THE ACTION OF DYSENTERY BACILLI ON NITRITES AND NITRATES.

By W. J. LOGIE, M.B., Ch.B.,
Carnegie Research Scholar.

(*From the Pathological Laboratory of the University and Western Infirmary, Glasgow.*)

In the course of work upon the inhibition of the cholera-red reaction in mixed cultures it was found that a marked difference exists between *B. dysenteriae* Shiga and *B. dysenteriae* Flexner in respect of their action on nitrites. This led to further investigations on the formation and destruction of nitrite by organisms of the dysentery group, which form the subject of the main part of this paper. In the first place, however, it has been necessary to examine the cultural methods hitherto employed for the identification and classification of organisms of the dysentery group.

It is convenient to separate the cultural and other characteristics into two divisions, (*a*) those which show that an organism belongs to the dysentery group, and (*b*) those which distinguish it as belonging to one or other of the various "types" or sub-divisions of the group. The general characteristics of dysentery organisms are as follows: they are short, plump, non-motile bacilli which give rise in the human subject to one form of the disease known clinically as dysentery. They may be isolated from the mucus occurring in the stools of dysentery patients or, after death, from the intestinal wall and mesenteric glands. From the organisms of the coli-typhoid group they are strikingly differentiated by the absence of flagella and consequently of motility. Their form is also plumper. In contrast to *B. coli* and *B. enteritidis* of Gaertner, they form no gas on any of the ordinary sugar media. None coagulate milk and none form acid from lactose, but almost all ferment

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glucose. On agar they form thin film-like growths while on potato the growth which is at first whitish or transparent assumes ultimately a brownish tint. Gelatine is not liquified. On Endo-agar the colonies are pale. In staining reactions these organisms from cultures resemble *B. coli* being Gram-negative, but staining readily with the usual aniline dyes.

While dysentery organisms present characters which mark them off as a distinct group, they differ amongst themselves both in regard to serum-reactions and in their action on mannite and certain sugars, so that various subdivisions of the group have been proposed. The exact number of subdivisions to be adopted is not yet finally settled and so many variations exist that classification becomes difficult. Hiss (1904) recognises four types, of which the first ferments glucose alone; the second glucose and mannite; the third glucose, mannite and saccharose; and the fourth, glucose, mannite, saccharose, maltose and dextrine. To these, Shiga (1907) adds a fifth group which differs from type four of Hiss in that the culture media return to an alkaline reaction after five or six days and also on account of "wide differences" in agglutination reactions. It may be remarked that both Hiss and Shiga find that *B. dysenteriae* Flexner ferments saccharose, a result which is not confirmed by other observers. In this connection it should be noted that an organism which has been cultivated for some time on artificial media may acquire the power of fermenting substances which it could not at first attack.

Otto Lentz (1909) in his table gives the differential reactions of four types of which "Shiga," "Y" and "Strong," correspond to types I, II and III of Hiss respectively, while type "Flexner" differs from type IV of Hiss in not fermenting saccharose.

The following table shows the types of Shiga and Hiss, and their relationship to those of Lentz.

Name adopted by Hiss & Shiga	O. Lentz	Reaction given on media containing						Indol formation
		Glucose	Mannite	Saccharose	Maltose	Dextrine	Lactose	
Type I	Shiga	Acid	–	–	–	–	–	–
Type II	"Y"	Acid	Acid	–	–	–	–	+
Type III	Strong	Acid	Acid	Acid	–	–	–	+
Type IV	—	Acid	Acid	Acid	Acid	Acid	–	+
—	Flexner	Acid	Acid	–	Acid	–	–	+
Type V (Shiga)	—	Acid	Acid	Acid	Acid	Acid	–	+

The various mannite-fermenting strains seem to be more closely related to each other than to bacilli of the Shiga type. This is shown

particularly by serum-reactions, the serum of patients infected with bacilli of the Flexner or "Y" types agglutinating both *B. Flexner*[1] and the "Y" bacillus in equal dilutions, so that a differential diagnosis between these two organisms by means of the serum-reaction becomes impossible, whereas *B. Shiga* is not agglutinated by the sera of patients infected with these organisms. (Jürgens (1903), Lentz, Auche and Campana (1905).)

