

## The Diagnosis of Chronic Streptococcus Mastitis.—Reaction, Chlorine, Methylene Blue and Hotis Tests, and Microscopic Examination.

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It is generally accepted that 90 per cent. or more of the cases of chronic mastitis in bovines are produced by streptococci, the principal one being *Streptococcus agalactiae* which is an obligatory parasite growing and multiplying in the udder and secreted in the milk from infected quarters.

The innumerable tests which have been devised for diagnosing the disease are based on identification of the causative organism, detection of the physical and chemical changes produced by the disease in the milk, and ascertaining the pathological changes in the udder tissue. Unfortunately for the diagnostician the changes produced by the disease are neither characteristic nor uniform, and the presence of mastitis streptococci cannot constantly be correlated with certain definite alterations in the milk or in the affected quarter.

### ABNORMAL CONDITIONS OF THE UDDER.

Examination of the affected bovine mammary gland and of its secretion may reveal any one or more of the following abnormal states in one or more of its quarters:—

(1) A clinically normal quarter (or quarters) secreting milk which is free from pathogenic bacteria but is nevertheless of abnormal composition. In studies on a group of 31 cows during their first lactation period Hastings and Beach found that 14 persistently yielded abnormal milk when pH, catalase and chlorine determinations were used as criteria. The cause of the abnormality was not determined. Unpublished data obtained by us in the regular examination (bacteriological, cytological, pH, chlorine, sugar, solids-not-fat, fats and milk yield) of a herd of 18 clean cows recruited from heifers free from tuberculosis, contagious abortion and mastitis show that the secretion of abnormal milk by quarters which have never been infected with mastitis streptococci or other pathogenic organisms and which show no induration of the udder tissue is by no means a rare occurrence.

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(2) Normal quarters secreting milk which is normal when judged by all the recognised physical and chemical tests but contains pathogenic bacteria. Such infected quarters may continue shedding streptococci for an indefinite period and there is as yet no unanimity of opinion as regards their ultimate fate. In many cases they may, probably under stimulus of certain predisposing factors, suddenly give rise to an acute mastitis which may subsequently subside and develop into the chronic form characterised by induration of the affected quarter and alteration in the composition of its milk. On the other hand some of these infected quarters may continue shedding streptococci for long periods without producing clinical manifestations in the quarter or affecting the composition of the milk in any way.

Many hold that streptococci may disappear spontaneously from such quarters. It is certain that streptococci are not being discharged continuously from them, but there is as yet no definite evidence to show that the disappearance of the streptococci from the milk is an indication of complete sterilisation of the quarter or whether it is merely due to a temporary cessation in the secretion of the streptococci which may nevertheless continue their existence in the udder tissue. Hucker (1937) reports that a study of 24 udders aseptically removed and cultured from cows known to be free from mastitis and to have passed through one or more lactations shows that all contain mastitis streptococci. In 18 of these cases however Hucker appears to have based his conclusion of "freedom from mastitis" merely on a physical ante- and post mortem examination of the udders, cultural examination of the milk having been made in only six cases. Bryan *et al* on the other hand carried out similar observations on 94 cows; 67 of these showed streptococci in the milk prior to slaughter and the streptococci were also found in the udder tissue collected immediately after slaughter. Streptococci were not found in the milk prior to slaughter in 27 of the 94 cows nor were the organisms recovered from the udder tissue, and it is concluded that the absence of streptococci in properly collected milk samples was evidence that streptococcus infection was not present in the udder tissue.

Whatever views may be held with regard to the possibility of auto-sterilisation in these cases of latent mastitis they constitute potential sources of infection for healthy animals, and from the point of view of control diagnosis is as important in latent mastitis as in the clinical form of the disease.

*Note.*—The term "normal quarters" in (1) and (2) is applied to quarters in which physical examination failed to reveal pathological changes. It is not contended that such quarters may not show microscopic changes. Probably no branch of mastitis research has received less attention than histopathology, and this aspect of the disease merits more consideration than it has received up to the present.

(3) Quarters showing pathological changes and secreting milk which is abnormal in composition but free from pathogenic bacteria. Clinical examination of the udders and the application of indirect



tests to the milk disclose in every infected herd a variable number of quarters which when judged by such criteria may rightly be regarded as classical examples of chronic streptococcus mastitis. Contrary to expectations bacteriological examination of milk from such quarters may reveal a total absence of streptococci or other pathogenic bacteria. It has been observed that the majority of such free quarters show well advanced induration and pronounced changes in the composition of the milk.

The cause of the absence of streptococci in the milk from such quarters is not known. Little (1939) has drawn attention to the possible presence in the milk of a bacteriostatic or inhibitory substance which may vary in individual cows and even in the different quarters of the same cow. Little's explanation of this protective mechanism is however offered more to account for the frequent failure to produce mastitis experimentally by the injection of streptococci through the teat canal and for the apparent harmlessness of mastitis streptococci in latent cases. It is more probable that the spontaneous disappearance of the streptococci in advanced clinical mastitis is due to the pronounced changes in the milk which may have a bacteriostatic or even bactericidal effect on the bacteria.

(4) Pathological quarters secreting milk which is abnormal in composition and which contains mastitis streptococci. This is the most characteristic form of the disease and the one which offers least difficulty in diagnosing as it can be detected by most of the more reliable indirect tests and bacteriological methods.

Consideration of the many implications may be associated with the different forms in which the disease may manifest itself immediately reveals the many difficulties that confront the investigator in search of the perfect test for the diagnosis of mastitis; and it is at once evident that there is no single test that will detect every form of the disease. From the point of view of control and eradication the only satisfactory method of diagnosis is one that will expose every quarter that sheds streptococci independent of the presence or absence of changes in the milk and in the udder tissue.

It thus appears obvious that the selection of a diagnostic method is confined to cultural and microscopic examinations. On their own however the reliability of the latter two methods is confined to control, and where the public health aspect has to be considered in addition the adoption of one or other of the indirect tests is essential. Further, the combination of bacteriological with one or more indirect methods is desirable on account of the fact that, especially in infected herds, detection of alteration in the composition of milk which may not contain streptococci is a valuable aid to diagnosis in that it immediately throws suspicion on the quarter in question. Not infrequently such a quarter is found to be infected at a subsequent retest.

The results here recorded were obtained in the testing of twenty herds. The five methods were not applied invariably in every instance, but the relative tables show the number of cases in which the various tests were applied.



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### COLLECTION OF SAMPLES.

The milk samples required for the tests were generally collected early in the afternoon. The udder and teats were wiped with a clean moist cloth and the teat orifice cleansed with a pledget of cotton wool soaked in alcohol. Thorough cleansing of the teat orifice in order to free it from all contaminating organisms is considered to be of much greater importance than wiping of the udder.

The first two or three streams of milk were discarded. After that two and sometimes three samples were taken from every quarter the first being for bacteriological examination and the other for the various indirect tests. It is generally accepted that streptococci are more frequent in the foremilk than in the middle milk or strippings. Therefore the samples required for bacteriological tests were taken first. For this purpose about 10 c.c. was milked from each quarter into properly sterilised test tubes. To avoid contamination of the milk from extraneous sources the tubes should be handled as little as possible after being opened, and for that reason it is not advisable to entrust the removal and retention of the cotton wool plugs to an assistant while the sample is being taken. This can all be done by the milker himself the plug being held between the first and second fingers on the back of the left hand which also holds the tube during the process of milking. As an additional precaution against particles of dust, dirt and hair from the udder falling into the tube the latter is held in a horizontal rather than a vertical position. Next about 50 c.c. was milked from each quarter into sterile bottles for the various indirect tests. Determination of alkalinity was usually performed in the stable immediately after milking except where it was possible to get the samples to the laboratory and examined for alkalinity within two hours after they were taken.

Samples from which smears were to be made after incubation were placed in the incubator immediately after arrival at the laboratory while all others were kept in a refrigerator overnight for the remaining tests to be done the following day.

### REACTION.

Storch in 1889 was the first to point out that mastitis milk was less acid than normal. Since then numerous investigators have drawn attention to the tendency toward increased alkalinity shown by mastitis milk. The pH of normal milk lies between 6.4 and 6.7 whereas in mastitis it increases to 8 or even 8.5.

The actual cause for this increased alkalinity has not yet been definitely determined though theoretically the phenomenon has been attributed to great variety of causes such as the passage by filtration of large amounts of unchanged blood serum into the milk, the presence in mastitis milk of alkali forming bacteria that more than counteracted the effect of the acid forming streptococci, and the passage into the milk of alkaline phosphates. Stableforth (1930) pointed out that in an infected quarter the reaction bears no relation to the bacterial content, and suggested that the fact that the change of reaction appears to be associated usually with some evidence of



mastitis, either acute or chronic, lends support to an interpretation of a different kind, namely that the change is due to actual damage of the alveolar membrane, causing a greater permeability with consequent passage into the milk of a substance such as serum or lymph or, if the damage is less severe, possibly only substances such as the salts of the blood.

Many indicators have been recommended for determining the degree of alkalinity, for instance, litmus and several other types of indicator papers, alcoholic solutions of rosolic acid, phenol red solution, phenolphthalein, brom-cresol-purple, brom-thymol-blue, and electrometric methods. While the latter are unquestionably the most accurate for determining changes in alkalinity their use is limited to the laboratory.

