to it 2 c.c. of an 8·1 per cent. solution of ferric chloride and 1 c.c. of a 12 per cent. solution of sodium hydrate and finally 6 c.c. of a 20 per cent. solution of sodium sulphite. The medium is delivered into tubes and forms a clear jelly with no obvious colour.

The Paratyphoid B group grow on this medium and stabs through the condensation water show very distinct blackening.

The addition of asparagin to the medium (Uschinsky's fluid) produces a slightly better growth.

With a view to obtaining uniform results as to the capacity of bacteria to

form hydrogen sulphide rapidly, I would suggest the use of medium No. I or some modification thereof. A Committee of the Society of American Bacteriologists under the Chairmanship of Dr H. J. Conn (1922) recommends as a provisional method an agar medium prepared according to standard peptone-beef-extract formula but containing 30 grms. instead of 5 grms. of peptone per litre. To 5 c.c. of this melted medium cooled to 50° C. 5 c.c. of a 0·1 per cent. solution of basic lead acetate are added. Incubation from 18 hours to a few days is recommended. The advantages that I claim for such a medium as No. I is that the results are constant and are obtained in a few hours.

Conclusions.

(1) In media containing sodium sulphite, glucose, and iron salts, reduction of sulphite to sulphide is effected by *B. typhosus*, *B. enteritidis* (Gaertner), *B. paratyphosus* B, and various other members of the Salmonella group. No reduction was shown by *B. paratyphosus* A, and the various dysentery bacilli.

(2) The use of certain media containing sodium sulphite is recommended in attempting to isolate typhoid and paratyphoid B bacilli from excreta, water, milk, etc.

(3) It is suggested that the blackening which is brought about by certain members of the Typhoid-Paratyphoid-Enteritidis group in nutrient agar containing lead acetate is due, at least in part, to the reduction of sulphite impurities and that in order to get constant results with different brands of peptone an addition of sodium sulphite and a suitable fermentable carbohydrate should be made to the medium.

REFERENCES.

Beijerinck, W. M. (1895). *Centralbl. f. Bakt.* Abt. II, I. 1, 49 and 104.
Braun, H. and Cahn-Bronner, C. E. (1921). *Centralbl. f. Bakt.* LXXXVI. 1, 196 and 380.
Bürger, Max (1914). *Arch. für Hyg.* LXXXII. 201.
Burnet, Ét. et Weissenbach, R. J. (1915). *Compt. rend. Soc. Biol.* LXXVIII. 565.
Ciani, G. (1920). Ann. d'ig. 30, 274; ref. *Med. Science*, 1921, 4, 354.
Conn, H. J. (1922). *J. Bact.* VII. 5.
Grosso, G. (1917). *Pathologica*, IX. 183; ref. *Bull. de l'Inst. Past.* XX. 30th Oct. 1917, 608.
Hollande et Beauverie (1915). *Compt. rend. Soc. Biol.* 68, 722.
Jordan, E. O. and Victorson, Ruth (1917). *Journ. Infect. Dis.* XXI. 554.
Kligler, I. J. (1917). *Am. Journ. Pub. Health*, 7, 1042.
Krumwiede, C., Kohn, L. A., and Valentine, E. (1918). *Journ. Med. Research*, XXXVIII. 89.
Kruse, Walther (1910). *Allgemeine Mikrobiologie*, Kap. XI. S. 634. Leipzig.
Morishima, Kan-Ichiro (1918). *J. Bact.* III. 1, 19.
Myers, John T. (1920). *J. Bact.* V. 231.
Orlowski, A. A. (1897). Beitrag zur Kenntniss der biologischen und pathogenen Eigenschaften der *Bacterium coli communis*. Diss. St Petersburg.
Sacquépée, E. and Chevrel, F. (1905). *Ann. de l'Inst. Past.* XX. 1. *C. r. Soc. Biol.* LIX. 535. Ref. *Bull. de l'Inst. Past.* IV. 1906, 112.
Saltet, R. H. (1900). *Centralbl. f. Bakt.* Abt. II, VI. 695.
Sasaki, T. und Otsuka, I. (1912). *Biochem. Zeitschr.* XXXIX. 208.
Tanner, F. W. (1917). *J. Bact.* II. 585.
Thompson, Luther S. (1921). *Journ. Med. Research.* XLII. 4, 383.
Tribondeau, L. (1918). *C. r. Soc. Biol.* LXXXI. 524.
van Delden, A. (1903). *Centralbl. f. Bakt.* Abt. II, XI. 81.
Wells, H. Gideon (1914). *Chemical Pathology*, W. B. Saunders, Philadelphia and London. Second edition, 526.