THE DETECTION OF STREPTOCOCCI IN AIR

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(With Plate 7)

INTRODUCTION

In 1904 Gordon described a 'bacterial test for estimating pollution of air' based, by analogy with comparable tests for water, on the cultivation of presumptive mouth streptococci from air samples, and he used this test in his investigations of the ventilation of the House of Commons (Gordon, 1906). Although Gordon's experimental observations were confirmed (Hamilton, 1905; Winslow & Robinson, 1910), no further work appears to have been reported until after the introduction of the 'air-centrifuge' (Wells, 1933) with which airborne bacteria could be collected more efficiently than by earlier methods. Wells & Wells (1936) demonstrated contamination of the air with α-haemolytic streptococci after sneezing and talking; and later Wells, Phelps, Robertson & Winslow (1941, 1942) proposed the use of a count of presumptive mouth streptococci—*Streptococcus viridans*—as an index of respiratory pollution of air.

The α-haemolytic *Streptococcus* count of the air of various occupied places was studied by Buchbinder, Solowey & Solotorovsky (1938) and by Torrey & Lake (1941); but no attempt has hitherto been made to justify the assumption, implied in the papers of Wells and his colleagues, that the number of mouth streptococci in the air of occupied places is a measure of the hazard of respiratory infection.

An extensive trial of ultra-violet irradiation as a method of aerial disinfection offered us an opportunity for investigating these points. Methods for detecting streptococci in air samples were therefore devised and tested; and these methods and tests form the subject of the present paper. An account of the results obtained,

and a discussion of the general implication of any index of respiratory pollution of air, will be presented elsewhere.

Methods used in previous work

Gordon collected airborne bacteria in carbohydrate-containing broth, which was incubated anaerobically, and relied on fermentation for recognition of the streptococci. At first Wells used α-haemolysis on a blood-agar medium to indicate the colonies of streptococci (Wells & Wells, 1936); Buchbinder and his co-workers used the same method. Later (Wells *et al.* 1941) the blood agar in the air-centrifuge tube was replaced by lactose broth containing gentian-violet, and fermentation was regarded as presumptive evidence of the presence of streptococci. Dorothy Wells (1941) suggested that tubes showing fermentation should be subcultured to blood agar containing crystal violet for confirmation. Torrey & Lake collected the airborne bacteria on glucose agar and picked all colonies resembling those of streptococci for further investigation. For reasons discussed later none of these methods seemed to us to be satisfactory.

General principles of the present work

It was clear from the previous work that streptococci generally make up no more than a very small proportion of the bacteria normally present in the air of occupied places, and the use of a selective culture medium was therefore desirable. Moreover, in view of the absence of any positive biochemical test by which streptococci could be readily distinguished from all staphylococci and other micrococci, it seemed likely that microscopic morphology might be a more useful diagnostic criterion than fermentation reactions and the like.

Preliminary investigations failed to discover a culture medium that would allow mouth streptococci to flourish and at the same time inhibit all the micrococci found in air samples. A routine was therefore devised in which the bacteria-carrying particles were collected on to a selective medium permitting the growth of streptococci but inhibiting as many as possible of the micrococci, and a random sample of the colonies developing on this medium was further examined to determine the proportion of streptococci present.

DEFINITIONS

The bacteriological terminology adopted in this paper is as follows:

Streptococcus. A Gram-positive coccus forming long or short chains when growing in broth; catalase negative. In routine practice, streptococci were very frequently diagnosed simply on the basis of chain formation by cocci in broth culture, as revealed in a nigrosin film, or of chain formation by cocci on a solid medium. Some difficulty was originally experienced with these simple methods in distinguishing enterococci from some micrococci; however, most other types of streptococci commonly formed longer chains on the selective medium described below than on blood agar.

Viridans-type *Streptococcus.* This term is used generally to refer to the typical streptococci of the mouth and saliva, i.e. to streptococci that are generally but not

always α-haemolytic on blood agar, and which fail to grow on serum agar containing 40 % ox bile.

Strep. salivarius. Streptococci forming soft mucoid colonies on agar containing 5 % sucrose (Sherman, Niven & Smiley, 1943).

Enterococcus. This term is used generally to cover streptococci—often predominantly diplococcal in morphology—α-, β- or non-haemolytic on blood agar, which flourish on agar containing 40 % bile. The majority give a positive precipitin reaction with Lancefield's Group D serum either with or without concentration of the extract by alcohol precipitation (Shattock, 1949). Both *Strep. faecalis* and *Strep. bovis* have been recognized in air samples.

Micrococcus. A Gram-positive coccus not forming chains in a fluid culture medium; catalase positive; coagulase-negative. In air samples on our selective medium one particular group of organisms, which we presumed to be micrococci, was very common. When transferred to blood agar these strains formed semi-transparent colonies 0·2–0·8 mm. in diameter, surrounded by a variable zone of greening; they grew mainly in pairs or small clusters both in broth and on solid media, and practically all strains flourished on bile agar. They were either very weak catalase producers or were catalase-negative, and strongly benzidine-positive. We refer to them as 'greening micrococci'; they may well be the organisms referred to as diplococci by Rabl & Seelemann (1949).

MATERIALS AND METHODS

Material. Our material consisted largely of samples of airborne bacteria collected either during routine surveys in the ultra-violet-irradiation investigation already mentioned, or in surveys in offices and factories, and in the open air. For various experiments using cultures of streptococci, samples were collected on swabs from the mouth or tonsils of healthy subjects; as a rule the specimen was emulsified in broth, which was then diluted appropriately and spread evenly over the surface of blood- or serum-agar plates. After incubation, colonies were picked from the plates in a strictly random fashion.

Methods. Air samples were usually obtained with various forms of slit-sampler (Bourdillon, Lidwell & Thomas, 1941; Bourdillon, Lidwell & Lovelock, 1948; Lidwell, 1950); in some cases open Petri dishes were exposed to collect bacteria-carrying particles settling from the air.

Viable counts with cultures on various media were performed by the method of Miles & Misra (1938); as a rule five replicate drops of each dilution were seeded on different plates.

A reading lens, 2½ in. in diameter giving about × 2 magnification, was used for counting the colonies on the plates, which were laid on an inclined glass plate ruled with a grid to allow illumination from above and below; a 'tally counter' was always used.