Brom-cresol-purple and brom-thymol-blue appear to enjoy preference over most other indicators, and of these two brom-thymol-blue seems to give the most accurate readings. Even this indicator does not however always give very clear colour changes especially when the milk contains a high percentage of butterfat, and to overcome this Stableforth has suggested first centrifuging the milk, which is a laborious procedure and renders the test unsuitable as a stable test.

#### *Brom-thymol-blue and BDH Universal Indicator.*

In the investigations under review the Universal Indicator prepared by British Drug Houses was used, but in four herds both brom-thymol-blue and Universal Indicator were applied. One drop of a 0.5 per cent. brom-thymol-blue solution in 10 per cent. alcohol was added to 1 c.c. milk in small test tubes. The colour in normal milk is greenish yellow and in mastitis it varies from yellowish green to bluish green. For the determination of the pH with the Universal Indicator the manufacturers recommend the addition of 0.1 c.c. of indicator to 10 c.c. of the liquid under test. For milk however this dilution was found to be too weak mainly on account of interference by the fat in the milk. This difficulty was overcome by increasing the concentration and the best results were obtained by adding one drop Universal Indicator to 3 or 4 drops of milk. Porcelain impression plates each measuring  $3\frac{1}{2}$  inches by 4 inches and having 12 depressions of about  $\frac{3}{4}$  inch diameter arranged in three rows were used. Quarter samples from three cows can therefore be done simultaneously on one plate. The colour changes take place immediately after the addition of the indicator to the milk in the depressions on the plate, but these changes are much more distinctive if the plate is allowed to stand for 5-10 minutes before the readings are taken. Normal milk with pH 6.4-6.7 is yellow. Increased alkalinity is indicated by greenish-yellow (pH 6.8-7.5), green (pH 7.5-8.0) or bluish green (8.0-8.5) in very bad cases.

In addition to the two indicator tests chlorine determination and microscopic examination of incubated samples of milk were also carried out on these four herds, and the results are summarised in Table 1.



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TABLE I.

*Comparison of Brom-thymol-blue and Universal Indicator.*

Herd No.	No. of Samples.	1	2	3	4	5	6	7	8	9
		ME- Cl <sub>2</sub> - UI- BTB-	ME+ Cl <sub>2</sub> + UI+ BTB+	ME+ Cl <sub>2</sub> + UI+ BTB-	ME+ Cl <sub>2</sub> + UI- BTB+	ME- Cl <sub>2</sub> + UI- BTB-	ME- Cl <sub>2</sub> + UI+ BTB+	ME+ Cl <sub>2</sub> + UI- BTB-	ME+ Cl <sub>2</sub> - UI- BTB-	ME+ Cl <sub>2</sub> - UI+ BTB+
9	32	2	3	4	2	3	2	13	3	0
10	19	7	2	1	0	5	2	2	0	0
11	38	13	3'	4	0	9	1	3	4	1
12	34	8	5	2	0	7	0	8	4	0
Total	123	30	13	11	2	24	5	26	11	1

Of the 123 samples examined 30 were negative and 13 positive to all four tests while 51 of the remaining 80 contained mastitis streptococci, thus bringing the total number infected to 64. Of these 25 (columns 2, 3, 9) or 39.1 per cent. were positive to Universal Indicator and only 16 (columns 2, 4, 9) or 25 per cent. to brom-thymol-blue. The Universal Indicator showed a rise in alkalinity in 11 (column 3) positive samples which were negative to brom-thymol-blue while the latter showed a positive reaction in only 2 (column 4) which were negative to Universal Indicator. Eleven (column 8) of the 64 infected samples showed no rise in chlorine and alkalinity and can therefore be regarded as coming from quarters with a latent infection. An active mastitis accompanied by alteration in the composition of the milk as suggested by the increase in chlorine but in which both indicators failed to disclose a rise in alkalinity was shown by 26 samples (column 7).

Of the 59 samples which were free from mastitis streptococci only 30 showed no apparent alteration in composition. An increase in chlorine not accompanied by a corresponding increase in alkalinity was revealed by 24. As will become apparent subsequently in the consideration of the chlorine variations this rise in chlorine only is probably physiological in the majority of these cases while in a small percentage it may be attributed to infection of one or more of the other quarters in the same udder. The remaining five samples (column 6) which were free from mastitis streptococci but showed increased alkalinity and chlorine were all derived from quarters with advanced clinical mastitis in which growth and multiplication of the streptococci had in all probability been stopped or inhibited for reasons already stated.

Very accurate determination of the pH of milk by means of the Universal Indicator cannot be claimed, but comparative tests carried out by this method and the Beckman pH meter show that the margin of error is small and not such as to detract from the value of the Universal Indicator in the diagnosis of mastitis, provided it is used in the concentration (1:3 to 1:4) recommended. When compared

with other popular indicators it provides a greater variation in colours depending on the degree of alkalinity, is more sensitive and affords a better method for the easy detection of changes in the reaction of milk.

TABLE 2.

*Comparison of Universal Indicator and Chlorine Determination.*

Herd No.	No. of Samples.	1	2	3	4	5	6	7
		ME- UI- Cl <sub>2</sub> -	ME+ UI+ Cl <sub>2</sub> +	ME- UI- Cl <sub>2</sub> +	ME+ UI- Cl <sub>2</sub> +	ME+ UI- Cl <sub>2</sub> -	ME- UI+ Cl <sub>2</sub> +	ME+ UI+ Cl <sub>2</sub> -
1.....	237	113	19	68	26	4	5	2
3.....	112	33	22	32	23	0	2	0
4.....	54	23	8	11	9	0	3	0
5.....	70	16	9	27	9	0	9	0
8.....	20	3	6	0	1	1	9	0
9.....	32	2	7	4	14	3	2	0
10.....	19	8	3	4	1	0	3	0
11.....	38	13	7	9	3	4	1	1
12.....	35	8	8	6	9	4	0	0
13.....	36	9	8	10	7	1	1	0
16.....	68	17	9	17	12	7	6	0
18.....	50	25	1	15	6	0	3	0
19.....	55	25	3	15	4	4	4	0
20.....	11	0	3	1	1	1	5	0
TOTAL.....	837	295	113	219	125	29	53	3

In 837 samples derived from 14 herds (Table 2) the Universal Indicator was used in conjunction with chlorine determination and microscopic examination of smears prepared from incubated samples of milk. Several other tests such as cultural examination, sediment, Hotis test and physical examination were also applied to many of these animals, but as these were not employed uniformly in all the herds the results obtained by their use are not included in the analysis in this table. They nevertheless furnished a valuable aid in assessing the presence or absence or the degree of mastitis in those cases where the results of the other three tests were inconclusive.

The total number of samples that were infected with mastitis streptococci (columns 2, 4, 5 and 7) was 270 the remainder of 567 being free from streptococci. Of the latter number, however, 272 (columns 3 and 6) showed changes in the composition of the milk according to the chlorine content and reaction, thus leaving 295 definitely negative to all tests. In the 270 infected samples the pH as determined by the Universal Indicator yielded indisputably positive results in 116 or 43 per cent. (columns 2 and 7) while another 42 (15.5 per cent.) samples, which for the sake of convenience are included in column 4, showed pH ranging from 6.7 to 7.0 and must therefore be regarded as suspicious. The reaction was accordingly within normal limits in 112 (41.5 per cent.) of the 270 infected samples.



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The secretion of abnormal milk by a large number of quarters which were free from mastitis streptococci will be discussed when the value of the chlorine test is considered.

### CONCLUSIONS.

(1) The Universal Indicator is superior to brom-thymol-blue for detecting the degree of alkalinity shown by mastitis milk.

(2) Estimation of the reaction of mastitis milk is only of limited value in the diagnosis of the disease, and although it may provide a useful aid to other tests, it cannot on its own be considered a reliable test.

### CHLORINE.

The tendency of mastitis milk to have a salty taste was one of the earliest abnormalities to be noted in such milk and the tasting of milk for saltiness as a test for mastitis was actually recommended by Rast in 1854. Since then all investigators with the solitary exception of Seel (1911) agreed that the chlorine content of the milk is increased in mastitis, and many hold that the determination of chlorine affords a very valuable method of diagnosis of the disease.

The chlorine content appears to be subject to greater variation than any other milk constituent and even in quarters free from infection it may show sudden changes, frequently for no apparent reason. The general belief about the origin of chlorine in milk is that it results from an exudation of serum from the blood, and it would appear that any factor which tends to produce an abnormal flow of blood to a quarter or to increased permeability of the alveolar epithelium may account for a rise in the percentage of the chlorine in the milk from that quarter. The mechanism controlling this exudation is remarkably sensitive to external influences, and a variation (generally an increase) in chlorine is usually the first perceptible indication of that mysterious group of udder abnormalities which are usually referred to as "secretory disturbances".

Increased chlorine is not only encountered in pathological or abnormal conditions of the udder but is also characteristic of various stages of lactation. It is high in colostrum milk, then declines to normal limits during the first two weeks after parturition and again shows a tendency to increase after six months lactation, this rise being particularly marked towards the end of lactation.

