INFLUENCE OF REFRIGERATED STORAGE ON DYE REDUCTION TIME OF MILKS

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Working with 100 weigh-can samples from 5 dairies it was found that icing for even 2 hours slightly retarded reduction of methylene blue; after 23 hours the effect was somewhat greater when the dye was present, but not when it was added just before incubation. With resazurin, on the other hand, there was better agreement when the dye was present during overnight storage. In no instance was the difference statistically significant. Methylene blue reduction times were in surprisingly close agreement with standard plate counts on the raw milk, while the correlation between reduction times (raw) and plate counts after laboratory pasteurization was better than was expected.

INTRODUCTION

In routine control work milk can rarely be tested immediately after sampling. Consequently, the manner in which samples are handled in the interim period is of some importance. In 1939, *Standard Methods for the Examination of Dairy Products*[2] stated that tubes of milk could be stored in ice water for up to 2 hours before starting their incubation. The purpose of limiting this period was to avoid too solid setting of the cream layer[22]. This was extended to 24 hours in 1948[3] because (a) the 2-hour requirement was inconvenient and was not being observed, and (b) if a storage period of 6 hours, in common practice, was not distorting the picture, overnight refrigeration might also be acceptable. Studies conducted by the Standard Methods Committee[1] on the effect of overnight refrigeration yielded results (unpublished) which were believed to warrant such a procedure and this was incorporated in the 9th edition of Standard Methods[3].

The desirability of overnight refrigeration has recently been questioned. Calbert and Wallenfeldt[5] expressly warn that tubes of milk, or milk plus dye, must not be held in ice water for over 2 hours before testing. These workers believe that great differences in results may occur when samples are held in ice water for long periods before incubation[23].

The influence of refrigerated storage on the reduction time of milk has been studied by a number of workers, [4, 6, 7, 9, 10, 11, 12, 13, 16, 18, 19, 20, 21, 24] most of whom have reported that the methylene blue reduction time was lengthened as a result of overnight refrigeration. Frayer[9] found an increase of about 9 percent for all grades of milk; Wilson [24] noted an average increase of 5 to 10 minutes for uncooled raw milk, and a decrease of 25 minutes for a poorer grade of previously cooled milk. Johns[13] found the average reduction time shortened by one hour in one series while in another series[12] there was an average time difference of only 3 minutes. Morton and Vincent[16] in Australia reported that icing for 24 hours had little effect with methylene blue, but was more serious with resazurin. Eddison *et al.*[6] in Britain found the reduction time with methylene blue was increased.

The effect on resazurin reduction has also been reported on. Thomas [20] found a considerable loss in resazurin-reducing ability. Galesloot [11] and also Revallier-Warffemius [18] reported a similar effect, which they attributed to the decreased activity of body cells. Frayer[10] also noted a definite retardation of activity. Thomas and Davies[21] however, found an increase of only 3 to 14 minutes in mean reduction times, which they regarded as insignificant.

The variable results noted probably reflect differences in the previous history of the milks, the presence or absence of the dye during the storage period, etc. Unfortunately, many of the papers fail to give sufficient details on these and other points. Furthermore, since the earlier studies were conducted, both the type and concentration of methylene blue have been changed and the periodical inversion of tubes during incubation has been adopted. The studies being reported in this paper were undertaken in the hope of providing more definite information concerning the influence of refrigerated storage under present conditions, using both methylene blue and resazurin tests[3] Although the shorter incubation period of the latter generally obviates the need for holding samples overnight, the earlier colour changes shown by resazurin suggested the desirability of including studies with this dye.

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EXPERIMENTAL

Unless otherwise specified, the *Standard Methods for the Examination of Dairy Products*[3] were followed. Fresh dye solutions were prepared for each series of tests. In the main experiment weigh-can samples were obtained from milk of the first 20 shippers at each of 5 local dairies between May 8 and June 12, 1951. Some milk for manufacturing purposes was included, in order to obtain a wider range of reduction times. Half-pint bottles

Contribution No. 329, Division of Bacteriology and Dairy Research, Science Service, Canada Department of Agriculture, Ottawa.
Presented at the 38th Annual Convention International Association of Milk and Food Sanitarians, Inc., Glenwood Springs, Colorado, September 27, 1951.

were half-filled, and each batch of 20 brought to the laboratory immediately. No attempt was made to keep the samples cool between sampling and subdividing in the laboratory. After thorough mixing of the samples, 10-ml portions were rapidly pipetted into sterile 16x150 mm test tubes identified as follows:

Treatment	Methylene Blue	Resazurin
Control - Incubated at once	A	B
In ice water 2 hrs. then incubated	C	D
In ice water 23± hr. then incubated	E	F
In ice water 23± hr. then incubated	G[1]	H

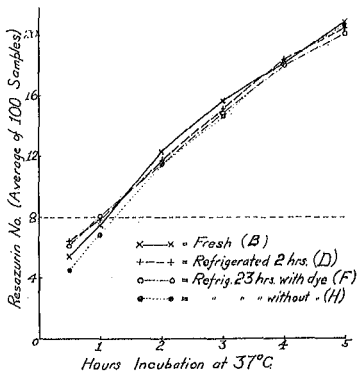
[1]This set was not prepared from the first series of 20 samples

For sets A to F inclusive, the dye solution was placed in the tubes before the milk was introduced: for sets G and H it was added immediately before starting incubation the following day. The ice water baths containing racks of tubes C to H, together with a further set for laboratory pasteurization, were held in a cold storage room at 3°C (37.4°F).

Approximately 5 minutes after placing the tubes in a thermostatically controlled water bath at 37°C they were closed with sterile rubber stoppers and each rack inverted 3 times to mix the milk and dye uniformly. Methylene blue tubes were examined every thirty minutes, with occasional further observations at 15-minute intervals; resazurin readings were recorded every 30 minutes for the first hour, then every hour. Munsell colour standards[3] were employed, and colour numbers recorded using the scale of Johns and Howson[14], where 0 is the initial colour, 16 a full pink, and 24 complete reduction. All tubes not showing obvious signs of reduction were inverted once each hour. In view of the more frequent observations, methylene blue reduction times were recorded for the exact period of incubation, rather than by the procedure outlined in Standard Methods[3].

RESULTS & DISCUSSION

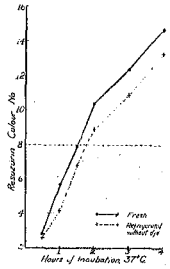
In figure 1 are shown curves representing the average resazurin colour number for all 100 samples at each reading for each of the four modifications (B, D, F, H) studied. Refrigeration overnight without the dye (H) had the greatest depressing effect on reduction during the first hour or two. The slightly greater colour change shown by the fresh milk (B) at the second and third hour may be attributable to non-bacterial reducing factors such as leucocytes (15) which tend to lose their reducing power when milk is stored in ice water, [11, 18, 20]).



1. Average resazurin colour numbers from 100 samples of raw milk subjected to the treatments indicated.

These results are in line with those obtained here in previous studies on 20 samples tested in May, 1950, and 20 in February, 1951, data from which are presented in Figure 2. Here, after setting up the tubes for immediate testing, the remainder of each sample (*ca* 150 ml) was placed in the refrigerator and held for 24 hours; next day, 10-ml portions were pipetted out, the dye added, and incubation commenced. Despite their being warm (62°-68°F.) on arrival at the laboratory, and cooling down slowly in air, the reduction rate was slower following refrigeration.

Another method of measuring the effect of refrigeration is by comparing the numbers of samples placed in the several grades by the "triple reading" test[3], [14]. Table 1 suggests that resazurin reduction was slowed down by all three modifications of refrigerated storage and especially by overnight storage of the milk without the dye. Results following overnight storage with the dye present agree very well with those following 2 hour storage, although the grading with both modifications was more lenient than



2. Average resazurin colour numbers for 40 samples of milk examined in May, 1950, and February, 1951.

that for the "fresh" sample. However, application of the anaylsis of variance to these data revealed that at the 1% level there was no significant difference between the various treatments.

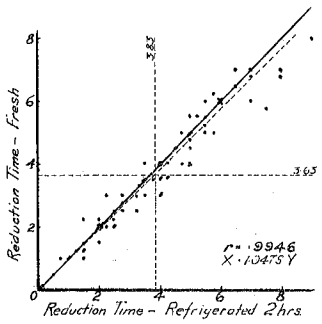
In figures 3, 4, 5 the results obtained with the several modifications of the methylene blue reduction test are presented as scatter diagrams so that the extent of variation can be more readily grasped. In these graphs the tubes incubated at once (A) were taken as the basis

