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Tetrazolium Reduction as a Means of Differentiating *Streptococcus faecalis* from *Streptococcus faecium*

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SUMMARY: Tetrazolium (2:3:5-triphenyltetrazolium chloride) was used in a glucose nutrient medium to show the considerable differences in reducing properties between some of the Lancefield group D streptococci. The ability to reduce tetrazolium to formazan, in a glucose-containing medium, at an initial pH 6.0, distinguishes strains of *Streptococcus faecalis* from those of *S. faecium* (Orla-Jensen) which for many years have been classified in the same species as *S. faecalis*. Differences in reducing properties correspond with many other tests now used to separate these species. Measurements of the oxidation-reduction potentials in growing cultures showed that *S. faecalis* produced an E_h value about 150 mV. lower than *S. faecium*. Confirmation of the differences in reducing properties of these species was obtained by testing 68 freshly isolated strains of *S. faecium* and 34 strains of *S. faecalis*.

The problem of differentiating the streptococci within the Lancefield group D has received considerable attention in the past few years because many of these organisms are important clinically and also because their unusual tolerance of salt, temperature and acid conditions renders them important in food spoilage. Considerable advances have been made in defining the species concerned, by correlating physiological reactions with serological types (Skadhauge, 1950; Seelemann & Carstens, 1951; Sharpe & Shattock, 1952). A case has been made for the re-introduction of the species *Streptococcus faecium* (Orla-Jensen, 1919) as a species separate from *S. faecalis* and its haemolytic and proteolytic variants *zymogenes* and *liquefaciens* (Shattock, 1955).

Streptococcus faecalis and *S. faecium* share the characteristics usually described as the 'Sherman criteria' for the enterococcus group (Sherman, 1937), but they may be distinguished by certain other properties (see Table 1). The necessity for distinguishing between these two species may be briefly explained as follows. (1) Both organisms have been isolated in human infections, but Skadhauge (1950) suggests that there may be a difference in the sites of infections with the two organisms. (2) Faecal streptococci have been implicated in a number of outbreaks of food-poisoning (Dack, 1944), and it is not yet known whether one or more types are responsible. (3) Although both species have been isolated in food spoilage, e.g. from canned hams, there is a suggestion that one (*S. faecalis*) may be indicative of poor hygiene, as this organism is mainly found in human faeces, whilst the other organism may get into the meat from the gut of the animal concerned, in this case, the pig (Ingram & Barnes, 1955; Barnes & Ingram, 1955). (4) *S. faecalis* is used as an indicator organism for water pollution.

Skadhauge (1950) and Barnes & Ingram (1955) found that there was a difference in the reducing properties of these two species as shown in litmus milk: *Streptococcus faecalis* usually produces reduction, acid, and clotting in 24 hr., but *S. faecium* shows less reduction and sometimes only acid production. The other two species in the Lancefield group D, *S. durans* and *S. bovis*, do not possess strong powers of reducing litmus milk, and usually cause only acidity or acidity and clotting.

Table 1. *The differentiation of Streptococcus faecalis from S. faecium*

S. faecalis and *S. faecium* share the following characteristics (Sherman criteria):

Lancefield group D.
Growth in the presence of 40 % bile.
Fermentation of glucose, lactose and maltose.
Growth at 10° and 45°.
Growth at pH 9·6 and in the presence of 6·5 % NaCl.
Survival at 60° for 30 min.
Growth in Sherman's methylene blue milk (0·1 %).

They differ as follows:

<i>S. faecalis</i> , <i>S. faecalis</i> var. <i>zymogenes</i> and <i>S. faecalis</i> var. <i>liquefaciens</i>	<i>S. faecium</i>
A number of serological types.	Several serological types but distinct from the <i>S. faecalis</i> types.
Growth in the presence of 1/2500 potassium tellurite.	Inhibited by 1/2500 potassium tellurite.
Strong fermentation reactions; mannitol and sorbitol always fermented, arabinose seldom.	More variable fermentation reactions, particularly with mannitol; arabinose always fermented, sorbitol seldom.
Strong reduction in litmus milk prior to acid and clot.	Less reduction of litmus milk and sometimes no reduction, only acidity.
Active production of tyrosine decarboxylase.	Some strains are active producers of tyrosine decarboxylase, whilst others do not produce it.