It is thus apparent that there may be considerable fluctuations in the chlorine percentage even in the case of milk from a normal healthy udder, and that it is consequently very difficult to determine the upper limit of the percentage of chlorine which should be allowed for normal milk. Rosell (1931) considers that the normal percentage ranges between 0.09 and 0.14 while Davies (1934) found that it varies from 0.045 to 0.15 per cent. with an average of 0.095, and he considers that a rise above 0.15 per cent. should be regarded as abnormal. Determinations made by us on a large number of samples of both normal and abnormal milk have shown that Rosell's limit of 0.14 per cent. is too low and consequently the margin fixed by



Davies at 0.15 per cent. was adopted as the maximum percentage for normal milk. The chlorine content of all samples in Table 2 was determined by titration according to the method described by Rosell. The chlorine test was positive (i.e. chlorine content of over 0.15 per cent.) in 238 (88.1 per cent.) of the 270 samples which showed the presence of mastitis streptococci on microscopic examination of incubated milk. It failed in 32 (11.9 per cent.) cases. In three of the latter (column 9) there was increased alkalinity but the remaining 29 (column 5) showed no evidence of clinical mastitis and it can therefore be assumed that these were cases of latent infection. 53 Samples (column 6) which were free from streptococci showed increased alkalinity and chlorine. It was not possible to ascertain the cause of this rise in 4 of these samples. In the remaining 49 the rise in chlorine and alkalinity was accompanied by other evidence of mastitis such as induration, abnormal sediment, and reaction to Hotis test. In the great majority of these there was advanced induration of the quarters and the reaction to the indirect tests was very marked, which suggests that they were old standing cases in which the pronounced changes in the milk probably rendered it an unsuitable habitat for the streptococci.

219 Samples (column 3) obtained from quarters free from mastitis streptococci showed an excessively high chlorine content without a corresponding increase in alkalinity. Over 50 per cent. of these samples were derived from quarters which, while showing no infection themselves nevertheless belonged to udders which had streptococci in one or more of the remaining quarters. In view of the sensitiveness of the chlorine in milk to external influences it is probable that the increase in chlorine in the milk from the healthy quarters of infected udders is due to the presence of infection in adjacent quarters. This is in accordance with the results obtained by Bryan and Trout (1935) who found that the average percentage of chlorine in the non-infected samples in two infected herds was 0.167 per cent. and 0.169 per cent. respectively, which is well above normal limits.

In the remaining samples which showed increase in chlorine content without other evidence of mastitis the cause was physiological in most cases. It was observed that in those samples in which the high chlorine must be attributed to physiological factors such as advanced lactation the rise was more or less uniform in all four quarters and was never very pronounced except during the first two weeks after parturition and right at the end of lactation. With the exceptions mentioned the highest percentage of chlorine which was found in cases where the rise was considered to be due to physiological causes was 0.189.

#### *Discussion.*

The detection by the chlorine test of 88.1 per cent. of mastitis quarters would suggest this method as the best of all the indirect tests. Severe limitations are however imposed on its utility for diagnostic purposes by its tendency to yield positive results in a very large percentage of quarters which are normal. Positive reaction to the chlorine test without further corroboration cannot therefore be accepted as proof that an udder is infected; and a test which, while



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detecting about 90 per cent. of the cases, at the same time gives a large percentage of false positives is indefinitely inferior to one which detects a smaller percentage but does not condemn as infected a large proportion of clean animals.

### *Conclusions.*

(1) The chlorine content of milk is subject to great variation and is very sensitive to both physiological and pathological factors.

(2) Chlorine determination detects a higher percentage of mastitis infected quarters than the alkalinity tests.

(3) The chlorine test also gives a large number of false positives.

### METHYLENE BLUE REDUCTION TEST.

Koning (1906-08) pointed out that the diastase content was invariably increased in milk from udders infected with mastitis. He accordingly suggested the use of this abnormality as a suitable means of diagnosing the disease and was thus the first to recommend the methylene blue test for mastitis.

According to a rough estimate by Prucha (1934) about 75 per cent. of the udders with some pathological condition were detected by the methylene blue test although in a few instances it was noted that milk which was decidedly gargety failed to reduce methylene blue in 5½ hours.

Devereux and Bryan (1935-36) in studying the effect of streptococcal mastitis on the methylene blue reduction test found that the addition of only 10 per cent. of mastitis positive milk to normal milk which is classed as Group I by the methylene blue test reduced this to Group III while the admixture of 40 per cent. mastitis milk caused normal milk to be classed in Group IV.

### *Theoretical.*

Methylene blue is a dye which exists in a blue oxidised state and its addition to a fluid containing a reducing system is liable to be followed by its reduction to a colourless state. The time taken for this reduction depends on the amount of oxygen present and the activity of the reducing agent. Normal milk has a natural reducing system which is largely dependent on its fat content, but the action of this system is expedited to a considerable extent by the presence of bacteria in milk, which utilise available oxygen at a very rapid rate during their growth and multiplication. It therefore follows that the more bacteria there are present in milk the shorter is the time required for the reduction of methylene blue.

### *Technique.*

Methylene blue tablets specially prepared for the reduction test by Messrs. Blauenfeldt and Tvede of Copenhagen were used in the tests under consideration. One tablet was dissolved in 200 c.c. warm sterile distilled water which after cooling was made up to



800 c.c., 1 c.c. of which was added to 10 c.c. milk in a test tube giving a concentration of 1:300,000. After shaking the tubes were kept at 37°C and observations made every half hour. According to Wilson the test as described is unsuitable since cream and associated organisms rise to the top and cannot therefore assist in the reduction of the dye. He therefore recommended the modified method which consists in corking and inverting the tubes at half hourly intervals. This procedure was followed in the present series and the end point taken when the dye was completely decolourised.

The modified test was applied to composite samples from 47 cows in four herds which all showed a high percentage of infection. The presence or absence of mastitis streptococci was determined by cultural methods and microscopic examination of incubated samples from the individual quarters of every cow. In addition to this the reaction and chlorine content of the individual quarters were determined, and the amount of sediment obtained by centrifuging 10 c.c. milk from individual quarters in Tromsdorff tubes was also recorded in three herds (33 cows).

Detailed results are given in Table 3 in which the quarters shedding streptococci are indicated in the third column. The samples were collected with due aseptic precautions during the course of ordinary routine testing for mastitis and in properly sterilised bottles. Contamination from extraneous sources such as is certain to occur in tests conducted on bulk samples of milk derived from producers or distributors was thus avoided, and it can therefore be assumed that the reduction was effected only by the ordinary reducing system of normal milk assisted by bacteria derived directly from the udders.

Of the 47 cows tested 32 were secreting mastitis streptococci from one or more quarters and 15 were clean. Fourteen of the latter showed a reduction time of over 8 hours. The milk from the remaining animal (No. 17 in herd 5) was also free from streptococci but cultural examination showed numerous cocci which may account for the low reducing period (5 hours).

The reduction time for the 32 infected samples was as follows:

8 hours and over	...	...	...	8 samples (25 per cent.)
7-8	„	...	...	10 „ (31.2 per cent.)
6-7	„	...	...	4 „ (12.5 per cent.)
4-5	„	...	...	5 „ (15.6 per cent.)
3-4	„	...	...	2 „ ( 6.3 per cent.)
2-3	„	...	...	2 „ ( 6.3 per cent.)
1-2	„	...	...	1 „ ( 3.1 per cent.)

Six of the 8 infected samples which showed a normal reduction period of 8 hours and over were derived from udders which showed infection in only one quarter while in the remaining two samples two quarters were infected in each case. Moreover with one exception (cow No. 15 in herd 5) the changes shown by the other tests were not so pronounced as to suggest a very active mastitis in these 8 cases and



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TABLE 3.  
Methylene Blue Reduction Test.

Hard No.	Cow No.	Quarters Infected.	CHLORINE PERCENTAGE.				pH.				SEDIMENT.				Reduction time in Hours.
			RF.	RH.	LF.	LH.	RF.	RH.	LF.	LH.	RF.	RH.	LF.	LH.	
5	1	RF, LF, LH	.294	.2275	.2134	.2065	7	7	6.8	7	+	+	+	+	6
	2	RF	.2905	.2135	—	—	8.5	7	—	—	.01	+	+	+	7½
	3	LF	.2205	.168	.2185	.182	6.8	6.5	6.8	6.5	+	+	+	+	7½
	4	NH	.269	.308	.2065	.35	8	8	6.5	8.5	+	+	+	+	Over 8
	5	RH	.14	.1505	.1435	.1295	6.5	6.5	6.5	6.5	+	+	+	+	Over 8
	6	NH	.133	.1295	.14	.217	6.5	6.5	6.5	7.5	+	+	+	+	Over 8
	7	LF	.147	.1435	.1575	.1505	6.5	6.5	6.8	6.5	+	+	+	+	6½
	8	LH	.1925	.182	.175	.203	6.5	6.5	6.5	7	+	+	+	+	7½
	9	LF, LH	—	—	.2695	.273	—	—	8	8	..	..	..	..	1½
	10	NH	.14	.1435	.1575	.147	6.5	6.5	6.5	6.5	—	+	+	+	Over 8
	11	RH, LH	.196	.1785	.175	.1645	6.5	6.6	6.5	6.8	—	+	+	+	7
	12	NH	.1435	.154	.147	.1645	6.5	6.5	6.5	6.5	+	+	+	+	8
	13	NH	.1785	.203	.2355	.189	6.8	6.8	7.5	6.8	+	+	+	+	Over 8
	14	NH	.231	—	.2205	.231	7	—	6.6	6.8	+	+	+	+	Over 8
	15	RF	.203	—	.21	.2135	6.5	—	6.8	6.5	—	+	+	+	Over 8
	16	LH	.14	.196	.1435	.182	6.5	6.5	6.5	6.5	—	+	+	+	Over 8
	17	NH	.161	.147	.1575	.1575	7	6.6	6.8	6.5	—	+	+	+	Over 8
	18	LF	.154	.1645	.175	.161	7	6.8	7	6.8	—	—	—	—	8
	19	RF	.35	.336	.322	.329	6.5	7	6.5	6.8	—	—	—	—	4½
8	1	RH	.1925	.168	.1715	.147	7.5	7.5	7.5	7.5	+	+	+	+	7
	2	LF	.189	.154	.14	.1645	7.8	7	7.3	7.3	.02	+	+	+	6
	3	RH, LF, LH	.175	.168	.1785	.175	7	6.8	7	7.3	+	+	+	+	4½
	4	RF, RH	.2275	.238	.231	.245	6.5	7.8	7.8	8	+	+	+	+	7½
	5	RH	.1155	.133	.1295	.14	6.5	6.5	6.5	6.5	.01	+	+	+	Over 8