Bacteriological media. Nutrient agar containing 5 % normal horse serum was the basis of all our air-sampling media; serum agar was preferred to blood agar because of the greater ease in counting colonies on a transparent medium, and of the difficulty in maintaining a stock of good quality blood-agar plates under the

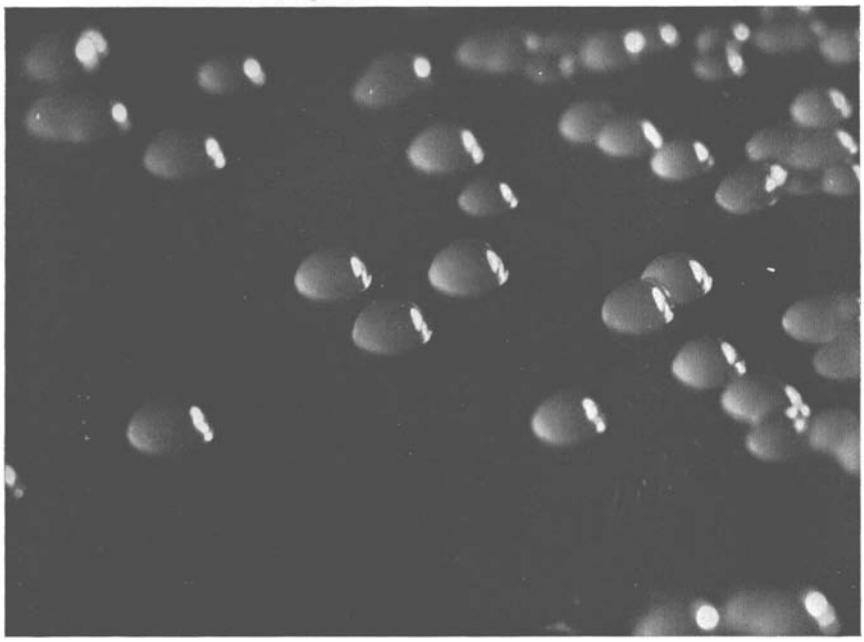

Fig. 1. Colonies of *Strep. salivarius* on the S1 medium, × 5.

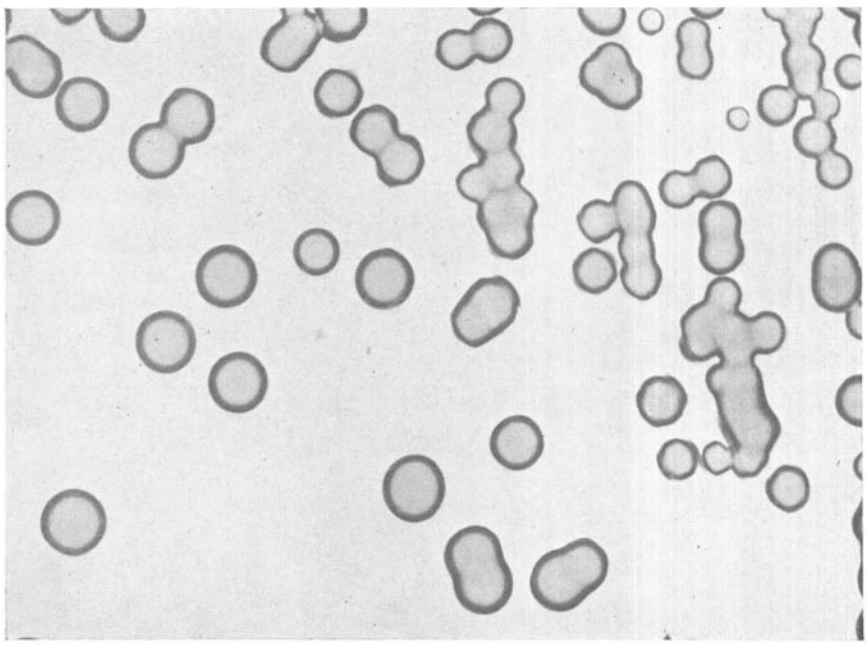

Fig. 2. Colonies of *Strep. salivarius* on S1 medium seen through the back of the plate, to show appearance of a dark ring at the periphery of the colony, × 4.

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conditions of a field experiment. All the selective media used contained 5 % sucrose to permit easy recognition of colonies of *Strep. salivarius*.

After some preliminary exploration crystal violet and potassium tellurite were adopted as selective agents; and a routine selective medium, designated S 1, was compounded as follows:

Nutrient agar with:	
Sucrose	5 %
Serum	5 %
Potassium tellurite	0·25 mg. %
Crystal violet	1·0 mg. %

For distinguishing mouth streptococci from enterococci, 'ditch plates' were used. A diametral incision was made in plain blood-agar plates; half the medium was removed and replaced by nutrient agar containing 5 % horse serum and Difco dehydrated ox-gall powder to give a final concentration corresponding to 40 % bile. Latterly the bile agar contained 0·1 % aesculin and 0·05 % ferric citrate for recognition of enterococci. Mouth streptococci typically fail to grow on the bile agar, but both enterococci and the greening micrococci flourish on it. Such ditch-plates could, of course, be prepared by pouring the two halves separately.

For examining morphology in fluid cultures, broth containing 5 % horse serum and 1% lactose was used.

General characters of routine medium. The S 1 medium adopted as a routine is a clear, greyish medium, which apparently permits the growth of most viable streptococci impinging upon it (see below), while inhibiting all but about 10 % of the general viable aerobic bacterial flora recognizable on, for instance, serum agar plates. The organisms other than streptococci cultivated on S 1 from the air of occupied places comprise a few Gram-negative bacilli, very few aerobic spore-bearing bacilli, and, predominantly, micrococci of the type already mentioned that produce greening on blood agar.

Colonies of *Strep. salivarius* are 1–3 mm. in diameter after 40 hr. incubation, standing boldly up from the surface of the agar, varying from translucent and colourless to greyish blue (Pl. 7, fig. 1). They can be readily distinguished from the few aerobic spore-bearing bacilli that produce watery colonies by the characteristic dark rim seen when the colony is inspected through the back of the dish (Pl. 7, fig. 2). On the selective medium described every one of over 100 of these mucoid colonies tested has proved to be a *Streptococcus*.

Most other streptococci form semi-transparent, grey or black colonies, 0·2–1·0 mm. in diameter after 40 hr. incubation. A small proportion form very hard glassy colonies about 0·5–1 mm. in diameter, which are deeply embedded in the agar. All of twenty-two such colonies subcultured to lactose broth proved to be streptococci.

The micrococci form grey or black colonies 0·2–1·5 mm. in diameter.