2:3:5-Triphenyltetrazolium chloride ('tetrazolium' subsequently) has often been used recently as a redox indicator. It is colourless in the oxidized form and is reduced to the insoluble red triphenylformazan in the presence of suitable reducing substances or systems. The E'_0 was shown by Jerchel & Mohle (1944) to be $-0\cdot08$ V. at pH 6·72. Laxminarayana & Iya (1953) found that certain unnamed strains of faecal streptococci varied in their ability to reduce tetrazolium. It was decided to adapt some of their methods to study tetrazolium reduction by known species of Lancefield group D streptococci, in particular *Streptococcus faecalis* and *S. faecium*.

METHODS

Estimation of tetrazolium reduction by faecal streptococci

Culture medium (subsequently called TG medium). The composition of the medium was (% w/v): peptone (Evans's), 1; Lab Lemco, 1; NaCl, 0·5; glucose, 1; distilled water; 2:3:5-triphenyltetrazolium chloride to give 375 µg/5 ml. medium. In the experiments described below the initial pH value was varied between 6·0 and 7·6.

The medium containing the peptone, Lemco and salt was adjusted to the required pH value and autoclaved at 15 lb./sq.in. for 20 min. in 95 ml. lots. After autoclaving, 5 ml. of a 20% (w/v) glucose solution (sterilized by autoclaving at 10 lb./sq.in. for 10 min.) were added together, with 0.75 ml. of a 1% solution of the tetrazolium salt. (The tetrazolium solution was sterilized by steaming for 30 min.). The TG medium was then aseptically pipetted in 5 ml. lots into 1 oz. screw-capped bottles.

Cultural conditions. The TG medium (5 ml.) was inoculated with 0.025 ml. of an 18 hr. broth culture to give $c. 1 \times 10^7$ organisms/ml. The inoculated bottles were incubated for 24 hr. at 37° and the reduced tetrazolium (insoluble red formazan) was extracted as follows.

Extraction of formazan from cultures. A culture (5 ml.) was shaken successively with 5, 2 and 2 ml. of *n*-butanol to extract all the formazan. The butanol extracts were withdrawn with a Pasteur pipette into marked centrifuge tubes and the volume made to 10 ml. with butanol. The solution was mixed and centrifuged to obtain a clear extract. The colour intensity of the extract was measured in an EEL. (Evans Electroselenium Ltd.) colorimeter with filter no. 625. The percentage reduction of tetrazolium was calculated by reference to a standard curve (see below) on the assumption that it was directly proportional to the amount of formazan produced.

Errors of the method. Care must be taken that the tetrazolium solution is freshly prepared and stored in the dark. In media where considerable reduction has taken place (e.g. with *Streptococcus faecalis*) sufficient formazan to give a visible colour adheres to the organisms during growth and cannot be removed by the butanol extraction. However, even in these cases virtually all the formazan may be recovered (see Fig. 2), the quantity adsorbed evidently being insufficient to interfere with the estimation.

Standard curve for estimation of tetrazolium reduction. Bottles of TG medium (5 ml., pH 7.0) containing 375, 300, 225, 150 or 75 µg. tetrazolium salt/bottle were treated with 1 ml. *N*-NaOH and several mg. ascorbic acid to reduce all the tetrazolium present (Fairbridge, Willis & Booth, 1951). The bottles were left for 30 min. and then adjusted to about pH 4.5 with *N*-HCl (to give a pH for extraction comparable to that obtained after the growth of the streptococci). The formazan was extracted from the broths with *n*-butanol (as above) and a calibration curve (Fig. 1) obtained by relating the colorimeter reading to the initial concentration of tetrazolium salt (when completely reduced to formazan).

Measurement of growth. The total number of organisms present was estimated either by a direct microscopical count or by a turbidimetric method using the EEL nephelometer, a standard curve having been made by relating direct microscopical count to nephelometer reading.

E_h measurements. The redox potentials in the broth cultures were measured by the method described by Hewitt (1950). In this case tall beakers containing 25 ml. medium were fitted with bungs carrying the sampling tube, the platinum electrode and a saturated KCl agar bridge connected to the calomel half-cell. The platinum electrodes were sterilized separately by leaving in

70% (v/v) ethanol overnight and then rinsing with sterile water and drying with sterile cotton-wool. In these experiments the object was not to eliminate all the air but to imitate as far as possible the conditions existing in TG medium in the 1 oz. bottles used for routine tests. The inoculum for these experiments was 1% (v/v) of an overnight broth culture so that the major part of the experiment was concluded in 8–10 hr., although readings were also made after 24 hr. When tetrazolium estimations were made on duplicate control solutions in 1 oz. bottles the inoculum was also increased to 1% (v/v) but the cultures were incubated for 24 hr. in the usual manner.