N.B.—Under "Sediment"—

+ Represents merely a trace of deposit.

++ Signifies deposit varying from a trace to 0.01 c.c. per 10 c.c.



TABLE 3—(continued).  
Methylene Blue Reduction Test.

Herd No.	Cow No.	Quarters Infected.	CHLORINE PERCENTAGE.				pH.				SEDIMENT.				Reduction time in Hours.
			RF.	RH.	LF.	LH.	RF.	RH.	LF.	LH.	RF.	RH.	LF.	LH.	
13	1	RH, LF....	.203	.1925	.168	.154	7	6.8	6.8	6.5	.03	.02	+	—	8
	2	LF.....	.1505	.1505	.161	.1575	6.5	6.8	6.5	6.8	+	+	+	+	Over 8
	3	RF, LH....	.168	.1505	.1575	.175	6.5	6.6	6.5	6.8	+	+	+	+	Over 8
	4	LF.....	.14	.1365	.1785	.14	6.5	6.5	7	6.5	+	+	+	+	7
	5	RF, RH, LF, LH.....	.2345	.3325	.2485	.287	7.5	8.5	6.8	8	.04	.01	+	.3	2½
	6	Nil.....	.1575	.14	.1435	.154	6.5	6.5	6.5	6.5	—	—	—	—	Over 8
	7	RF, LF, LH	.308	.14	.1525	.196	8	6.5	6.5	6.8	.03	+	+	+	2½
	8	RF, RH, LH	.182	.1785	.175	.175	7	6.8	6.6	6.5	+	.03	+	+	7
	9	LH.....	.1505	.1435	.147	.147	6.5	6.5	6.5	6.5	.02	.01	+	.07	6
19	1	RF, LH....	.176	.1505	.147	.14	7	6.8	6.6	6.5	7	6.8	6.6	6.5	4
	2	RF.....	.1365	.126	.1295	—	6.5	6.8	6.8	—	6.5	6.8	6.8	4½	
	3	LF, LH....	.1855	.119	.133	.119	7.5	6.8	6.8	6.8	7.5	6.8	6.8	4	
	4	Nil.....	.1365	.133	.112	.1085	6.5	6.5	6.5	6.5	6.5	6.5	6.5	Over 8	
	5	Nil.....	.189	.1295	.119	.119	7	6.8	6.5	6.5	7	6.5	6.5	Over 8	
	6	Nil.....	.126	.1295	.126	.1575	6.5	6.5	6.5	6.5	6.5	6.5	6.5	Over 8	
	7	Nil.....	.1855	.1575	.154	.1435	7	6.8	6.8	6.5	7	6.8	6.8	Over 8	
	8	RF, LH....	.1505	.154	.1645	.1645	6.5	6.8	6.5	6.8	6.5	6.5	6.8	7½	
	9	LF, LH....	—	—	.1715	.1435	—	—	6.8	6.8	—	—	6.8	3½	
	10	RH.....	.1575	.182	.238	.505	6.5	6.8	7.5	6.5	6.5	6.5	6.5	8	
	11	LF.....	.133	.1295	.168	.1365	6.5	6.5	6.5	6.5	6.5	6.5	6.5	7	
	12	Nil.....	.1575	.161	.1785	.1645	6.8	6.8	6.8	6.6	6.8	6.8	6.6	Over 8	
	13	Nil.....	.154	—	.154	.1435	6.5	—	6.6	6.5	6.5	6.6	6.5	Over 8	
	14	Nil.....	.1505	—	.133	.154	6.5	—	6.5	6.5	6.5	6.5	6.5	Over 8	

N.B.—Under "Sediment";—

+ Represents merely a trace of deposit.

++ Signifies deposit varying from a trace to 0.01 c.c. per 10 c.c.



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it is probable that the infection was not heavy. On the other hand one quarter of cow No. 15 in herd 5 was functionless while the milk from the other three quarters showed such a marked rise in chlorine as to justify the conclusion that this is a very advanced case of mastitis, and one would therefore expect an appreciable lowering of the reduction period. That this did not actually take place is probably due to the fact that, on account of the advanced mastitis and for reasons suggested elsewhere in this paper, there was no gross infection or multiplication of streptococci in the one infected quarter.

### *Discussion.*

Although in this series the methylene blue reduction test revealed 24 out of 32 (75 per cent.) infected udders it can, nevertheless, not be recommended as a suitable test for mastitis. Lowering of the reduction time is brought about not only by mastitis streptococci nor solely by pathogenic bacteria, but may be effected by various contaminating organisms as well. Decrease in the reduction time cannot therefore be accepted as positive proof of the presence of mastitis organisms in the milk. Consequently the test cannot be regarded as specific for mastitis but should rather be considered as a useful indicator of general bacterial activity in the milk. On this account its use has been adopted first in Scandinavian countries and the United States and subsequently also in most other countries for the routine grading of raw milk.

According to the results shown in Table 3 the most characteristic changes in the composition of milk which are usually associated with mastitis, such as increased chlorine, alkalinity, and sediment consisting mainly of leucocytes, have no appreciable effect on the reduction of methylene blue. Milk from many of the udders free from streptococci but showing marked abnormalities in composition (e.g., Nos. 4, 6, 13 and 14 in herd 5 and No. 7 in herd 19) shows a reduction period of over eight hours. On the other hand this period may be markedly reduced in milk in which there is no great variation from normal but which contains mastitis streptococci (e.g., No. 7 in herd 5, and Nos. 1, 2 and 3 in herd 19). When therefore the reduction test is used for determining the quality of milk its efficiency is limited to an estimation of bacterial content and bacterial activity, and like the plate count it cannot be relied upon to detect the abnormalities produced in the composition of the milk by pathological conditions of the udder.

### *Conclusions.*

(1) In aseptically drawn samples of milk the reduction time of methylene blue is markedly lowered by mastitis streptococci.

(2) In streptococcus free samples there appears to be no correlation between the reduction time and the chlorine content, alkalinity and number of leucocytes in the milk.

(3) The methylene blue reduction test cannot be considered a specific test for mastitis.



## HOTIS TEST.

Hotis and Miller (1936) described a new test which in a series of 753 samples gave reactions which agreed with blood agar cultures in 94.6 per cent. of samples.

The test is carried out by adding 0.5 c.c. of a sterile 0.5 per cent. aqueous solution of brom-cresol purple to 9.5 c.c. milk in a sterile test tube. The test tube is inverted several times to mix the contents and is then incubated for 24 hours at 37° C. When the dye and milk are first mixed the mixture is a purple colour. If streptococci are present the colour changes during the incubation period to a greenish or yellow shade as a result of acid production from the lactose. Furthermore if *Streptococcus agalactiae* is present small yellow flakes from 0.5 to 4 mm. in diameter form in the side of the tube. These are stated to be quite distinctive. The method is applied only to quarter samples.

Bryan and Devereux (1937) found that the test will detect only 64.3 per cent. samples that are positive to the microscopic test and that it gives rise to many suspicious reactions. They further ascertained that the results of the Hotis test are not constant on repeated testing of milk from positive or negative cows.

McCulloch and Fuller (1939) produced evidence to suggest that the flakes are due to the growth of organisms in the presence of specific agglutinins in blood elements which have infiltrated through the mammary tissue.

A study made by them of pure cultures of various streptococci obtained from cows with mastitis, from the nasal discharge of normal cows and from normal human throats shows that organisms capable of giving a positive Hotis test are widely distributed, and that a reaction may in fact be produced by any lactose fermenting organism which produces agglutinins and forms such clumps when grown in their presence.

In the investigations under review the Hotis test was applied as directed to herds Nos. 6, 7, 9, 10 and 11 comprising 69 animals of which 269 individual quarters were examined. In every instance microscopic examination of duplicate samples of milk incubated overnight was also carried out, and in addition one or more of the other indirect tests (alkalinity, chlorine, physical examination) was applied.

