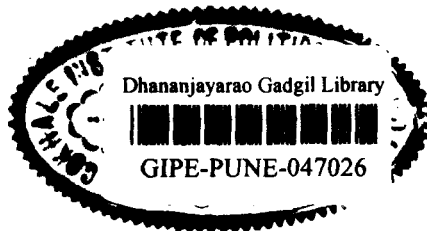
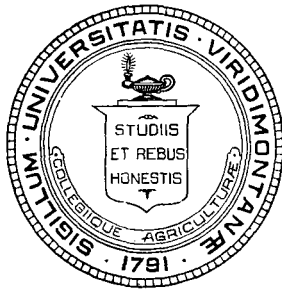


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DYES AND METHODS AS THEY AFFECT  
THE METHYLENE BLUE TEST

By J. M. FRAYER



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Three methods of conducting the methylene blue reduction test were compared, 50 to 100 replicate tests of each sample of milk being made.

1. Substantially as directed in the sixth edition of "Standard Methods of Milk Analysis."

2. Essentially according to proposed changes to be embodied in the seventh edition of "Standard Methods for the Examination of Dairy Products."

3. Fundamentally the English modified method as suggested by Wilson et al.

The data in hand suggest that the English method, which prescribes the careful inversion (not shaking) of each tube at 30-minute intervals, results in shortening the reduction times as well as in rendering them significantly more reliable as indicators. It is commonly recognized that the accuracy of the test as usually operated is impaired by the rising of the bacteria to the surface as the creaming process proceeds. The modified test yields more accurate results mainly because of the more uniform bacterial dispersion brought about by frequent inversion.

The reduction times were much the same when methylene blue thiocyanate and methylene blue chloride were used in approximately equal concentrations. Significantly longer reduction times elapsed when these dyes were used in concentrations of 1:300,000 than when used at concentrations of 1:700,000.

# BULLETIN 424: DYES AND METHODS AS THEY AFFECT THE METHYLENE BLUE TEST

J. M. FRAYER,

## INTRODUCTION

As long ago as 1887 Duclaux observed the reducing action of incubated milk on certain dyes. Later Neisser and Wechsberg (1900) found that a reduction test, using methylene blue as a dye, was serviceable in milk quality work especially as an indication of bacterial content. As time went on, others contributed to the standardization of the test and studied it from many angles in an effort the better to understand the underlying reasons for observed phenomena.

Hastings et al. (1920), Thornton and Hastings (1930), Ellenberger and Moody (1930), Fay and Aikens (1932), Wilson et al. (1935), and others in recent years have made careful studies of the relative importance of certain practices involved in the operation of the test, usually with a view of attaining simplicity without impairing accuracy. As might be expected, complete agreement has not always been attained. However, it has been shown that, as conducted in research laboratories, it may safely be simplified in ways which make it better adapted for use as a routine test by milk control officials and even at relatively small milk plants. Since its introduction here, about 20 years ago, and despite criticism, it has come into popular and scientific favor as a helpful tool in milk quality work, being preferred by some investigators to the plate count. Of course the utility of either depends upon the exactitude of its conduct and the judgment exercised in the interpretation of results.

Although the special appeal of the reduction test lies in the relative simplicity of its operation, the underlying factors involved are somewhat complex, a situation holding in any case where actions or reactions depend upon biologic processes. Obviously, no permanent changes should be made in test technique unless accuracy is at least maintained or, better, enhanced.

The studies here reported deal with two important phases of the problem.

1. A simple statistical comparison of the results obtained by three methods differing somewhat in detail with a view of showing something of their relative dependability.

2. A comparison of the results obtained when two dye compounds are used at two concentrations. Unless otherwise specified, tests were made by the English modified method.