A further property which differentiates the Shiga strain is its much greater power of producing soluble toxins. This has led to a classification into a toxin producing type (giftiger Typus)—*B. Shiga*, and a relatively non-toxic group (giftarmen Typen)—the other dysentery bacilli.

While most writers agree that dysentery due to the Shiga type of bacillus is more severe and more fatal than the disease caused by the other members of the group, it is impossible to say from the clinical phenomena which type of bacillus is responsible for a particular case of dysentery. In this connection it may be mentioned that mixed infections occur and both Shiga and Flexner organisms may be obtained from the same case. No doubt this helps to confuse the clinical picture and make any differentiation of types due to different bacilli more difficult. Böse (1908) has even found amoebic and bacillary dysentery combined.

Of the organisms studied by the author three, which are all of the Shiga type, have been isolated by Dr Eyre from cases of asylum dysentery, while for six, the author is indebted to Professor Neisser of Frankfurt. One bacillus was isolated from a case of dysentery occurring in a Lascar, and the remaining four were obtained from Král of Prague.

Most of these had been cultivated on artificial media for some years, but the bacillus from the Lascar was examined when isolated and has preserved its properties unchanged during the two months it has been under observation.

With the exception of *B. Neisser* Ac.[2] all these organisms form acid readily from glucose. Some form acid (though not so readily as *B. coli*) in litmus-whey, but in lactose agar the reaction remains alkaline. This depends of course upon the fact that the litmus-whey is neutral to start with, while the agar medium besides being slightly alkaline con-

[1] The different types of *B. dysenteriae* hereinafter referred to are, for brevity's sake, named *B. Flexner*. *B. Shiga*, *B. Neisser*, etc.

[2] This organism was isolated from a severe sporadic case of dysentery. It is peculiar also in that peptone water cultures assume a distinct brown colour.

tains peptone from which alkali is liberated by the organism. The effect of the peptone is perhaps more strikingly shown by the fact that all the one per cent. mannite, maltose, and saccharose media, which had been fermented within 48 hours by *B. Flexner*, *B. Celli*, etc., had become alkaline by the end of 30 days. This return to an alkaline reaction has been noted also by other writers.

No gas is formed by these organisms on any of the usual media and milk is not coagulated.

Microscopically they appear as plump bacilli, of variable length, the tendency to the production of long forms being more marked in some strains than in others. *B. Flexner* for instance is on the whole a longer organism than *B. Jürgens*. In hanging drop preparations from young cultures the organisms are often seen joined end to end in pairs. Although Brownian movement is often marked, motility is absent.

The following table gives the reactions on one per cent. mannite, saccharose, maltose and dextrine agar[1], after 48 hours and also the results of testing for indol in 2 per cent. peptone water cultures after two weeks incubation at 37° C.

Organism	Mannite	Saccharose	Maltose	Dextrine	Litmus-whey	Indol
B. Eyre 7	–	–	–	–	–	–
,, 9	–	–	–	–	–	–
,, 10	–	–	–	–	–	–
B. Neisser Ac.	–	–	–	–	–	–
B. Shiga (Kral)	–	–	–	–	–	–
B. from Lascar	A	–	–	–	A	–
B. Celli	A	–	–	–	A faint	+
B. Neisser Mc.	A	–	–	–	A	–
B. Jürgens	A	–	A	A	–	+
B. Flexner	A	–	A	A	A	+
B. Neisser Lb.	A	–	A	A	A	–
,, Mb.	A	–	A	A	A	–
,, Nb.	A	–	A	A	A	–
,, Nc.	A	A	–	–	–	–

A = acid, – = no change.

B. Neisser Mc. ultimately (after about 10 days at 37° C.) forms acid from maltose, and *B. Neisser* Nc. after three or four days gives an acid reaction in litmus-whey. The Neisser organisms Lb., Mb., Mc., and Nb., all acidify litmus-whey more rapidly than *B. Flexner*, producing marked acidity in fourteen hours, whereas *B. Flexner* after that period gives only a very faint trace of acidity.

[1] 1 % peptone-water was used in place of bouillon in the preparation of the agar.

