STUDIES ON ENDO'S MEDIUM, WITH OBSERVATIONS ON THE DIFFERENTIATION OF BACILLI OF THE PARATYPHOID GROUP.*

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Of the many media at present in use for the routine isolation of typhoid bacilli from stools and urine, the medium of Endo is probably the one most widely employed. While this medium is easy to apply and reliable, its production is fraught with a certain amount of difficulty, and irregularities in the character of the finished medium often result.

Kastle and Elvove[1] have pointed out that the chief cause of irregularity is the varying purity of sodium sulfite as commercially obtainable. They overcame this difficulty by using anhydrous sodium sulfite instead of the crystallized variety which is hydrated.

The work here recorded was begun with the purpose of determining the chemical principles on which depend the color reactions taking place in this medium in the hope that, after fully understanding these, changes might be introduced which would lead to simplification of production, and greater stability of the end product.

Endo, in his original article,[2] said: "Der Säurekomponent des roten Rosanilin Salzes wird durch Reductionsmittel, wie Na_2SO_3, leicht reduziert. Das dadurch entfärbte Rosanilin verbindet sich mit der durch Coli Bacterium produzierten Säure, und der Nährboden färbt sich schön rot." This statement, while offering a plausible explanation, which seems to have been accepted, was apparently not based on actual experimental investigation.

The colorless solution obtained by treating fuchsin with Na_2SO_3,

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[1] *Jour. Infect. Dis.*, 1909, 6, p. 619.

[2] *Centralbl. f. Bakt.*, I, Orig., 1904, 35, p. 109.

$NaHSO_3$, or SO_2 in solution is used extensively in chemical analysis for the detection and even colorimetric quantitative determinations under the name of "Schiff's aldehyde reagent."[1] Acids do not give any color test with this reagent and, if present in sufficient quantity, prevent aldehydes from giving it. These well known facts contradict Endo's statement. The reaction taking place between fuchsin, Na_2SO_3, and aldehydes as given by Nietski[2] is as follows: "Rosanilin and pararosanilin form with sulfurous acid and the alkali bisulfites colorless, easily decomposed compounds. By the action of aldehydes upon these bodies peculiar violet dyes are formed."

This explains why the medium is red when hot, and colorless when cold, since the compound upon dissociating would liberate the free fuchsin.

It is reasonable to suppose that the same reaction takes place in Endo's medium since it is to be expected that aldehydes are intermediate products in the oxidation of carbohydrates to acids by bacteria.

Preliminary experiments were made to determine whether the reaction of various bacteria on a medium made up like that of Endo, except that various carbohydrates were substituted for lactose, would correspond to the production of acid by these bacteria from the same carbohydrates.

TABLE 1.

MEDIUM MADE UP WITH SODIUM SULFITE AND FUCHSIN AS IN ENDO'S MEDIUM, CONTAINING VARIOUS CARBOHYDRATES.

	Dextrose	Mannite	Maltose	Lactose	Sucrose	Dextrin
Fecalis alkaligenes	Pink	Pink	Pink	Pink	Pink	Pink
B. dysenteriae, Shiga-Kruse	Red	White	White	White	White	White
" " , Hiss-Russell	"	Red	"	"	"	"
" " , Flexner	"	"	Red	"	Red	Light Red
B. typhosus	"	"	"	"	White	"
B. schottmüller-müller	"	"	"	"	"	White
B. coli communis	"	"	"	Red	"	"
B. coli communior	"	"	"	"	Red	Red

Compare with:

[1] S. P. Mulliken, *Identification of Pure Organic Compounds*, 1904, 1, p. 15.

[2] *Chemie der Organischen Farbstoffe*, 1906, p. 163; Victor Meyer, *Berichte*, 13, p. 2343.

ACID FORMATION.

(From Hiss and Zinsser, *A Textbook of Bacteriology*, p. 443.)