**Gokhale Institute of Politics  
& Economic, Poona**

## GENERAL PROCEDURE

From 120 to 300 tests were made of each milk sample according to the number of methods or factors being compared, the results being analyzed by simple statistics. In order to control as many sources of variation as possible, a more strict routine was followed than would be necessary in practical usage. All glassware was made bacteriologically sterile and rubber stoppers were steamed in an Arnold atmospheric sterilizer for at least one hour. The dye solutions were freshly prepared every other day, and kept in tightly stoppered, brown-glass bottles. They were measured into sterile tubes with a burette calibrated at each 0.1 ml, after which the milks were measured into the tubes with sterile, straightsided 10-ml pipettes. One thermostatically controlled and two manually controlled water baths, held at  $37^{\circ}\text{C.} \pm 1$ , were used. Care was taken to cover each bath tightly so as to exclude light. Tubes were examined and temperatures checked at 15-minute intervals. Tubal examination by transmitted rather than by reflected light greatly facilitated the determination of end points. Tubes were deemed to be "out" only when complete reduction had taken place. Preliminary trials satisfied the writer that complete reduction could not be assumed until the last blue area had disappeared. This conviction seems to be corroborated by observations of others to the effect that the human eye is not sensitive enough to determine the points at which reduction starts and ends, and that workers differ in their ability to make fine color discriminations. An effort was made to maintain some degree of homogeneity in the series sampled by gently agitating the milk at frequent intervals while the sub-samples were being drawn.

Skar (1913) attributes certain variations in results to the rising of a large proportion of the bacteria and leucocytes which are swept to the surface by the butterfat and to the settling of some of the cells to the bottom of the tube. Thornton and Hastings (1930) state that: "It is probable that the two most important sources of inaccuracy are (1) the different rates of oxygen uptake of different species of bacteria; (2) the sweeping of bacteria out of the body of the milk by the rising butterfat during the operation of the test." They then proceed to say that: "It is evident that mixing at intervals will lead to more accurate results with good milks. With the poorer milks the effect on accuracy would be slight." Wilson (1935) indicates that inversion of the tubes at half-hour intervals increases accuracy and materially shortens the reduction time, and states that this procedure makes the test better suited to the examination of high-grade milks.

## PROCEDURE

Three methods of procedure were compared.

Method 1 is essentially that specified in the 1934 edition of "Standard Methods of Milk Analysis." The tubes were stoppered and protected from light to prevent possibility of catalysis and were not touched after they were placed in the water baths, homogeneous bacterial dispersion being thus prevented.

Method 2 conforms closely to the revisions suggested for the seventh edition of "Standard Methods for the Examination of Dairy Products," the tubes being carefully inverted once, after five minutes' immersion in the water bath.

Method 3 (English modified) was devised by G. S. Wilson and his assistants (1935), working at the London School of Hygiene and Tropical Medicine. Their modification consists essentially of half-hourly inversions of the tubes so as to keep the fat and microorganisms in more or less homogeneous dispersion. The tubes are inverted but not shaken. It may be felt that this is a time-consuming procedure which complicates the test. However, the writer found that if adequate equipment was used it took only 30 seconds to invert 100 tubes, which does not seem to be an unreasonable requirement in view of the advantages resulting from this modification in procedure. As used by the writer certain unessential details were slightly altered.

The dye concentration as used by Wilson, after being diluted by the milk, was 1:300,000, whereas that now used in this country is approximately 1:700,000. Methylene blue chloride was used (certification number NA 2), the concentration of the dye in the milk being approximately 1:700,000.

## RESULTS

Table 1 shows a frequency distribution of all reduction times (4,347) and percentages of total for each series, together with the number of tests involved, the reduction time ranges and the averages. A cursory examination indicates the advantage of making many tests on few representative milks rather than making single or duplicate tests on many samples.

Perhaps the most important deduction has to do with the relative reliability of the three methods of conducting the test. With the exception of series 7, 11, and 15, the greatest dispersion of tests by methods 1 and 2 is located in rather high reduction time milks. However, when the English modified method was used, the extent of this

dispersion was lessened. In 14 cases reduction of all tubes took place simultaneously and in four of the six series where coincident reduction did not occur, more than 90 percent reduced at the same time.

Averages are shown, but they are valueless in the absence of the frequency distributions. In only one instance (series 20), a very poor milk, did simultaneous reduction occur by either method 1 or 2.

If single or duplicate tests had been made on these milks by either method 1 or 2, it would often have been impossible to predict in which of two or more classes they might have been placed. This observation is significant in view of the proposed change in minimum reduction time from five and one-half to eight hours for class 1 milk. Probable reliability of any given method is most important.