Routine method of examining air samples

The S 1 plates were incubated at 37° C. for about 40 hr. The total colonies and the *Strep. salivarius* colonies were then counted, and a random sample of all the colonies

not *Strep. salivarius* was picked for identification. In the early stages of the work the air sampler was one that distributed airborne bacteria evenly over an annular area on the plate (Bourdillon *et al.* 1941); and since the plate rotated under the slit several times during the exposure it could be assumed that all sectors of the plate were comparable. For the selection of the sample of colonies the plate was laid, at random, on a glass plate ruled with a number of concentric circles and divided into thirty-two equal sectors, numbered 1–32 in a strictly random order. Colonies were then picked from the periphery of the plate towards the centre in each of the numbered sectors in order until the proper sample had been collected. The sampling fractions finally adopted were as follows:

No. of colonies on plate	No. picked for identification
1–20	All
21–30	20
31–75	25
76–120	30
120–200	35
More than 200	40

After the introduction of the 'spiral' sampler (Lidwell, 1950) the method of picking was modified; although no longer strictly random, it was considered to give the most appropriate sample. The dish was laid on a glass plate which was ruled with a spiral corresponding to the sampler track and divided into areas for each of the 24 minutes of exposure. Colonies were picked from the areas of the 3rd, 8th, 13th, 18th and 23rd min., proceeding in each case from the periphery of the spiral track on the plate towards the centre, until one-fifth of the quota of colonies had been picked. If these five areas did not give sufficient colonies, the procedure was repeated using the areas of the 2nd, 7th, 12th, 17th and 22nd min.; and so forth.

With both routines of picking, care was taken that the colonies to be picked were determined solely by their position on the plate and not by their size or prominence.

We use the term 'estimated streptococcal count' to denote the count on the whole plate calculated from the proportion of streptococci in the sample and the number of colonies on the plate.

Identification of colonies

Three different routines were employed for identification of the colonies picked.

In the first, *routine A*, the colonies were simply transferred to drops of nigrosin on a glass slide for subsequent microscopic identification.

When our investigations had proceeded for some time we found that enterococci were not uncommon in the air of occupied rooms and, since the presence of enterococci cannot be regarded as indicating respiratory pollution, it was desirable to eliminate them from the estimate of respiratory tract streptococci. Two rough methods—one depending on the fact that enterococci usually form larger colonies than viridans streptococci, and one on the predominantly larger size of the individual cocci—were considered but found inadequate. Nevertheless, the experience gained in these investigations showed that the proper segregation of the enterococci would be of great interest in our wider study of the ecology of airborne streptococci, and

we decided to adopt the relatively laborious methods involving subculture to bile-agar 'ditch-plates' (routines B and C below). *Strep. salivarius*, *Strep. viridans*, *Strep. pyogenes* and the like are unable to grow on agar containing 40 % bile, while enterococci and practically all the micrococci present on the S 1 plates flourish. This offers a simple presumptive test for mouth streptococci.

In *routine B* therefore we transferred the random sample of colonies to ditch plates and to nigrosin films. Colonies having a streptococcal morphology in the film and failing to grow on the bile agar were regarded as 'presumptive mouth streptococci' (*viridans* and β-haemolytic types) and were not as a rule subjected to further confirmation. The following strains were transferred to lactose serum broth for examination of microscopic morphology: (*a*) colonies morphologically resembling streptococci that had not been regarded as streptococci in the original nigrosin film, but which grew on the blood agar and not on the bile agar; and (*b*) colonies that had had a streptococcal morphology in the nigrosin film but were bile-tolerant, i.e. presumptive enterococci.

From the experience of the use of the bile-ditch-plates it was clear that, if a cultural method of distinguishing enterococci from the bile-tolerant micrococci could be devised, we could limit the examination of nigrosin films to the colonies suspected of being streptococci from the growth on the ditch plate, so saving much time while still obtaining a precise diagnosis of the streptococci. Most enterococci hydrolyse aesculin and produce a black colour in a medium devised by Edwards (1933), and we adapted Edwards' principle by adding 0·1 % aesculin and 0·05 % ferric citrate to the bile agar. As a check on the reliability of this reaction the routine described in the last paragraph was at first modified only to the extent that aesculin and ferric citrate were incorporated in the bile agar. Of 140 bile-tolerant streptococci recognized by their morphology either in the direct film or in a broth subculture 117 (83·6 %) hydrolysed aesculin; of the twenty-three strains not fermenting aesculin fifteen could be distinguished from the bile-tolerant micrococci by their colonial morphology. In a method designed primarily for the recognition of presumptive mouth streptococci the failure to recognize some 6 % of the enterococci was considered no great loss, and in *routine C* the colonies were picked only to the ditch plates; after overnight incubation the following strains were picked to lactose serum broth for confirmation by microscopic morphology: (*a*) all bile-intolerant strains not obviously coliform or spore-bearing organisms, including any strains showing poor growth on the bile agar; (*b*) all colonies blackening the aesculin-bile-agar; (*c*) any non-haemolytic, bile-tolerant colonies colonially resembling enterococci, but not fermenting aesculin.

TESTS OF EFFICIENCY OF ROUTINE METHODS

The routines just described have been submitted to a number of tests to measure: (1) possible inhibition of the growth of streptococci resulting from the incorporation of the selective agents, (2) the effect of variations in the routine on the streptococcal count obtained, and (3) the efficiency of recognition of streptococci by the three methods used.

Investigations of possible inhibition of streptococci

Tests with cultures of streptococci

The concentrations of crystal violet and potassium tellurite used in the S1 medium were based in the first place on a number of tests with streaked cultures of streptococci from normal mouths. The tentative results obtained in this way were studied further in eight experiments, each with a different strain of *Streptococcus* from mouth cultures, designed to reveal quantitatively any inhibition due to the crystal violet or potassium tellurite; and to discover whether the count was affected by the addition of proteose peptone, or by the substitution of horse blood for horse serum. By using a factorial design (Fisher, 1948) it was possible to investigate in addition possible interactions between these four factors. Batches of media, in a nutrient agar base, were compounded with each of the sixteen possible combinations of the four factors (crystal violet, potassium tellurite, proteose peptone, and serum), and five plates of each medium were poured. On each of the eighty plates, rearranged in a random order, was seeded with standard dropping pipettes (delivering 0·02 ml. drops) one drop of each of three dilutions of the culture under test. Colonies were counted after 48 hr. incubation.

Table 1. In-vitro *comparisons of inhibitors for streptococcal selective medium*

Exp. no.	1	2	3	4	5	6	7	8
Strain*	P1	V1	S1	V2	V3	S2	V4	V5
Mean count	831	265	348	482	852	288	526	2246
S.E. of diff.	46	27	27	20	58	24	31	74
Factors (mg./100 ml.)								
Crystal violet:								
0·2	+46	+3	+6	—	—	—	—	—
0·25	—	—	—	−3	+23	−8	—	—
0·17	—	—	—	—	—	—	−31	+11
Potassium tellurite:								
1·25	+42	+1	−10	—	—	—	−17	+43
1·6	—	—	—	—	*−231*†	*−70*	—	—
2·0	—	—	—	*−121*	—	—	—	—
Blood (vs. serum)	+24	−1	−4	*−375*	−11	+28	−37	−75
Proteose peptone	−66	+29	−18	+17	+41	−14	−37	+45
Interaction, blood and tellurite	+4	+41	−22	*−59*	−65	−26	−29	−39

* P = *Strep. pyogenes*; V = *Strep. viridans*; S = *Strep. salivarius*.