Fig. 1. Calibration curve relating the percentage reduction of 375 µg. of tetrazolium (as formazan) to the reading on the EEL colorimeter.

RESULTS

For a preliminary survey of the ability of group D streptococci to reduce tetrazolium to formazan, named strains were obtained from Dr P. M. Frances Shattock (University of Reading) and Dr M. Elisabeth Sharpe (National Institute for Research in Dairying). The tests were carried out by the method described above; the pH value of the TG medium was 7.2–7.4. At the same time a comparison was made with the reaction in litmus milk. The results are given in Table 2. The three strains of *Streptococcus faecalis* showed over 80% reduction of tetrazolium to the insoluble red formazan and produced rapid reduction, acid and clot in litmus milk. The five strains of *S. faecium* produced less than 45% reduction of tetrazolium, and litmus milk was only partially reduced in 24 hr. *S. bovis* and *S. durans* reduced the tetrazolium by less than 10%, and produced little change in litmus milk in 24 hr.

These results encouraged further experiments with tetrazolium and freshly isolated strains of *Streptococcus faecium* and *S. faecalis*. The identifications were based on the properties shown in Table 1. Over a period of several months 68 strains of *S. faecium* and 34 strains of *S. faecalis* were tested in TG medium (pH 6.8–7.0) and the differences in their reducing powers was found to be quite characteristic. These results are shown in Fig. 2. Only 6 strains of *S. faecium* gave over 50% reduction of tetrazolium, whilst 2 strains of *S. faecalis* gave less than 50% reduction.

Table 2. *A comparison of the reaction in litmus milk with the reduction of tetrazolium for several species of Lancefield group D streptococci*

Species	Litmus milk			TG medium	
	6 hr.	24 hr.	3 days	Total count × 10 ⁹ /ml. after 24 hr.	% reduction to formazan
<i>S. faecalis</i> (N. 83)	R*	Rc	RAC	2.0	86
<i>S. faecalis</i> var. <i>liquefaciens</i> (GB. 122)	r	RCAIq.	RCAIq.	2.0	91
<i>S. faecalis</i> var. <i>zymogenes</i> (N. 37)	R	RAC	RAC	2.0	85
<i>S. faecium</i> 2766/2	nc	A	ARC	4.0	24
<i>S. faecium</i> 2767	nc	Ar	ARC	2.5	31
<i>S. faecium</i> 2678	nc	Ar	ARC	3.0	35
<i>S. faecium</i> S. 748	r	Ar	ARC	3.0	39
<i>S. faecium</i> 2766/1	nc	Ar	ARC	3.0	43
<i>S. durans</i>	nc	A	AR	2.5	10
<i>S. bovis</i>	nc	nc	A	2.5	9

* nc, no change; A, acid; r, some reduction; R, strong reduction; c, slight clot; C, clot, Iq., liquefaction.

Relationship of redox potential to tetrazolium reduction

In order to determine whether differences in tetrazolium reduction were related to different reducing powers of these organisms, measurements were made of the actual oxidation-reduction potentials of cultures in TG medium. Tests which were carried out separately in tetrazolium glucose medium of different initial pH values showed that after the first few hours the growth rates and pH changes were comparable for strains of *Streptococcus faecalis*, *S. faecium*, *S. durans* and *S. bovis*, as shown in Table 3. Thus any recorded ultimate differences in redox potential would be a reflexion of different reducing properties of the organisms concerned, rather than a function of the pH value of the culture or of the numbers of organisms present.

In the redox potential experiments described below only the pH changes are recorded as an index of the growth of the organisms, as it was difficult to obtain homogenous samples for total count without disturbing the redox potential of the medium. Samples were, however, removed at the end of the experiment and these, together with a few samples taken at intervals, showed that the counts were not significantly different from those recorded in Table 3.

Fig. 2. A comparison of the percentage reduction of tetrazolium to formazan by strains of *Streptococcus faecalis* and *S. faecium*.

Fig. 3. Changes in the redox potential and pH during the growth of *Streptococcus faecalis*, *S. faecium* and *S. bovis*.

+ — +, E_h } *S. faecalis* ▽ — ▽, E_h } *S. faecium* □ — □, E_h } *S. bovis*.
 + - - - +, pH } *S. faecalis* ▽ - - - ▽, pH } *S. faecium* □ - - - □, pH } *S. bovis*.