The colour changes and the presence or absence of the characteristic clumps or floccules in the Hotis tubes were noted after 24 hours incubation. Furthermore, smears were made from each one of the Hotis tubes after the prescribed incubation period. These were stained by Newman's method and examined microscopically with the other smears.

The results tabulated in Table 4 show that no less than twelve different combinations were obtained by the examination of the two classes of smears and the recording of colour and flocculence readings

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of Hotis tubes. There was definite agreement in the results yielded by the four methods in only 98 (33·8 per cent.) cases, 51 samples being negative and 47 positive to all four tests.

Microscopic examination of smears prepared from milk samples incubated overnight and from the Hotis tubes after 24 hours incubation showed that mastitis streptococci were secreted by 138 (51·3 per cent.) quarters. Smears made from incubated milk detected 129 (i.e., 92·8 per cent.) of the 138 positives and smears from the Hotis tubes 113 (81·9 per cent.). Streptococci in the Hotis tube smears showed some degree of disintegration probably the result of prolonged incubation.

In the samples from the 138 positive quarters the Hotis colour test yielded positive results in 125 (90·6 per cent.) and was negative in 13 (9·4 per cent.). Flocculence was shown in 59 (42·8 per cent.) and was absent in 79 (57·2 per cent.). Of the 131 streptococcus free samples the Hotis tubes showed colour changes which must be regarded as positive reactions in 73 (55·7 per cent.) while colour changes and flocculence were present in 7 (5·3 per cent.).

### *Discussion.*

The high percentage of false positives given by the colour change eliminates this factor forthwith from all consideration as a suitable test. The formation of floccules in the tubes proved far more reliable in this respect since these were present in only 5·3 per cent. streptococcus free samples, but on the other hand the absence of flocculation in 57·2 per cent. of infected samples condemns this too as a most unreliable factor. The combination of colour changes and the tendency to flocculation originally recommended by Hotis and Miller yielded positive results in only 59 (42·8 per cent.) of the 138 infected samples. These two factors on which the test is based are therefore, when considered individually as well as in combination, very unreliable—not only on account of their failure to detect a large percentage of positives but rather because they show positive or suspicious reactions in a very large percentage of clean samples. Reliance can only be placed on the colour change and flocculation if these are corroborated by microscopic examination of smears made from the samples after incubation. Even then the smears from the Hotis samples do not reveal the same high percentage of infected quarters as those prepared from milk alone after incubation overnight.

### *Conclusions.*

- (1) The Hotis test fails to reveal a large percentage of infected samples.
- (2) The test yields a very large number of false positives.
- (3) Reliance can only be placed on the Hotis test if its findings are confirmed by microscopic examination.



TABLE 4.  
*Hotis Test.*

Herd No.	No. of Cows.	No. of Samples Tested.	1 Colour - Floculence - Microscopic Examination (Hotis) - Incubd. -	2 Colour + Floculence + Microscopic Examination (Hotis) + Microscopic Examination (Incubd.) +	3 Colour + Floculence - Microscopic Examination (Hotis) - Microscopic Examination (Incubd.) -	4 Colour + Floculence + Microscopic Examination (Hotis) - Microscopic Examination (Incubd.) -	5 Colour + Floculence + Microscopic Examination (Hotis) - Microscopic Examination (Incubd.) +	6 Colour + Floculence - Microscopic Examination (Hotis) + Microscopic Examination (Incubd.) +	7 Colour - Floculence - Microscopic Examination (Hotis) + Microscopic Examination (Incubd.) -	8 Colour - Floculence - Microscopic Examination (Hotis) - Microscopic Examination (Incubd.) +	9 Colour - Floculence - Microscopic Examination (Hotis) + Microscopic Examination (Incubd.) +	10 Colour + Floculence - Microscopic Examination (Hotis) - Microscopic Examination (Incubd.) +	11 Colour + Floculence - Microscopic Examination (Hotis) + Microscopic Examination (Incubd.) -	12 Colour + Floculence + Microscopic Examination (Hotis) + Microscopic Examination (Incubd.) -
6.....	28	111	13	20	34	6	3	23	2	1	3	4	2	0
7.....	17	66	8	12	20	1	4	17	0	0	0	3	0	1
9.....	8	32	2	12	5	0	0	7	1	0	2	2	0	1
10.....	5	19	12	1	3	0	0	0	0	1	0	2	0	0
11.....	11	41	16	2	11	0	2	5	1	2	0	1	0	1
TOTAL.....	69	269	51	47	73	7	9	52	4	4	5	12	2	3

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### MICROSCOPIC EXAMINATION.

Ernst (1909) was the first to attempt to differentiate between those streptococci in milk which are derived from extraneous sources (air, dust, straw, etc.) and those associated with pathological conditions of the udder. This was done by growing cultures and by examining smears made from the milk sediment. He described the morphological characteristics of the mastitis streptococci, and noted their tendency to form long chains composed of cocci which appear to be elongated transversely and are arranged in palisade fashion. In 1840 samples of market milk he established the presence of mastitis streptococci in 336 (18.26 per cent.) by microscopic examination of smears made from sediment.

Klimmer, Haupt and Roots (1928) cultured typical mastitis streptococci from 109 milk samples, but microscopic examination of sediment smears made from these samples yielded unmistakably positive results in only 35 per cent.

Minett, Stableforth and Edwards (1930) stated that a positive diagnosis can be made with safety in cases where long streptococcus chains composed of cocci which are slightly elongated transversely are present in milk smears, and that the presence of diplococci or short chains also indicates with fair certainty the existence of streptococci mastitis. Their observations, however, showed the unreliability of the method in comparison with cultural examination. Of 223 instances in which streptococci were cultivated from milk, these organisms were undetected in deposit smears in 106 (47.5 per cent.)

Ehrlich and Bischoff (1930) examined 585 quarter samples both culturally and microscopically. Of these 51 (8.7 per cent.) were positive to both tests, 8 (1.3 per cent.) showed streptococci only on microscopic examination of sediment smears, and 85 only on cultural examination. Thus of 144 positives microscopic examination revealed only 59 (41 per cent) while 136 (94 per cent.) were detected in the cultures.

Hucker, Trudell and Jennings (1932) found that direct microscopic examination of milk made immediately following milking may have only a limited significance in isolating infected udders of the sub-clinical type and assert that by this procedure it is only possible to isolate individuals that are actively infected. They, however, suggested that microscopic examination of fresh unincubated milk might be a very valuable means of eliminating in an initial survey all of the cows which are infected beyond question. In order to detect the large percentage of cows which discharge only small numbers of streptococci and which may thus serve as a focus of infection for the rest they incubated milk overnight at 37° C. before subjecting it to microscopic examination, and concluded that this procedure was the most accurate and reliable for use in detecting cows discharging streptococci even in small numbers.

They made a study of 221 cows of which 48 per cent. of all the quarters were found to discharge streptococci in the milk. Of the bacteriological tests available for the detection of sub-clinical



mastitis the following proved to them to be efficient in the order named: Microscopic examination of samples incubated overnight at 37° C., veal infusion agar plates, veal infusion blood agar plates and direct microscopic examination of milk samples.

Bryan and Huber (1935) tried various dilutions for inhibiting extraneous bacteria and found brilliant green in a final dilution of 1:50,000 to be the optimum for this purpose. This still permits subsequent detection of mastitis streptococci upon incubation for 12 hours at 37° C. They found that when the streptococci were detected in producer samples of milk infected cows were always found in the herd, and such producer samples showed the streptococci were found in the producer samples the cows in the herds were free from streptococcus mastitis.

Trossbach (1935) came to the conclusion that no single test or combination of tests is efficient in 100 per cent. cases but that the most reliable method is a combined microscopic and cultural examination of the secretion. This he considered reliable in 95 per cent. cases.

Plastridge, Anderson and Weirether (1935) compared six methods of examination and found that microscopic examination of films prepared from incubated milk showed streptococci in a larger number of instances than (1) microscopic examination of films made from (a) whole milk, or from (b) sediment, or (2) blood agar plates inoculated with (a) a 4 mm. loopful of whole milk, or (b) sediment, or (c) 1 c.c. of a 1:10 dilution of the sample.

They, however, maintain that in the absence of other laboratory evidence of mastitis the finding of streptococci in incubated samples should not be taken as conclusive evidence of infection.

Hucker and Udall (1933) on the other hand hold that when streptococci are found in milk aseptically drawn the quarter is always indurated.

Plastridge and Anderson (1936) made the following determinations on 970 quarter samples from 15 herds: Physical examination, brom thymol blue reaction, leucocyte count, sediment, types of colonies on blood agar plates, and microscopic examination of incubated samples. The latter was found to be the most efficient of the six methods used and it revealed the presence of streptococci in 98.7 per cent. of samples collected from animals affected with chronic streptococcus mastitis. However, about 13.5 per cent. of the samples from healthy quarters also yielded chains of streptococci by this method, and it is concluded that the significance of the finding of streptococci in incubated samples from healthy quarters, in the absence of other evidence of mastitis, can be determined only by isolation and identification of the streptococci found.