† Differences in italic figures exceed the value of twice their standard error.

The figures in the body of Table 1 give the difference between the total number of colonies from the five 0·02 ml. drops on each of the eight media containing the specified factor, and the total number of colonies on the eight media not containing the factor. The mean count is the average of these two sets of counts, each on eight media. Thus in Exp. 1, the mean number of colonies per set of eight media was 831, and the standard error of the difference between the two sets was 46. The eight plates containing crystal violet had, in all, forty-six more colonies than the eight plates containing no crystal violet; and this difference is clearly not significant since it is no greater than its standard error.

These experiments showed that neither crystal violet, in the strength tested, nor proteose peptone had any effect on the count. In tests where the tellurite concentration was above 1·25 mg./100 ml. there was obvious inhibition, but there

was no evidence of ill-effect with the five strains tested at that level. In one experiment (Exp. 4) the blood was found to be somewhat inhibitory, and in this case the tellurite + blood plates showed a greater depression of the count than either tellurite or blood plates alone. An observation that may be analogous to this has been recorded by Tinsdale (1947).

On the basis of these results, further semi-quantitative tests were made of serum agar plates containing 0·25 mg. crystal violet, and 1·0 mg. potassium tellurite per 100 ml. One hundred and twenty colonies of streptococci isolated from the mouth swabs of two subjects on a number of occasions were streaked on S 1 and blood agar. Only two of the colonies failed to grow on S 1 and no more than about six showed any obvious sign of inhibition.

However, after we had used the S 1 medium for a period, further tests, described in the next section, indicated that some streptococci are inhibited. An additional experiment was therefore carried out with streptococci recovered from air samples. Colonies macroscopically resembling streptococci were picked from serum agar plates to lactose serum broth which was incubated for 18 hr. and then examined microscopically. Streptococci recovered in this way were seeded into buffered glucose broth (modified from Todd & Hewitt, 1932); after overnight incubation 10^{-4} and 10^{-5} dilutions of the broth were seeded on five replicate blood agar and S 1 plates, and the colonies counted after 18 and 40 hr. incubation respectively. Forty-four strains were examined; these represented 72 % of the strains isolated from the serum agar plates during this experiment, the remainder failing to survive in the interval between isolation and counting. Some 18 % of the forty-four strains tested failed to grow to any noteworthy extent on S 1, although growing well on blood agar (Table 2); most of the rest of the strains were no more than slightly inhibited on the S 1. No detailed examination of the susceptible strains was made, but none was a levan-producing *Strep. salivarius*.

Table 2. *Viable counts on S 1 and on blood agar of cultures of streptococci recovered from the air*

Count on S 1 as percentage of count on blood agar	No. of strains	Percentage of total strains
less than 20	8	18
20–	0	0
40–	1	2
60–	5	11
80–	14	32
100–	9	21
120–	5	11
140–	2	5
	44	100

Tests with airborne streptococci

On the basis of the preliminary laboratory experiments the S 1 medium was adopted for the field investigations, and, since no great defects were detected, it was used for the full 3-year period of the ultra-violet irradiation experiment. Further tests for possible inhibition of streptococcal growth have, however, been carried out in the course of the field work. These tests were of two varieties. In the first, colonies from serum agar plates exposed simultaneously with S 1 plates,

either in a slit sampler or as 'settling' plates, were picked to determine the proportion of streptococci. In the second, variously modified selective media were exposed in a slit sampler alternately with S 1 and the streptococcal counts estimated in the usual way; as a rule two modifications were tested in any one experiment, and the order of sampling was arranged in a 'Latin square' form, e.g. A, B, C; B, C, A; C, A, B, so that, although all three media appear once in each set of three plates, the order in each set of three is different. In almost all experiments of the second type, serum agar plates were exposed simultaneously to detect major variations in the count of airborne bacteria, so that the validity of observed differences in the counts on the selective media might be to some extent checked by covariance methods. In fact, no noteworthy differences in the mean serum agar counts were observed, and this refinement was not thought necessary.

Simultaneous samples on serum agar and on S 1. In the first series of experiments a random sample of colonies from serum agar plates exposed in a spiral sampler was picked to blood agar for identification of species; the estimated number of streptococci per cu.ft. of air on these plates was compared with that derived by routine B or C from the S 1 plates exposed simultaneously in the same sampler (Table 3). All but seven of twenty-six such comparisons favoured the serum agar, and the mean excess of the serum agar count over the S 1 count was 0·93 colonies per cu.ft., or if the first rather divergent sample is excluded, 0·67 colonies per cu.ft. The mean count on the S 1 plates was only about 60 % of the mean count on the serum agar plates. This deficiency must have been due partly to inhibition by the medium and partly to the fact that some of the viable streptococci would not be recognized on the plates (see below).

Serial sampling on various media. Media compounded with various concentrations of crystal violet and potassium tellurite were compared with the standard S 1 medium; the total streptococcal count was estimated by routine A, with the modification that the sampling fractions were considerably increased above the routine. In each experiment the estimated total number of streptococci on three plates of the selective medium under test was expressed as a percentage of the number on the three S 1 plates exposed in the same series. The means of these percentage values, with their standard errors, are set out in Table 4. Although no individual mean differs significantly from 100, the general trend of the results appears to indicate that a reduction in either the crystal violet or the potassium tellurite concentration led to some increase in the streptococcal count, and an increase in either inhibitor decreased the count. However, it was clear that small alterations in the concentrations of the inhibitors had no great effect. It was further noted that reduction in the crystal violet concentration below 0·17 mg./100 ml. often led to a considerable growth of moulds on the plates after the 2 days' incubation, which made accurate counting of the colonies impossible. In the absence of potassium tellurite the colonies were practically colourless and consequently difficult to count and pick.