	Dextrose	Mannite	Maltose	Lactose	Sucrose	Dextrin
Fecalis alkaligenes	.....	.....	.....	.....	.....	.....
B. dysenteriae, Shiga-Kruse	Acid	.....	.....	.....	.....	.....
" " , Hiss-Russell	"	Acid	.....	.....	.....	.....
" " , Flexner	"	"	Acid	.....	Acid	Acid
B. typhosus	"	"	"	.....	.....	"
B. schottmüller-müller	"	"	"	.....	.....	.....
B. coli communis	"	"	"	Acid	.....	.....
B. coli communior	"	"	"	"	Acid	Acid

It is evident that color production on fuchsin-sulfite agar follows the acid chart with the one exception that *B. fecalis alkaligenes* in all cases gave pink colonies. This is in direct agreement with the fact that alkalis react with "Schiff's reagent" to give a pink color.[1]

After the second or third day the plates became pink throughout, as is usual with Endo's medium when exposed to the light and air. In from five to 10 days later, when the red colonies had attained considerable size, they began to decolorize the medium surrounding them, the body of the colony remaining red owing to the fact that the bacteria had taken up the stain. We account for this in the following manner: The aldehyde, as fast as produced, combines with the reagent and is fixed, thus being prevented from oxidizing to acid. But upon continued growth of the colony, all of the fuchsin sulfite in the immediate neighborhood is used up. As soon as this occurs, the aldehyde, still being formed, is oxidized to acid and this acid decolorizes the medium. A part of the decolorized medium was tested with litmus and the leukobase of "soluble blue"[2] and showed an acid reaction. By placing a drop of dilute acid on that portion of a plate which had been colored by the growth of the bacteria, we produced a similar effect. A drop of dilute alkali, on the other hand, intensified the color.

Owing to the fact that Na_2SO_3 is very easily oxidized and that most samples therefore vary in SO_2 content, we prepared our fuchsin sulfite reagent by adding a solution of the sulfite to a

[1] *Loc. cit.*

[2] "Soluble blue," the commercial term for an anilin blue soluble in water and easily reduced to its leukobase by treatment with ammonia and zinc dust.

measured quantity (5 c.c. saturated alcohol solution per liter) of fuchsin until we obtained the maximum delicacy of reaction with a dilute solution of formaldehyde. This solution of the reagent was then poured into the hot 3 per cent lactose agar. The medium thus prepared gave us the best color reaction with the bacteria. This we take as an additional proof that the color is due to aldehyde formation. To keep the color from spreading, however, it was found necessary to add a slight excess of sulfite.

The differentiation of typhoid and colon bacilli on Endo's medium on the basis of aldehyde formation is, as far as we can ascertain, the only application of this principle to the separation of bacterial species. It seemed to the writers that this principle might serve to base differentiation on aldehyde formation from the various carbohydrates in the same way that such differentiations are now made on the basis of acid formation. For this reason tests were made with other members of the colon-typhoid-dysentery group as follows:

TABLE 2.

MEDIUM MADE UP OF FUCHSIN-SULFITE AGAR AS IN ENDO'S MEDIUM—WITH ADDITION OF CARBOHYDRATES AS FOLLOWS:

	Inulin	Dulcite	Rhamnose	Raffinose	Arabinose	Xylose	Glycogen
Fecalis alkaligenes..	Pink	Pink	Pink	Pink	Pink	Pink	No growth
B. dysenteriae, Shiga-Kruse.....	White	White	White	White	White	White	"
B. dysenteriae, Hiss-Russell.....	"	"	"	"	"	"	"
B. dysenteriae, Flexner.....	"	"	"	"	Late Red	"	"
B. typhosus.....	"	"	"	"	White	Red	"
B. schottmüller-müller.....	"	Late Red	"	"	Red	"	"
B. coli communis...	"	Red	Red	"	"	"	"
B. coli communior..	Red	"	"	Red	"	"	"

The colors produced on inulin, dulcite, and rhamnose were rather weak and indefinite, and therefore of no differential value. Raffinose differed from sucrose only in its behavior toward the dysentery bacillus of the Flexner type. In the glycogen medium no increase in growth took place and after two days the bacteria were evidently all dead, since we could obtain no growth upon agar slants on transplantation. But when cultures were planted upon media containing the same amount (10 gms. per liter) of

glycogen but no fuchsin sulfite, they grew excellently, showing that the bactericidal effect was not due to the glycogen itself.

The reactions with the xylose medium appeared to us particularly interesting in that they showed differences between *B. typhosus* and *B. dysenteriae*, the former giving colored, the latter white colonies.

Arabinose in the same way seemed to offer a means of differentiating *B. typhosus* from the paratyphoid group.

Wishing to determine whether this was true for all of the members of the paratyphoid group we made a more thorough study of the behavior of the paratyphoid bacilli which were available at that time in this laboratory. The arabinose and xylose media together divided our cultures into three groups, viz.: (1) those that gave color with both; (2) those that gave color with arabinose and not with xylose; (3) those that gave color with xylose and not arabinose.