TABLE I

EFFECT OF REFRIGERATED STORAGE ON GRADE BY RESAZURIN TRIPLE READING TEST

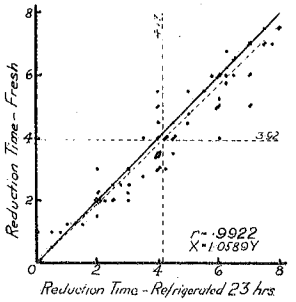
Treatment prior to incubation	% Distribution of Samples			
	Class 1	Class 2	Class 3	Class 4
B Fresh	19	23	24	34
D Refrigerated 2 hrs	25	21	23	31
F Refrigerated 23 hrs with dye	24	20	24	32
H Refrigerated 23 hrs without dye.	29	19	25	27

for comparison. The *average* reduction time for all samples is indicated by a dotted line, horizontal for the fresh and vertical for the refrigerated. Fig. 3 shows that reduction

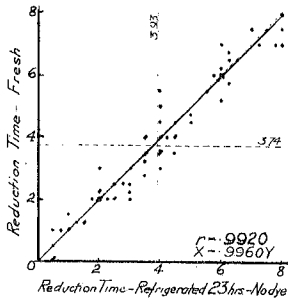


3. Corresponding methylene blue reduction times of fresh and iced milk samples.

time was prolonged slightly following even 2 hours refrigeration; after 23 hours the effect was more pronounced when the dye was present (fig. 4), but when stored without the dye (fig. 5) the effect was even less than that following the 2-hour storage with the dye present. This is in contrast to the effect noted with resazurin (fig. 1) where better agreement was obtained



4. Corresponding methylene blue reduction times of fresh and iced milk samples.

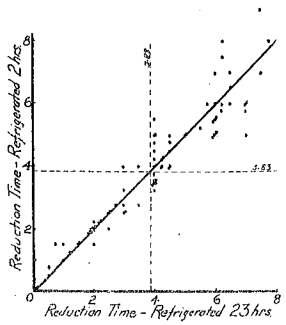


5. Corresponding methylene blue reduction times of fresh and iced milk samples.

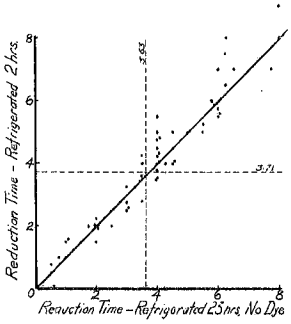
when the dye was present during overnight refrigeration.

Here again, despite the apparent differences, statistical anaylsis has revealed that in none of the three cases (fig. 3-5) is the calculated slope of the fitted equation $X = mY$ significantly different from the theoretical slope of 1.0000 which would be found if refrigeration caused no change in the reduction time of milk. The t values of 0.4097, 0.5338 and 0.0355 for the three sets of comparisons are also far below the values (2.64, 2.63 and 2.64) required for significance at the 1 percent level. The standard errors of the estimate are $\pm$ 0.2078, $\pm$ 0.2700 and $\pm$ 0.2513 respectively for the three comparisons, while the correlation coefficients are 0.9946, 0.9922 and 0.9920 respectively. It is concluded, therefore, that for these samples *there is no significant difference between the methylene blue reduction times for fresh and refrigerated milk.*

In actual practice, samples are not tested immediately but are held in ice water for varying periods. Consequently, the results obtained from portions iced for 2 hours are probably more representative of of those obtained in practice than are those from portions run immediately. Taking the former as the basis for comparison, the results are as shown in figures 6 and 7. Here it will be noted that the deviations are more evenly distributed on either side of the line denoting perfect agreement, with very little difference between the average values for 2-hour and 24-hour refrig-



6. Corresponding methylene blue reduction times of milks iced for two hours vs. 23 hours.



7. Corresponding methylene blue reduction times of milks iced for two hours vs. 23 hours.

eration. It would appear, therefore, that in actual practice overnight refrigeration has even less effect than was indicated in figs. 3-5.

The effect of the several modifications may also be studied by comparing the percentage distribution of reduction times. From the data in table II, it will be seen that the tubes refrigerated overnight without the dye (G) gave results in closest agreement with those from the tubes run immediately (A). Overnight refrigeration with

TABLE II

EFFECT OF REFRIGERATED STORAGE ON GRADING BY THE METHYLENE BLUE REDUCTION TEST

Treatment prior to incubation	No. of pairs	% Distribution of Reduction Times (hrs.)			
		6 & >	4 - 5¾	2 - 3¾	< 2
A Fresh	90	21.1	27.8	36.7	14.4
C Refrigerated 2 hrs		20.0	34.5	31.1	14.4
A Fresh	94	24.5	26.6	35.1	13.8
E Refrigerated 23 hrs with dye		31.9	30.9	24.5	12.8
A Fresh	80	22.5	23.8	38.8	15.0
G Refrigerated 23 hrs without dye		23.8	27.5	32.5	16.3

the dye (E) increased the proportion of samples requiring over 6 hours to reduce, while icing for 2 hours (C) increased the proportion reducing between 4 and 6 hours.