We may conclude, first, that alterations in the concentrations of inhibitors likely to arise through minor errors in measuring while preparing the medium will have little effect on the streptococcal count; and, secondly, that no notable increase in

Table 3. *Comparison of recognition of streptococci on serum agar and on S 1*

Sample no.	Serum agar plates: Total colonies	Serum agar plates: No. picked	Serum agar plates: Estimated no. of streptococci per cu.ft.	S 1 plates: Total colonies	S 1 plates: No. of *Strep. salivarius*	S 1 plates: No. picked	S 1 plates: Estimated no. of streptococci per cu.ft.	Difference, estimated count on serum agar—count on S 1
1	489	48	8·5	300	5	39	1·0	+7·5
2	218	39	5·6	112	7	17	2·8	+2·8
3	156	20	3·9	65	1	28	1·1	+2·8
4	277	54	5·1	207	1	39	2·4	+2·7
5	316	69	3·1	187	2	31	0·9	+2·2
6	435	18	4·0	270	13	34	2·1	+1·9
7	318	68	3·1	409	4	39	1·2	+1·9
8	109	50	1·7	81	6	30	0·2	+1·5
9	207	68	2·0	151	0	32	0·5	+1·5
10	209	32	2·2	111	5	30	1·8	+1·4
11	457	36	2·1	87	0	24	0·7	+1·4
12	183	30	1·0	32	0	21	0·1	+0·9
13	348	37	1·6	51	0	25	0·8	+0·8
14	254	30	1·3	146	2	28	0·6	+0·7
15	194	24	1·4	61	1	32	0·7	+0·7
16	320	33	3·2	187	7	36	2·6	+0·6
17	248	32	1·3	86	1	30	0·9	+0·4
18	376	31	2·0	249	2	29	1·8	+0·2
19	80	40	0·4	130	3	40	0·2	+0·2
20	193	30	0	118	5	29	0·2	−0·2
21	889	42	0	716	1	49	0·8	−0·8
22	111	31	0·6	85	4	23	1·5	−0·9
23	240	20	0	136	2	31	1·2	−1·2
24	412	60	0	415	6	40	1·3	−1·3
25	314	23	0	167	6	30	1·7	−1·7
26	376	39	0	161	13	23	1·9	−1·9
Means			2·08				1·19	+0·93

Note. The volume of air sampled was 6 cu.ft. on the serum agar and 20·4 cu.ft. on the S 1 in all cases except samples 8 and 19 in which the volumes were 5 and 200 respectively; the samples are arranged in descending order of the difference.

Table 4. *Total number of colonies, and estimated number of streptococci (in italic figures), on various selective media, as a percentage of the number on the standard S 1 selective medium exposed alternately*

Potassium tellurite (mg./ml.)	Crystal violet (mg./100 ml.): 0·1	0·17	0·25	0·33
0	—	—	138·9 ± 20·8 (10)* *131·2 ± 24·4*	—
0·5	—	—	112·1 ± 15·9 (4) *108·0 ± 19·3*	—
1·0	107·2 ± 11·1 (4) *92·6 ± 9·1*	101·7 ± 10·0 (6) *113·5 ± 13·5*	(standard) S 1	90·9 ± 12·8 (4) *113·5 ± 10·2*
1·2	—	—	97·6 ± 4·7 (7) *78·4 ± 13·4*	—

* Figures in brackets give the number of experiments, each comprising three pairs of plates, on which the mean is based; means are tabulated with their standard errors.

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the streptococcal count could be obtained by any practicable reduction in either inhibitor.

Effect of variation in time of incubation

Using the method of random allocation of plates followed in the tests of various media, a number of comparisons were made of the streptococcal count estimated from plates read after 24, 40 and 48 hr. incubation. The mean of the six counts at 40 hr., expressed as a percentage of the corresponding six 24 hr. counts, was 181 % which is significantly greater ($t = 2{\cdot}7$, $P = < 0{\cdot}01$) than the value of 100 that would be expected if the additional incubation had no effect. The corresponding value at 48 hr. compared with the 40 hr. count was 129, which is suggestively but not fully significantly greater than 100 ($t = 1{\cdot}9$, $P =$ about $0{\cdot}05$). It is clear that the time of incubation is fairly critical if constant results are to be obtained. We were reluctant to increase the time of incubation above 40 hr. because we had evidence that some strains of streptococci failed to survive for any greater period; these results suggest, however, that 48 hr. incubation might really be preferable to 40 hr.

Effect of storage of selective plates before and after use

In field investigations it is often necessary to store poured plates for a few days before use; and it would often be an advantage if plates could be stored overnight after exposure in the sampler but before incubation. A few tests were therefore carried out to measure the effect of such storage on the streptococcal count.

In the first test, twelve S 1 plates were stored in the refrigerator for 7 days before exposure; and a further twelve were poured 1 day before exposure. Four plates of each batch were exposed in each of three rooms, and after use two of the plates from each room were incubated immediately, and two were stored in a refrigerator for 24 hr. and then incubated. The streptococcal counts were estimated by routine A for the plates from two rooms and by routine C for those from the third. From the summarized figures (Table 5) it was clear that the storage before incubation had

Table 5. *Estimated streptococcal counts on S 1 plates stored at* 4° C. *before and after exposure*

(Sum of counts on two replicate plates in each room.)

Duration of storage of plates before exposure (days)	Plates incubated without preliminary storage			Plates incubated after 24 hr. storage at 4° C.			Totals
	Room A 1*	Room A 2	Room C	Room A 1	Room A 2	Room C	
7	14·9	25·1	112·2	3·2	36·9	85·5	277·8
1	16·5	13·9	84·0	5·0	32·6	78·3	230·3
Totals		266·6			241·5		508·1

* Plates were examined by routine A in rooms A 1 and A 2 and by routine C in room C.

no detectable effect on the streptococcal count. This conclusion was supported by results of an experiment in which viable counts of streptococcal cultures were carried out on plates stored 1, 2, 3 and 4 weeks.

The plates stored after exposure had a slightly lower count than those incubated immediately. Further inspection of the results from the plates examined by routine C suggested that the presumptive mouth streptococci were partly inhibited;

and a second experiment with eight pairs of plates was carried out to investigate this point. The sums of the estimated streptococcal counts on four plates exposed in each of three rooms and incubated within a few hours were 119·9, 99·4 and 170·0; the counts for the parallel plates stored at 4° C. for 24 hr. before incubation were 60·4, 51·2 and 99·0 or 50·4, 51·4 and 58·2 % respectively of the control plates.

We conclude, therefore, that storage of poured plates before use is unlikely to have any effect on the streptococcal count; but that storage between exposure and incubation leads to a considerable reduction in the count.

Efficiency of methods of recognizing streptococci

Reliability of direct nigrosin film. In a number of preliminary experiments in which we tested the reliability of our recognition of streptococci in the direct nigrosin film by a comparison with the morphology of the strain in a lactose serum broth culture, we estimated that about 15 % of the streptococci were missed in the direct film and perhaps 2 % of the colonies were falsely identified as streptococci.