Significantly, all average reduction times determined by the English modified method, except one (series 12), were less than those arrived at by the other two methods. These differences in serial averages ranged from over four hours to one minute, and in individual tests were much greater. In series 12, which showed a lower average reduction time by method 2 than by the English modified method, simultaneous reduction was attained when the latter was used and some dispersion when the former was employed, a result which leaves little doubt as to which average is the more reliable.

These data indicate that the English modified method, as here conducted, furnished more dependable figures than its mates and that the averages were uniformly but variably lower, an observation which is in line with Wilson's findings.

Figures setting forth a different viewpoint are shown in tables 2 and 3. The results from 20 series, involving 3,697 tests by methods 2 and 3, are set forth in table 2, and results involving 1,950 tests by methods 1, 2, and 3 in table 3. Comparisons are made between high, low, and average reduction times for each series, together with serial ranges and a figure showing the relation between the serial ranges and averages, using the average as 100. This reduces the absolute ranges to a value which is mathematically more equitable, and permits comparisons between the ranges found in each series by the three methods. High and low reduction times are included because they are an essential step in determining the ranges and range relatives. They serve as a background for judging the relative importance of the ranges, etc.

Grand average reduction times obtained by three methods, as shown in table 3, do not indicate any vital difference between the results secured when methods 1 and 2 were used, whereas when method 3 was

employed they were consistently and significantly lower. If one compares the absolute ranges of either serial or grand averages (tables 2, 3) the distinctly greater dependability of the results obtained by method 3 becomes manifest.

Average absolute ranges of 97 minutes for samples tested by method 2 (table 2), and of 82 and 98 minutes for samples tested by methods 1 and 2 (table 3), are significant. When individual ranges run as high as 330 minutes, their meaning can not be ignored. The relative unreliability of present methods stands out. When these figures are compared with those secured when the English modified method was used, one's confidence in them is shaken. Averages in table 2 (20 series, 1,847 tests) were 16 minutes, 14 out of 20 series being zero, while the average in table 3 (8 series, 650 tests) was six minutes, seven out of eight series being zero.

The relative ranges, while being mathematically adequate, sometimes convey a wrong impression. For example, in series 19 the relative range was 33 while the actual range was 15 minutes, an unimportant difference, but in series 18 the one was 20 and the other 105 minutes, a variation which is significant. However, these relative ranges do serve a purpose when one is comparing two or more methods. The divergence between the average relative of 24 (table 2) by method 2 and that of 4 by the English modified method is striking; and the relatives when three methods are compared (table 3) indicate substantially similar ratios.

Thus again the English modified method is shown to be more dependable than its competitors.

The coefficients of variability and the figures necessary for their computation for all series as conducted by methods 1 and 2, are shown in table 4. The same values for reduction times obtained by methods 1, 2, and 3 for eight series are found in table 5. Here, as with the relative ranges, these coefficients are somewhat misleading. For example, that for series 20 (table 4) is 13 while the average deviation from the mean of reduction times is only 6.5 minutes; that for series 11 is 15, while the mean deviation is approximately 60 minutes. However, when the average coefficient of variability for method 2 of 4.2 (table 4) is compared with that of 0.3 by the English modified method, the conclusion is obvious. These coefficients for tests conducted by the English modified method (20 series, table 4) are zero in 14 instances, the remaining six ranging from 0.3 to 2. Similar results were found for the three methods as shown in table 5.

These comparisons of the figures secured in this study, made from three angles, by the three methods, indicate that the English modified method is significantly more dependable than the other two.

#### EFFECT OF DYE COMPOUND AND CONCENTRATION ON REDUCTION TIME

Considerable confusion exists as to the exact dye concentration which should be present in the milk as tested.

"Standard Methods of Milk Analysis" (1923 edition) points out that "Those who do not have the tablets will obtain approximately the same results by preparing solutions of methylene blue of such concentration that when 1 cc of the solution is added to 10 cc of milk, the dye will be present in the proportion of 1 part dye to 200,000 parts of the mixture of milk and the solution of dye. A stock solution can be prepared by dissolving 1.1 grams of the dry dye in 500 cc of the distilled water." The inference is that the tablets then available when used as directed would furnish a solution of the same concentration.