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In the above table, seven groups may be distinguished. The most striking is that formed by the strains which do not ferment mannite, viz. *B. Shiga*, *B. Eyre* 7, 9, and 10, and *B. Neisser* Ac. The organisms which do ferment mannite fall into five groups: (1) the Neisser organisms Lb., Mb., and Nb. which all ferment mannite, maltose, and dextrine, but do not form indol; (2) *B. Flexner* which differs from these in forming indol; (3) *B. Jürgens*, which differs from *B. Flexner* in not forming acid in litmus-whey; (4) *B. Neisser* Mc., and the bacillus isolated from a Lascar, which ferment only mannite ("Y" type); (5) *B. Celli*, also "Y" type but differing from group (4) in that it produces indol; (6) *B. Neisser* Nc., which ferments mannite and saccharose (Strong type).

Formation and Destruction of Nitrite.

The medium used in these experiments was peptone water (peptone 1 %, sodium chloride ·5 %) to which ·00023 % sodium nitrite, or ·000283 % sodium nitrate $\left(\frac{n}{30{,}000}\right)$ was added. The test used for nitrite was that with α-napthylamin acetate and sulphanilic acid.

(1) Dissolve ·5 grm. sulphanilic acid in 150 c.c. dilute acetic acid (sp. gr. 1040); (2) Boil ·1 grm. naphthylamin acetate in 20 c.c. distilled water; filter and add filtrate to 180 c.c. dilute acetic acid (sp. gr. 1040). Mix these two solutions and keep protected from the air. At the time of using, the solution should be quite colourless.

A red colour, due to the formation of an "azo" dye, indicates the presence of nitrous acid or nitrites.

In studying the formation of nitrite two fallacies must be guarded against. In the first place, ordinary Witte's peptone as supplied for bacteriological purposes contains a trace of nitrite. In fresh 1 % solution the reaction may be very faint, but in 2 % solution it is more marked and in 5 % solution it is quite apparent. To obviate this source of error it is advisable to make all the peptone water for a single experiment at one time and to use an uninoculated tube of the medium (incubated along with the others) as a control. This "control tube" is useful also as a standard since in an experiment with plain peptone water organisms which destroy nitrite give a paler solution, while those which form nitrite give a stronger pink when tested with α-napthylamin solution. It is thus possible by a single experiment to determine whether an organism forms or destroys nitrite.

The second source of error is the circumstance that nitrite and nitrate are present in the air. It has long been known for instance, that rain water contains traces of both, and the great difficulty of keeping the α-naphthylamin solution colourless serves to show how easily atmospheric nitrogen may complicate a reaction. In the course of these experiments it was found that under certain circumstances nitrite may be absorbed from the air in sufficient quantity to affect the result. The principal factors affecting the absorption are time, temperature, and amount of surface exposed.

Five c.c. of sterile water kept at 57° C. in a 3″ × ½″ test tube, plugged in the usual way with cotton wool, give even after 19 hours a strong nitrite reaction. If kept at 37° C. a much longer time is required before the same depth of tint is obtained, only a very faint trace of nitrite being detectable after 24 hours. On the other hand if 5 c.c. of sterile water be placed in a 300 c.c. Erlenmeyer flask, plugged with cotton wool as usual, so that it forms a thin layer and exposes a large surface to the air quite a marked reaction is obtained even after 24 hours at 37° C.

When water is boiled for a short time in air, *e.g.* to sterilise it or in dissolving substances in it, no appreciable reaction is obtained, nor does sterilising for 1½ hours in a Koch's steriliser lead to the absorption of a detectable amount of nitrite.

That the nitrite reaction is due to absorption and not simply to concentration of nitrites already in solution, may be shown either by the use of distilled water or by taking 5 c.c. of fresh tap water and boiling till its bulk is reduced to below that of the water (originally 5 c.c.) which has been incubated. The simple concentration by boiling causes no nitrite reaction. Moreover, sealing the tube in a blow-pipe flame or in the case of incubation at 37° C. by means of paraffin wax, prevents the appearance of a reaction even after several weeks.

In the experiments in which the action of dysentery bacilli on nitrate and nitrite was tested all the tubes were sealed with paraffin wax before being placed in the incubator, and uninoculated tubes of the same medium were always incubated as controls along with the cultures. The following table gives the results obtained on testing with α-naphthylamin acetate and sulphanilic acid after 24 hours' incubation at 37° C.