After a study of diagnostic methods and subjecting 15,000 samples to the microscopic test Giltner (1936) concluded that microscopic examination of smears made from properly collected milk samples incubated for at least 12 hours at 37° C. permits an accurate diagnosis of infectious mastitis.



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In a comparative study of various tests Bryan and Devereux (1937) found that blood agar cultures detected on an average only 89 per cent. of all milk samples that were shown to contain mastitis streptococci in the microscopic test.

Bryan (1938) claims that microscopic examination of milk samples incubated for at least 12 hours at 37° C. detects 99 per cent. of cows infected with streptococcus mastitis whereas the blood agar method reveals only 90 per cent. and physical examination of the udder only 61 per cent.

Many of the workers referred to have attempted to compare microscopic examination with one or more of the indirect tests. In view however, of the exceptionally large number of factors, both physiological and pathological, which may produce alterations in the milk, which are indistinguishable from those encountered in streptococcus mastitis, such a comparison does not give a fair indication of the value or otherwise of the tests concerned. The only true and reliable criterion of a streptococcus infection is detection and recognition of the causative organism, and when it is desired to determine the efficacy of any method which has this object in view the comparison should be limited to those tests the results of which are dependent solely on the presence or absence of the streptococci in the udder and its secretion.

Microscopic examination was performed in every case in the 20 herds included in this survey and although it was usually accompanied by one or more of the indirect tests a comparison to determine its reliability has been intentionally limited to cultural examination which is the method more commonly employed.

### *Technique:*

When milk smears are made from the individual quarters all four smears from the same cow are prepared on one slide by marking four incomplete divisions on the slide with a diamond pencil (Figure 1). The four divisions on the slide correspond with the four quarters of the udder and they are made more to the one side of the slide leaving space at the other end for the number of the cow and the date of the test. When this method is followed there is no need to mark the different quarters on the slide because when the slide is held so that the examiner can read the number of the cow and the date of the test. The two divisions on the right represent the right fore and right hind quarters respectively, and the same applies to the left side.

This method not only effects marked economy in slides, stain, labour and time, but it also greatly facilitates the subsequent examination under the microscope.

The smears are airdried and then immersed in Newman's stain (Formula I). This solution consists of:—

Methylene blue powder	... ..	2.0 gm.
Ethyl Alcohol 95 per cent.	... ..	60.0 ccm.
Xylene	... ..	40.0 ccm.
Glacial Acetic Acid	... ..	6.0 ccm.



It combines fat extraction, fixation and staining. After about 5 minutes immersion (there is no danger of overstaining) smears are taken out and allowed to airdry before being washed, otherwise there is a danger of the smear being washed off the slide. After washing it is again airdried and is then ready for examination. Newman's solution can be kept for a long time and used repeatedly.

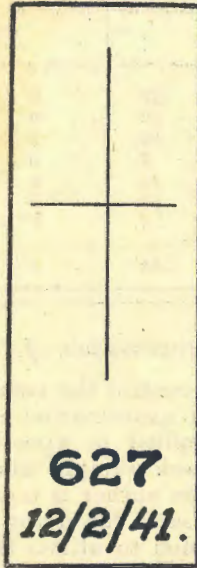


Fig. 1.—Glass slide.

#### *Microscopic Examination of Fresh Milk.*

The examination of films prepared from freshly drawn milk has been tested out repeatedly and thoroughly by many workers all of whom consider this method to have very limited value. Such smears were also examined at the commencement of the present series of tests, but the results were so disappointing that it was discarded in favour of other more reliable methods.

Centrifuging the fresh milk and examining smears prepared from the sediment detected a far higher percentage of infected quarters although when compared with the results obtained by examining smears made from milk incubated overnight it also shows severe limitations. This was carried out on 389 samples obtained from 7 herds (Table 5). Mastitis streptococci were present in 166, the remainder of 223 being negative. The sediment smears revealed only 41 (7 per cent.) and failed in 125 samples which showed streptococci after incubation overnight, while the latter was negative in only one case which was positive in the sediment smear. In one herd only (herd 16) were over 40 per cent. infected cases detected in the sediment smears, and this may be accounted for by the fact that in this instance the samples were kept at room temperature for an unusually long period before being centrifuged and smears prepared.

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TABLE 5.  
*Examination of Deposit Smears.*

Herd No.	No. of Samples Examined.	Incubated +		Incubated -		Total Positive.	TOTAL POSITIVES SHOWN BY.	
		Sediment +	Sediment -	Sediment +	Sediment -		Incubated Smears.	Sediment Smears.
1	108	8	37	0	45	45	8	
3	40	8	27	0	35	35	8	
5	66	3	16	1	20	19	4	
8	20	2	8	0	10	10	2	
13	36	3	16	0	19	19	3	
16	69	13	16	0	29	29	13	
18	50	3	5	0	8	8	3	
TOTAL.	389	40	125	1	166	165	41	

*Microscopic Examination of Incubated Milk.*

It was not possible to control the results obtained by microscopic examination with cultural examination in all of the 20 herds but the latter method was applied to nine herds. This was done by centrifuging 10 c.c. of each sample and growing cultures of the sediment on blood agar. The author is indebted to Mr. A. S. Canham and the late Mr. A. E. Lund for doing the cultural examinations and for their kind permission to utilise their results in this comparison. The two methods combined were applied to 267 samples (137 composite and 130 quarter samples). The total number infected with mastitis streptococci was 125. There was complete agreement between the two methods in 103 (82.4 per cent.) cases (Table 6.). Microscopic

TABLE 6.  
*Microscopic and Cultural Examinations.*

Herd Number.	Number of Samples.	Kind of Samples.	Microscopic + Cultural +	Microscopic + Cultural -	Microscopic - Cultural +	Total Number containing Streptococci.	Percentage infected as shown by.	
							Microscopic Examination.	Cultural.
5	19	Composite..	12	0	0	12	100	100
8	5	Composite..	4	1	0	5	100	80
9	24	Quarter.....	13	4	2	19	89.5	68.4
11	31	Quarter.....	10	0	1	11	90.9	100
13	9	Composite..	7	2	0	9	100	77.7
14	53	Composite..	22	1	0	23	100	95.6
15	51	Composite..	19	5	5	29	82.8	82.8
19	55	Quarter.....	11	0	0	11	100	100
20	12	Quarter.....	5	1	0	6	100	83.5
TOTAL..	267		103	14	8	126	93.6	88.8



examination detected another 14 which were not revealed in the cultures while on the other hand the latter showed up 8 which were negative on microscopic examination. Of the 125 positive cases microscopic examination thus detected 117 (93.6 per cent.) and cultural examination 111 (88.8 per cent.).

These results appear to confirm the conclusions arrived at by the many workers who found microscopic examination of incubated milk a more reliable method of diagnosis than cultural examination. However, a more critical investigation into the results obtained raises the question as to why two reliable methods which can confidently be expected to reveal 100 per cent. infected cases should fail in 6.4 per cent. and 11.2 per cent. cases respectively. In the literature referred to no attempt is made to explain this anomaly, and there appears to be a tendency to arrive at conclusions merely on the results obtained without due regard being paid to possible factors which may favour one test and militate against another. The result of this may be that of two tests which are probably equally efficient one may be unjustly regarded as being less reliable than the other.

A factor which does not appear to be taken into account when the value of various bacteriological methods is assessed is that the samples which are subjected to different comparative tests are not in fact the same although they are derived from the same quarter or the same udder. The method usually followed in collecting the samples is to milk the required quantity (about 10 c.c.) for one test into a test tube while that for the other test is drawn immediately afterwards into another test tube or bottle. It is generally accepted that the organisms are more numerous in the foremilk than in the middle milk or strippings. In a bacteriological examination of equal amounts (10 c.c.) of foremilk, middle milk and strippings Little found streptococci in all, but they were 20 times more numerous in foremilk than in middle milk and 14 times more in foremilk than in strippings. When taking samples Little accordingly does not discard the first 2 or 3 streams of the foremilk. It is therefore probable that in quarters in which the infection is slight a few streptococci may be present in the sample that is drawn first but that the second sample may be free from streptococci.

The two types of streptococcus mastitis in which the organisms are usually only present in small numbers are the quiescent or latent form and the very advanced cases in which there are pronounced changes in the milk. Consideration of the results obtained by the indirect tests in the 22 samples in which there was no agreement between microscopic and cultural examination showed that one case was apparently in an active stage while two were advanced and the remainder showed no appreciable changes in the milk, which factor would place them in the latent group. It is therefore probable that with one exception the infection in all cases was very mild. It is suggested that there is a strong possibility that in mild infections a streptococcus free sample may be obtained especially in the sample that is drawn last and that this may be responsible for infection being determined microscopically but not culturally or vice versa. It is also an accepted fact that infected quarters do not always shed streptococci, but show a tendency to secrete organisms intermittently

especially in the case of latent infection. Hence it is most desirable not to consider a cow clean on one test, and the necessity for regular testing is evident.