A further comparison was made with the results of the examination of 2246 colonies by routine B, that is, transfer to nigrosin and to a bile-agar ditch plate (Table 6). Of the 287 colonies identified in the nigrosin film as streptococci, and growing on the ditch plates, 27 (9·4 %) could not be confirmed as streptococci; however, 21 of the 27 (78 % of the total) were bile-tolerant and it may be that some of these were in fact enterococci. Of the 1781 strains seen in the nigrosin films, but thought not to be streptococci, 20 (1·1 %) proved to be streptococci on subculture. There remained 113 colonies where no organisms had been visible in the nigrosin film and of these 56 (49·5 %) proved to be streptococci. Thus, of the total 1894 colonies for which the nigrosin films showed organisms thought not to be streptococci or showed no organisms, 76 (4·0 %) yielded viable streptococci on subculture.

These results were consonant with those already mentioned, and suggest that the nigrosin film diagnosis of a '*Streptococcus*' is false on perhaps 5–9 % of occasions; and the diagnosis of a coccus as 'not a *Streptococcus*' is false in about 4 % of cases. It may be noted that the diagnosis of streptococci in these films is often made difficult by the small numbers of cocci present due to the small size of the colony, which has to be divided between the film and the ditch plate, and the fact that many streptococci form rather hard colonies.

Bile-agar ditch plates. The results in Table 6 also show that of 2115 colonies on which a nigrosin film diagnosis could be made, 47 (2·2 %) failed to grow on the ditch plates; of these 21 (44·7 %) were streptococci. Since the percentage of streptococci among the strains that did grow on the plates was only 13·2 it is clear that the streptococci were less viable than the micrococci. Exclusive reliance on the ditch plate therefore has the disadvantage that a proportion (about 6 %) of the streptococci will fail to be recognized. On the other hand, the ditch plate does permit recognition of a number of streptococci that would be missed by the direct film, as well as permitting more precise identification.

It is noteworthy that of the 1845 strains that grew on the ditch plates and were

Table 6. *Recognition and distinction of streptococci by nigrosin film and bile ditch plate (Routine B)*

	Growth on bile ditch plates												
	Bile tolerant					Bile intolerant							
			Colonies subcultured					Colonies subcultured					
Appearance in direct nigrosin film	Total colonies	No. not sub-cultured*	No.	No. proving to be strepto-cocci	No. not strepto-cocci	Total colonies	No. not sub-cultured*	No.	No. proving to be strepto-cocci	No. not strepto-cocci	Total growing on ditch plate	No. not growing on ditch plate	Grand total
Streptococcus	65	5	60	39	21	222	180	42	36	6	287	21	308
Not *streptococcus*	1690	1678	12	3	9	91	33	58	17	41	1781	26	1807
Nothing seen	36	2	34	3	31	77	1	76	53	23	113	18†	131
Totals	1791	1685	106	45	61	390	214	176	106	70	2181	65	2246
Total streptococci		50						286			336		
Total not streptococci		1741						104			1845		

* Strains were not always subcultured if the colonial appearance on the ditch plate fully confirmed the diagnosis made on the nigrosin film; or if it was characteristic of, for instance, aerobic spore-bearing or coliform organisms (see text).

† It is likely that many of these eighteen 'colonies' were really pieces of black dust, etc.

N.B. Colonies of *Strep. salivarius*, being recognizable on the original sample plates, are not included in this table.

not streptococci, 1741 (93%) were bile-tolerant; and conversely of the 390 bile-intolerant strains, 286 (71·8 %) were streptococci and 34 (8·7 %) were readily distinguishable from streptococci by their colonial morphology. If a search were being made only for bile-intolerant streptococci, i.e. presumptive mouth types, 70 (3·3 %) of the 2181 colonies growing on the ditch plates would be wrongly diagnosed as streptococci from the fact that they failed to grow on the bile; another 34 colonies, although failing to grow on the bile, were distinguishable from streptococci by their colony form. The 70, therefore, represent 19·8 % of the 356 bile-intolerant colonies morphologically resembling streptococci.

In addition it may be noted that of the 336 streptococci growing on the ditch plates, 50 (14·9 %) were bile-tolerant and regarded, therefore, as enterococci.

Aesculin-bile-agar ditch plates. Similar results obtained by the use of routine C, that is, transfer of colonies simply to aesculin-bile-agar ditch plates with subsequent confirmation, as set out on p. 509, are presented in Table 7.

Table 7. *Recognition and distinction of streptococci by ditch plate alone (Routine C)*

Growth on bile agar	Hydrolysis of aesculin	Morphology on subculture		Totals
		Streptococcus	Not streptococcus	
+	+	64	77	141
+	−	15	1641	1656
−	.	278	66	344
Totals		357	1784	2141

One hundred and forty-one strains hydrolysed aesculin and 64 (45·4 %) of these proved to be streptococci; 15 streptococci were recognized among the 1656 bile-tolerant colonies that did not hydrolyse aesculin. The proportion of aesculin-negative strains among the enterococci (15/79) does not differ significantly from the proportion (23/140) observed in the earlier test (p. 509); and from the results of the latter it can be estimated that some five α-haemolytic aesculin-negative enterococci may have been missed by the omission of the direct film in routine C. Of the total 2141 colonies, 344 (16·0 %) were bile-intolerant and 278 of these (80·8 or 13·0 % of all the colonies) proved to be streptococci.

A direct comparison was made of the proportions of streptococci recognized in samples from single plates examined by routines A and C. A random sample of colonies was picked from each of eighteen sets of three plates to aesculin-bile ditch plates (routine C), and a further random sample of the same size was then picked from the same plates by routine A, that is, directly to nigrosin drops. There was some suggestion in the results (Table 8) that the nigrosin film gave a slightly higher count than the ditch plate, and in two cases the difference was significant at the 5 % level; on eighteen samples, however, this range of variation is not unexpected ($\Sigma t^2 = 21{\cdot}01$; $0{\cdot}3 > P > 0{\cdot}2$), and we conclude that, so far as counts of total streptococci are concerned, the defects of the respective routines balance their advantages.

Table 8. *Proportion of streptococci recognized in successive samples from single plates examined by routines A and C*

(Each entry represents the sum of the number of colonies from three plates.)