Thornton and Hastings (1929) say that: "The standard concentration of methylene blue used in the reduction test is 1 part of dye to 200,000 parts of milk." Fay and Aikins (1932) divided "A sample of market milk . . . into three parts, to two of which was added the standard amount of methylene blue ordinarily employed in the reductase test (1:200,000)." This was generally accepted as the resultant dye concentration used in the test as conducted in this country when use was made of either weighed amounts of methylene blue chloride or of the certified tablets. From time to time question has been raised as to the purity of the dye. Grimes et al. (1927) observe that: "The influences of impurities in methylene blue or variations in commercial grades of this product do not appear to have been fully investigated." Hastings (1924) reports that: "The American tablets were found to be 40-46 percent of their intended strength. It may be assumed that in the Standard American test the dye concentration in milk is approximately 1:465,000."

A corrected mimeographed sheet inclosed with a personal letter (1937) from Dr. H. J. Conn, Chairman of the Commission on Standardization of Biological Stains, sets forth a chart showing the dye content of methylene blue tablets, both Danish and American, which have been used in this country from 1922 to 1934, together with the resultant dilutions in milk. Apparently certified tablets have never been available which, when used as directed, furnished a dye concentration of more than 1:280,000. Indeed since 1923 the certified tablets have given an approximate dye concentration of from 1:635,000 to 1:710,000.



The situation has not been much better when methylene blue solution has been made from the crystals. Thornton (1935) has shown that seven lots of methylene blue chloride crystals have ranged from 59.3 to 87.5 percent total dye content in them.

Then, too, opinions have differed as to the importance of variations in dye concentration. Thornton and Hastings (1929) state that "data show that there may be a slight increase in the reduction time with increase in the concentration of methylene blue. The concentration must be approximately doubled before any appreciable increase in reduction time is observed." On the other hand Fay (1930) observes that: "The direct relationship between reduction time and the amount of dye solution added indicates that gross errors in measuring would introduce variations of sufficient magnitude to impair the accuracy of the test. Since there is no particular end gained by careless measuring or the use of inaccurate measuring devices, and since the amount of methylene blue added has such a direct bearing, it is advisable to use care in measuring the dye." Of course variations in the amount of methylene blue solution would exert the same effect as variation in dye concentration. In case where both dye concentration and measurement solution may vary, opportunities are offered for major discrepancies.

Clearly a rather chaotic condition has existed for many years. In many cases the exact dye concentrations which have been used are not known. Obviously there is need for a more dependable dye. Such information as is in hand as to the exact dye concentration to be expected should be widely disseminated. The effect upon results of the use of dyes of different concentrations should be better understood. Doubtless some of the discrepancies in results heretofore secured may be thus explained. They are now recognized and are being studied. Clarke et al. (1925) state that methylene blue chloride is difficult to purify, but they did not suggest an alternative. However, Drew and Head (1933) have pointed out that methylene blue thiocyanate might prove superior to the chloride for test purposes. Thornton et al. (1935) carefully compared the two and found the thiocyanate to be as good a means of measuring reduction in milk as the chloride and that it was more easily standardized to an approximate 100 percent dye content. It is now proposed to use the former in tablet making thus eliminating one source of inaccuracy encountered in the past; and it is proposed to raise the dye concentration as used in the milk to 1:300,000.

In view of these facts, the short study hereinafter reported was projected in order to compare these phases of the proposed changes.

## PROCEDURE

A solution of methylene blue chloride was prepared from tablets (certification number NA 2) as directed in the 1934 edition of "Standard Methods of Milk Analysis," with the exceptions noted. The methylene blue thiocyanate was received through the courtesy of Dr. R. S. Breed of the New York (Geneva) Station, and was similarly prepared for use. The English modified method (p. 5), was used. Forty replicate tests were made on each sample of milk for each variation noted. Table 6 compares the effect of two dye compounds and two dye concentrations on the average reduction times of replicate tests of four samples of milk. Tests were made using both dyes in approximate concentrations of 1:700,000 and 1:300,000, these being selected because the one represents the concentration resulting when the tablets in current use are dissolved and the other because it represents the proposed concentration.