Ellenberger and Moody[7] reported that reduction times increased progressively up to a maximum of 65 minutes as the icing of samples was continued from 0 to 2 hours. To check on the effect of icing over this period, 40 more samples were obtained and 10-ml portions treated as indicated in table III. Since

TABLE III

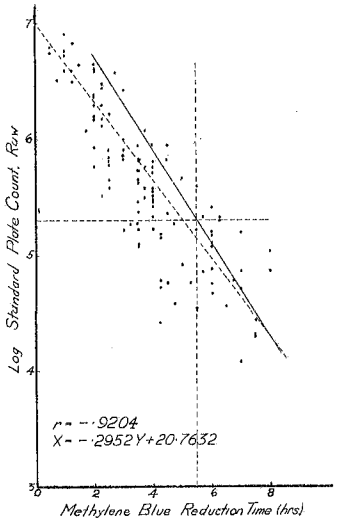
EFFECT OF REFRIGERATED STORAGE ON METHYLENE BLUE REDUCTION TIMES. 40 SAMPLES

Treatment	Average Reduction Time (hrs)
A Control - incubated at once	3.59
B Iced 1 hr. with dye	3.73
C " 1 hr. without dye	3.69
D " 2 hrs. with dye	3.69
E " 2 hrs. without dye	3.65

readings were made at 15-minute intervals, the results are more precise than those in the main experiment. They fail to confirm those of Ellenberger and Moody in that the 1-hour icing prolonged the reduction time on an average slightly more than did the 2-hour icing. Here again the presence of the dye shows a slight tendency to slow down subsequent reduction. However, grading on the basis of the classes used in table II shows very slight differences (table IV).

While differences in methylene blue reduction time as a result of refrigerated storage lack statistical signficance, it seems preferable to minimize such differences by holding samples overnight without the dye being added. With resazurin however, if samples are to be stored overnight, the dye should be added before storage.

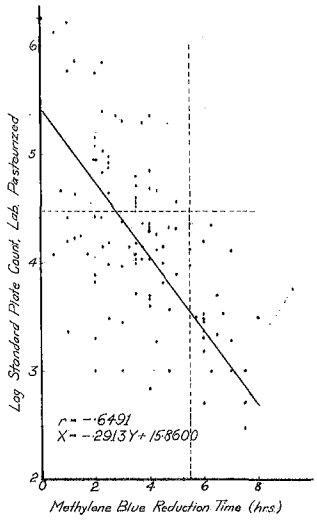
Incidental to the study of the effect of refrigerated storage, standard plate counts at 35°C (95°F) were made on the samples (a) as received at the laboratory, and (b) after laboratory pasteurization of 10-ml portions in open 16 x 150 mm test tubes in a thermostatically controlled water bath at 61.7°C (143°F) ± 0.5° for 35 minutes. Fig. 8 shows the relationship between raw plate counts and the



8. Log. plate counts vs. methylene blue reduction times for 116 milks.

methylene blue reduction times of the fresh milk. The solid diagonal line represents the relationship between these two tests specified in the revised Milk Ordinance recommended by the U. S. Public Health Service[8], while the dotted diagonal line represents the slope of the fitted curve when X = −0.2952Y + 20.7632. Although the number of samples -116- is not large, the agreement is surprisingly good, with a correlation coefficient of −0.9204. Had the high count samples been plated on a higher dilution, overcrowding would have been avoided and an even closer agreement obtained. It will be noted that only three samples with counts over 200,000 per ml failed to reduce methylene blue in 5½ hours.