The changes in E_h and pH were followed for 7–8 hr. at 37° when beakers of TG medium (pH 6·5–6·6) were inoculated with 1% (v/v) of an overnight broth culture of *Streptococcus faecalis* (N. 83), *S. faecium* (S. 748) and *S. bovis* (S. 194). The results are shown in Fig. 3. The lowest potential reached in the *S. faecalis* culture was about E_h –90 mV., that of *S. faecium* was +90 mV.

Table 3. *The effect of initial pH on the growth and total population in glucose broth of Streptococcus faecalis, S. faecium, S. bovis and S. durans*

		Initial pH							
		6·1		6·6		7·1		7·4	
		Time (hr.)	pH	Total count × 10 ⁹ /ml.	pH	Total count × 10 ⁹ /ml.	pH	Total count × 10 ⁹ /ml.	pH
<i>S. faecalis</i>	4	5·6	0·5	6·5	0·7	6·9	0·7	7·0	1
	6	4·5	2·5	4·5	4·0	4·5	5·5	4·5	5·5
	24	4·1	4·0	4·1	5·5	4·1	6·5	4·1	6
<i>S. faecalis</i> var. <i>liquefaciens</i>	4	5·8	0·5	6·4	0·9	6·8	1·0	6·9	0·9
	6	4·5	2·0	4·5	3·5	4·5	4·5	4·5	4·5
	24	4·1	3·0	4·1	4·5	4·1	5·0	4·1	5·0
<i>S. faecalis</i> var. <i>zymogenes</i>	4	5·8	0·5	6·4	0·7	6·4	1·0	6·9	0·9
	6	4·5	2·5	4·5	4·0	4·5	5·0	4·5	5
	24	4·1	4·0	4·1	5·0	4·1	6·0	4·1	6
<i>S. faecium</i> 2766/1	4	6·0	0·4	6·5	0·4	6·9	0·5	7·1	0·4
	6	4·5	3·5	4·5	5·0	4·5	5·5	4·5	5·5
	24	4·1	4·0	4·1	5·5	4·1	6·5	4·1	6
<i>S. faecium</i> S. 748	4	5·9	0·4	6·5	0·5	6·9	0·5	7·1	0·5
	6	4·5	3·0	4·5	4·5	4·5	5·5	4·5	5·5
	24	4·1	4·0	4·1	5·5	4·1	6·0	4·1	6·0
<i>S. faecium</i> 2678	4	6·0	0·4	6·4	0·5	6·9	0·6	7·0	0·6
	6	4·5	3·5	4·5	5·0	4·5	5·5	4·5	5·9
	24	4·1	4·5	4·1	5·5	4·1	6·0	4·1	6·0
<i>S. durans</i>	4	5·9	0·4	6·4	0·6	6·9	0·6	7·0	0·6
	6	4·5	3·0	4·5	4·4	4·5	5·0	4·5	5·5
	24	4·1	4·0	4·1	5·0	4·1	6·0	4·1	6
<i>S. bovis</i>	4	6·1	0·3	6·5	0·4	7·0	0·4	7·3	0·4
	6	4·5	1·5	4·5	3·0	5·7	2·5	5·8	2·5
	24	4·1	3·5	4·1	5·5	4·1	6·0	4·1	5·0

and *S. bovis* +180 mV. Thus the reducing conditions were much greater in the *S. faecalis* culture than in either the *S. faecium* or *S. bovis* cultures. This experiment was repeated on several occasions, and the marked difference between *S. faecalis* and *S. faecium* always obtained. Although the *S. faecium* strain used was usually more reducing than *S. bovis*, occasionally it showed little difference from *S. bovis* in the TG medium. As this investigation was concerned mainly with the differences between *S. faecium* and *S. faecalis* detailed investigations of the relationship of *S. faecium* to *S. bovis* were not made.

Effect of changes in the medium on tetrazolium reduction

Alteration in the initial pH value of the glucose tetrazolium medium. The initial value of samples of TG medium was adjusted to 6.1, 6.6, 7.1 and 7.4 respectively, and inoculated with a number of strains of *Streptococcus faecalis*, *S. durans* and *S. bovis*. The results obtained from a number of experiments are shown in Fig. 4 where tetrazolium reduction is related to the initial pH value of the

Fig. 4. The effect of the initial pH of glucose broth on the reduction of tetrazolium to formazan by (A) *S. faecalis*, (B) *S. faecium*, (C) *S. durans* and (D) *S. bovis*.