Both individual and grand average reduction times indicate close agreement when approximately the same dye concentrations are used, particularly 1:700,000. However, significant differences occurred between the reduction times when dye concentrations of 1:700,000 and of 1:300,000 were employed. They are much greater than those reported by Thornton and Sandin in 1935. Of course, only a few samples were tested; but, on the other hand, the many replicate tests made on each one in some degree compensate for this shortcoming. In view of these results, one finds it hard to agree with the contention that the deeper color resulting from a greater dye concentration is responsible for such great differences. However, some factor of undetermined importance may have played a part. It is a well-known fact that individuals differ in their ability to determine shades of color. Be that as it may, these average differences will occur if the test is generally conducted using the suggested higher dye concentration.

Comparisons are shown in table 7 involving 50 replicate tests from three samples by the three methods previously described, making a total of 900 individual tests, the methylene blue chloride of approximately the present concentration (1:700,000) and the methylene blue thiocyanate of approximately the 1:300,000 concentration being used.

The use of the higher dye concentration resulted in materially longer reduction times by either method. The modified method gave consistently lower reduction times. Dye concentration had no material effect on coefficients of variability.

## CONCLUSIONS

1. Comparisons indicate that the English modified method, involving careful inversion of each tube at 30-minute intervals, gives shorter and significantly more reliable reduction times than the other two methods studied, due in the main to more uniform bacterial dispersion.
2. Methylene blue thiocyanate and methylene blue chloride, when used in about the same concentrations, afforded similar results.
3. Significantly longer reduction times elapsed with both dyes used at a concentration of 1:300,000 than when used at a concentration of 1:700,000.

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TABLE I.—FREQUENCY DISTRIBUTION OF REDUCTION TIMES AND PERCENTAGES OF TOTALS FOR EACH SERIES. METHODS 1, 2, 3.

Series numbers	1		2		3		4		5		6		7		8		9		10		11		12		
	Reduction time ranges in hours and minutes																								
	6:00-10:45		4:00-8:00		5:30-8:45		7:15-10:45		3:45-4:45		6:15-12:30		2:45-5:15		6:45-8:30		5:15-5:45		6:00-7:15		4:30-8:00		2:45-3:15		
Method	II	III	II	III	II	III	II	III	II	III	II	III	II	III	II	III	II	III	II	III	II	III	II	III	
..	93	..	100	..	30	..	1	..	100	..	100	..	2	..	100	40	..	..	91	8	..	28	..	..	
..	..	..	..	..	64	..	..	..	..	..	..	..	98	..	..	49	..	..	8	3	..	72	..	..	
..	6	..	..	..	6	..	..	..	52	..	..	..	..	..	2	..	11	100	8	1	3	..	..	100	
..	1	..	..	..	..	..	..	..	35	..	..	..	..	..	6	..	..	..	29	..	4	100	..	..	
..	..	..	..	..	..	..	..	..	13	..	..	..	..	..	52	..	..	..	42	..	3	..	..	..	
2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	36	..	..	..	21	..	..	..	..	..	
..	..	..	..	..	..	..	..	98	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	
2	..	..	..	..	..	..	..	..	..	..	..	..	33	..	3	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..	38	..	1	..	..	..	..	..	..	..	..	..	..
15	..	..	..	..	1	..	..	..	..	..	..	..	..	..	18	..	..	..	..	..	1	..	..	..	..
49	..	1	..	..	6	..	..	..	..	..	..	..	1	..	10	..	..	..	..	..	..	..	..	..	..
25	..	2	..	..	10	..	7	1	..	..	..	..	1	..	1	..	..	..	..	..	9	..	..	..	..
6	..	5	..	..	30	..	29	..	..	..	..	..	2	..	..	..	..	..	..	..	3	..	..	..	..
..	..	8	..	..	48	..	41	..	..	..	..	..	6	..	..	..	..	..	..	..	13	..	..	..	..
..	..	20	..	..	5	..	15	..	..	..	..	..	17	..	..	..	..	..	..	..	36	..	..	..	..
..	..	44	..	..	..	..	8	..	..	..	..	..	8	..	..	..	..	..	..	..	17	..	..	..	..
..	..	14	..	..	..	..	..	..	..	..	..	..	11	..	..	..	..	..	..	..	..	..	..	..	..
..	..	6	..	..	..	..	..	..	..	..	..	..	3	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..	8	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..	12	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..	10	..	..	..	..	..	..	..	..	..	..	..	..
1	..	..	..	..	..	..	..	..	..	..	..	..	11	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..	3	..	..	..	..	..	..	..	..	..	..	..	..
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..	..	..	..	..	..	..	..	..	..	..	..	..	3	..	..	..	..	..	..	..	..	..	..	..	..
8:17	6:02	7:24	4:00	8:20	5:41	10:13	8:45	4:25	3:45	10:23	6:15	4:31	3:00	7:50	6:45	5:26	5:45	6:59	6:02	6:54	5:15	2:56	3:15	..	..