From this it appears (1) that none of the organisms of Shiga type destroy nitrite; (2) that of the others, *B. Jürgens* and *B. Neisser* Nc. alone fail to destroy nitrite; (3) all except *B. Neisser* Ac. reduce nitrate. The number of types has now become eight since *B. Neisser* Ac. differs from the other Shiga strains in not reducing nitrate. *B. Jürgens* differs from *B. Flexner* in failing to destroy nitrite as

well as in giving no acid reaction in litmus-whey. As *B. Jürgens* forms both indol and nitrite, and fails to destroy nitrite it gives the cholera-red reaction.

Organism	Result of growth in medium containing ·000283 % nitrate	·00023 % nitrite
B. Eyre 7	Reduction to nitrite	Nitrite not destroyed.
,, 9	,, ,,	,, ,,
,, 10	,, ,,	,, ,,
B. Neisser Ac.	No nitrite	,, ,,
B. Shiga	Reduction to nitrite	,, ,,
B. Celli	,, ,,	Nitrite destroyed.
B. Flexner	,, ,,	,, ,,
B. Neisser Lb.	,, ,,	,, ,,
,, Mb.	,, ,,	,, ,,
,, Mc.	,, ,,	,, ,,
,, Nb.	,, ,,	,, ,,
B. from Lascar	,, ,,	,, ,,
B. Neisser Nc.	,, ,,	Nitrite not destroyed.
B. Jürgens	,, ,,	,, ,,

B. Flexner forms nitrite in 1 % peptone solution but destroys it within 24 hours. This may be shown by the following experiment.

200 c.c. of 1 % peptone water are inoculated with *B. Flexner* and placed at 37° C. Samples withdrawn by means of a sterile pipette are tested at intervals of an hour. During the first few hours no nitrite seems to be formed; but after about five hours the samples begin to show an increase in the amount of nitrite present and ultimately a strong nitrite reaction developes, the maximum being attained about 8—10 hours after inoculation. The amount of nitrite then rapidly diminishes and after about 18 hours the nitrite reaction has entirely disappeared.

Cultures of *B. Shiga* still give the nitrite reaction in full strength even after incubating for as long as four weeks.

The Effect of Oxygen upon Reduction.

It has been shown by Burri and Stutzer (1895), Weissenberg (1897) and others that free exposure to oxygen prevents certain organisms from reducing substances which under less aerobic conditions they may even reduce with ease. Cultivation of organisms in thin layers of medium or in a current of air or oxygen are methods which have been used. In the case of the dysentery organisms studied by the author the following method was adopted.

Five c.c. of nitrite peptone solution (peptone 1 %, sodium chloride ·5 %, sodium nitrite ·00023 %) were placed in a conical Jena-glass flask of 300 c.c. capacity. The layer of fluid thus formed had a maximum depth of about 5·mm. and exposed a surface of about 45 sqr. cm. The flask was fitted with a two hole indiarubber stopper through which passed two glass tubes one to within about $\frac{1}{2}$″ of the surface of the medium; the other but a short distance beyond the lower surface of this stopper. As they emerged from the stopper these tubes were bent at right angles and the horizontal portion was drawn out at one point to capillary dimensions in order to facilitate sealing. After the flask and its contained medium had been sterilised it was inoculated with the organism to be tested and the stopper tied in to prevent its being expelled by the expansion of the contained gas when the flask was placed in the incubator. The junction between the stopper and the neck of the flask and the points of emergence of the glass connecting tubes, were then luted with paraffin and the flask was connected in series with its fellows. As a rule from six to a dozen flasks were inoculated with different strains and treated at the same time, one flask being always kept sterile as a control. Sterilised rubber tubing was used to join up the series and from the last flask a longer piece of tubing led the escaping gases under water, thus obviating all risk of air re-entering the last flask, and at the same time affording a means of readily ascertaining whether gas was really passing. The importance of this may be understood when it is mentioned that the surface tension of a bubble of water such as often condenses in the capillary parts of the connecting tubes (especially during sterilisation) enables it to oppose such resistance to the passage of the gas that a flask will burst before the bubble yields. Gentle heating with a Bunsen-flame usually dispels such drops of water.