*Prolonged Incubation and Centrifuging Incubated Samples.*

The detection of mastitis streptococci by microscopic examination of smears from samples incubated for 12 to 18 hours has proved to be just as efficient—or even more so—as cultural methods of diagnosis. However, according to results shown in Table 6 it does not yet provide that acme of perfection which is the dream of the diagnostician although, as has been suggested above the failure of both this method and cultural examination in a small percentage of cases may not actually be due to any imperfection of the methods employed but rather to the possible complete absence of infection in the particular sample subjected to the test concerned. The question nevertheless arises as to whether the efficiency of this method cannot be increased by incubation for a longer period or by centrifuging the sample after incubation and preparing smears from the sediment.

In addition to the smears made from the Hotis tubes the advisability of prolonging the period was tested in a number of cases by making smears at varying intervals from 12 to 36 hours during incubation, but it was found that prolonging the period beyond 18 hours, which appears to be the optimum time, in no way enhanced the value of this method. Prolonged incubation in fact appears to decrease the efficiency of the test. For instance, according to the results in Table 4 the Hotis smears prepared after 24 hours incubation detected only 81·9 per cent. as against 92·8 per cent. revealed in smears made after incubating overnight. It further tends to produce disintegration of the streptococci which renders their recognition more difficult.

Centrifuging of the samples after incubation and preparation of the smears from the sediment did not improve the reliability of the test either. At most this merely ensures more rapid detection in cases in which the infection is very slight, but since it never appears necessary to examine more than approximately 20 fields before the detecting evidence of infection the slight advantage offered in a small percentage of cases by centrifuging does not justify the additional labour and time involved.

*Composite vs. Quarter Samples.*

Where curative treatment is to be applied it is essential that the milk from individual quarters be examined separately in order to discover the infected quarters. Where, however, the object of the test is merely to expose the individual cows that secrete streptococci material saving in labour, time and equipment would be effected if the examination of composite samples were just as reliable as that of individual quarter samples.

Kleckner reports that in the examination of 464 composite samples from 85 cows he found this to be fully as accurate as individual quarter samples. The results recorded in Table 7 however indicate that the same 100 per cent. success was not obtained in the



present series in which both quarter and composite samples from 71 cows were examined; 46 of these cows were discharging streptococci in their milk and the composite samples detected 43 (93.5 per cent.) of these while the quarter samples revealed infection in 45 (97.8 per cent.) and failed in one which was positive in the composite sample.

TABLE 7.  
*Quarter and Composite Samples.*

Herd No.	No. of Cows	qt. - comp. -	qt. + comp. +	qt. + comp. -	qt. - comp. +
9.....	8	0	8	0	0
10.....	5	2	2	1	0
11.....	9	4	4	1	0
12.....	8	1	7	0	0
13.....	9	0	8	0	1
18.....	14	9	5	0	0
19.....	15	8	6	1	0
20.....	3	1	2	0	0
TOTAL.....	71	25	42	3	1

Kleckner does not indicate what quantity of milk was used nor how the samples were obtained, but in our tests approximately 10 c.c. foremilk was first drawn from each quarter into four sterile test tubes and subsequent to this about 5 c.c. from each quarter was milked into another sterile test tube for the composite test. It is thus possible that the latter test would not have failed in 6.5 per cent. cases if 10 instead of 5 c.c. were taken from each quarter for the composite test or if these samples were drawn before those for the individual quarter test.

#### *Cytology.*

The cytology of milk will receive full consideration in a subsequent paper, but it is deemed opportune to point out here that the value of microscopic examination is not confined to recognition of the streptococci, but that this method also affords an opportunity of observing the cell content of the milk, and this may yield useful information on the extent of the changes in the quarter concerned as well as in the milk derived from it, and on the sanitary condition of the milk.

If in the preparation of the smear approximately 0.01 c.c. of the incubated milk taken after the tube has been thoroughly shaken is spread over about 1 sq. cm. on the slide, cells and cellular elements will be seen only very occasionally in smears made from milk of a perfectly healthy quarter. When examined under an oil immersion lens an average of one or more cells per field should be regarded with suspicion, though in the absence of streptococci this does not justify a positive diagnosis. Such an increase of cells is the result of cellular and circulatory response to alveolar irritation which may

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be due to many different factors besides streptococcus infection, and is generally seen in all types of secretory disturbances. Accurate differentiation of the cells is not always possible on account of degenerative changes which take place during incubation but the great majority of the cells in these cases of so called secretory disturbances of unknown aetiology appears to be epithelials and mononuclear types.

A preponderance of polymorphonuclear leucocytes is more suggestive of an active infection, and this combined with the long chain streptococci constitutes the most characteristic picture of streptococcus mastitis (Fig. 4). It is not however uncommon to find a large number of cells with a majority of polymorphonuclears but no streptococci. This is generally seen in cases of advanced mastitis in which there is marked induration of the quarter accompanied by the secretion of large amounts of pus in the milk. As has already been suggested it is probable that the absence of organisms in such samples is due to the bacteriostatic or even bactericidal effect of the milk (Fig. 7). On the other hand, a smear may show numerous long chain streptococci but no cells, and it has been observed that this picture is most typical of latent infections in which there is no clinical evidence of mastitis (Figures 2 and 3).

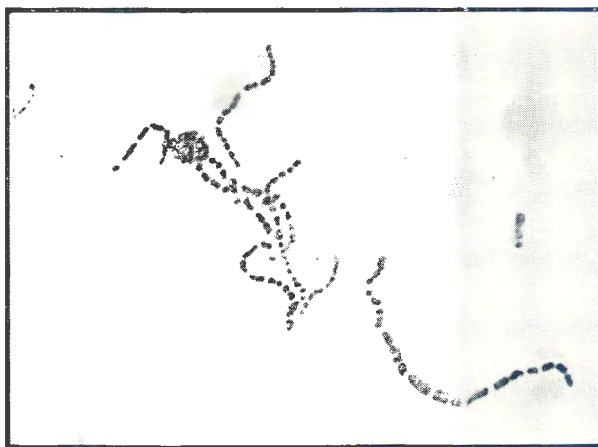


Fig. 2.- *Streptococcus agalactiae*; 1,200x.

#### *Discussion.*

All those investigators who have compared microscopic examination of incubated samples of milk with cultural methods hold that the former is the most efficient method of diagnosing streptococcus mastitis. As is however indicated in the review of the literature the question which appears to cause some degree of uneasiness or suspicion in the minds of many of these workers is the possibility of false positives being given by the microscope. Some express concern, not over the ability of this method to detect mastitis streptococci, but over the probability of it showing long chain streptococci in milk from clean quarters. Plastridge and Anderson, for instance, found



that it revealed 98·7 per cent. of cases but at the same time about 13·5 per cent. of the samples from healthy quarters also showed chains of streptococci by this method. They do not, however, state by what standards the quarters in question were judged to be healthy. If this conclusion was arrived at merely because the milk from these quarters yielded negative results to other tests, it still is no proof that the quarters were not infected.

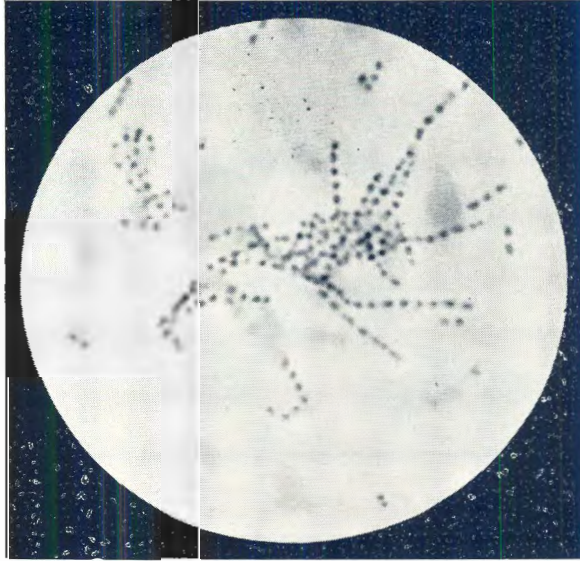


Fig. 3.—Mastitis streptococci of Group III type; 2,000×

*Note.*—The absence of cells in figures 2 and 3 suggests that these smears are derived from recently infested quarters or from latest cases.

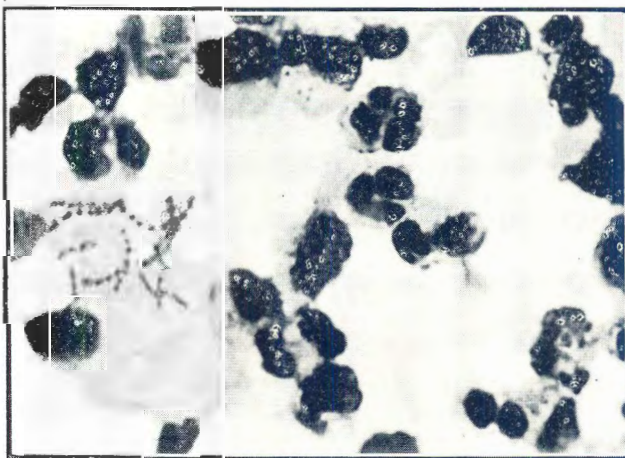


Fig. 4.—Mastitis streptococci and many cells (poly-morphonuclears predominating; 1,200×.