Class intervals 15 minutes throughout.

Number of cases, Series Nos. 1-17, 100 except that in No. 3, Method 3 and No. 7, Method 3, 98 and 99 respectively, were used; Nos. 18-20, 50. Hence in all cases Nos. 1-17, the data indicate both numbers of cases and percentages; while in Nos. 18-20 the percentages always represent twice the number of cases.

TABLE 1.—FREQUENCY DISTRIBUTION OF REDUCTION TIMES AND PERCENTAGES OF TOTALS FOR EACH SERIES. METHODS 1, 2, 3—concluded

Series numbers	13			14			15			16			17			18			19			20			
Reduction time ranges in hours and minutes																									
6:15-7:30			8:30-14:15			2:00-5:45			4:00-5:45			5:45-6:15			5:45-11:00			0:45-1:30			0:45-1:00				
Method	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	
..	..	100	..	1	..	..	1	..	..	..	100	79	92	..	..	..	4	..	96	100	..	70	..	..	
..	12	..	..	100	..	..	..	..	..	..	..	..	21	6	..	..	78	..	4	..	100	30	100	..	..
..	64	..	..	..	..	..	1	..	..	14	..	..	..	2	100	..	14	6	..	..	..	..	..	..	..
40	24	..	..	1	..	..	1	..	..	56	..	..	..	..	..	..	4	94	..	..	..	..	..	..	..
49	..	..	..	..	..	..	..	..	..	26	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
11	..	..	..	1	..	..	..	..	..	28	4	..	..	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	1	2	..	..	..	..	32	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	1	..	..	1	1	..	40	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	1	1	..	2	..	..	..	..	..	..	..	..	6	..	..	..	..	..	..	..	..	..
..	..	..	..	3	..	..	..	..	100	..	..	..	..	..	..	8	10	..	..	..	..	..	..	..	..
..	..	..	..	1	..	..	2	..	..	..	..	..	..	..	..	10	4	..	..	..	..	..	..	..	..
..	..	..	..	3	1	..	5	96	..	..	..	..	..	..	..	12	28	..	..	..	..	..	..	..	..
..	..	..	..	4	2	..	5	..	..	..	..	..	..	..	..	6	20	..	..	..	..	..	..	..	..
..	..	..	..	3	3	..	..	..	..	..	..	..	..	..	..	4	26	..	..	..	..	..	..	..	..
..	..	..	..	14	5	..	4	..	..	..	..	..	..	..	..	4	4	..	..	..	..	..	..	..	..
..	..	..	..	6	11	..	81	..	..	..	..	..	..	..	..	12	4	..	..	..	..	..	..	..	..
..	..	..	..	9	4	..	..	..	..	..	..	..	..	..	..	6	4	..	..	..	..	..	..	..	..
..	..	..	..	13	24	..	..	..	..	..	..	..	..	..	..	2	..	..	..	..	..	..	..	..	..
..	..	..	..	13	16	..	..	..	..	..	..	..	..	..	..	14	..	..	..	..	..	..	..	..	..
..	..	..	..	12	8	..	..	..	..	..	..	..	..	..	..	4	..	..	..	..	..	..	..	..	..
..	..	..	..	12	9	..	..	..	..	..	..	..	..	..	..	10	..	..	..	..	..	..	..	..	..
..	..	..	..	5	3	..	..	..	..	..	..	..	..	..	..	2	..	..	..	..	..	..	..	..	..
..	..	..	..	2	4	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
..	..	..	..	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Averages	7:11	6:47	6:15	12:43	12:34	8:45	5:34	5:24	5:00	5:32	4:48	4:00	5:48	5:46	6:15	9:17	8:44	6:03	1:29	:46	:45	1:00	:50	1:0	..