medium. With the strains of *S. faecalis* there was over 50% reduction of tetrazolium to formazan at all pH values, whilst with *S. durans* and *S. bovis* there was less than 10%. In the case of *S. faecium* the initial pH value considerably influenced the amount of formazan produced during growth. It can be seen that in order to get the maximum differentiation between *S. faecium* and *S. faecalis* the test should be carried out at pH 6.0–6.2. With a strain of *S. faecium* an attempt was made to correlate the amount of formazan produced in the media of different initial pH values with the lowest redox potential obtained in that particular medium. Although there was no absolute correlation the lowest potentials recorded varied from $E_h + 210$ mV. in medium of initial pH 6.0 to $E_h + 120$ mV. when the initial pH was 7.6. In the same experiment the reduction of tetrazolium varied from less than 2.0 to 28%.

Effect of alterations in the composition of the medium. The effect on tetrazolium reduction of growing the organisms in media where there was only a slight change in pH value was tested in the following manner. Samples of Hartley's tryptic digest broth containing the usual concentration of tetrazolium were adjusted to pH 6.0, 6.4, 6.8 and 7.2. The broths were inoculated with strains of *Streptococcus faecalis*, *S. faecium* and *S. bovis*, and incubated for 24 hr. In every case there was more than 50% reduction of tetrazolium to formazan, i.e. no difference was detected in the reducing properties of the organisms. In order to explain this a redox potential experiment was set up where TG medium and digest broth at pH 6.1–6.2 were compared under

Fig. 5. Changes in E_h and pH of (A) *S. faecalis*, (B) *S. faecium* and (C) *S. bovis* when grown in glucose broth and digest broth.

+ — +, E_h } in glucose broth Δ — Δ, E_h } in digest broth.
 O — — O, pH } ▽ — — ▽, pH }

identical conditions, with inocula of the three different species. Changes in redox potential and pH are shown in Fig. 5. In the digest media the pH value rose and the actual oxidation-reduction potentials were much lower, by 80 or more mV., than those reached in the glucose media. Even in the case of *S. bovis* the E_h of the digest broth was as low as +74 mV. so that all three species then reduced the tetrazolium.

Reduction of tetrazolium at pH 6.0. From the above experiments it was apparent that the best medium to use for obtaining the maximum differentiation between *Streptococcus faecalis* and *S. faecium* was a TG medium of initial pH 6.0–6.2, as the redox potentials reached in this medium were such that there was no reduction with cultures of *S. faecium* but over 50% reduction with *S. faecalis*. In view of a statement by Jámboř (1954) that at pH values below 6.0 tetrazolium is partially reduced to a colourless compound, it was decided to estimate the residual unreduced tetrazolium in the cultures to see whether all the initial tetrazolium could be accounted for.

Samples of TG medium were inoculated in quadruplicate with *Streptococcus faecalis*, *S. faecium* or *S. bovis*, and incubated for 24 hr. Two bottles were taken

for the estimation of formazan in the usual manner. The other two bottles were treated with 1 ml. of N-NaOH followed by a few mg. ascorbic acid to reduce all the residual tetrazolium to formazan. The broths were then acidified by the addition of 1 ml. of N-HCl and the total formazan extracted from the solution as before. The results are shown in Table 4. With *S. faecium* and *S. bovis* almost all of the tetrazolium was recovered from the medium so that it had not been reduced by the organisms. In the case of *S. faecalis* about 30% of the tetrazolium was not recovered; the reduction of tetrazolium had

Table 4. *The reduction of tetrazolium by Streptococcus faecalis, S. faecium and S. bovis in media of initial pH 6.0*

	% of tetrazolium reduced to formazan	Residual unreduced tetrazolium (%)	% of tetrazolium unaccounted for in the medium
Control solution	0	100	0
<i>S. faecalis</i> (N. 83)	59 } 45 } 54 59 }	8 } 19 } 14 16 }	32
<i>S. faecalis</i> var. <i>zymogenes</i>	46 } 54 } 50	22 } 27 } 25	25
<i>S. faecium</i> (S. 748)	1.5 } 0.5 } 1.0	90 } 92 } 91	8
<i>S. bovis</i>	0.5 } 0.5 } 0.5	97.5 } 97.5 } 97.5	2

either resulted in a mixture of the formazan and a colourless compound, or the formazan had been reduced further. It would therefore be unwise to attempt quantitative estimations of tetrazolium reduction in culture media with an initial pH of 6.0; but qualitatively, sufficient formazan is produced by strains of *S. faecalis* to give a bright red colour when extracted with butanol as opposed to an almost colourless solution with strains of *S. faecium*.