Exp. no.	No. of streptococcal colonies/no. of colonies examined		Difference between percentage of streptococcal colonies recognized by C and A	t = difference ÷ standard error
	Routine C	Routine A		
1	32/95	25/99	+8·4	1·3
2	14/117	8/120	+5·3	1·5
3	31/120	25/120	+5·0	0·9
4	7/88	5/88	+2·3	0·5
5	36/97	32/98	+3·4	0·5
6	7/80	6/80	+1·2	0·3
7	24/106	25/112	+1·4	0·2
8	21/120	19/110	+0·2	0·04
9	35/120	32/107	−0·4	0·1
10	27/102	28/102	−0·9	0·1
11	5/99	7/104	−1·7	0·5
12	2/98	4/105	−1·4	0·6
13	16/93	20/95	−3·9	0·7
14	13/112	17/117	−3·0	0·7
15	11/103	19/109	−6·7	1·4
16	10/95	18/105	−6·6	1·8
17	3/79	11/80	−10·0	2·1
18	19/75	34/83	−15·6	2·1

$\Sigma t^2 = 21{\cdot}01$. $0{\cdot}3 > P > 0{\cdot}2$.

TIME TAKEN FOR VARIOUS ROUTINES

We have made a rough estimate of the time taken to examine plates by the three routines described (Table 9). These times are those actually taken in the various steps and do not include the time required for preparing the media and so forth; they can therefore be used to estimate the increase in time that may be required by an increase in the number of colonies examined, since such an increase will not as a rule greatly affect the time taken in media-preparation.

Table 9. *Approximate time (in minutes) taken to examine fifty plates by various routines*

Operation	Routine A	Routine B	Routine C
Counting original plates	60	60	60
Picking colonies to ditch plate or nigrosin film	330	—	330
Picking colonies to ditch plate and nigrosin films	—	505	—
Examination of growth on ditch plates	—	105	105
Picking colonies from ditch plates to lactose broth	—	100	130
Making nigrosin films from lactose broth	—	80	100
Examination of nigrosin films	300	480	100
Totals	690	1360	825

The fifty plates were assumed to have the same total numbers of colonies as those summarized in the text on p. 520, and proportions of streptococci, etc., as in Tables 6 and 7.

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ESTIMATION OF THE SAMPLING ERRORS OF STREPTOCOCCAL COUNT

The practice of determining the streptococcal count on the plates by examining only a sample of the colonies introduces some complications into the determination of standard errors of the counts. The size of the sample to be examined should be in part governed by the extent to which it is desired to reduce this standard error. When our own investigations were commenced we had insufficient experience to make any precise estimates on this point and the sampling fractions were chosen arbitrarily; we picked as many colonies from the plates as could conveniently be examined with the staff available. We have, however, examined the standard errors obtained with our routine sampling fractions, and investigated the effect to be expected from an increase in the fractions.

It was suggested to us by Mr P. Armitage, of the Medical Research Council Statistical Research Unit, that one should regard the number of streptococci found on our plates as samples of an infinite population at the site sampled; the counts might then be regarded as distributed in a Poisson fashion and the sampling variance of any particular count would be estimated as having the same numerical value as the count itself. This argument may be applied directly to our *Strep. salivarius* counts, which are the actual numbers of colonies observed on the plates. In the case of the streptococci other than *Strep. salivarius*, which are recognized by the sampling procedure, the variance (V') of the estimated number of colonies per plate is approximately

$$V' = (N/n)^2 \times s,$$

where $N =$ the number of colonies on the plate, $n =$ the number of colonies picked for identification, and $s =$ the number of streptococci found in the sample n. The variance V of the estimate of the total number of streptococci per plate is given as the sum of the variance of the *Strep. salivarius* count (equal to the count itself) and that of the count of other streptococci, which we have denoted by V'.

It is not, as a rule, an estimate of the precision of any particular streptococcal count that is needed, but rather an estimate of the precision of the mean of a number of counts taken, for instance at one or more sites on a number of days. In such cases the variation between different counts becomes important, and we need therefore to investigate the relative importance of the variation due to differences between counts and that due to the sampling element in the counting method. We have estimated the sampling error attached to the mean of fifty consecutive samples taken in schoolrooms during the summer of 1949, and examined by the routine C described above.

The mean number of colonies per plate (representing the number collected from 20·4 cu.ft of air in 24 min.) was 173·650; the mean count of *Strep. salivarius* was 5·440; and the mean of the fifty estimates of the streptococci other than *Strep. salivarius*, derived from the routine sampling of each of the fifty plates, was 12·708. The estimate of the mean total number of streptococci per plate was therefore 18·148. The routine sampling of the fifty plates involved the subculture and examination of a total of 1,572 colonies.

The variance associated with the mean of the fifty total streptococcal counts,

calculated in the ordinary way as the variance of the fifty total counts divided by fifty, was 6·537. Of this total variance a part was due to the sampling errors involved in the recognition of the streptococci in individual rooms, and this was estimated by the method outlined above as

$$\Sigma V/(50)^2,$$

where ΣV is the sum, over all the fifty plates, of the quantities V defined above. This variance due to sampling had the value of 2·235, and we may say therefore that the sampling element constituted about 34·2 % of the total variance of the mean of the set of fifty counts. The remaining 65·8 % was due to variation between numbers of streptococci at different sites.

To obtain some idea of the effect on the precision of our estimate of the mean of such a set of fifty counts to be expected from an increase in the sampling fraction, the data were recalculated on the assumption that the same proportions of streptococci were found in larger samples picked from the same plates. The sampling fractions used were:

Number of colonies	Number sampled	Number of of colonies	Number sampled
1–	All	91–	35
21–	20	121–	40
31–	25	161–	50
61–	30	201–	60

The new sampling fractions would have necessitated the examination of 2037 colonies instead of 1572, and the new variance due to sampling had the value of 1·552. If we may assume that the variance due to differences between the plates was unaffected by the change in sampling fraction, the total variance would only be reduced from 6·537 to 5·854. These two variances give percentage standard errors of the mean of 14·09 and 13·33 respectively. It is clear, therefore, that in the circumstances of this investigation, where there were considerable differences between the counts in the different rooms, no reasonable reduction in the standard error would result from any practicable increase in the size of the sample of colonies picked from individual plates for identification.

DISCUSSION

The ideal method for recognizing streptococci in air samples would clearly be one in which the streptococcal colonies could be recognized on the original sample plates. This is possible for that proportion of airborne streptococci (about 25 % in our schoolroom samples) that produce a levan on sucrose agar. We have found no comparable method for recognizing other streptococcal colonies.

Previous workers (Wells; Buchbinder *et al.*) employed α-haemolysis on blood agar as their diagnostic criterion, but we feel that this is quite unreliable, partly because a significant proportion of mouth streptococci are non-haemolytic, but largely because of the great number of α-haemolytic micrococci that are present in the air of occupied places. These micrococci seem to us to be the organisms referred to by Buchbinder and his colleagues as 'putative streptococci'. Although we are doubtful of their exact taxonomic position, we think that it is most unlikely that

they are really streptococci; they are certainly extremely rare in the mouths of normal people.