Class intervals 15 minutes throughout.

Number of cases, Series Nos. 1-17, 100 except that in No. 3, Method 3 and No. 7, Method 3, 98 and 99 respectively, were used; Nos. 18-20, 50. Hence in all cases Nos 1-17, the data indicate both numbers of cases and percentages; while in Nos. 18-20 the percentages always represent twice the number of cases.

TABLE 2.—COMPARISONS INVOLVING ALL TESTS—TWO METHODS  
Arranged according to magnitude of reduction times by method 2

Series number	Number tubes involved in each series		High reduction times (minutes)			Low reduction times (minutes)			Average reduction times (minutes)			Ranges (minutes)		Relation range to average reduction time. Average = 100	
	2†	3‡	2†	3‡		2†	3‡		2†	3‡		2†	3‡	2†	3‡
19	50	50	60	45		45	45		46	45		15	0	33	0
20	50	50	60	60		45	60		50	60		15	0	30	0
12	100	100	180	195		165	195		176	195		15	0	9	0
5	100	100	285	225		255	225		265	225		30	0	11	0
7	100	100	315	180		255	165		271	180		60	15	22	8
16	100	100	315	240		270	240		288	240		45	0	16	0
15	100	100	330	300		120	300		324	300		210	0	65	0
9	100	100	345	345		315	345		326	345		30	0	9	0
17	100	100	375	375		345	375		346	375		30	0	9	0
13	100	99	420	375		390	375		407	375		30	0	7	0
11	100	100	480	315		270	315		414	315		210	0	51	0
10	100	100	435	390		390	360		419	362		45	30	11	8
2	100	100	480	240		375	240		444	240		105	0	24	0
8	100	100	510	405		435	405		470	405		75	0	16	0
1	100	100	645	405		435	360		497	362		210	45	42	12
3	100	98	525	360		450	330		500	341		75	30	15	9
18	50	50	585	390		480	345		524	363		105	45	20	12
4	100	100	645	585		585	435		613	525		60	150	10	29
6	100	100	750	375		510	375		623	375		240	0	39	0
14	100	100	840	525		510	525		754	525		330	0	44	0
Totals	1,850	1,847													
Averages			429	316		332	301		388	308		97	16	24	4

† = Method 2.  
‡ = English modified method.

TABLE 3.—COMPARISONS INVOLVING ALL TESTS—THREE METHODS  
Arranged according to magnitude of reduction times by method 2

Series number	Tubes involved in each series			High reduction times (minutes)			Low reduction times (minutes)			Average reduction times (minutes)			Ranges (minutes)			Relation range to average reduction time. Average = 100		
	1*	2†	3‡	1*	2†	3‡	1*	2†	3‡	1*	2†	3‡	1*	2†	3‡	1*	2†	3‡
19	50	50	50	90	60	45	75	45	45	89	46	45	15	15	0	17	33	0
20	50	50	50	60	60	60	60	45	60	60	50	60	0	15	0	0	30	0
16	100	100	100	345	315	240	315	270	240	332	288	240	30	45	0	9	16	0
15	100	100	100	345	330	300	225	120	300	334	324	300	120	210	0	36	65	0
17	100	100	100	360	375	375	345	345	375	348	346	375	15	30	0	4	9	0
13	100	100	100	450	420	375	420	390	375	431	407	375	30	30	0	7	7	0
18	50	50	50	660	585	390	465	480	345	557	524	363	195	105	45	35	20	12
14	100	100	100	855	840	525	600	510	525	763	754	525	255	330	0	33	44	0
Totals	650	650	650															
Averages				396	373	289	313	276	283	364	342	285	82	98	6	18	28	2

\* = Method number 1.  
† = Method number 2.  
‡ = English modified method.