In fig. 9 similar log counts, after laboratory pasteurization, are plotted against reduction times *when raw* for 112 milks, all but 20 of



9. Log. plate counts after laboratory pasteurization vs. methylene blue reduction times of raw milks (112 samples).

which were those shown in fig. 8, plus an additional 20 for which raw counts were not obtained. The horizontal and vertical dotted lines represent a count of 30,000 per ml and a reduction time of 5½ hours respectively, while the solid diagonal line indicates the slope of the fitted curve where X = −0.2913Y + 15.8600. In view of the popular opinion that thermoduric bacteria are unlikely to be detected by the dye reduction tests, it it is interesting to note that none of these milks with a plate count after laboratory pasteurization in excess of 30,000 showed a methylene blue reduction time, when raw, of over 5 hours.

TABLE IV

EFFECT OF REFRIGERATED STORAGE ON GRADING BY THE METHYLENE BLUE REDUCTION TEST. 40 SAMPLES

Treatment	% Distribution of Reduction Times (hrs)			
	6& >	4 - 5⅝	2 - 3⅜	< 2
A Incubated at once	7.5	32.5	52.5	7.5
B Iced 1 hr. with dye	7.5	35.0	50.0	7.5
C " 1 hr. without dye	5.0	32.5	55.0	7.5
D " 2 hrs. with dye	5.0	32.5	52.5	10.0
E " 2 hrs. without dye	7.5	32.5	50.0	10.0

SUMMARY

Our findings confirm previous reports that storing tubes of milk in ice water between collecting and testing may affect the results of the dye reduction tests. With resazurin, the effect was greatest where tubes of milk without dye were stored overnight. When stored overnight with the dye present, the agreement with the controls was at least as good as that where tubes were refrigerated for 2 hours only. With methylene blue, however, the milks, without dye refrigerated overnight showed the closest agreement with the controls. Both the 2-hour and overnight holding with the dye slowed down the rate of reduction sufficiently to result in slightly more lenient grading than with the control. However, none of the differences were statistically significant, hence the 2-hour limitation for icing samples before testing does not appear to be justified.

Where it is difficult to conduct the test on the day of sampling, tubes of milk for the methylene blue reduction tests should preferably be stored overnight without the dye, whole those for the resazurin test should contain the dye.

The relationship between raw milk reduction times and plate counts (a) before and (b) after laboratory pasteurization was surprisingly good.

ACKNOWLEDGMENTS

The author is grateful to Mr. G. B. Oakland and Miss C. E. Cox of the Biometric Unit, Science Service, Department of Agriculture, for statistical advice and analysis of data, and to Misses B. Brawn and C. Darby and Messrs. T. W. Humphreys and J. G. Desmarais for technical assistance.

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WISCONSIN FIELDMEN'S CONFERENCE

The annual Dairy Plant Fieldmen's Conference will be held on the University of Wisconsin Campus at Madison on February 7 and 8, 1952.

On the morning of February 7 at 11:00 o'clock it is planned to dedicate Babcock Hall, the new Dairy and Food Industries Building. Fieldmen who attend this conference will therefore have an opportunity of being present at the Dedication Ceremonies. It is planned to hold Open House at Babcock Hall on the evening of that day.

During that week, on Wednesday afternoon, February 6, another program is being held which will be of considerable interest to fieldmen. This relates to discussions on Brucellosis. All fieldmen are invited to attend it, as well as the regular Fieldmen's Conference on Thursday and Friday, February 7 and 8.

What Effect Does Grass Silage Have on Quality of Milk and Milk Products? By W. V. Price, Department of Dairy and Food Industries

Cow Barn Ventilation: By C. H. Neitzke, Department of Agricultural Engineering.

The Milkhouse Problem:

The Milkhouse Regulation: By H. J. Weavers, Dairy Division, Wisconsin Department of Agriculture.

Getting Action: By William Kasakaitas, Wisconsin Farm Bureau Federation.

Plans and Costs: By M. J. LaRock, Department of Agricultural Engineering.

Financing Milkhouses: By Jim Judd, Consolidated Badger Cooperative.

Questions and Answers by Panel of Speakers.

The conference program follows:

Effects of Mastitis on the Cow: By S. M. McNutt, Department of Veterinary Science.

Causes and Prevention:

Bacterial Phases: By E. M. Foster, Department of Bacteriology.

Management Phases by V. R. Smith, Department of Dairy Husbandry

Control of Mastitis:

Tests and General Control: By J. Simon, Department of Veterinary Science.

Organized Programs: By C. A. Brandly, Department of Veterinary Science.

The Mastitis Problem as it Affects the Dairy Plant: By H. E. Calbert, Department of Dairy and Food Industries.

Questions and Answers: By Panel of Speakers.

Evening Program

"Open House" at Babcock Hall.