The oxygen was obtained from a cylinder and was passed through a series of wash-bottles, the first of which contained a saturated solution of urea acidified with hydrochloric acid, while the next two contained a strong solution of caustic soda. Two Drechsel wash bottles containing lead acetate and silver nitrate solution as used in purifying hydrogen were included on empirical grounds as it was found at the first attempt that the organisms did not grow, whereas on the second when these two wash-bottles were included growth was quite as good as that in 4″ × $\frac{1}{2}$″ test tubes, sealed with paraffin in the manner already described. Samples of the gas issuing from the last flask were collected from time to time and tested with a glowing splinter of wood, or the glowing end of a piece of twine. When the sample of gas was able to re-ignite a glowing match, the gas was allowed to continue passing for some time and then the flow was stopped. The series was left from ten minutes to quarter of an hour to allow the pressure to become equalised, and then the flasks were sealed off one by one beginning with the flask nearest the oxygen cylinder. Similar flasks containing nitrate peptone solution (peptone 1 %, sodium chloride ·5 %, sodium nitrate ·000283 %) were treated in the same way.

The results are included in the following table (p. 151).

In other experiments flasks were kept three or four days at 37° C., but no reduction had taken place even after that period.

It thus appears that oxygen completely inhibits the destruction both of nitrate and of nitrite. The growth in these flasks as judged by the degree

of turbidity produced was much the same as that of a similar series of cultures made in the same medium at the same time, but in $4'' \times \frac{1}{2}''$ test tubes which were sealed with paraffin. Lest the failure to destroy should be due to the death of the organisms, flasks inoculated with *B. Flexner*, *B. Neisser* Mc., *B. Neisser* Lb. and the bacillus isolated by the author were incubated at 37° C. for 48 hours in an atmosphere of oxygen, and agar slopes were then inoculated from them. Subcultures were obtained from all four flasks though no destruction of nitrite had taken place.

	Result of testing for nitrite after 36 hours, at 37° C.		
Organism	Nitrate series	Nitrite series	
B. Eyre 7	−	+	Same as control.
,, 9	−	+	,, ,,
,, ·10	−	+	,, ,,
B. Neisser Ac.	−	+	,, ,,
B. Shiga	−	+	,, ,,
B. Celli	−	+	,, ,,
B. Flexner	−	+	,, ,,
B. Neisser Lb.	−	+	,, ,,
,, Mb.	−	+	,, ,,
,, Mc.	−	+	,, ,,
,, Nb.	−	+	,, ,,
,, Nc.	−	+	,, ,,
B. Jürgens	−	+	,, ,,
Control (uninoculated medium)	−	+	

In this connection it may be mentioned that Moore and Williams (1909) have studied the growth of *B. Shiga*, *B. Flexner*, and *B. Kruse*, in an atmosphere containing about 90 % oxygen. Following somewhat different methods they found that *B. Flexner* grew almost as well in 90 % oxygen as in air. The other two strains however showed wide variations in different experiments. The important point is that in the author's experiments growth as judged by turbidity was equal to that in the paraffin sealed tubes.

Effect of Anaerobic Conditions (Hydrogen).

With a view to testing whether anaerobic conditions might cause the non-reducing strains to attack the nitrite, the following experiments were performed.

A series of Jena-glass test tubes each containing 5 c.c. of nitrite peptone (peptone 1 %, sodium chloride ·5 %, sodium nitrite ·00023 %) were inoculated with the various strains of dysentery bacilli and hydrogen was passed through the tubes for a considerable time after the escaping gas burnt with a steady flame. They were

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then sealed and incubated at 37° C., a control tube of uninoculated medium being treated in the same way. A similar series of tubes containing nitrate peptone solution was also treated in the same fashion.

The following table gives the results.

Organism		Result of testing for nitrite after 36 hours at 37° C.		
		Nitrate series		Nitrite series
B. Flexner (Kral)	+	Like nitrite series control		—
B. Celli ,,	+	,, ,, ,,		—
B. Jürgens ,,	+	,, ,, ,,		Same as control.
B. Shiga ,,	+	,, ,, ,,		,, ,,
B. Eyre 7	+	,, ,, ,,		,, ,,
B. Neisser Ac.	−	Like nitrate series control		,, ,,
,, Lb.	+	Like nitrite series control		—
,, Mb.	+	,, ,, ,,		—
,, Mc.	+	,, ,, ,,		—
,, Nb.	+	,, ,, ,,		—
,, Nc.	+	,, ,, ,,		Same as control.
Control tube of uninoculated medium treated in the same way	−	—		+

It thus appears that under anaerobic conditions both the reducing and the non-reducing strains behave exactly as in the paraffin sealed $4'' \times \frac{1}{2}''$ test tubes, the non-reducing strains still failing to destroy nitrite though they reduce nitrate as usual. In some experiments the tubes were allowed to stand for several days, in one case for a week, before testing, but no reduction was effected even in that period.