TABLE 4.—COEFFICIENTS OF VARIABILITY—TWO METHODS  
 Arranged according to magnitude of reduction times by method number 2

Series number	Number tubes		Average reduction times (minutes)		Average deviations (minutes)		Coefficients of variability	
	2†	3‡	2†	3‡	2†	3‡	2†	3‡
19	50	50	46	45	1.52	0	3.30	0
20	50	50	50	60	6.50	0	13.00	0
12	100	100	176	195	5.96	0	3.39	0
5	100	100	265	225	9.55	0	3.60	0
7	100	99	271	180	11.52	0.45	4.25	0.25
16	100	100	288	240	8.40	0	2.92	0
15	100	100	324	300	11.52	0	3.56	0
9	100	100	326	345	8.45	0	2.59	0
17	100	100	346	375	2.34	0	0.68	0
13	100	100	407	375	6.44	0	1.58	0
11	100	100	414	315	60.96	0	14.72	0
10	100	100	419	362	10.16	3.14	2.42	0.87
2	100	100	444	240	12.76	0	2.87	0
8	100	100	470	405	9.25	0	1.97	0
1	100	100	497	362	11.34	3.97	2.28	1.10
3	100	98	500	341	12.15	6.99	2.43	2.05
18	50	50	524	363	19.96	5.82	3.81	1.60
4	100	100	613	525	11.77	1.50	1.92	0.29
6	100	100	623	375	45.28	0	7.27	0
14	100	100	754	525	42.83	0	5.68	0
Total	1,850	1,847						
Average			388	308	15.43	1.09	4.21	0.31

† = Method number 2.  
 ‡ = English modified method.

TABLE 5.—COEFFICIENTS OF VARIABILITY—THREE METHODS  
 Arranged according to magnitude of reduction times by method 2

Series number	Number tubes			Average reduction times (minutes)			Average deviations (minutes)			Coefficients of variability		
	1*	2†	3‡	1*	2†	3‡	1*	2†	3‡	1*	2†	3‡
19	50	50	50	89	46	45	1.78	1.52	0	2.00	3.30	0
20	50	50	50	60	50	60	0	6.50	0	0	13.00	0
16	100	100	100	332	288	240	10.60	8.40	0	3.19	2.92	0
15	100	100	100	334	324	300	17.51	11.52	0	5.24	3.56	0
17	100	100	100	348	346	375	4.89	2.34	0	1.41	0.68	0
13	100	100	100	431	407	375	8.45	6.44	0	1.96	1.58	0
18	50	50	50	557	524	363	52.20	19.96	5.82	9.91	3.81	1.60
14	100	100	100	763	754	525	37.56	42.83	0	4.92	5.68	0
Total	650	650	650									
Average				364	342	285	16.62	12.44	0.73	3.58	4.32	0.20

\* = Method number 1.  
 † = Method number 2.  
 ‡ = English modified method.

TABLE 6.—COMPARISON OF THE EFFECT OF DYE COMPOUND AND CONCENTRATION ON AVERAGE REDUCTION TIMES OF REPLICATE TESTS FROM SAME SAMPLE

Series number	Number of tests	Reduction times in minutes			
		Methylene blue chloride	Methylene blue thiocyanate	Methylene blue chloride	Methylene blue thiocyanate
		1: 300,000*	1: 300,000*	1: 700,000*	1: 700,000*
21	160	349	308	161	179
22	160	787	772	315	315
23	160	196	179	136	135
24	160	184	178	88	93
Total	640				
Average		379	359	175	180

\* Approximately.

TABLE 7.—METHYLENE BLUE CHLORIDE VS. METHYLENE BLUE THIOCYANATE (900 tubes)

Method	Reduction times in minutes					
	I		II		III	
	Series number	Chloride 1: 700,000	Thiocyanate 1: 300,000	Chloride 1: 700,000	Thiocyanate 1: 300,000	Chloride 1: 700,000
18	557	1,076	524	1,063	363	650
19	89	170	46	144	45	150
20	60	123	50	111	60	105
Average	235	456	207	439	156	302
Average deviations (minutes)						
18	52.20	40.62	19.96	34.92	5.82	9.50
19	1.78	15.20	1.52	9.30	0	0
20	0	4.80	6.50	14.40	0	0
Average	17.99	20.21	9.33	19.54	1.94	3.17
Coefficients of variability						
18	9.91	3.78	3.81	3.28	1.60	1.46
19	2.00	8.94	3.30	6.46	0	0
20	0	3.90	13.00	12.97	0	0
Average	3.97	5.54	6.70	7.57	0.53	0.49