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The possibility of milk from clean udders showing long chain streptococci when examined microscopically cannot be determined with certainty by the testing of infected herds, but rather by repeated and long continued study of the milk from animals that are known never to have shown any indication of a streptococcus infection to a number of tests. Such an investigation is at present being carried out at Onderstepoort on a herd of clean cows which were obtained

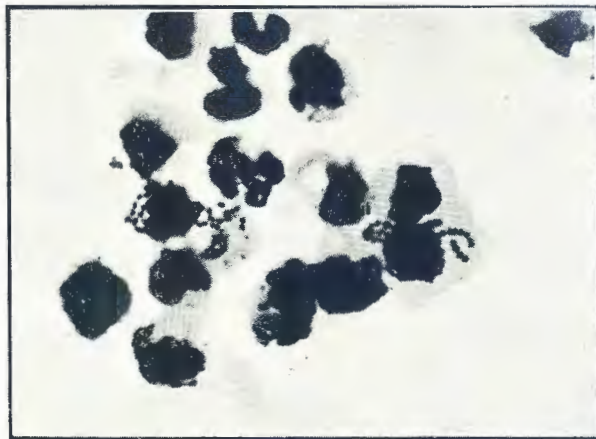
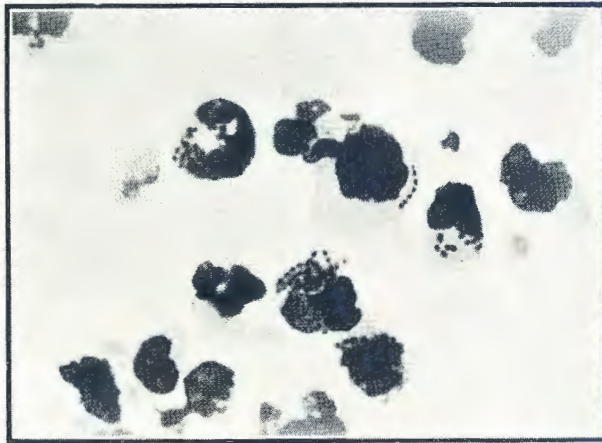


Fig. 5 and 6.—Mastitis streptococci and cells showing phagocytosis; 1,200 $\times$ .

as heifers heavy in calf. These are kept isolated in a paddock and are being milked in a new milking shed which has never housed any other cows. Quarter samples of milk from these animals are subjected to various indirect tests and to cultural and microscopic examination at four weekly intervals. A full report on these investigations is to be published in due course, but it will be relevant to



state here that of over 1,000 samples examined during the past 18 months none has thus far shown mastitis streptococci culturally and that microscopic examination of incubated milk has detected only one sample containing long chain streptococci but even in this one case the morphology of the organism in question did not correspond with that of mastitis streptococci in that it did not show the transverse elongation which is characteristic of the latter (Fig. 9). From these results it would appear that there is very little likelihood of an experienced examiner wrongly condemning a clean udder as infected, and the percentage of false positives which may be given by this method is certainly not as high as suggested by Plastridge and Anderson.

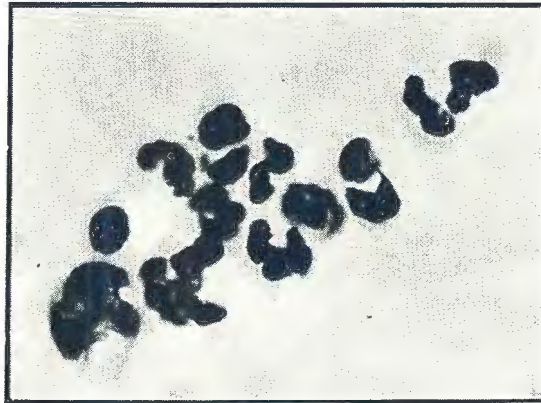


Fig. 7.—Many cells (mainly polymorphonuclears) and chromatin granules but no infection—probably from an advanced case; 1,200 $\times$ .

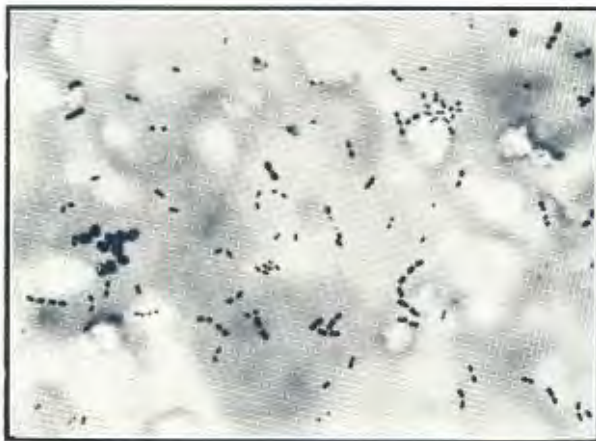


Fig. 8.—A negative smear from a healthy quarter showing marked contamination; 1,200 $\times$ .

*Conclusions.*

(1) Microscopic examination of smears prepared from fresh milk is valueless as a means of diagnosing streptococcus mastitis, and smears made from the deposit of fresh centrifuged milk detect infection in only a very small percentage of cases.

(2) Microscopic examination of smears made from milk incubated overnight revealed streptococcus infection in 93·6 per cent. as against 88·8 per cent. disclosed by cultural examination.

(3) Examination of smears made from composite samples of milk detected 93·5 per cent. as compared with 97·8 per cent. in quarter samples.

(4) The reliability of the microscopic examination of milk incubated overnight is not improved by incubating for periods in excess of 18 hours or by centrifuging after incubation.

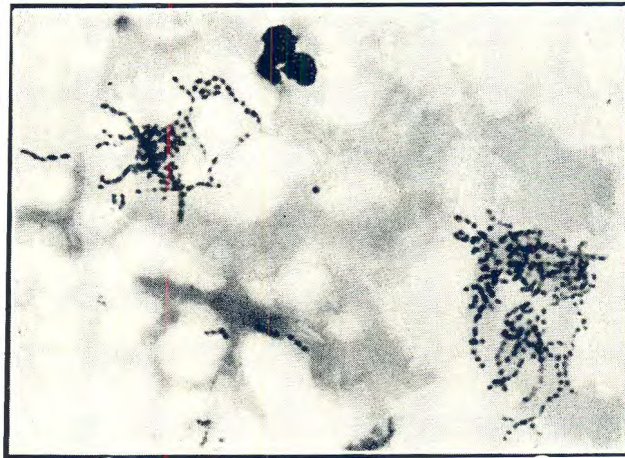


Fig. 9.- A non haemolytic long chain streptococcus not associated with mastitis and found occasionally in milk smears. Note that the individual units are smaller and thinner than those of mastitis streptococci (figures 2 and 3): 1,200 $\times$ .

GENERAL SUMMARY AND CONCLUSIONS.

(1) An account is given of the abnormal conditions which may be encountered in the bovine udder and its secretion in chronic streptococcus mastitis. It is pointed out that, on account of these different manifestations of the disease, it is not possible for any single test to reveal the condition in all its forms.

(2) The results obtained by the application of the alkalinity, chlorine, methyleneblue and Hotis tests and by microscopic examination are detailed.



(3) For the estimation of the alkalinity of milk the British Drug Houses' Universal Indicator was compared with brom-thymol-blue in 123 samples and revealed 39.1 per cent. of infected samples as against 25 per cent. shown by brom-thymol-blue.

(4) Chlorine determination was compared with alkalinity in 837 samples and detected a larger number of abnormal quarters, but its value as a test for mastitis is nullified by a tendency to show a large percentage of false positives on account of its sensitiveness to physiological as well as pathological factors.

(5) The methylene blue reduction test was applied to composite samples from 32 infected and 15 clean cows. The reduction time was under 8 hours in 24 (75 per cent.) infected udders and in 1 (6.7 per cent.) streptococcus free udder.

(6) Although reduction time is greatly reduced by mastitis streptococci, the reduction test cannot be regarded as specific for mastitis since reduction is also brought about by organisms other than mastitis streptococci.

(7) There appears to be no correlation in streptococcus free milk between the reduction time and the chlorine content, alkalinity and sediment.

(8) The Hotis test was applied to 269 individual quarter samples from 69 animals. It gives a high percentage of false positives and reliance can only be placed on its results if these are substantiated by microscopic examination of smears prepared from the milk after incubation.

(9) A review of the literature dealing with microscopic examination of milk for the detection of mastitis streptococci is given.

(10) The technique for preparing smears for microscopic examination is described.

(11) It is concluded that microscopic examination of smears from fresh milk is unreliable while that of smears taken after centrifuging fresh milk is of limited value. On the other hand, smears prepared from 267 samples after incubation overnight detected 93.6 per cent. positives as against 88.8 per cent. revealed by cultural methods.

(12) The lack of agreement between cultural and microscopic examinations in a small percentage of cases is attributed to the absence of infection in the particular sample in which the test concerned apparently failed.

(13) In an examination of over 1,000 milk smears from quarters known to be free from mastitis streptococci long chain streptococci were detected in only one case. These, however, were not the same morphologically as mastitis streptococci.

(14) The cell content of the milk as revealed in the smears provides a useful indication of the extent of the changes in the quarter and in the milk.

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(15) For the detection of mastitis streptococci microscopic examination of smears made from milk that has been incubated overnight at 37° C. is just as reliable as cultural methods. It requires less time, labour and equipment and can easily be adapted to testing under field conditions.

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