The selection of streptococcal colonies from cultures on a non-selective medium on a basis of colonial morphology, as used by Torrey & Lake, has the great disadvantage that the streptococci form no more than 1–3 % of the total colonies and are by no means easily recognized. In fact, in a series of twenty-seven serum agar plates in which we attempted to use this method to compare with S 1 routine, we recognized fewer streptococci on the serum agar than on the S 1 plates, and this despite the fact that the S 1 inhibits a proportion of the streptococci.

Lactose fermentation has often been used to distinguish streptococci from micrococci (e.g. Wells *et al.* 1941). It is true that streptococci ferment lactose more vigorously than the micrococci, but in our experience a great number of the greening micrococci that offer our chief diagnostic difficulty ferment lactose, and we do not therefore regard this test as satisfactory.

Most strains of *Strep. viridans* produce hydrogen peroxide, as can be shown by the benzidine test (M'Leod & Gordon, 1922), but the greening micrococci that are far the commonest of the strains other than streptococci on our air sample plates, also give this reaction. At the same time their very weak catalase production makes the use of the latter test impracticable.

It is possible that other selective agents than those that we have used might be more efficient. We have made a few tests with sodium azide (Packer, 1943) and with thallium acetate (Rantasalo, 1947), but neither proved satisfactory. The chief difficulty seems to arise from the fact that the greening micrococci resemble streptococci very closely in their tolerance of the agents that are ordinarily used to select streptococci.

The routines that we have used suffer from a number of disadvantages. In the first place our selective medium undoubtedly inhibits a proportion, certainly 20 % and perhaps up to 30 %, of the viable streptococci found in the air of occupied places. Secondly, the routines requiring subculture to bile-agar ditch plates are time-consuming and fail to recognize the small proportion (about 7 %) of the streptococci on the original plate that are non-viable at the time of picking. Thirdly, as a consequence of the laborious methods used for identification of the streptococci, we are able to examine only a proportion of the colonies on our original plates; and this introduces the uncertainty necessarily associated with sampling methods.

Of our three methods for recognizing the streptococci on the plate, the second is clearly the most complete since the examination of the direct nigrosin film enables one to recognize streptococci that are not viable on subculture, and at the same time the characters of the viable streptococci are determined by the ditch plate. Moreover, the use of the ditch plate greatly facilitates the recognition of enterococci, especially if aesculin is added to the bile agar. Our third routine, in which the direct film is omitted, is a convenient compromise, but it necessarily recognizes fewer of the mouth streptococci.

The reduction in the streptococcal count that results from the defects of the method is probably, as already indicated, of the order of 25–40 %, but it is of course selective: a greater proportion of the mouth streptococci than of the

enterococci are lost; and this may be of some importance in tests of aerial disinfectants and in other circumstances in which the relative prevalence of mouth streptococci and enterococci may vary. Moreover, streptococci are often so rare in the air of occupied places that any reduction in the efficiency of collection represents a serious loss.

The comparative assessment of the various methods that we have described for the detection of airborne streptococci must clearly take account of the purpose for which the count is required and the relative importance to be attached to precise definitions of the varieties of streptococci, and to a precise estimate of their numbers.

Determination of the streptococcal content of air may be required for two reasons: first, as suggested by Gordon, Wells, Winslow, and others, as an index of the extent to which the air has suffered pollution from respiratory sources; and, secondly, as part of a determination of the general bacterial content of air as such. It is clear that for estimating respiratory contamination of air we need to estimate only the number of streptococci that can reasonably be assumed to come from the respiratory tract; and enterococci, which are for practical purposes not found in the mouth, must be classed with micrococci and eliminated from the streptococcal count. For this purpose a routine, such as our first, which gave a 'total' streptococcal count based solely on morphology is not really satisfactory. Transfer of the sample of colonies to bile-agar ditch plates allows distinction of enterococci and β-haemolytic streptococci from other streptococci, and although microscopic confirmation of the bile-intolerant strains is desirable, it is clear from Table 6 that reliance on macroscopic morphology would not very often be misleading. The addition of aesculin and ferric citrate to the bile-agar enables one to recognize a large proportion of the enterococci, subject to microscopic confirmation; but, since some enterococci fail to hydrolyse aesculin, these strains will be missed without microscopic examination of all colonies, as in our routine B.

Increase in the precision of the numerical estimate can come only from increase in the sampling fraction. Where a number of plates with different mean counts of streptococci are being examined, however, no practicable increase in the sampling fraction will give a noteworthy increase in the precision of the count: in our example a 30 % increase in time spent in picking colonies and examining the subcultures only reduced the standard error of the mean of fifty plates from 14·1 to 13·3 %.

Clearly the routines that we have described, as well as being somewhat defective, are so time-consuming that one would have to seek other methods of detecting mouth streptococci if their detection were to be considered a matter of general hygienic importance. One obvious method, which we have ourselves explored, is the exclusive use of *Strep. salivarius* as an indicator organism, coupled with a great increase in the volume of air sampled to offset the smaller numbers. This problem will, however, be better deferred to a subsequent report in which we propose to discuss the general hygienic significance of air streptococcal counts; the methods described in the present paper, despite their defects, have been adequate for the preliminary surveys on which such a discussion can usefully be based.

SUMMARY

To estimate the number of viable streptococci present in the air of occupied places, airborne bacteria were collected in a slit-sampler on a nutrient agar medium containing 5 % sucrose, 5 % horse serum, 0·25 mg./100 ml. crystal violet, and 1·0/100 ml. potassium tellurite. This medium inhibits the great majority of the staphylococci and micrococci found in the air. On it colonies of *Strep. salivarius* are readily recognized by their mucoid form. The number of other streptococci was estimated by picking and examining a random sample of the colonies.

Streptococci were recognized in the sample by three different methods: (1) by microscopic examination of a nigrosin film made from the original colony; (2) by examination of a nigrosin film and also subculturing the colony to a ditch plate having blood agar on one side and serum agar containing 40 % ox bile on the other; or (3) by subculture to bile-agar ditch plates alone, the bile agar in this case containing aesculin and ferric citrate. By method (1) streptococci could be distinguished from micrococci; by methods (2) and (3) this distinction could be made with more confidence, and in addition enterococci could be distinguished from other streptococci.

A number of tests of the efficiency of the method were carried out, and it was concluded that some 20–40 % of the viable streptococci in the air were missed.

The standard errors of the mean of a number of streptococcal counts in school-rooms were calculated and found to be of the order of 14 % of the mean when our standard routine was followed; increasing the size of the sample of colonies picked for examination by 30 % would probably have had only a trivial effect on the standard error of the mean, since the greater part of the variation was due to differences between the counts in different rooms.

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