Qualitative methods for the separation of Streptococcus faecalis and S. faecium

As a result of the above experiments the following media were developed to assist in the separation of *Streptococcus faecalis* and *S. faecium*.

(a) *TG medium at pH 6.0–6.1*. This medium was prepared as described above, but was adjusted to an initial pH of 6.0–6.1. Instead of extracting the formazan after overnight incubation with the test organisms, the cultures were shaken with 5 ml. of *n*-butanol and allowed to settle. Positive reactions were indicated by a bright red butanol layer whilst negative reactions were either colourless or very pale pink. About 70 of the isolates of *Streptococcus faecalis* and *S. faecium*, which had previously been tested in TG medium at pH 6.8–7.0 with the results shown in Fig. 2, were re-tested in the TG medium at pH 6.0 by this qualitative method. The strains could now be divided easily into *S. faecalis* with the bright red reaction and *S. faecium* with a colourless

reaction. The only exceptions were those few strains which had previously been atypical—in the case of *S. faecalis* having a low reducing power, and with *S. faecium* having an exceptionally high one.

(b) *Tetrazolium* agar for isolating *Streptococcus faecalis* and *S. faecium*. A useful extension of the methods described above was the preparation of a solid medium for separating these two organisms. This medium had the same composition as TG medium but the concentration of tetrazolium was increased to 0.01 %, and it was solidified by addition of 1.2 % (w/v) New Zealand agar; the pH value was adjusted to 6.0–6.1. The agar, peptone, Lemco and salt was autoclaved at 15 lb./sq.in. for 20 min. in 95 ml. lots and the glucose and tetrazolium solutions added immediately before the plates were poured. The plates were streaked with cultures of the test organisms and incubated for 1 or 2 days at 37°. Isolated colonies of *Streptococcus faecalis* were deep red, whilst those of *S. faecium* were colourless. When cultures of *S. faecalis* were mixed with *S. faecium* it was quite easy to separate the two species again by this method.

DISCUSSION

The results given in this paper emphasize the great differences in the reducing powers of the two related species, *Streptococcus faecium* and *S. faecalis*, and substantiate the position of *S. faecium* (Orla-Jensen) as a species distinct from *S. faecalis* and its variants *zymogenes* and *liquefaciens*. Within the Lancefield group D, *S. faecium* appears to occupy an intermediate position between *S. faecalis* on the one side and *S. durans* and *S. bovis* on the other.

As there was a difference in E_h of over 100 mV. between the cultures of *Streptococcus faecalis* and *S. faecium* grown under the same conditions, it was possible to adjust the initial pH value of TG medium so that with *S. faecium* there was no reduction of tetrazolium during growth, whilst with *S. faecalis* the tetrazolium was almost entirely reduced.

This test with TG medium at pH 6.0 augments the other distinguishing tests given in Table 1. The advantage of using tetrazolium in such a test is that the production of the insoluble formazan is an irreversible reaction. This is important as it was observed in all the redox potential experiments with TG medium that the E_h rose rapidly before growth had ceased. Hewitt (1950) also found this with other streptococci, the rise in potential being attributed to the production of hydrogen peroxide. Hence, with a reversible redox indicator such as methylene blue, reduction is liable to be only temporary, making the test ambiguous.

In the experiments described above it is evident that the reduction of tetrazolium took place in cultures of a much higher redox potential than would be expected from the E_0 of –80 mV. at pH 6.72 given by Jerchel & Mohle (1944). From the information available it seems that once the potential falls below about $E_h + 150$ mV. reduction of tetrazolium begins.

The tetrazolium glucose agar medium at pH 6.0 has been found most valuable for distinguishing the colonies of *Streptococcus faecalis* from *S. faecium* and the other Lancefield group D streptococci.

Cooper & Ramadan (1955) showed that true *Streptococcus faecalis* organisms which strongly reduced litmus milk and also Janus Green were typical of human faeces. They isolated a number of strains from bovine and sheep faeces which they listed as atypical *S. faecalis* strains and which were shown to have low reducing powers. These organisms were probably similar to the *S. faecium* strains described in this paper. Unfortunately the concentration of potassium tellurite (1/5000) used by Cooper & Ramadan was too low to inhibit their atypical strains if indeed they were *S. faecium*. Skadhauge (1950) showed that 1/2500 tellurite was the optimal concentration for separating *S. faecalis* and *S. faecium*; at higher dilutions *S. faecium* would grow.

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