The Effect of Glucose on Nitrite Destruction.

Although as has been already shown, *B. Shiga*, *B. Jürgens*, and *B. Eyre* 7, 9, and 10, fail to destroy nitrite which has been added to ordinary 1 % peptone solution, the further addition of 1 % glucose to the medium enables them to do so quite as thoroughly as *B. Flexner* or *B. Celli*. Since all of these organisms form acid from glucose, this resulting destruction is no doubt due, as Grimbert and Bagros suggest, to the formation of acid which displaces the nitrous acid from its compound and so enables it to react with amide nitrogen, according to the equation :

$$R{\cdot}NH_2 + HNO_2 = R{\cdot}OH + H_2O + N_2.$$

The mechanism of this reaction will be dealt with in a future communication.

Effect of Oxygen on Nitrite Destruction in Glucose-containing Media.

To test the effect of oxygen upon nitrite destruction in glucose-containing media the following experiment was carried out.

Six flasks fitted with indiarubber stoppers and glass connecting tubes were prepared as before and in each was placed 4·5 c.c. nitrite peptone solution. After sterilisation, ·5 c.c. of a sterile 20 % solution of glucose was added to each of three, and ·5 c.c. of sterile water to each of the other three. Of each set of three, one flask was inoculated with *B. Shiga*, one with *B. Flexner*, and one was kept sterile as a control. After inoculation the flasks were connected in series and oxygen passed as before, the flasks being finally sealed and placed in the incubator at 37° C. After 80 hours each was tested for nitrite.

The following table gives the results.

	The result of testing for nitrite the flask containing	
Organism	Glucose	No glucose
B. Flexner	Faint colour (destruction)	Marked colour (like control).
B. Shiga	,, ,,	,, ,,
Medium control treated in the same way	Marked colour	,, ,,

It is thus apparent that marked destruction had occurred with both organisms in the presence of glucose although not to quite so marked an extent as is the case in sealed test-tubes. This destruction on the part of *B. Flexner* contrasts with the total absence of destruction in the corresponding flask inoculated with *B. Flexner*, and treated in precisely the same way, but containing no glucose. With both organisms abundant growth had occurred in the glucose medium as was shown both by turbidity and on microscopic examination. To test whether the conditions of growth had affected the vitality of the organisms sub-cultures were made on agar and growth resulted except from the glucose peptone culture of *B. Shiga*. It is most likely that the acid produced had caused the death of this organism, which is, in general, more delicate than *B. Flexner*. At any rate, growth was more abundant in this flask than in any of the others and the death of the organism cannot therefore be due to inhibition by the oxygen.

SUMMARY AND CONCLUSIONS.

(1) Dysentery strains from various sources have been examined with respect to their power of reducing nitrates to nitrites and of further destroying nitrites. All the strains with one exception (*B. Neisser* Ac.) reduced nitrates to nitrites. None of the strains which fail to ferment mannite (Shiga type) destroyed nitrites. Those dysentery strains which

form acid from mannite differ amongst themselves in regard to their action on sugars and also in their effect on nitrites. Of the eight mannite-fermenting strains examined, two (*B. dysenteriae* Jürgens and *B. Neisser* Nc.) failed to destroy nitrites.

(2) *B. dysenteriae* Jürgens, although closely related to *B. dysenteriae* Flexner, differs from it both in its action on litmus-whey and in failing to destroy nitrite. It must therefore be considered a different strain. *B. dysenteriae* Jürgens is remarkable as being the only one of the strains examined which forms indol and at the same time fails to destory nitrites. It is consequently the only one which gives the cholera-red reaction.

(3) The addition of glucose (*i.e.* of a carbohydrate from which acid can be formed) enables Shiga strains to destroy nitrite.

(4) In the presence of an abundant supply of oxygen all the strains fail to destroy nitrites and nitrates.

(5) In glucose-containing media the inhibitory effect of oxygen is less marked.

(6) Under anaerobic conditions Shiga strains and *B. dysenteriae* Jürgens still fail to destroy nitrites.

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