Later we were able to repeat these tests with a larger number of cultures of the intermediate group from various laboratories.[1] The cultures we obtained in addition to our own and their source were as follows:

B. paratyphosus "A" No. 25, University of California
B. paratyphosus "B" No. 62, " " "
B. libman, Mt. Sinai Hospital, New York
B. seeman, " " " " "
B. gärtner, " " " " "
B. paratyphosus No. 7, Loomis Laboratory New York
B. paratyphosus No. 116, " " " "
B. paratyphosus schottmüller, " " " "
B. enteritidis No. 18, " " " "
B. enteritidis No. 69–5, " " " "
B. enteritidis No. 61–9, " " " "
B. enteritidis No. 132, " " " "

All the cultures used in our experiments were plated and fished to avoid errors of contamination. They were all gram negative bacilli of characteristic appearance, motile, and reacted as follows on the media mentioned:

[1] We take pleasure in expressing our gratitude to Professor Ravenel of Wisconsin, Professors Winslow, Libman, Torrey of New York, and Professor Fitzgerald of Berkeley for cultures kindly sent to us.

Gelatin—no liquefaction.
Hiss tube medium—motility and gas.
Dextrose broth—gas.
Lactose broth—no gas; no acid.
Sucrose broth—no gas; no acid.

Our results on xylose and arabinose fuchsin-sulfite agar were as follows:

TABLE 3.

COLOR REACTION OF MEMBERS OF THE SO-CALLED PARATYPHOID GROUP OF BACILLI ON FUCHSIN-SULFITE AGAR CONTAINING ARABINOSE AND XYLOSE RESPECTIVELY:

	Arabinose	Xylose
Group 1, Color Reaction on Both Sugars		
B. schottmüller-müller (Columbia University)	Red	Red
B. schottmüller-seeman (Columbia University)	"	"
B. schottmüller (Loomis)	"	"
B. seeman (Mt. Sinai)	"	"
B. enteritidis (Am. Museum Nat. Hist.)	"	"
B. enteritidis "No. 18" (Loomis)	"	"
B. enteritidis "No. 69–5" (Loomis)	"	"
B. enteritidis "No. 61–9" (Loomis)	"	"
B. enteritidis "No. 132" (Loomis)	"	"
B. gärtner (Enteritidis) (Mt. Sinai)	"	"
B. paratyphosus "B" (University of Chicago)	"	"
Group 2, Color on Arabinose but Not on Xylose		
B. typhi murium (Columbia University)	"	White
B. paracolon gwynn (Columbia University)	"	"
B. paratyphosus "A" (University of Chicago)	"	"
B. paratyphosus "No. 7" (Loomis)	"	"
B. paratyphosus "No. 116" (Loomis)	"	"
B. libman (Mt. Sinai)	"	"
Group 3, Color on Xylose but Not on Arabinose		
B. hog cholera (University of Wisconsin)	White	Red

Twenty-five strains from the colon-typhoid-dysentery group were tried out on xylose and arabinose broth to see whether aldehyde and acid formation always occurred together. This was found to be true for all the strains we tried. The indicator we used for showing acid formation was the leukobase of "soluble blue." The use of this compound as an indicator for the fatty acids and our results concerning the identification of *B. typhosus* by the use of xylose broth, in which it is the only member of the whole group which ferments the sugar without gas formation, will be discussed in our next communication.

SUMMARY AND CONCLUSIONS.

It is seen from our results that by the use of the sugars xylose and arabinose in media, the bacilli of the so-called paratyphoid or intermediate group may be divided into definite subgroups.

We regret that we had only 18 strains with which to work but believe that the definite and constant variations observed should serve to indicate a method by which the organisms of this group, now so difficult to classify, may be eventually differentiated. We believe that these differentiations as well as others, when obtained on the fuchsin-sulfite media of Endo, depend not on acid formation but on aldehyde production from the carbohydrates.[1] Indeed a differentiation may be possibly further obtained when strong acid formation occurs, by subsequent decolorization of the medium surrounding the individual colonies.

When arabinose and xylose are used, however, differentiations within this group may be obtained with a simple acid indicator.

[1] H. Kahlenberg, *Ueber die Bildung und Vergärung von Ameisensäure durch Bacterium Coli Commune*. Heidelberg, 1911, p. 14.